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

# World Journal of *Gastroenterology*

*World J Gastroenterol* 2017 February 21; 23(7): 1119-1324

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 1375 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 (25), Chile (4), China (165), Croatia (2), Cuba (1), Czech (6), Denmark (2), Egypt (9), Estonia (2), Finland (6), France (20), Germany (58), Greece (31), Guatemala (1), Hungary (14), 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*
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*
Yan-Chang Lei, *Hangzhou*
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*

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*
Ferenc Sipos, *Budapest*
Miklós Tanyi, *Debrecen*
Tibor Wittmann, *Szeged*

**Iceland**

Tryggvi Bjorn Stefánsson, *Reykjavík*

**Indiad**

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*

World Journal of Gastroenterology

Contents

Weekly Volume 23 Number 7 February 21, 2017

## META-ANALYSIS



## CASE REPORT

*World Journal of Gastroenterology*
Volume 23 Number 7 February 21, 2017

# Contents





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 21, 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/

*World J Gastroenterol* 2017 February 21; 23(7): 1119-1124

DOI: 10.3748/wjg.v23.i7.1119

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

EDITORIAL

# Common controversies in management of biliary strictures

Mansour A Parsi

Mansour A Parsi, Center for Endoscopy and Pancreatobiliary Disorders, Department of Gastroenterology and Hepatology, Cleveland Clinic, Cleveland, OH 44195, United States

Author contributions: Parsi MA solely designed, wrote and edited the manuscript.

Conflict-of-interest statement: No conflicts of interest related to this manuscript.



Manuscript source: Invited manuscript

Correspondence to: Mansour A Parsi, MD, MPH, Center for Endoscopy and Pancreatobiliary Disorders, Department of Gastroenterology and Hepatology, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, United States. parsim@ccf.org
Telephone: +1-216-4454880
Fax: +1-216-4446284

Received: September 14, 2016
Peer-review started: September 16, 2016
First decision: November 21, 2016
Revised: December 10, 2016
Accepted: January 18, 2017
Article in press: January 18, 2017
Published online: February 21, 2017

## Abstract

Biliary strictures are caused by a heterogeneous group of benign and malignant conditions, each requiring a specific treatment approach. Management of biliary strictures often involves endoscopy either for definite treatment, as a bridge to surgery or for palliative purposes. Endoscopic treatment of various types of biliary strictures is not standardized and there are multiple areas of controversy regarding the best treatment options. These controversies are mainly due to lack of well-designed comparative studies to support a specific therapy. This paper reviews three common areas of controversy in the endoscopic management of biliary strictures. The areas discussed in this editorial include the role of biliary drainage in resectable malignant strictures and whether such drainage should be performed routinely prior to surgery, the best endoscopic palliation for unresectable hilar strictures and whether unilateral or bilateral stenting should be attempted, and the optimal endoscopic management for dominant strictures in patients with primary sclerosing cholangitis. The goal of this editorial is twofold. The first is to review the current literature on management of the aforementioned strictures and offer recommendations based on available evidence. The second goal is to highlight the gaps in our knowledge which in turn can encourage future research on these topics.

**Key words:** Biliary stricture; Benign; Primary sclerosing cholangitis; Malignant; Controversy; Biliary drainage; Preoperative; Hilar stricture



**Core tip:** Based on available evidence preoperative biliary drainage is not routinely indicated in resectable malignant strictures. However, it is appropriate in acute cholangitis, in severely symptomatic patients and in those with delayed surgery. In patients with unresectable hilar stricture, cross-sectional imaging is advised prior to attempt at palliative drainage. In such patients unilateral stenting during endoscopic retrograde cholangiopancreatography is adequate in most cases. Routine stenting of dominant strictures in primary sclerosing cholangitis patients is not recommended. Stenting of dominant strictures is appropriate

if there is poor drainage of contrast after dilatation or concern for collapse of the bile duct compromising biliary drainage.

Parsi MA. Common controversies in management of biliary strictures. *World J Gastroenterol* 2017; 23(7): 1119-1124 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1119.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1119

## INTRODUCTION

### *Preoperative biliary drainage in resectable malignant strictures*

The rationale behind preoperative biliary drainage is the belief that cholestasis is associated with higher postoperative morbidity and mortality and that preoperative biliary drainage may improve surgical outcomes by improving the liver synthetic function, increasing clearance of endotoxins and improving the gastrointestinal mucosal barrier function which in turn may reduce the risk of bacterial translocation[1].

Increased risk of surgery in jaundiced patients has been suggested for decades. In fact, the first report of preoperative biliary drainage has been attributed to Allen Whipple who in 1935 described performance of cholecystogastrostomy to relieve jaundice prior to pancreatoduodenectomy for treatment of periampullary cancers[2]. The issue of preoperative biliary drainage, however, has remained controversial.

### *Preoperative drainage in distal biliary strictures*

In an attempt to shed some light on the role of preoperative drainage for distal biliary strictures, a clinical trial randomized patients with pancreatic head cancer and obstructive jaundice with bilirubin levels between 2.3-14.6 mg/dL to preoperative biliary drainage or early surgery. The preoperative biliary drainage group underwent endoscopic retrograde cholangiopancreatography (ERCP) with plastic stent placement followed by surgery 4-6 wk later. The early surgery group had surgery within a week of randomization. The investigators compared serious adverse events between the two groups within 120 d of randomization. In all, 102 patients were randomized to preoperative biliary drainage while 94 patients were randomized to early surgery. Surgery-related complications and mortality did not differ between the two groups. Overall serious complications, on the other hand, were significantly higher in patients who had undergone preoperative biliary drainage compared to those who had early surgery[3]. The study concluded that preoperative biliary drainage with stent placement has no beneficial effect on surgical outcomes in patients with distal biliary stricture[3].

The study was criticized for exclusion of patients with severe jaundice (bilirubin > 14.6 mg/dL) and higher than expected stent-related complications[4-6]. Some experts argued that if the study investigators had used metallic rather plastic stents, the stent-related complications would have been lower, possibly leading to different study results[7].

The investigators therefore conducted a follow up study in which patients with pancreatic cancer and obstructive jaundice who could not undergo early surgery were assigned to undergo ERCP with metallic stent placement[8]. The observed outcomes in these patients were compared to the patients in the original study[3,8]. Comparison of the patients who received plastic stent with those who received metallic stent showed that the drainage-related complications were significantly lower in the metallic stent group mostly because of a significant decrease in the stent-related complications[8]. However, when the study investigators compared the metallic stent group with early surgery group, the early surgery group still had significantly less serious complications. The authors concluded that early surgery is preferable to preoperative biliary drainage independent of the type of the stent used[8].

A recent meta-analysis identified six randomized studies assessing the role of preoperative biliary drainage in patients with distal biliary strictures[9]. Four of these studies had used a percutaneous approach while 2 had used an endoscopic approach for biliary drainage. The studies included in this meta-analysis had a total of 520 patients, of whom 265 had received preoperative biliary drainage and 255 did not. The meta-analysis showed that although there were no differences in mortality rate or hospital length of stay, preoperative biliary drainage was associated with significantly higher morbidity[9].

**Recommendations for preoperative drainage in distal biliary strictures:** Based on available evidence preoperative biliary drainage is not routinely indicated in patients with resectable distal strictures[10]. However, preoperative biliary drainage is appropriate in patients with acute cholangitis, those who are severely symptomatic and also those who have delayed surgery either because of logistical issues or need for neo-adjuvant therapy. If preoperative biliary drainage is to be performed, metallic stents are preferable to plastic stents. Both covered and uncovered metallic stents can be used in this setting. If uncovered metallic stents are to be used, shortest possible stent length should be utilized in order to prevent interference with surgery.

### *Preoperative biliary drainage in hilar strictures*

There are no randomized controlled trials assessing the role of preoperative biliary drainage in hilar strictures. There are, however, multiple retrospective studies available[11-15]. With the exception of one study which suggested a decreased incidence of intra-abdominal

abscess formation[14], other studies have shown a deleterious effect associated with preoperative biliary drainage in the form of increased rates of postoperative infections or increased length of hospital stay[11-13,15]. None of the studies showed any survival benefit associated with preoperative biliary drainage[11-15]. A systematic review and meta-analysis of studies up to 2010 showed no clinical benefit associated with preoperative biliary drainage in patients with hilar stricture and suggested that preoperative biliary drainage in such strictures may increase postoperative adverse events and infectious complications[16].

**Recommendations for preoperative biliary drainage in hilar strictures:** Based on available evidence preoperative biliary drainage is not recommended routinely in patients with malignant hilar stricture. Preoperative biliary drainage, however, is appropriate in patients with acute cholangitis, those who require neo-adjuvant therapy and those who have hyperbilirubinemia induced malnutrition, hepatic insufficiency or renal insufficiency[17]. Severely symptomatic patients and those with delays in surgery should also be considered for preoperative biliary drainage.

## PALLIATION OF UNRESECTABLE HILAR STRICTURES

Regardless of histology less than 30% of malignant hilar strictures are suitable for curative resection. Palliation is therefore needed in the majority of patients and biliary drainage is a major component of palliation in such patients. Whether biliary drainage for palliation of unresectable hilar strictures is best achieved by unilateral or bilateral stenting remains a controversial issue. Two randomized controlled trials have assessed unilateral versus bilateral drainage for palliation of hilar strictures[18,19].

In the first trial, 157 patients with hilar strictures were randomized to ERCP with unilateral plastic stent placement (79 patients) or ERCP with bilateral plastic stent placement (78 patients). Technical success was significantly higher in patients with unilateral stent placement[18]. Complications including infectious complications were significantly lower in patients with unilateral stent placement. Drainage success, defined as at least 75% reduction in pre-procedure bilirubin levels, were significantly higher in patients with unilateral stent placement compared to those who had bilateral stenting[18]. Survival did not differ between the two groups. The investigators of the study, therefore, concluded that routine bilateral stenting is not advised[18].

The second trial included 60 patients with hilar strictures who were randomized to ERCP with plastic stent placement (30 patients) or ERCP with metallic stent placement (30 patients)[19]. Within each group approximately half of the patient's received unilateral while the other half received bilateral stenting. Although the main goal of the study was to compare metallic with plastic stents, subgroup analysis of unilateral versus bilateral stenting were performed. This study did not show any difference in patency time between unilateral versus bilateral stenting[19]. Re-intervention success, however, was significantly higher in patients with unilateral stent placement compared to those who had bilateral stenting[19]. The study also showed that metallic stents had longer patency time and less need for re-intervention compared to plastic stents[19].

One prospective and multiple retrospective studies have also looked at this topic[20-24]. Although none of the studies showed any survival benefit with unilateral or bilateral stenting, they showed mixed results in other areas. A study by Chang *et al*[20] utilizing plastic stents showed that survival is worse if the ducts in both liver lobes are injected during ERCP but only one lobe drained. A small prospective study by Freeman *et al*[21] utilizing metallic stents showed that unilateral stenting is adequate in most patients. A retrospective study by Naitoh *et al*[22] utilizing metallic stents suggested longer patency time with bilateral stenting although survival time and complication rates were the same as with unilateral stenting. A retrospective study by Iwano *et al*[23] utilizing metallic stents showed that unilateral stenting is associated with lower infection rates but the same survival and stent patency compared to bilateral stenting. A retrospective study using both metallic stents and plastic stents by Liberato *et al*[24] suggested that bilateral stenting is associated with longer stent patency time without affecting survival time. Metallic stents in that study had longer patency time than plastic stents[24].

A French retrospective study suggested that drainage of more than 50% of liver volume confers longer survival[25]. It also suggested that injection of contrast during ERCP and stenting of an atrophic lobe in the liver is associated with higher complication rates. That study suggested that preprocedural cross-sectional imaging would be of value to avoid stenting or injecting an atrophic lobe during ERCP[25].

A recent meta-analysis, including 7 studies with 634 patients, did not find any difference in mortality, stent occlusion rate or cholangitis rate between those who had unilateral versus bilateral stenting for palliation of malignant hilar strictures[26]. The meta-analysis concluded that there are no benefits to routine bilateral stenting for palliation of unresectable hilar strictures[26].

### *Recommendations for endoscopic palliation of unresectable hilar strictures*

A cross-sectional imaging study such as CT or MRI is advised prior to attempt at palliative drainage of unresectable malignant hilar strictures as a roadmap to better define the biliary anatomy and avoid injecting

or stenting an atrophic liver lobe. Unilateral stenting during ERCP in such patients seems to be adequate in most cases. Bilateral injection of contrast, however, requires bilateral stenting to assure drainage. For palliation of hilar strictures, metallic stents seem to have longer patency and require less re-intervention that plastic stents.

## MANAGEMENT OF DOMINANT STRICTURES IN PRIMARY SCLEROSING CHOLANGITIS

Primary sclerosing cholangitis (PSC) is a chronic progressive disease that can affect both intrahepatic and extrahepatic bile ducts[27]. According to epidemiologic studies, intrahepatic and extrahepatic bile duct involvement are seen in approximately 69% of the patients while involvement of only intrahepatic or only extrahepatic ducts are seen in 25% and 4% of the patients respectively[27,28]. Endoscopic therapy in the form of ERCP is effective in patients with strictures localized to the extrahepatic and large intrahepatic bile ducts, described as dominant strictures. On cholangiography, dominant strictures are defined as stenoses measuring < 1.5 mm in the common bile duct or < 1.0 mm in the hepatic ducts[27,29]. Between 45%-58% of patients with PSC develop dominant biliary strictures during their course of disease[27]. In addition to cholangiocarcinoma, dominant strictures have been associated with increased risk of ascending cholangitis, stone formation and hepatic decompensation[27]. Treatment of dominant strictures is therefore recommended[27].

Endoscopy for treatment of dominant strictures is the preferred mode of therapy and multiple observational studies have shown that endoscopic treatment of dominant strictures not only leads to clinical improvement but also can lead to biochemical and radiological improvements[27,29]. Endoscopic approaches to treatment of dominant strictures include ERCP with stent placement, balloon dilatation or both. The optimal endoscopic approach to treatment of dominant strictures in patients with PSC, however, is not known. There are no randomized controlled studies assessing effectiveness of balloon dilatation *vs* stenting in patients with PSC and dominant stricture. There are, however, a few retrospective studies available. In a retrospective study 34 patients were treated with only balloon dilatation, while 37 patients had balloon dilatation and stenting of dominant strictures[30]. During a median follow-up of 24 mo, there were 30 complications associated with balloon dilatation plus stenting compared to 6 complications in the group who had balloon dilatation only. Postprocedure cholangitis rates were significantly higher in patients who had stenting compared to those who had only balloon dilatation[30]. In another retrospective study 64 ERCP procedures were performed in 30 patients with PSC associated dominant stricture[31]. Thirteen of those ERCP procedures were performed with only balloon dilatation and 51 with stenting. Although the difference in the rate of complications between the groups who had dilatation versus dose who had stenting did not reach statistical significance, percentagewise there were twice as many complications with stenting compared to dilatation[31]. It is likely that statistical significance could not be reached due to low number of patients. Two other retrospective studies have compared balloon dilatation with stenting in patients with PSC related dominant strictures[32]. In a retrospective study involving 75 patients, the investigators reported that stenting was not associated with an increased risk of adverse events[32]. On the other hand, another retrospective study including 72 patients found that even short-term stenting was associated with higher likelihood of adverse events[33].

### *Recommendations for endoscopic treatment of dominant strictures in PSC*

Based on the available evidence ERCP with balloon dilatation is effective in most patients with PSC and dominant stricture. Routine stenting of dominant strictures in PSC patients is not recommended. Stenting of dominant strictures is appropriate if there is poor drainage of contrast after dilatation and concern for collapse of the bile duct comprising biliary drainage. If stenting is performed, short-term stenting should be considered. These recommendations are in line with the American College of Gastroenterology clinical guidelines[29].

## CONCLUSION

Preoperative biliary drainage in patients with resectable malignancies has been a controversial issue for many years. Based on current evidence preoperative biliary drainage is not routinely indicated. However, it is appropriate in patients who suffer from acute cholangitis, are severely symptomatic or in cases of delayed surgery. Another point of controversy has been whether unilateral or bilateral stenting would offer the best palliation for patients with unresectable hilar strictures. In such patients unilateral stenting during ERCP seems to provide adequate palliation in most cases while maintaining minimal risk.

Finally, the best management strategy for endoscopic treatment of dominant strictures in PSC patients remains unknown. Based on currently available evidence routine stenting of such strictures is not recommended. However, stenting of dominant strictures may become necessary if there is poor drainage of contrast after dilatation or concern for collapse of the bile duct compromising biliary drainage.

## REFERENCES


1 **van der Gaag NA**, Kloek JJ, de Castro SM, Busch OR, van Gulik TM, Gouma DJ. Preoperative biliary drainage in patients with obstructive jaundice: history and current status. *J Gastrointest Surg* 2009; **13**: 814-820 [PMID: 18726134 DOI: 10.1007/s11605-008-0618-4]

2 **Whipple AO**, Parsons WB, Mullins CR. Treatment of carcinoma of the ampulla of vater. *Ann Surg* 1935; **102**: 763-779 [PMID: 17856666]

3 **van der Gaag NA**, Rauws EA, van Eijck CH, Bruno MJ, van der Harst E, Kubben FJ, Gerritsen JJ, Greve JW, Gerhards MF, de Hingh IH, Klinkenbijl JH, Nio CY, de Castro SM, Busch OR, van Gulik TM, Bossuyt PM, Gouma DJ. Preoperative biliary drainage for cancer of the head of the pancreas. *N Engl J Med* 2010; **362**: 129-137 [PMID: 20071702 DOI: 10.1056/NEJMoa0903230]

4 **Dawwas MF**, James MW, Aithal GP. Preoperative drainage in pancreatic cancer. *N Engl J Med* 2010; **362**: 1342-1343; author reply 1345 [PMID: 20375415 DOI: 10.1056/NEJMc1001847]

5 **Wang CC**, Kao JH. Preoperative drainage in pancreatic cancer. *N Engl J Med* 2010; **362**: 1343; author reply 1345 [PMID: 20380027]

6 **Gorard DA**. Preoperative drainage in pancreatic cancer. *N Engl J Med* 2010; **362**: 1344-1345; author reply 1345 [PMID: 20380031]

7 **Baron TH**, Kozarek RA. Preoperative biliary stents in pancreatic cancer--proceed with caution. *N Engl J Med* 2010; **362**: 170-172 [PMID: 20071708 DOI: 10.1056/NEJMe0908773]

8 **Tol JA**, van Hooft JE, Timmer R, Kubben FJ, van der Harst E, de Hingh IH, Vleggaar FP, Molenaar IQ, Keulemans YC, Boerma D, Bruno MJ, Schoon EJ, van der Gaag NA, Besselink MG, Fockens P, van Gulik TM, Rauws EA, Busch OR, Gouma DJ. Metal or plastic stents for preoperative biliary drainage in resectable pancreatic cancer. *Gut* 2016; **65**: 1981-1987 [PMID: 26306760 DOI: 10.1136/gutjnl-2014-308762]

9 **Fang Y**, Gurusamy KS, Wang Q, Davidson BR, Lin H, Xie X, Wang C. Meta-analysis of randomized clinical trials on safety and efficacy of biliary drainage before surgery for obstructive jaundice. *Br J Surg* 2013; **100**: 1589-1596 [PMID: 24264780 DOI: 10.1002/bjs.9260]

10 **Dumonceau JM**, Tringali A, Blero D, Devière J, Laugiers R, Heresbach D, Costamagna G; European Society of Gastrointestinal Endoscopy. Biliary stenting: indications, choice of stents and results: European Society of Gastrointestinal Endoscopy (ESGE) clinical guideline. *Endoscopy* 2012; **44**: 277-298 [PMID: 22297801 DOI: 10.1055/s-0031-1291633]

11 **Hochwald SN**, Burke EC, Jarnagin WR, Fong Y, Blumgart LH. Association of preoperative biliary stenting with increased postoperative infectious complications in proximal cholangiocarcinoma. *Arch Surg* 1999; **134**: 261-266 [PMID: 10088565]

12 **Figueras J**, Llado L, Valls C, Serrano T, Ramos E, Fabregat J, Rafecas A, Torras J, Jaurrieta E. Changing strategies in diagnosis and management of hilar cholangiocarcinoma. *Liver Transpl* 2000; **6**: 786-794 [PMID: 11084070 DOI: 10.1053/jlts.2000.18507]

13 **Ferrero A**, Lo Tesoriere R, Viganò L, Caggiano L, Sgotto E, Capussotti L. Preoperative biliary drainage increases infectious complications after hepatectomy for proximal bile duct tumor obstruction. *World J Surg* 2009; **33**: 318-325 [PMID: 19020929 DOI: 10.1007/s00268-008-9830-3]

14 **Grandadam S**, Compagnon P, Arnaud A, Olivié D, Malledant Y, Meunier B, Launois B, Boudjema K. Role of preoperative optimization of the liver for resection in patients with hilar cholangiocarcinoma type III. *Ann Surg Oncol* 2010; **17**: 3155-3161 [PMID: 20593243 DOI: 10.1245/s10434-010-1168-z]

15 **Farges O**, Regimbeau JM, Fuks D, Le Treut YP, Cherqui D, Bachellier P, Mabrut JY, Adham M, Pruvot FR, Gigot JF. Multicentre European study of preoperative biliary drainage for hilar cholangiocarcinoma. *Br J Surg* 2013; **100**: 274-283 [PMID: 23124720 DOI: 10.1002/bjs.8950]

16 **Liu F**, Li Y, Wei Y, Li B. Preoperative biliary drainage before resection for hilar cholangiocarcinoma: whether or not? A systematic review. *Dig Dis Sci* 2011; **56**: 663-672 [PMID: 20635143 DOI: 10.1007/s10620-010-1338-7]

17 **Mansour JC**, Aloia TA, Crane CH, Heimbach JK, Nagino M, Vauthey JN. Hilar cholangiocarcinoma: expert consensus statement. *HPB* (Oxford) 2015; **17**: 691-699 [PMID: 26172136 DOI: 10.1111/hpb.12450]

18 **De Palma GD**, Galloro G, Siciliano S, Iovino P, Catanzano C. Unilateral versus bilateral endoscopic hepatic duct drainage in patients with malignant hilar biliary obstruction: results of a prospective, randomized, and controlled study. *Gastrointest Endosc* 2001; **53**: 547-553 [PMID: 11323577]

19 **Mukai T**, Yasuda I, Nakashima M, Doi S, Iwashita T, Iwata K, Kato T, Tomita E, Moriwaki H. Metallic stents are more efficacious than plastic stents in unresectable malignant hilar biliary strictures: a randomized controlled trial. *J Hepatobiliary Pancreat Sci* 2013; **20**: 214-222 [PMID: 22415652 DOI: 10.1007/s00534-012-0508-8]

20 **Chang WH**, Kortan P, Haber GB. Outcome in patients with bifurcation tumors who undergo unilateral versus bilateral hepatic duct drainage. *Gastrointest Endosc* 1998; **47**: 354-362 [PMID: 9609426]

21 **Freeman ML**, Overby C. Selective MRCP and CT-targeted drainage of malignant hilar biliary obstruction with self-expanding metallic stents. *Gastrointest Endosc* 2003; **58**: 41-49 [PMID: 12838219 DOI: 10.1067/mge.2003.292]

22 **Naitoh I**, Ohara H, Nakazawa T, Ando T, Hayashi K, Okumura F, Okayama Y, Sano H, Kitajima Y, Hirai M, Ban T, Miyabe K, Ueno K, Yamashita H, Joh T. Unilateral versus bilateral endoscopic metal stenting for malignant hilar biliary obstruction. *J Gastroenterol Hepatol* 2009; **24**: 552-557 [PMID: 19220678 DOI: 10.1111/j.1440-1746.2008.05750.x]

23 **Iwano H**, Ryozawa S, Ishigaki N, Taba K, Senyo M, Yoshida K, Sakaida I. Unilateral versus bilateral drainage using self-expandable metallic stent for unresectable hilar biliary obstruction. *Dig Endosc* 2011; **23**: 43-48 [PMID: 21198916 DOI: 10.1111/j.1443-1661.2010.01036.x]

24 **Liberato MJ**, Canena JM. Endoscopic stenting for hilar cholangiocarcinoma: efficacy of unilateral and bilateral placement of plastic and metal stents in a retrospective review of 480 patients. *BMC Gastroenterol* 2012; **12**: 103 [PMID: 22873816 DOI: 10.1186/1471-230X-12-103]

25 **Vienne A**, Hobeika E, Gouya H, Lapidus N, Fritsch J, Choury AD, Chryssostalis A, Gaudric M, Pelletier G, Buffet C, Chaussade S, Prat F. Prediction of drainage effectiveness during endoscopic stenting of malignant hilar strictures: the role of liver volume assessment. *Gastrointest Endosc* 2010; **72**: 728-735 [PMID: 20883850 DOI: 10.1016/j.gie.2010.06.040]

26 **Sawas T**, Al Halabi S, Parsi MA, Vargo JJ. Self-expandable metal stents versus plastic stents for malignant biliary obstruction: a meta-analysis. *Gastrointest Endosc* 2015; **82**: 256-267.e7 [PMID: 25982849 DOI: 10.1016/j.gie.2015.03.1980]

27 **Chapman R**, Fevery J, Kalloo A, Nagorney DM, Boberg KM, Shneider B, Gores GJ. Diagnosis and management of primary sclerosing cholangitis. *Hepatology* 2010; **51**: 660-678 [PMID: 20101749 DOI: 10.1002/hep.23294]

28 **Tischendorf JJ**, Hecker H, Krüger M, Manns MP, Meier PN. Characterization, outcome, and prognosis in 273 patients with primary sclerosing cholangitis: A single center study. *Am J Gastroenterol* 2007; **102**: 107-114 [PMID: 17037993 DOI: 10.1111/j.1572-0241.2006.00872.x]

29 **Lindor KD**, Kowdley KV, Harrison ME. ACG Clinical Guideline: Primary Sclerosing Cholangitis. *Am J Gastroenterol* 2015; **110**: 646-659; quiz 660 [PMID: 25869391 DOI: 10.1038/ajg.2015.112]

30 **Kaya M**, Petersen BT, Angulo P, Baron TH, Andrews JC, Gostout CJ, Lindor KD. Balloon dilation compared to stenting of dominant strictures in primary sclerosing cholangitis. *Am J Gastroenterol* 2001; **96**: 1059-1066 [PMID: 11316147 DOI: 10.1111/j.1572-0241.2001.03690.x]

31 **Etzel JP**, Eng SC, Ko CW, Lee SD, Saunders MD, Tung BY, Kimmey MB, Kowdley KV. Complications after ERCP in patients with primary sclerosing cholangitis. *Gastrointest Endosc* 2008; **67**: 643-648 [PMID: 18061595 DOI: 10.1016/j.gie.2007.07.042]

32 **Alkhatib AA**, Hilden K, Adler DG. Comorbidities, sphincterotomy,

and balloon dilation predict post-ERCP adverse events in PSC patients: operator experience is protective. *Dig Dis Sci* 2011; **56**: 3685-3688 [PMID: 21789539 DOI: 10.1007/s10620-011-1830-8]

33 **Navaneethan U**, Jegadeesan R, Hammel J, Parsi MA. Endoscopic Treatment of Dominant Biliary Strictures in Primary Sclerosing Cholangitis Patients: Comparison of Adverse Events Associated With Balloon Dilation With and Without Stenting. *Gastrointest Endosc* 2015; **81**: AB364

**P- Reviewer**: Wan XJ **S- Editor**: Gong ZM
**L- Editor**: A **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/ *World J Gastroenterol* 2017 February 21; 23(7): 1125-1138

DOI: 10.3748/wjg.v23.i7.1125 ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

# Therapeutic mechanism of Yīn-Chén-Hāo decoction in hepatic diseases

Jian-Yuan Li, Hong-Yan Cao, Lin Sun, Run-Fei Sun, Chao Wu, Yan-Qin Bian, Shu Dong, Ping Liu, Ming-Yu Sun

Jian-Yuan Li, Hong-Yan Cao, Run-Fei Sun, Chao Wu, Yan-Qin Bian, Shu Dong, Ping Liu, Ming-Yu Sun, Key Laboratory of Liver and Kidney Diseases, Institute of Liver Diseases, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

Jian-Yuan Li, Hong-Yan Cao, Run-Fei Sun, Chao Wu, Yan-Qin Bian, Shu Dong, Ping Liu, Ming-Yu Sun, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

Jian-Yuan Li, Department of Traditional Chinese Medicine, Huzhou Central Hospital, Huzhou 313000, Zhejiang Province, China

Lin Sun, Liaoning University of Traditional Chinese Medicine, Shenyang 110033, Liaoning Province, China

Author contributions: Li JY and Sun MY wrote the manuscript and were involved with project concept and submission; Cao HY, Sun L, Sun RF, Wu C, Bian YQ and Dong S performed data collection; Liu P and Sun MY revised the manuscript; all authors contributed to this manuscript.

Supported by the National Natural Science Foundation, No. 81273729; Major Project of Shanghai Municipal S and T Commission, No. 15DZ1900104; Innovative Research Team in Universities, Shanghai Municipal Education Commission, Shanghai Key Laboratory of Traditional Chinese Clinical Medicine and Key Disciplines of Liver and Gall Bladder Diseases of State Administration of Traditional Chinese Medicine of the People's Republic of China.

Conflict-of-interest statement: Authors declare no conflicts of interest.



Manuscript source: Unsolicited manuscript

Correspondence to: Dr. Ming-Yu Sun, Professor, Key Laboratory of Liver and Kidney Diseases, Institute of Liver Diseases, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China. mysun248@hotmail.com
Telephone: +86-21-20256520

Received: October 15, 2016
Peer-review started: October 19, 2016
First decision: December 2, 2016
Revised: December 16, 2016
Accepted: January 4, 2017
Article in press: January 4, 2017
Published online: February 21, 2017

## Abstract

Yīn-Chén-Hāo decoction (YCHD) is a traditional Chinese medicine formula composed of capillaris (*Artemisia capillaris*), gardenia (*Gardenia jasminoides*), and rhubarb (*Rheum rhabarbarum*) that is used for the treatment of damp-heat jaundice. In modern clinics, YCHD is mostly used for hepatic diseases. This review summarizes the biological activities of YCHD and its medical applications. The main active compounds of YCHD are chlorogenic acid, rhein, geniposide, emodin, and scoparone. The pharmacological actions of YCHD include inhibition of hepatic steatosis, apoptosis, necrosis, anti-inflammation, and immune regulation. YCHD could be developed as a new therapeutic strategy for the treatment of hepatic diseases.

**Key words:** Yīn-Chén-Hāo decoction; Hepatic disease; Clinical application; Effector mechanism; Review



**Core tip:** Yīn-Chén-Hāo decoction (YCHD) was a classical prescription for more than 1800 years in China. This review summarizes the efficacy of YCHD in

liver disease from clinical trials and its mechanisms of action *in vitro* and *in vivo*. Studies indicate that YCHD can modulate various molecular pathways in liver disease. YCHD is widely used in clinical settings for the treatment of liver diseases, and could be a safe and novel therapeutic drug for liver injury worldwide.

Li JY, Cao HY, Sun L, Sun RF, Wu C, Bian YQ, Dong S, Liu P, Sun MY. Therapeutic mechanism of Yīn-Chén-Hāo decoction in hepatic diseases. *World J Gastroenterol* 2017; 23(7): 1125-1138 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1125.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1125

## INTRODUCTION

Yīn-Chén-Hāo decoction (YCHD) was first described in the "*Treatise on Febrile Diseases*" by Zhong-Jing Zhang during the Eastern Han Dynasty (AD 25-220). It a classic prescription in traditional Chinese medicine (TCM) that is mainly used for internal stasis heat and jaundice arising from Yang Ming disease and malnutrition, with doctors also widely using this prescription for heat jaundice. Modern usage of YCHD includes treatment for acute icteric infectious hepatitis along with other herbal medicines. YCHD is also used for hepatic diseases and has some uses in internal medicine, surgery, and pediatrics (Table 1). This paper discusses studies on YCHD and its mechanisms in the treatment of hepatic diseases.

Classic Chinese formulae include four elements: the monarch drug, the minister drug, the assistant drug, and the servant drug; the monarch drug plays the most important role in the formula, the assistant drug increases the effectiveness of the monarch drug, the minister drug helps the monarch and minister drugs reach their target positions, and the servant drug can reduce the adverse effects or increase the potency of the entire formula[1].

YCHD (Figure 1) is comprised of 12 g capillaris (*Artemisia capillaris*), 9 g gardenia (*Gardenia jasminoides*), and 9 g rhubarb (*Rheum rhabarbarum*). Capillaris is the monarch drug, and can clear heat and dampness and remove jaundice. Gardenia is the minister drug, and can clear heat-fire and damp-heat in the triple burner, and remove pathogenic factors from the urine. Rhubarb is both the assistant and servant drug, and acts to purge heat from the bowels, cool the blood, detoxify, dispel stasis, and dredge the meridians. The three drugs together prevent stasis, promote bowel movement, and guide stagnant heat to be excreted alongside the stool. YCHD is usually used twice daily. In an animal study with rats, total bilirubin (TBIL), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were improved and the blood level of scoparone was higher for longer when YCHD was administered once *vs* twice or three times daily. The exact mechanism of this effect has yet to be determined[2].

## CHEMICAL COMPOSITION OF YCHD

YCHD has broad prospects for application in the field of liver disease, particularly in the treatment of hepatitis with jaundice; for example, it promotes bilirubin metabolism, can prevent liver damage, and inhibits hepatic apoptosis, hepatic stellate cell (HSC) activation, and collagen synthesis[3]. Studies on its chemical composition have determined some effective compounds found in YCHD. Capillaris mainly contains five classes[4] of active compounds: coumarins (*e.g.,* scoparone); flavonoids (*e.g.,* arcapillin, 5,3′,4′,hydroxy-6,7-dimethoxycoumarin, cirsimaritin, 3′-methoxy thistle flavin); chromones (*e.g.,* capillarisn, 7-a methyl wormwood color ketone, 4′-a methyl wormwood color ketone, 1-methoxy 4′ methyl 6 gall color ketone); organic acids (*e.g.,* chlorogenic acid, coumaric acid A and B); and alkaloids (*e.g.,* alkynes, monoterpenes, sesquiterpenes, water-soluble polypeptides). Rhubarb mainly contains five classes of active compounds: anthraquinones (including two types of free anthraquinones like rhein, emodin, aloe emodin, chrysophanol, and physcion; and bound anthraquinones like anthraquinone glycoside and dianthrone glucosides); tannins (*e.g.,* gallic acid, D-catechin); stilbenes (*e.g.,* rhaponticin, piceatannol, resveratrol, 3′,4′,5-hydroxyl stilbene, rhaponticin-2″-O-gallate, rhaponticin-6″-O-gallate); volatile oils (*e.g.,* palmitic acid, hexacosanoic acid, palmitic ethyl ester, dibutyl phthalate); and rhubarb polysaccharides. Gardenia mainly contains five classes of active compounds: gardenosides (*e.g.,* geniposide, hydroxyl gardenoside, caryptoside, gardoside, scandoside methyl ester, geniposidic acid); pigments (*e.g.,* crocin, crocetin); organic acids (*e.g.,* chlorogenic acid, bitter saffron acid, alicyclic acid, 3-oxygen α coffee α mustard acyl α 4-oxygen quinic acid); volatile oils (*e.g.,* linoleic acid, palmitic acid); and gardenia polysaccharides.

One study identified 45 compounds from YCHD, with 21 found in rat blood after oral administration. After studying the influence of different herbs alone compared with the whole YCHD decoction, investigators found eight compounds that are selectively absorbed into the bloodstream only after administration of all herbs from the YCHD decoction. Each compound had a significant effect on protecting the liver and gallbladder[5] (Figure 2).

The biological effects of many ingredients in YCHD reflect the therapy of the entire formula (Table 2). For example, rhein may relieve cellular insult through its anti-inflammatory activity in combination with nitric oxide (NO) from L-arginine[6]. Rhein can improve liver function and remove hepatic fibrosis *via* anti-inflammatory and antioxidant pathways, and inhibit TGF-α activity and HSC activation[7]. Chlorogenic acid, another critical active ingredient of YCHD, can counteract liver injury at various levels by preventing apoptosis and oxidative stress damage. More specifically, both the glutathione and thioredoxin antioxidant systems and the mitogen-activated protein kinase (MAPK) signaling cascade appear to be engaged in the protective


**Figure 1 Composition of Yīn-Chén-Hāo decoction.**

mechanism of chlorogenic acid[8]. Geniposidic acid, the critical active ingredient of gardenia, alleviates liver injury by enhancing the antioxidative defense system and slowing the apoptotic signaling pathways[9].

One study used high-performance liquid chromatography-UV to test scoparone in rat plasma after intragastric administration of YCHD or water decoctions of capillaris, gardenia, and rhubarb separately. The study found that the scoparone level after YCHD administration was significantly higher than that of water decoctions alone. Moreover, the therapeutic effect of YCHD was found to be better than that of the herbs alone[10]. Another study used scoparone, geniposide, or rhein alone or in combination, and investigated their immunohistochemistry, biochemistry, metabolomics, and proteomics. The results showed that the scoparone, geniposide, and rhein combination exerts a more robust therapeutic effect than any one or two of the three individual compounds in a rat model of hepatic injury. Furthermore, scoparone, geniposide, and rhein synergistically cause intensified dynamic changes in metabolic biomarkers, regulate molecular networks through target proteins, have a synergistic effect, and activate both intrinsic and extrinsic pathways[11].

## MECHANISMS OF YCHD EFFECTS

### *Liver injury repair*

The liver is a major location for the metabolism and elimination of drugs, as well as being involved in their absorption. Therefore, the liver greatly influences the pharmacokinetics of compounds. Studies demonstrate that liver injury significantly influences the pharmacokinetics of scoparone, not only *via* changes in intestinal absorption, but also *via* changes in hepatic metabolism. In one study, the absorption and distribution of scoparone was accelerated in liver-injured rats at the cost of slowed metabolism and elimination. Changes in parameters like metabolism can explain some molecular mechanisms of YCHD in the treatment of liver injury[12].

Research into different drug-induced liver injury forms have been conducted with YCHD. One study explored liver injury induced by α-naphthyl isothiocyanate (ANIT) and carbon tetrachloride ($CCl_4$). ANIT intoxication groups were given ANIT (in corn oil at a ratio of 10:1) at a single dose of 80 mg/kg orally, while $CCl_4$ intoxication groups were given $CCl_4$ (in corn oil at a ratio of 1:4) at a single dose of 1 mL/kg orally. In the ANIT group, the levels of ALT, alkaline phosphatase (AKP), and TBIL were significantly higher than those in the control group. In the $CCl_4$ group, the malondialdehyde content was significantly higher and superoxide dismutase and glutathione peroxidase activities were significantly lower compared with those in the control group. YCHD significantly reduced AST, ALT, and AKP levels in the ANIT group and significantly reduced AST, ALT, AKP, and malondialdehyde levels in the $CCl_4$ group. In addition, YCHD was also found to increase the ratio of liver weight to body weight[13].

Scoparone is an important compound found in capillaris that has been shown to be hepatoprotective, effective in the treatment of liver diseases, and contribute directly to the therapeutic effect of YCHD[14]. The metabonomic characteristics of $CCl_4$-induced hepatotoxicity and intervention with scoparone illustrate that scoparone could have hepatoprotective effects *via* multiple pathways, including primary bile acid biosynthesis and pyrimidine metabolic pathways[15]. The hepatoprotective effects of scoparone in rat liver injury were associated with regulated expression of six proteins that appear to be involved in antioxidation and signal transduction, energy production, immunity, metabolism, and chaperoning[16].

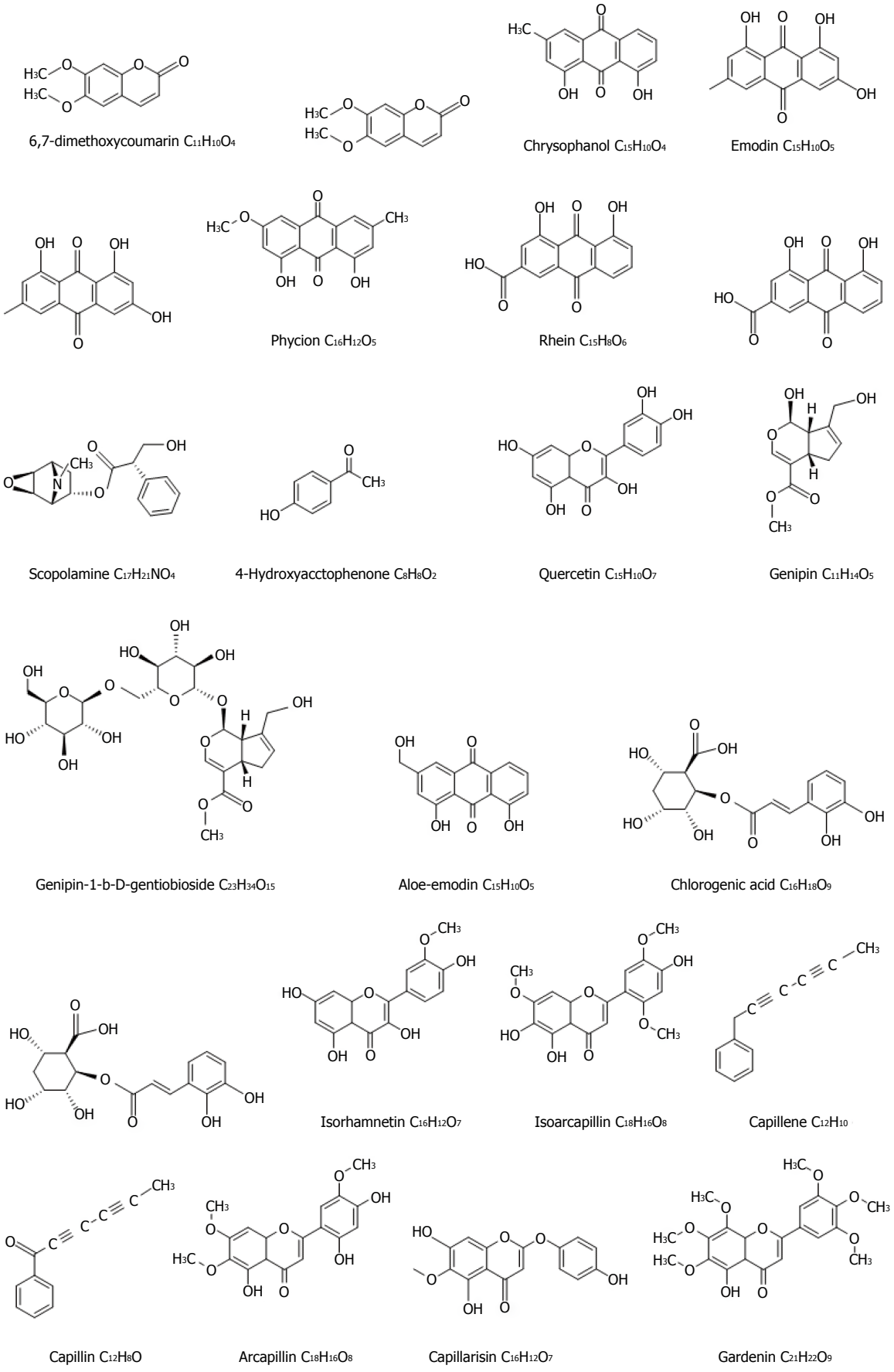



**Figure 2 Molecular structures of compounds found in Yīn-Chén-Hāo decoction.**

**Table 1 Clinical trials using Yīn-Chén-Hāo decoction**

| Herb or compound | Dose and course of treatment | Combined medication | Case/control | Disease type | Symptoms | Efficacy | Positive control drug | Side effects | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| YCHD | From 28th wk of pregnancy, decocted with water, daily, 4 wk | From 36th wk of pregnancy, both groups, + administration of 30 mg phenobarbital, twice a day, orally | 81/76 | Maternal-fetal ABO blood group incompatibility | IgG anti-A/B antibody titer, growth in fetus, neonatal jaundice | Reduced antibody titer, reduced jaundice incidence rate, reduced TBIL | Vitamin C 0.5 g + 100 IU coenzyme A + 40 mg adenosine triphosphate, IVGTT | None | [61] |
| YCHD | Modified YCHD, decocted with water daily; orally, twice a day | 1000 mg transmetil injection, IVGTT | 38/30 | Hyperbilirubinemia induced by viral hepatitis | Clinical symptoms, liver function, bilirubin | Symptoms, bilirubin levels significantly improved due to liver function | Vitamin C, vitamin B6, diammonium | None | [62] |
| Modified YCHD | Decocted with water, daily, 10 d | Routine Western medical treatment | 34/34 | Pathological neonatal jaundice | Time of jaundice disappearance, jaundice degree, TBIL | Time jaundice disappearance is shut down, liver TBIL significantly decreased | Routine Western medicine therapy, etiological treatment, light therapy, phenobarbital, albumin for patients with high bilirubin, gamma globulin for patients with hemolytic disease | None | [63] |
| Modified YCHD | Decocted with water, daily, 14 d | Plasma exchange | 20/20 | Hepatitis B combined with hyperbilirubinemia | Liver function, prothrombin activity | TBIL significantly decreased effective rate of treatment group compared with significantly increased control group | Plasma exchange | None | [64] |
| Modified YCHD | Decocted with water, daily, orally, 28 d | Mask with the function of removing heat and eliminating stasis | 31/29 | Gastrointestinal damp-heat acne | Acne coalescence condition | Increased total effective rate of acne coalescence, average efficacy index statistically significantly decreased | Danshentong capsules for oral use, vitamin E emulsifiable paste for external use | None | [65] |
| Modified YCHD | Decocted with water, daily, orally, 7 d | None | 32/34 | Juvenile bronchial asthma | Asthma symptoms | Curative effect of treatment group significantly better than control group; fade time of cough, sputum, and wheezing in treatment group significantly shorter than control group ($P < 0.05$) | Normal saline, dexamethasone injection, ambroxol hydrochloride injection, aerosol inhalation, twice a day, 3-7 days, additional antibiotics for infected individual | None | [66] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Modified YCHD | Decocted with water, daily, orally | EST + endoscopic stone extraction + ENBD | 24/24 | Choledocholithiasis | TBIL, DBIL, AKP, GGT, ALT, daily bile reflux | Observed indicators improved significantly, daily bile reflux significantly higher than control group ($P < 0.05$) | Anti-infection, hepatoprotective, prevents fluid and electrolyte imbalance, symptomatic supportive care | None | [67] |
| Yin-Zhi-Huang injection | 10 mL + 20 mL glucose solution, IVGTT, daily, 15 wk | Kadai surgery, antibiotic treatment, hormone treatment, +vitamin K1, +ursodeoxycholic acid | 18/14 | Biliary atresia | TBIL, DBIL, ALT, AST | Observation target significantly improved ($P < 0.05$) | None | None | [68] |
| Yin-Zhi-Huang granule | 1.0 g, 3 times a day, orally, 7 d | Blue light irradiation, 8 h daily + 105 mg bifid-triple viable capsule, twice a day orally, 7 d | 150/133/141 | Neonatal jaundice | Time of jaundice disappearance, transcutaneous bilirubin | Time of jaundice disappearance significantly reduced, transcutaneous bilirubin level significantly reduced ($P < 0.05$) | Blue light irradiation | None | [69] |
| Yin-Zhi-Huang granule | 3.3 mL, 3 times a day, orally, 5 d | None | 120/120/120 | Pathological neonatal jaundice | Bilirubin concentration, time of jaundice disappearance | Plasma bilirubin level significantly reduced, total effective rate significantly increased | Tuihuang mixture | None | [70] |
| Yin-Zhi-Huang injection | 5 mL + 10% glucose solution 50 mL, IVGTT, daily, 10 d | ATP, coenzyme A, inosine, vitamin C, glucuronolactone | 46/12 | Infantile hepatitis syndrome | Treatment results (cured, improved, invalid), serum TBIL, ALT value, size of liver and spleen | Cure rate, total effective rate significantly increased, plasma bilirubin level significantly reduced, obvious change to liver and spleen size | ATP, coenzyme A, inosine, vitamin C, glucuronolactone | None | [71] |
| Modified compound Yin-Chén recipe | Twice a day | None | 215/120 | Maternal-fetal ABO blood group incompatibility-induced neonatal jaundice | Antibody titer, neonatal jaundice incidence, jaundice degree | Antibody titer and neonatal jaundice incidence much lower post-treatment, TCM treatment before delivery failed to reduce jaundice degree | None | None | [72] |
| ICKT | 7.5 g, daily, orally | None | 1 | Acute cholestatic hepatitis | Clinical course of patient | TBIL level began to decrease, liver biopsy showed chronic active hepatitis with mild fibrosis | Predonine | None | [73] |
| ICKT | 7.5 g, daily, orally | None | 50/50 | Persistent hyperbilirubinemia as a symptom of post-operative liver failure after hepatectomy | Choleretic effect | ICKT group showed significant decrease compared with control group in indirect bilirubin levels | None | None | [74] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Modified compound Yīn-Chén recipe | Modified compound Yīn-Chén recipe decoction, orally | Routine Western medical treatment | 30/30 | Chronic severe hepatitis | Liver function indices, complication incidences, score of TCM syndromes, clinical effects | Treatment group better than control group ($P$ < 0.05) | Basic Western medical treatment | None | [75] |
| Modified YCHD | Retention enema, daily | Routine comprehensive treatment | 30/30 | Chronic severe hepatitis | Liver function indices, endotoxin, blood ammonia | Treatment group better than control group ($P$ < 0.05) in improvement of clinical symptoms, endotoxin, blood ammonia, ALT, AST, and TBIL | Lactulose medicated by retention enema | None | [76] |
| Modified Artemisiae Scopariae decoction | Twice a day | Ursodeoxycholic acid | 30/30 | Primary biliary cirrhosis | Clinical effect, biochemistry indicators, immunoglobulin levels | Treatment group better than control group ($P$ < 0.05) in clinical effect and biochemistry indicators, insignificantly decreased immunoglobulin levels ($P$ > 0. 05) | Ursodeoxycholic acid | None | [77] |
| Modified YCHD | Twice a day | Conventional treatment, phenobarbital (blue light-struck) | 30/29 | Neonatal hyperbilirubinemia | Clinical effect | Treatment group better than control group ($P$ < 0.05) in clinical effect | Conventional treatment (phenobarbital, blue light-struck) | None | [78] |
| Lidan Xiaohuang decoction | 50-100 mL 2-3 times a day, orally | Routine Western medical treatment | 35/31 | Obstructive jaundice, post-operative persistent jaundice | Bilirubin | Treatment group better than control group ($P$ < 0.01) | Acupuncture therapy | None | [79] |
| Qing-Re-Li-Shi formula | Twice a day, 90 d | None | 60 | Chronic hepatitis B | Liver function comparison, hepatitis B virus marker comparison | Contents of ALT, AST, AKP, G, TBIL, DBIL improved ($P$ < 0.01); negative conversion rates of 16.7% HbsAg, 21.2% HbeAg, and 35.0% HBV | None | None | [80] |
| Modified compound Yīn-Chén recipe | Twice a day | Triple anti-Hp therapy | 30/30 | Hp-positive rosacea | Clinical effective rate, Hp- positive rate, serum IL-8, TNF-α | Clinical effective rate in treatment group better than control group ($P$ < 0.05); Hp- positive rate, serum IL-8, and TNF-α in treatment group significantly lower than control group ($P$ < 0.01) | Triple anti-Hp therapy | None | [81] |
| Modified compound Yīn-Chén recipe | Twice a day | Routine Western medical treatment | 120/90 | Viral hepatitis jaundice | Jaundice disappearance, liver function improvement | Clinical effective rate and liver function in treatment group better than control group ($P$ < 0.05) | Routine Western medical treatment | None | [82] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Obstructive jaundice | Twice a day | Obstructive jaundice | 28/26 | Obstructive jaundice | TBIL, DBIL | More obvious decline of TBIL and DBIL in experimental group ($P$ < 0.05) | Obstructive jaundice | None | [83] |
| Supplement YCHD | Twice a day | Routine Western medical treatment for protective liver, plasma exchange | 30/30 | Severe chronic hepatitis B | Liver function, gross effective rate, mortality rate | YCHD significantly improved liver function and gross effective rate, mortality rate lower than control group ($P$ < 0.05) | Routine Western medicinal treatment for protective liver, plasma exchange | None | [84] |
| YCHD | Twice a day | Routine Western medical treatment for protective liver | 30/30 | Intrahepatic cholestasis of pregnancy | Pruritus score, serum bile acid level, Apgar score, body weight | Pruritus score and serum bile acid level lower than control group ($P$ < 0.05, $P$ < 0.01, respectively); Apgar score and body weight better than control group ($P$ < 0.01) | Routine Western medical treatment for protective liver | None | [85] |

YCHD: Yīn-Chén-Hāo decoction; TBIL: Total bilirubin; IVGTT: Intravenous glucose tolerance test; EST: Endoscopic sphincterotomy; ENBD: Endoscopic nasobiliary drainage; DBIL: Direct bilirubin; AKP: Alkaline phosphatase; GTT: Gamma-glutamyl transferase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ATP: Adenosine triphosphate; TCM: Traditional Chinese medicine; ICKT: In-Chin-Ko-To; HBV: Hepatitis B virus; Hp: Haptoglobin.

Network reconstruction techniques were used to study the treatment of alcoholic hepatitis with scoparone. The metabolites after scoparone treatment in rats with alcoholic hepatitis were observed. The results showed that scoparone could regulate dysfunctions in the citrate cycle, sphingolipid metabolism, and taurine and hypotaurine pathways[17]. In addition, scoparone was distributed and eliminated rapidly in rats. Tissue distribution was highest in the liver, followed by the kidney and spleen. There were lower concentrations observed in the muscle, thyroid, and adrenal gland. Scoparone was not detected in the brain, which indicates that it does not cross the blood-brain barrier after oral administration[18].

YCHD combined with Da-Cheng-Qi decoction can reduce Bax and caspase-3 protein expression, increase Bcl-2 protein expression, regulate the balance of Bcl-2/Bax, and treat endotoxin-induced liver cell apoptosis[19].

### *Reverse steatosis*

Recent studies have reported the efficacy of YCHD in reducing hepatic fat accumulation. Enhanced adiponectin and endothelial progenitor cells and upregulation of PPAR-γ might be responsible for the therapeutic effect of YCHD in the treatment of non-alcoholic fatty liver disease (NAFLD). In addition, the antioxidative effect of YCHD might be associated with the inhibition of hepatic-free fatty acid (FFA) concentrations and the elevation of glutathione levels in hepatic tissues. Furthermore, YCHD might promote senescence marker protein-30 metabolism, which increases resistance to hepatic oxidative stress[20].

In steatohepatitis experiments with rats, those treated with YCHD had lower serum ALT activity, tumor necrosis factor (TNF-α) levels, hepatic triglycerides, and FFA levels. Moreover, there was significantly less fat deposition in hepatocytes than in the steatohepatitis rats. Therefore, YCHD has good therapeutic effects on non-alcoholic steatohepatitis, can protect liver function, and can reduce fatty deposition in the liver. These effects are possibly related to reductions in FFA content and inhibition of TNF-α expression[21].

YCHD contains scoparone, geniposide, and rhein, with the three causing dynamic changes in metabolic biomarkers, regulating molecular networks through target proteins, having synergistic effects, and activating both intrinsic and extrinsic pathways to exert a robust therapeutic effect in a rat model of hepatic injury[11]. A meta-analysis was conducted on the role of YCHD in NAFLD. The results showed that YCHD can significantly regulate ALT, AST, gamma-glutamyl transpeptidase (GGT), triglycerides, total cholesterol, low-density lipoprotein cholesterol, and syndrome score. Total effective rate, syndrome effective rate, and B-type ultrasonography effective rate were significantly better in the test group than those in the control group. Therefore, YCHD has a satisfactory therapeutic effect on NAFLD[22].

**Table 2 Mechanisms of action of compounds found in Yīn-Chén-Hāo decoction**

| Compound | Pharmacological activities | Mechanisms of action | Ref. |
|---|---|---|---|
| Chlorogenic acid | Antibacterial, antiviral, antioxidation, free radical scavenging, mutation suppression, anti-tumor | Reversed liver reduced GSH levels and expression of TRX, triggering GSH and TRX antioxidant systems and MAPK signaling cascade | [8] |
| Scoparone | Anti-inflammatory, analgesia, antioxidant, immunosuppressive, cholagogue, blood pressure, hypolipidemic, anti-asthmatic | Inhibition of protein tyrosine kinase and release of arachidonic acid metabolites, reduced expression of tissue factor at mRNA level and thrombus generation; enhancement of prostacyclin release, protection against endothelium derived relaxing factor inactivation, and activating guanylate cyclase, relaxed bronchial smooth muscle | [86-89] |
| Geniposide | Anti-inflammatory, analgesia, cholagogue, laxness, soft tissue injury repair, gastric acid secretion inhibition, amylopsin secretion reduction | Enhancing antioxidative defense system and reducing apoptotic signaling pathways; regulating adipocytokine release and the expression of PPARα expression | [9,90] |
| Rhein | Antineoplastic, anti-inflammatory, antimicrobial, immunosuppressive, diuresis and purgation, improve glycometabolic | Inhibiting cytochrome P450 enzymes in liver microsomes | [91] |
| Emodin | Antineoplastic, anti-inflammatory, antimicrobial, immunosuppressive, diuresis and purgation | Inhibiting HSC activation | [92] |

GSH: Glutathione; TRX: Thioredoxin; MAPK: Mitogen-activated protein kinase; PPARα: Peroxisome proliferator activated receptor-α; HSC: Hepatic stellate cell.

### *Anti-inflammatory and anti-viral effects*

Hepatitis B is a major global health problem caused by the hepatitis B virus (HBV). Many clinical studies have reported that YCHD can reduce serum transaminase activity, elevate serum albumin, reduce the ratio of albumin and globulin (A/G), improve liver function, and provide satisfactory long-term effects[23,24]. These effects could help in the treatment of hepatitis B.

The pathogenic process of HBV is immune-mediated inflammation. Cytotoxic T lymphocytes (CTL) recognize and destroy target cells by antigen presentation, and cause target-cell apoptosis when fas ligand protein on the membrane surface unites with target-cell Fas antigen. HBV infection activates the immune system to produce and release cytokines, which promote liver inflammation and cause liver damage.

One study found that, in the treatment of chronic hepatitis B, YCHD combined with Western medicine was significantly better than Western medicine treatment alone[25]. In an experiment with concanavalin A-induced acute immune liver injury in mice infected with hepatitis B, YCHD was found to reduce AST, interferon-γ, and Fas gene levels, and increase the level of interleukin (IL)-4 in the liver. YCHD was also able to repair damage to liver cells, decrease the frequency of liver cell apoptosis, and reduce inflammatory response[26].

Drug-induced hepatitis refers to liver damage caused by drugs or their metabolites. In recent years, increased usage of new drugs and drug combinations has increased the incidence of drug-induced hepatitis. YCHD plus glycyrrhizinate or transmetil was found to be more effective than Western medicine in the treatment of drug-induced hepatitis. A control group was treated with glycyrrhizinate capsules and transmetil, while a treatment group received the same drugs plus modified YCHD. The levels of ALT, TBIL, and globulin in the treatment group were lower than those in control group after treatment ($P < 0.05$). In addition, the level of albumin in the treatment group was higher than that in the control group[27].

Chronic severe hepatitis is another common type of hepatitis. The treatment of chronic severe hepatitis is difficult, and usually results in such complications as ascites, hepatic encephalopathy, gastrointestinal bleeding, and hepatorenal syndrome. Clinical research indicates that YCHD can reduce liver cell inflammation, expand intrahepatic bile capillaries, promote bile secretion, increase bile flow, improve hepatic microcirculation, reduce the absorption of intestinal toxins, and increase the excretion of bilirubin. These effects can reduce symptoms and significantly improve liver function in patients with chronic severe hepatitis[28]. Liver dialysis combined with YCHD is an especially effective therapeutic regimen for severe hepatitis[29].

### *Anti-fibrotic effects*

Fibrosis is a wound-healing response that engages a range of cell types and mediators to encapsulate an injury. During fibrogenesis, pathological factors including inflammation from Kupffer cells (KCs), angiogenesis, and HSC activation lead to collagen deposition[30]. Cirrhosis, an advanced liver disease, is characterized by fibrosis, nodule formation, and inflammation[31]. Despite the high incidence of hepatic fibrosis worldwide, no generally accepted antifibrogenic therapy is available. However, TCM has been widely used for treating chronic liver hepatitis and liver cirrhosis, with said treatments appearing to improve clinical symptoms, liver function, and patient quality of life[32].

YCHD can significantly inhibit apoptosis in liver cells, inhibit HSC activation, and inhibit KC activation, thereby protecting liver function and preventing

fibrosis. In a rat model of dimethylnitrosamine (DMN)-induced liver fibrosis, YCHD significantly improved liver function, liver pathology, and reduced collagen content in liver tissue[33]. In addition, a DMN-induced liver fibrosis study in rats found that YCHD could decrease abnormal ALT, AST, TBIL, GGT, hydroxyproline, hyaluronic acid, laminin, collagen type IV, and amino-terminal type III procollagen peptide levels. The study also found that YCHD treatment could improve mRNA and protein levels of α-SMA (a marker of activated HSCs), restore normal albumin levels, and change amino acid metabolism. The molecular mechanism of the anti-fibrotic effects of YCHD might operate *via* suppression of HSC activation[34,35]. In addition, the therapeutic effect of YCHD is better than that of other classic TCM formulae, such as Yīn-Chén-Si-Ni-Tang[36] and Gan-Lu-Xiao-Du-Dan[37] in the treatment of dampness-heat with liver fibrosis.

KCs are liver macrophages generally believed to be involved in liver damage *via* inflammation. There are two ways to activate KCs: the classical pathway and the alternative pathway[38,39]. KC activation *via* the classical pathway releases inflammatory cytokines, which further activate HSCs and lead to a phenotypic transition to myofibroblasts. This transition results in excessive proliferation, as well as the synthesis and secretion of ECM components, which leads to fibrosis. The inhibition of HSC activation can reduce the biosynthesis of collagen, and therefore inhibit fibrosis.

YCHD administration attenuates liver fibrosis partially by inhibiting HSC activation, rather than promoting cell apoptosis of activated HSCs or suppressing the activation of KCs[40]. YCHD reduced the expression of IL-1β, CD68, Tnfrsf14, Tnfrsf9, COL1α2, MMP2, MMP23, TNF-α, and Prkcb, and increased the expression of CD14, Tf, and Igf1. These gene expression changes indicate that YCHD can inhibit liver inflammation, HSC activation, liver sinusoidal endothelial cell activation, and liver parenchymal damage *via* inhibition and regulation of the classical KC activation pathway. These effects in combination result in anti-fibrotic effects from YCHD[41].

YCHD can also reduce the expression of TNF, FAS, and Prkcb, and regulate the expression of CD14 genes, indicating that it can block the MAPK pathway, which inhibits hepatocyte apoptosis to prevent fibrosis[42].

One study found YCHD plus Huang-Qi decoction reversed liver cirrhosis in rats *via* a reduction in oxidative stress. YCHD was found to eliminate hepatic lipid peroxide formation and Huang-Qi decoction enhanced the antioxidative ability of the liver[43].

The pathogenesis of DMN-induced liver cirrhosis corresponds to the syndrome of interior dampness-heat with qi deficiency[44], and the pathogenesis of liver cirrhosis corresponds to the syndrome of interior dampness-heat[35].

### *Relief of ascites*

Cirrhosis with ascites often occurs during chronic hepatitis, with ascites often being observed in end-stage cirrhosis. The clinical manifestations of ascites are tympanites and edema. One clinical study found that YCHD combined with Jijiaolihuang bolus could clear heat and expel dampness, induce diuresis to remove edema, and reduce ascites[45].

### *Effects in other liver diseases*

Since YCHD can clear heat, promote diuresis, and remove jaundice, it also plays an important role in the treatment of primary liver cancer[46].

Liver failure is caused by extensive liver cell necrosis, which results in severely impaired liver function, while chronic liver failure results from decompensation of the liver during cirrhosis and is accompanied by a poor prognosis and high mortality rate.

HBV is the most common cause of liver failure in China. Although nucleoside analogs can inhibit viral replication in the short-term, they usually result in drug resistance in the long-term. TCM enema can solve issues such as difficulties in oral administration, effectively removing harmful bacteria in the gut, improving intestinal endotoxemia, and promoting the recovery of liver function. In patients with HBV-induced liver failure, an enema of YCHD plus colon lavage was found to result in liver function and symptom improvement, jaundice improvement, TBIL reduction, and increased prothrombin time[47].

One study established a rat model with hepatic failure after 70% liver resection to research the therapeutic effect of capillaris. The results showed that the herb can improve IL-6 levels, increase serum IL-6 levels, and improve survival rate in rats with liver failure after surgery[48].

## OTHER PHARMACOLOGICAL ACTIVITIES

YCHD-ameliorated alloxan (ALX) induced hyperglycemia in mice and significantly reduced fasting blood glucose (FBG) in normal mice and ALX-diabetes in mellitus model mice and rats. YCHD also improved impaired glucose tolerance in a dexamethasone-induced insulin resistance rat model and reduced 2-h post-prandial blood glucose after an oral glucose tolerance test. This suggests that YCHD has hypoglycemic effects like sulfonylurea or biguanide[49].

Clinical studies indicate that YCHD plus Bai-Tou-Weng decoction and hormones have a better treatment effect than Western medicine in the clinical treatment of Behçet's disease. In one study, a control group was treated with prednisone, while a treatment group was treated with the same drug plus YCHD and Bai-Tou-Weng decoction. According to the diagnostic criteria of the International Society for Behçet's Disease, the therapeutic effect in the treatment group was better than in the control group ($P < 0.05$)[50].

YCHD has also been used clinically in neonatal hyperbilirubinemia[51], neonatal jaundice[52], maternal-

**Table 3 Patents containing Yīn-Chén-Hāo decoction**

| Patent | Patent number |
|---|---|
| YCHD preparation methods | CN101371882 |
| Damp-proof TCM granule and its preparation method | CN1781499 |
| Composition for improving the composition/kind of composition of crude drug powder containing rhubarb and/or its extract that can improve constipation | JP2005179316 |
| Medical treatment for heterotopic calcification/ medicine root in TCM prescription treatment for heterotopic calcification | JP5000961 |
| Kind of formula granules, including YCHD and its preparation and detection methods | CN103230453 |
| Method which can filtrate the material foundation of YCHD efficacy | CN104101674 |

YCHD: Yīn-Chén-Hāo decoction; TCM: Traditional Chinese medicine.

fetal ABO blood type incompatibility[53-56], intrahepatic cholestasis of pregnancy[57], newborn pathological jaundice[58], and acne[59].

Compared with insulin and insulin analogs, combined therapy of NovoRapid and modified YCHD is an effective, safe, and economical approach to the treatment of chronic hepatitis B with diabetes[60].

## DRUGS THAT INCLUDE THE HERBS FROM YCHD

Drugs that include the herbs found in YCHD have been on the market for many years and most have important therapeutic uses. Xiong-Dan-Yīn-Chén Oral Solution® (XDYCKFY, Hei-Bao-Yao-Ye, Hei-Long-Jiang, China) is an effective and safe treatment for chronic liver diseases, and can slow down the progress of cholecystitis and cholelithiasis. Moreover, Ling-Zhi-Yīn-Chén Capsule® (LZYC capsule, Zhong-Long-Yi-Yao, Hei-Long-Jiang, China) and Huang-Dan-Yīn-Chén Granule® (HDYCKL, Fo-Ci-Zhi-Yao, Lan-Zhou, China) are used to improve the symptoms of idiopathic pain, abdominal distension, anorexia, malaise, fatigue, and greasy yellow tongue coating. Yīn-Chén-Tui-Huang Capsule® (YCTH capsule, De Shang Yao Ye, Jilin, China) is often used in the treatment of jaundice caused by acute and chronic liver disease.

## CONCLUSION

This review summarized the efficacy of YCHD in liver disease from clinical trials and its mechanisms of action *in vitro* and *in vivo*. Studies indicate that YCHD can modulate various molecular pathways in liver disease. YCHD is widely used in clinical settings for the treatment of liver diseases, and could be a safe and novel therapeutic treatment for liver injury worldwide (Table 3). Future studies on YCHD could help define its various effective constituents, molecular mechanisms, and targets that help prevent inflammation and fibrosis. Although YCHD has been used clinically for thousands of years, most studies on it are basic, and so its material basis and mechanisms remain unclear. At present, there are few multicenter, large, randomized, double-blind, controlled clinical trials on YCHD. Extensive clinical research is warranted to evaluate the safety and efficacy of YCHD alone or in combination with other drugs.

## REFERENCES


1 **Wang L**, Zhou GB, Liu P, Song JH, Liang Y, Yan XJ, Xu F, Wang BS, Mao JH, Shen ZX, Chen SJ, Chen Z. Dissection of mechanisms of Chinese medicinal formula Realgar-Indigo naturalis as an effective treatment for promyelocytic leukemia. *Proc Natl Acad Sci USA* 2008; **105**: 4826-4831 [PMID: 18344322]

2 **Lv JL**, Jin SY, Yuan HL, Han J, Fu SS, Jin SX, Guo JJ, Xiao XH. Rational Daily Administration Times of Yinchenhao Decoction in Rats with Jaundice Based on PD/PK. *Zhong Cao Yao* 2012; **4**: 150-156 [DOI: 10.3969/j.issn.1674-6384.2012.02.009]

3 **Mu YP**, Liu P, Wang L. Evolution and Modern Research of Yinchenhao Decoction. *Zhongguo Shiyan Fangjixue* 2006; **12**: 67-71 [DOI: 10.3969/j.issn.1005-9903.2006.02.023]

4 **Meng S**, Xing S. Classical prescription - Chemical Research of Yin-chen-hao Decoction. *Yatai Chuantong Yixue* 2009; **5**: 173-175

5 **Wang X**, Zhang B. Elucidation of compatibility principle and scientific value of Chinese medical formulae based on pharmacometabolomics. *Zhongguo Zhong Yao Za Zhi* 2010; **35**: 1346-1348 [PMID: 20707212]

6 **Zhang YM**, Yu F, Dai DZ, Gao J, Cong XD, Dai Y. Hypoxia alters pharmacokinetics of argirein because of mitochondrial dysfunction that is alleviated by apocynin. *J Pharm Pharmacol* 2013; **65**: 1360-1372 [PMID: 23927475 DOI: 10.1111/jphp.12098]

7 **Guo MZ**, Li XS, Shen DM, Guan XQ, Xu HR, Gao J. Effect of Rhein on the development of hepatic fibrosis in rats. *Zhonghua Gan Zang Bing Za Zhi* 2003; **11**: 26-29 [PMID: 12546737]

8 **Ji L**, Jiang P, Lu B, Sheng Y, Wang X, Wang Z. Chlorogenic acid, a dietary polyphenol, protects acetaminophen-induced liver injury and its mechanism. *J Nutr Biochem* 2013; **24**: 1911-1919 [PMID: 24011717 DOI: 10.1016/j.jnutbio.2013.05.007]

9 **Kim SJ**, Kim KM, Park J, Kwak JH, Kim YS, Lee SM. Geniposidic acid protects against D-galactosamine and lipopolysaccharide-induced hepatic failure in mice. *J Ethnopharmacol* 2013; **146**: 271-277 [PMID: 23298456 DOI: 10.1016/j.jep.2012.12.042]

10 **Yu ZG**, Wang Q, Li K, Li YQ, Gao XX. Determination and pharmacokinetics of 6,7-dimethoxycoumarin in rat plasma after intragastric administration of different decoctions of yinchenhao tang. *J Chromatogr Sci* 2007; **45**: 544-548 [PMID: 18019567]

11 **Wang X**, Zhang A, Wang P, Sun H, Wu G, Sun W, Lv H, Jiao G, Xu H, Yuan Y, Liu L, Zou D, Wu Z, Han Y, Yan G, Dong W, Wu F, Dong T, Yu Y, Zhang S, Wu X, Tong X, Meng X. Metabolomics coupled with proteomics advancing drug discovery toward more agile development of targeted combination therapies. *Mol Cell Proteomics* 2013; **12**: 1226-1238 [PMID: 23362329 DOI: 10.1074/mcp.M112.021683]

12 **Lv JL**, Li RS, Jin SY, Yuan HL, Fu SS, Han J, Jin SX, Xiao XH. Changes of pharmacokinetics of 6,7-dimethoxycoumarin in a rat model of alpha-naphthylisothiocyanate-induced experimental hepatic injury after Yinchenhao Decoction () treatment. *Chin J Integr Med* 2012; **18**: 831-836 [PMID: 23086487 DOI: 10.1007/s11655-012-1272-8]

13 **Cao HX**, Sun H, Jiang XG, Lu HT, Zhang GM, Wang XJ, Sun WJ, Wu ZM, Wang P, Liu L, Zhou J. Comparative study on the protective effects of Yinchenhao Decoction against liver injury induced by alpha-naphthylisothiocyanate and carbon tetrachloride. *Chin J Integr Med* 2009; **15**: 204-209 [PMID: 19568713 DOI: 10.1007/s11655-009-0204-y]

14 **Zhang A**, Sun H, Yuan Y, Sun W, Jiao G, Wang X. An in vivo

analysis of the therapeutic and synergistic properties of Chinese medicinal formula Yin-Chen-Hao-Tang based on its active constituents. *Fitoterapia* 2011; **82**: 1160-1168 [PMID: 21835230 DOI: 10.1016/j.fitote.2011.07.014]

15 **Zhang A**, Sun H, Dou S, Sun W, Wu X, Wang P, Wang X. Metabolomics study on the hepatoprotective effect of scoparone using ultra-performance liquid chromatography/electrospray ionization quadruple time-of-flight mass spectrometry. *Analyst* 2013; **138**: 353-361 [PMID: 23152956 DOI: 10.1039/c2an36382h]

16 **Zhang A**, Sun H, Wu G, Sun W, Yuan Y, Wang X. Proteomics analysis of hepatoprotective effects for scoparone using MALDI-TOF/TOF mass spectrometry with bioinformatics. *OMICS* 2013; **17**: 224-229 [PMID: 23514563 DOI: 10.1089/omi.2012.0064]

17 **Zhang A**, Sun H, Wang X. Urinary metabolic profiling of rat models revealed protective function of scoparone against alcohol induced hepatotoxicity. *Sci Rep* 2014; **4**: 6768 [PMID: 25341677 DOI: 10.1038/srep06768]

18 **Yin Q**, Sun H, Zhang A, Wang X. Pharmacokinetics and tissue distribution study of scoparone in rats by ultraperformance liquid-chromatography with tandem high-definition mass spectrometry. *Fitoterapia* 2012; **83**: 795-800 [PMID: 22465507 DOI: 10.1016/j.fitote.2012.03.010]

19 **Wang FL**, Yang HZ, Li YM, Wu WK, Zou ZC. Prevention and treatment mechanism of qingxia therapy (based on yinchenhao decoction and dachengqi decoction) on hepatocyte apoptosis in rats with acute hepatic injury induced by lipopolysaccharide/D-galactosamine. *Zhong Yao Cai* 2014; **37**: 848-852 [PMID: 25335295]

20 **Lee TY**, Chang HH, Lo WC, Lin HC. Alleviation of hepatic oxidative stress by Chinese herbal medicine Yin-Chen-Hao-Tang in obese mice with steatosis. *Int J Mol Med* 2010; **25**: 837-844 [PMID: 20428786]

21 **Chen SD**, Fan Y, Xu WJ. Effects of yinchenhao decoction (see text) for non-alcoholic steatohepatitis in rats and study of the mechanism. *J Tradit Chin Med* 2011; **31**: 220-223 [PMID: 21977865]

22 **Liang DZ**, Wei W, Yao KW. Yinchenhao decoction adjustment for treatment of nonalcoholic fatty liver disease: A systematic review and meta-analysis of randomized controlled trials. *Shijie Huaren Xiaohua Zazhi* 2014; **22**: 2327-2337

23 **Zhang J**, Li HT, Chen P. 48 cases of Modified Yin-Chen-Hao Decoction Treating for Chronic Hepatitis B. *Hunan Zhongyi Za Zhi* 2014; **30**: 52-53

24 **Zhou X**. Clinical Observation for 48 cases of Modified Yin-Chen-Hao Decoction Treating for Jaundice Hepatitis B. *Taishan Yixueyuan Xuebao* 2014; **35**: 925-926

25 **Guo SF**. 36 cases of Modified Yin-Chen-Hao Decoction Treating for Chronic Hepatitis B. *Fujian Zhongyi Zazhi* 2014; **45**: 16-17

26 **Zhang YQ**, Tang XD, Wang FY, Yang B, Liu YL, Guo P, Wang P, Bian LQ, Zhao YP. Effect of ronggan mixture on immunoregulation and hepatocyte apoptosis-related factors in concanavalin A induced acute immunological liver injury mice. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 2013; **33**: 1500-1506 [PMID: 24483111]

27 **Xu XM**, Xu N. Clinical observation of Yinchenhao decoction combined with western medicine therapy on the treatment of drug-induced hepatitis. *Hebei Zhongyiyao Xuebao* 2014; **36**: 845-854

28 **Zhu DZ**. Observation of the curative effect of Xinjia Yinchenhao Decoction in treating severe chronic hepatitis. *Zhong Xi Yi Jie He Xue Bao* 2003; **1**: 77-78 [PMID: 15339628]

29 **Zhao AL**, Zheng ZG. Clinical Analysis for 50 cases of Modified Yin-Chen-Hao Decoction combine with Plasma exchange Treating for Severe Hepatitis. *Beijing Yixue* 2014; **36**: 506-507

30 **Hernandez-Gea V**, Friedman SL. Pathogenesis of liver fibrosis. *Annu Rev Pathol* 2011; **6**: 425-456 [PMID: 21073339 DOI: 10.1146/annurev-pathol-011110-130246]

31 **Friedman SL**. Hepatic fibrosis -- overview. *Toxicology* 2008; **254**: 120-129 [PMID: 18662740 DOI: 10.1016/j.tox.2008.06.013]

32 **Liu C**, Wang G, Chen G, Mu Y, Zhang L, Hu X, Sun M, Liu C, Liu P. Huangqi decoction inhibits apoptosis and fibrosis, but promotes Kupffer cell activation in dimethylnitrosamine-induced rat liver fibrosis. *BMC Complement Altern Med* 2012; **12**: 51 [PMID: 22531084 DOI: 10.1186/1472-6882-12-51]

33 **Bian YQ**, Ning BB, Cao HY, Lu Y, Liu C, Chen GF, Liu J, Liu P, Sun MY. Formula-syndrome correlation study of three classical anti-jaundice formulas in inhibition of liver fibrosis induced by dimethylnitrosamine in rats. *Zhong Xi Yi Jie He Xue Bao* 2012; **10**: 1405-1412 [PMID: 23257134 DOI: 10.3736/jcim20121212]

34 **Liu C**, Sun M, Wang L, Wang G, Chen G, Liu C, Liu P. Effects of Yinchenhao Tang and related decoctions on DMN-induced cirrhosis/fibrosis in rats. *Chin Med* 2008; **3**: 1 [PMID: 18237412 DOI: 10.1186/1749-8546-3-1]

35 **Wang YH**, Zhao CX, Chen BM, He M, Liu LQ, Li CY, Chen X. Reverse effect of Yinchenhao decoction in dimethyl nitrosamine-induced hepatic fibrosis in rats. *Zhongguo Zhong Yao Za Zhi* 2014; **39**: 1473-1478 [PMID: 25039185]

36 **Bian YQ**, Liu P, Sun MY. Recipe-syndrome Correlation Study of Yinchenhao Tang and Yinchen Sini Tang on Inhibiton of Liver Fibrosis Induced by Dimethylnitrosamine in Rats. *Liaoning Zhongyiyao Daxue Xuebao* 2013; **5**: 48

37 **Bian YQ**, Cao HY, Sun MY. Recipe-syndrome correlation study of Yinchenhao Tang and Ganluxiaodu Dan on inhibition of liver fibrosis induced by dimethylnitrosamine in rats. *Zhongguo Zhongyiyao Xuebao* 2013; **28**: 1396-1401

38 **Goerdt S**, Politz O, Schledzewski K, Birk R, Gratchev A, Guillot P, Hakiy N, Klemke CD, Dippel E, Kodelja V, Orfanos CE. Alternative versus classical activation of macrophages. *Pathobiology* 1999; **67**: 222-226 [PMID: 10725788]

39 **Song E**, Ouyang N, Hörbelt M, Antus B, Wang M, Exton MS. Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. *Cell Immunol* 2000; **204**: 19-28 [PMID: 11006014 DOI: 10.1006/cimm.2000.1687]

40 **Liu C**, Sun M, Yan X, Han L, Zhang Y, Liu C, El-Nezami H, Liu P. Inhibition of hepatic stellate cell activation following Yinchenhao decoction administration to dimethylnitrosamine-treated rats. *Hepatol Res* 2008; **38**: 919-929 [PMID: 18371158 DOI: 10.1111/j.1872-034X.2008.00346.x]

41 **Liu C**, Liu P, Tao Q. [Recipe-syndrome correlation and pathogenesis mechanism of Yinchenhao Decoction in intervening dimethylnitrosamine induced liver cirrhosis progress in rats]. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 2010; **30**: 845-850 [PMID: 21038655]

42 **Cao HY**, Li JY, Liu P, Sun MY. Antifibrotic mechanism of action based on the corresponding on Traditional Chinese Medicine formula-syndrome to explore Yin-Chen-Hao Decoction regulation of Kupffer cell function and MAPK pathways. *Shijie Zhongyiyao* 2015; **2**: 5-10 [DOI: 10.3969/j.issn.1673-7202(sj).2015.02.005]

43 **Wang L**, Liu P, Wang CS. [Effects of 5 classical recipes on anti-oxidative stress in rat liver with cirrhosis]. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 2008; **28**: 435-439 [PMID: 18672772]

44 **Wang L**, Liu P, Mu YP, Li FH, Long AH, Gu HT, Chen GF. Study on TCM recipe and syndrome of dimethylnitrosamine-induced hepatic fibrosis in rats. *Zhongyi Za Zhi* 2006; **47**: 929-932 [DOI: 10.13288/j.11-2166/r.2006.12.031]

45 **Wang XD**, Zhang CZ. Clinical Research on the Treatment of Ascites Due to Cirrhosis by Modified Yinchenhao Decoction and Jijiaolihuang Pill. *Zhongguo Zhongyao Zazhi* 2014; **29**: 1373-1374 [DOI: 10.16368/j.issn.1674-8999.2014.09.035]

46 **Liu X**, Li N. Regularity analysis on clinical treatment in primary liver cancer by traditional Chinese medicine. *Zhongguo Zhong Yao Za Zhi* 2012; **37**: 1327-1331 [PMID: 22803386]

47 **Guo XJ**, Dang D. 36 cases of Modified Yin-Chen-Hao Decoction retention enema combine with Colonic irrigation Treating for Chronic Hepatitis B and hepatic failure. *Shanxi Zhongyi Zazhi* 2014; **35**: 858 [DOI: 10.3969/j.issn.1000-7369.2014.07.58]

48 **Li J**, Mao DW, Zhang RZ. Artemisia capillaris impact the level of interleukin 6 in Postoperative hepatic failure in rats. *Henan Zhongyi* 2014; **34**: 1251-1252

49 **Pan J**, Han C, Liu H, Du J, Li A. Effects of yinchenhao decoction on normal animals and animal models of diabetes mellitus. *Zhong Yao Cai* 2001; **24**: 128-131 [PMID: 11402731]

50 **Zhang BX**, Liu SZ, Peng XH. Clinical Analysis of Yin-Chen-Hao Decoction Combine with Hormone Treating for Behcets disease. *Zhongguo Zhongyiyao Keji* 2014; **21**: 412
51 **Wen HR**, Zhang JM, Zhu LJ. 232 cases of Modified Yin-Chen-Hao Decoction Preventive Against Neonatal Hyperbilirubinemia. *Zhonguo Zhongyiyao Xiandai Yuancheng Jiaoyu* 2014; **12**: 39-40 [DOI: 10.3969/j.issn.1672-2779.2014.01.018]
52 **Wang Y**, Ning YT. Effect analysis of modified Virgate wormwood decoction treating infant jaundice in 69 cases. *Zhongguo Xiandai Yixue* 2014; **21**: 104-105
53 **Zhu QY**, Zhu WZ. Clinical Observation for 80 cases of Modified Yin-Chen-Hao Decoction Treating for Maternal-fetal ABO Incompatibility. *Zhejiang Zhongyi Zazhi* 2014; **49**: 737
54 **Cai XQ**, Cheng HJ. Clinical Observation for 50 cases of Modified Yin-Chen-Hao Decoction Treating for Maternal-fetal ABO Incompatibility. *Zhejiang Zhongyi Zazhi* 2014; **49**: 267
55 **Li H**, Yu MM, Zhou HY. Effects of Yinchenhao decoction on TNF-α level of BALB/c mouse immunized with A blood group antigen. *Zhongguo Shuxue Zazhi* 2014; **2**: 572-575
56 **Zhang Y**, Zhang YC, Tian M. Clinical Observation for 17 cases of Modified Yin-Chen-Hao Decoction Treating for Maternal-fetal ABO Incompatibility. *Nei Mongol Zhongyi Zazhi* 2014; **33**: 11
57 **Liao YH**, Sun RF. Yin-Chen-Hao Decoction Impact on the Balance of Intrahepatic Cholestasis in Patients during Pregnancy. *Zhongguo Zhongxiyi Jiehe Zazhi* 2014; **34**: 1017-1018
58 **Sang YQ**, Tian WQ, Feng J. Clinical study on adjuvant treatment of neonatal pathological jaundice by Yinchenhao decoction. *Zhongguo Dangdai Yisheng* 2014; **52**: 85-87
59 **Liu CC**, Zhu PH, Sun Y. Clinical Research of Yinchenhao Decoction in Treating Adolescent Female Acnes. *Zhongguo Zhongyao Zazhi* 2014; **29**: 1382-1384
60 **Liu HX**, Cheng M, Wang XM. Clinical effect of combination therapy with NovoRapid 30 and modified Yinchenhao decoction in treatment of chronic hepatitis B with diabetes. *Linchuang Gandanbing Zazhi* 2014; **30**: 311-313 [DOI: 10.3969/j.issn.1001-5256.2014.04.006]
61 **Xu BH**, Li MQ. 81 cases of Modified Yin-Chen-Hao Decoction Treating for Maternal-fetal ABO Incompatibility. *Zhongyi Zazhi* 2011; 1418-1419
62 **Gan LM**, Li P. Clinical Observation of Modified Yin-Chen-Hao Decoction combine with Transmetil Treating for hyperbilirubinemia induced by viral hepatitis. *Xin Zhong Yi* 2009; **41**: 20-21
63 **Yang FR**. Clinical Observation for 68 cases of Modified Yin-Chen-Hao Decoction combine with conventional western treatment Treating treating for pathological jaundice of newborn. *Zhongguo Shiyan Fangjixue* 2011; **17**: 260-261
64 **Yi XD**, Luo YW. Clinical Observation of Modified Yin-Chen-Hao Decoction combined with Plasma exchange Treating treating for Hepatitis B merger hyperbilirubinemia. *Shandong Yiyao* 2011; **51**: 63-64
65 **Zhang CY**, Gao Z. Clinical Observation for 31 Cases of Modified Yin-Chen-Hao Decoction Combine with Traditional Chinese Medicine Face Pack Treating for Acne with Gastrointestinal Dampness-Heat. *Xin Zhong Yi* 2011; **43**: 62-63
66 **Sang Y**, Zhang P. 32 cases of Modified Yin-Chen-Hao Decoction Treating for Children Bronchial Asthma. *Zhongyi Zazhi* 2012; **53**: 876-877
67 **Feng J**, Kong D. Clinical research of Traditional Chinese Medicine Combine with Duodenoscopy Treating for Choledocholithiasis. *Shizhen Guoyi Guoyao* 2011; **22**: 1533-1534 [DOI: 10.3969/j.issn.1008-0805.2011.06.121]
68 **Huang L**, Feng JX, Wei MF, Li MJ. The Adjuvant Therapeutic Effect of Yinzhihuang Injection Before or After Biliary Atresia Surgery in Children. *Shiyong Erke Linchuang Zazhi* 2007; **11**: 811-812
69 **Chen Y**, Zheng YN. Clinical Analysis of Yinzhihuang Injection and Bifid-Triple Viable Capsule Combine with Light Therapy Treating for Neonatal Jaundice. *Shiyong Erke Linchuang Zazhi* 2012; **15**: 2480-2483 [DOI: 10.3969/j.issn.1007−9572.2012.07.121]
70 **Gao XC**, Shen JH, Ni YhYH. Clinical Observation of Yinzhihuang Injection Treating for Pathological Jaundice of Newborn. *Zhongguo Yaofang* 2011; **22**: 1872-1873
71 **Pen H**, Dong GQ, Ni YH. Observation of curative effect of yinzhihuang injection for Infantile infantile hepatitis syndromeJ. *Zhongyaocai* 2003; **26**: 534-535
72 **Zhang Y**, Song D. Effect of Traditional Chinese Medicine Treatment on Neonatal Jaundice induced by Maternal- Fetal ABO Blood Group Incompatibility. *Zhonghua Zhongyiyao Xuekan* 2013; **31**: 1713-1715
73 **Ohwada S**, Kobayashi I, Harasawa N, Tsuda K, Inui Y. Severe acute cholestatic hepatitis of unknown etiology successfully treated with the Chinese herbal medicine Inchinko-to (TJ-135). *World J Gastroenterol* 2009; **15**: 2927-2929 [PMID: 19533820 DOI: 10.3748/wjg.15.2927]
74 **Kaiho T**, Tsuchiya S, Yanagisawa S, Takeuchi O, Togawa A, Okamoto R, Saigusa N, Miyazaki M. Effect of the herbal medicine Inchin-Ko-To for serum bilirubin in hepatectomized patients. *Hepatogastroenterology* 2008; **55**: 150-154 [PMID: 18507096]
75 **Ai XY**, Fu LC, Tan XH, Zhang AM, Fan HM. Therapeutic Effect of Modified Compound Yinchen Recipe for Chronic Severe Hepatitis. *Guangzhou Zhongyiyao Daxue Xuebao* 2013; **30**: 145-148
76 **Sun JG**. Effect of Modified Yinchenhao Decoction Medicated by Retention Enema on Endotoxin and Blood Ammonia of Chronic Severe Hepatitis Patients. *Sichuan Zhongyiyao Zazhi* 2013; **31**: 75-77
77 **Zheng YJ**, Tang HH, He JS. Clinical efficacy of ursodeoxycholic acid combined with modified Artemisiae Scopariae decoction for treating 30 cases of primary biliary cirrhosis. *Zhongguo Zhongxiyi Jiehe Zazhi* 2012; **22**: 89-91 [DOI: 10.3969/j. issn.1005-0264.2012.02.008]
78 **Li X**, Zhu JL. Modified Yinchenhao Decoction in the treatment of 30 cases of neonatal hyperbilirubinemia. *Zhongguo Erke Xuebao* 2013; **9**: 42-43
79 **Jin H**, Zhao YF, Dai XH. Lidan Xiaohuang Decoction and Acupuncture Treatment of Obstructive Jaundice Postoperative Persistent Jaundice of 35 cases. *Shiyong Zhongyi Neike Zazhi* 2011; **25**: 52-53 [DOI: 10.3969/j.issn.1671-7813.2011.12.29]
80 **Chen XT**. Clinical Study of Qingre Lishi Forfula in Treating Chronic Hepatitis B. *Zhongguo Zhongyao Zazhi* 2011; **26**: 207-208
81 **Zhang HY**, Chen L, Yu C. Clinical Observation of Integrated Chinese and Western Medicine Sequential Therapy for Treatment of Hp-positive Acne Rosacea. *Xin Zhong Yi* 2013; **45**: 81-83
82 **Li SZ**, Xu FM, Li SB. Tarragon Decoction Treat Jaundice Virus Hepatitis. *Zhejiang Zhongyiyao Daxue Xuebao* 2012; **36**: 267-269
83 **Feng W**, He L, Dong X. Effects of Capillaris Decoction on Postoperative Bilirubin of Patients with Obstructive Jaundice. *Zhongguo Zhongyi Chuangxin* 2012; **9**: 12-13 [DOI: 10.3969/j.issn.1674-4985.2012.17.006]
84 **Cao JL**, Wu CM, Zhu XQ, Ou YQ. Enhanced therapeutic effect of Yinchenhao decoction supplemented to plasma exchange in the treatment of severe chronic hepatitis B patients. *Zhongguo Bingduxue Zazhi* 2012; **2**: 142-144
85 **Yang YF**, Diao XD, Zhao YZ. Therapeutic Effect of Yinchenhao Decoction for Intrahepatic Cholestasis of Pregnancy: An Analysis of 30 Cases. *Xin Zhong Yi* 2013; **45**: 71-74
86 **Huang HC**, Huang YL, Chang JH, Chen CC, Lee YT. Possible mechanism of immunosuppressive effect of scoparone (6,7-dimethoxycoumarin). *Eur J Pharmacol* 1992; **217**: 143-148 [PMID: 1425935]
87 **Amarnath A**, Lenin V, Archunan G. Evaluation of the hypolipidemic activity of 6,7-dimethoxycoumarin on placental tissue factor mRNA expression in experimental anti-phospholipid syndrome. *Pharmacogn Mag* 2013; **9**: 264-270 [PMID: 23930012 DOI: 10.4103/0973-1296.113287]
88 **Huang HC**, Lee CR, Weng YI, Lee MC, Lee YT. Vasodilator effect of scoparone (6,7-dimethoxycoumarin) from a Chinese herb. *Eur J Pharmacol* 1992; **218**: 123-128 [PMID: 1327821]
89 **Liu YJ**, Li Z, Lin ZB. Scoparone's effects on the airway smooth muscle of asthmatic guinea pigs. *Zhongguo Yike Daxue Xuebao*

2001; **30** Suppl: 12-13

90 **Ma T**, Huang C, Zong G, Zha D, Meng X, Li J, Tang W. Hepatoprotective effects of geniposide in a rat model of nonalcoholic steatohepatitis. *J Pharm Pharmacol* 2011; **63**: 587-593 [PMID: 21401612 DOI: 10.1111/j.2042-7158.2011.01256.x]

91 **Tang JC**, Yang H, Song XY, Song XH, Yan SL, Shao JQ, Zhang TL, Zhang JN. Inhibition of cytochrome P450 enzymes by rhein in rat liver microsomes. *Phytother Res* 2009; **23**: 159-164 [PMID: 18814214 DOI: 10.1002/ptr.2572]

92 **Dong MX**, Jia Y, Zhang YB, Li CC, Geng YT, Zhou L, Li XY, Liu JC, Niu YC. Emodin protects rat liver from CCl(4)-induced fibrogenesis via inhibition of hepatic stellate cells activation. *World J Gastroenterol* 2009; **15**: 4753-4762 [PMID: 19824107 DOI: 10.3748/wjg.15.4753]

**P- Reviewer**: Daliry A, Im SS **S- Editor**: Yu J
**L- Editor**: Rutherford A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/ *World J Gastroenterol* 2017 February 21; 23(7): 1139-1146

DOI: 10.3748/wjg.v23.i7.1139 ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

# Anatomical resection of hepatocellular carcinoma: A critical review of the procedure and its benefits on survival

Koo Jeong Kang, Keun Soo Ahn

Koo Jeong Kang, Keun Soo Ahn, Division of Hepatobiliary and Pancreatic Surgery, Department of Surgery, School of Medicine, Keimyung University Dong-san Medical Center, Daegu 41931, South Korea

Author contributions: Kang KJ and Ahn KS designed the review and collected and analyzed data; Kang KJ wrote the manuscript; Ahn KS revised the manuscript.

Conflict-of-interest statement: No potential conflicts of interest.



Manuscript source: Invited manuscript

Correspondence to: Koo Jeong Kang, MD, PhD, Division of Hepatobiliary and Pancreatic Surgery, Department of Surgery, School of Medicine, Keimyung University Dong-san Medical Center, 56 Dalseong-Ro, Jung-Gu, Daegu 41931, South Korea. kjkang@dsmc.or.kr
Telephone: +82-53-2507655
Fax: +82-53-2507322

Received: August 27, 2016
Peer-review started: August 31, 2016
First decision: September 20, 2016
Revised: October 18, 2016
Accepted: November 14, 2016
Article in press: November 16, 2016
Published online: February 21, 2017

## Abstract

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer and the third most frequent cause of cancer-related death. Advances in preoperative assessment of HCC (*e.g.*, imaging studies and liver function tests), surgical techniques, and postoperative care have improved the surgical outcomes and survival of patients who undergo hepatic resection for HCC. However, in the last 20 years, the long-term survival after hepatectomy has remained unsatisfactory owing to the high rates of local recurrence and multicentric occurrence. Anatomical liver resection (AR) was introduced in the 1980s. Although several studies have revealed tangible benefits of AR for HCC, these benefits are still debated. Because most HCCs occur in patients with liver cirrhosis and poor hepatic function, there are many factors that affect survival, including the surgical method. Nevertheless, many studies have documented the perioperative and long-term benefits of AR in various conditions. In this article, we review the results of several recently published, well-designed comparative studies of AR, to investigate whether AR provides real benefits on survival outcomes. We also discuss the potential pitfalls associated with this approach.

**Key words:** Hepatocellular carcinoma; Cirrhosis; Curative; Anatomical resection; Prognosis



**Core tip:** Anatomical liver resection (AR) has been widely used for two decades in hepatocellular carcinoma and although many studies have shown the perioperative benefits, long term survival benefit of AR is still debated. For evaluation of benefits of AR, many factors should be considered, such as degree of cirrhosis, anatomical variation and surgical techniques. Moreover, critical review of previous studies considering bias is necessary. In this article, we review the results of several recently published, well-designed comparative studies of AR to investigate whether AR provides real benefits on survival outcomes. We also discuss the potential pitfalls associated with this approach.

Kang KJ, Ahn KS. Anatomical resection of hepatocellular carcinoma: A critical review of the procedure and its benefits on survival. *World J Gastroenterol* 2017; 23(7): 1139-1146 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1139.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1139

## INTRODUCTION

Primary liver cancer is the second leading cause of cancer death worldwide[1]. The most common histologic types of liver cancer are hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC). HCC is the sixth most common type of cancer and the third most frequent cause of cancer-related death[2]. Advances in preoperative assessment of HCC (*e.g.,* imaging studies and liver function tests), surgical techniques, and postoperative care have markedly improved the surgical outcomes and survival of patients who undergo hepatic resection for HCC. However, in the last 20 years, the long-term survival after hepatectomy has remained unsatisfactory owing to the high rates of local and multicentric recurrence.

The oncologic principles of hepatic resection, especially in cirrhotic liver, are to resect all of the malignant tissue (tumor, satellite nodules, and portal vein territory) completely with effective clearance while preserving enough nontumorous liver parenchyma to prevent postoperative liver failure[3]. Regarding hepatectomy, modern surgical techniques were developed and important insight was gained in the late 1980s and through the 1990s. In the mid-1980s, hepatic surgeons gained more knowledge about liver anatomy, including its segments, leading to the introduction of systematic segmentectomy. Several strategies for reducing blood loss during hepatic transection were also introduced. They included keeping central venous pressure (CVP) as low as possible, the Pringle maneuver, and establishment of ischemia and reperfusion injury[4-6]. As a consequence of these developments, the surgical outcomes improved markedly in this era and the mortality rate decreased from 5%-10% to < 1%[7].

Although most hepatic surgeons estimate the optimal tumor-free margin during hepatic resection of the primary hepatic tumor, the concept of anatomical resection (AR) was introduced, in which the tumor-free margin is independent of margin length. This approach may improve the survival rate by reducing local recurrence[8]. AR was first proposed by the distinguished Japanese hepatic surgeon, Dr. Makuuchi[9,10]. The concept involves resection of the entire hepatic parenchymal tissue supplied by the portal venous system draining the tumor tissue. To achieve this, the liver surface is tattooed by injecting indigo carmine dye into the portal vein under intraoperative ultrasound guidance. A similar approach, Glissonean pedicle transection, was introduced by Dr. Takasaki[11]. Both groups suggested that AR confers a survival benefit. However, the outcomes of AR reported for other case-series differed between institutions when compared with non-anatomical resection (NAR). Accordingly, the benefits of AR are still debated. Different results may be due to patient selection bias and the use of different surgical techniques at each institution. To date, no prospective randomized studies have compared the outcomes of AR and NAR, for example. Several case-controlled studies have been published in which cases and controls were matched by propensity score matching. Although this statistical method is retrospective, it may provide valuable data in lieu of randomized studies.

Here, we perform a review of well-designed comparative studies, including case-series, meta-analyses, and case-control studies with propensity score matching, to investigate whether AR confers real clinical benefits relative to other resection methods. We also discuss the possible pitfalls of AR in this setting.

## DEFINITION AND THEORETICAL BACKGROUND

AR is defined as resection of the tumor together with the portal veins draining the tumor and the corresponding hepatic territory, as determined by dye injection into the feeding portal vein, or Glissonean pedicle transection[8,11]. AR involves either segmentectomy or sectionectomy, which includes bisectionectomy, hemihepatectomy, and trisectionectomy. NAR is defined as resection of a lesion regardless of the anatomical segment or section of the lobar anatomy, and includes limited resection or enucleation[8,11-15]. In the case of subsegmentectomy, which involves resection of the hepatic parenchyma fed by the fourth-order portal venous branch or one of several third-order branches, it is debated whether resection of parenchyma fed by one or several fourth-order branches should be classified as either AR or NAR.

Liver tumors are thought to invade the portal venous branches, allowing tumor cells to be carried to other regions of the liver in the portal venous flow. These disseminated tumor cells grow into microscopic tumor thrombi and then into daughter nodules[8]. Accordingly, it is theorized that AR confers a survival benefit by removing possible microscopic tumor thrombi or hitherto undetected daughter nodules in other parts of the liver. Several rigorous comparative analyses of the survival benefits of patients who underwent hepatic resection by AR or NAR have been conducted in many different centers. Most of these studies were performed in Eastern countries, and the results were inconsistent. This theory to explain the survival benefit of AR was subsequently reinforced by a well-designed imaging study, in which the authors used CT angiography to monitor the intratumoral hemodynamic changes associated with hepatocarcinogenesis[16]. The study showed that early

deterioration of arterial blood flow and an increase in neovascularized arterial blood flow was followed by a decrease in portal flow. Therefore, these intratumoral vascular and hemodynamic changes allow intratumoral blood containing free cancer cells to flow into the portal vein.

## SURGICAL TECHNIQUES OF ANATOMICAL RESECTION

There are two main types of AR technique, the Glissonean pedicle transection method and transection guided by dye injected into the portal venous branches.

### *Systematic segmentectomy with ultrasound-guided dye injection*

In segmentectomy or subsegmentectomy, it is important that the ligations are made at a point inside the liver parenchyma. In this method, the tumor-bearing portal pedicle is punctured and dye is injected under ultrasound guidance[8]. There are some tips to consider when performing ultrasound-guided puncture. The tip of the 21-gauge needle used in percutaneous ethanol injection therapy is easily visible on ultrasound. The needle tip has three holes, and its visibility can be improved by moving the needle forward and backward by millimeters, which sends vibrations towards the target portal vein. If the tip does not reach the target portal vein, the angle of the needle should be changed. Once the needle has punctured the vein, a blue dye (indigocarmine) should be injected very slowly without regurgitation. To stain the liver surface, the blood flow of the hepatic artery should be temporarily clamped with a Bulldog-type clamp[10]. The stained area must be carefully marked with electrocautery, and transection should gradually proceed from the liver surface towards the portal pedicle stained by the dye. Finally, the target segment should be removed after cutting the pedicle[8,9].

### *Glissonean pedical transection*

This procedure is based on the three ramifications of the Glissonean pedicle, namely the left, middle, and right, as initially proposed by Takasaki[11]. The hepatic parenchymal territory of each ramification includes the relevant sector or segment, and is now referred to by section according to the Brisbane terminology[17]. Resection of the hepatic area corresponding to one ramus was originally referred to as "systematized hepatectomy"[11,18]. In this procedure, right posterior sectionectomy and anterior sectionectomy correspond to the right ramus and middle ramus, respectively. Meanwhile, left hemihepatectomy of the left ramus can be divided into left medial and lateral sectionectomy. The ramifications of the Glissonean pedicles are located outside the liver. The sheath can be easily detached from the liver tissue without injuring the hepatic parenchyma or the portal vein or duct inside the pedicle, especially when the surgeon uses a Yankauer suction device plus a periosteal elevator. The trunks of the secondary or tertiary branches run inside of the liver. The area fed by each tertiary branch is cone-shaped, and is termed a "cone unit".

### *Anatomical variation of the portal pedicles*

The pattern and number of third-order portal branches in each ramification differ between patients. In a study of 16 cadavers, it was found that the segmental branches of the anterior pedicle arose from the main trunk of the anterior pedicle, while branches of the posterior pedicle arose from the main trunk of the posterior pedicle in 55.9% of the cadavers[19]. Meanwhile, anterior pedicle branches arose from the posterior dominant branches in 26.5% of cadavers and posterior pedicle branches arose from the anterior dominant branch in 17.6% of cadavers. These findings were confirmed in multidetector computed tomographic (MD-CT) study of 20 liver donors[19]. In most cases, the tertiary branches of the portal pedicle are supplied by one pedicle in a cone unit. However, in 33%-70% of cases, a single Couinaud segment is supplied by $\geqslant$ 2 tertiary branches arising from the same or different secondary branches, especially in segments 7 and 8[20,21]. In these variations, the sliding branch is not included in one Couinaud segment, instead crossing into a neighboring segment. In anatomical segmentectomy with ultrasound-guided dye injection, 54.8% percent of lesions were fed by a single main portal vein and 23.3% by adjacent double portal vein branches. In addition, 15.1% of lesions were partially stained and the opposite side could not be distinguished after dye injection because the resection line was demarcated through counterstaining of the adjacent segment in some cases. Furthermore, 7.1% of lesions were supplied by several small distributed portal veins or the lesions were difficult to see, in which case it is impossible to stain the tributaries. In these patients, AR is unfeasible[22].

### *Selection of appropriate surgical method for AR*

Glissonean pedicle transection is a feasible and safe method for AR, especially in cases requiring hemihepatectomy and sectionectomy. However, in cases requiring segmentectomy, the Glissonean approach is an invasive and technically demanding modality for patients with complicated portal anatomical variations or tumors located in the posterosuperior segments (segments Ⅶ and Ⅷ). In these cases, a larger volume of parenchyma in the noninvolved segment needs to be dissected to determine the involved Glissonean pedicle. Ultrasound-guided dye injection might be superior to Glissonean pedicle transection in cases requiring systematic segmentectomy, especially for tumors located farther from the main portal branches, tumors located in superioposterior segments (Ⅶ or Ⅷ), and if the tumor is fed by several branches. However, the ultrasound-guided dye injection method is a technically

demanding procedure that may be invasive, cause bleeding and prick the tumor. In addition, it is possible to inject the dye into the wrong portal vein branch. Aside from AR of the portal vein, some tumors span two or more segments and sections. If major hepatectomy is not indicated, a combination of the Glissonean pedicle approach and ultrasound-guided dye injection may allow the surgeon to remove the tumor with a cone-shaped resection. Therefore, the surgical method(s) can be selected based on tumor location and the surgeon's preference. Moreover, a combination of both techniques might be helpful in complex cases.

For safe AR, it is essential that the surgeon has good understanding of the anatomical variability of the Glisson pedicle, particularly the right hepatic vein, which serves as the reference structure for dividing the right anterior and right posterior sections. Careful imaging studies are very important; in addition to intraoperative ultrasound, careful review of the relation between portal venous branches and tumor looking at MD-CT that taken before hepatectomy.

### *Other issues regarding AR and what is real AR?*

There is another issue of importance to segmentectomy. For segmentectomy of Couinaud's segments 7 or 8, it is essential to expose the adjacent hepatic vein(s): the right hepatic vein for segment 7, and the right and mid-hepatic veins for segment 8[9,12]. This is reasonable when we consider the concept of Couinaud's classification of liver segments in patients with normal anatomy. However, in cases with sliding pedicles, for example the anterior pedicular branch has slid from the posterior ramus and posterior pedicle branch from the anterior ramus, the slid branch may bypass the hepatic venous limitation of the corresponding segment[19]. In patients with these variations, hepatic segmentectomy should include partial resection of the neighboring segment, including the relevant peripheral branch of the hepatic vein.

Some questions arise regarding how to perform AR and what technique should be used if the branching pattern of the portal vein does not match the Couinaud's segment and the tumor location is not confined to the Couinaud's anatomical segment.

Does cone-shaped parenchymal resection of tissue fed by one or several fourth-order branches represent AR or NAR? If the sliding branches cross over another neighboring segment[19], resection of the entire cone unit of the Glissonean pedicular branch, including partial resection of the neighboring segment, can be included in AR. If one cone unit partially fills a segment, resection of the cone unit without exposing the neighboring hepatic vein (for right upper segments) corresponds to resection of less than one segment. Therefore, does this constitute NAR? Should we always expose the segment bordering the hepatic veins in anatomical segmentectomy of segments 7 or 8? Does resection of the cone unit fed by the Glissonean pedicle cross over a segment included in AR? If the tumor is fed by two or more different segmental branches, is resection of all of the feeding Glissonean pedicles, including the parenchymal territory for AR? We suggest that AR procedures also include resection of one cone unit, whether or not the target tissue is smaller than a segment (*i.e.,* subsegmentectomy), is larger than the anatomical segment, or is fed by several branches when resection includes the hepatic parenchyma with the tumor-feeding Glissonean pedicle.

## BENEFICIAL OUTCOMES OF AR

### *Perioperative results*

HCC is usually associated with liver cirrhosis and many patients with HCC have poor liver function. Therefore, for curative resection with preservation of liver function, an adequate amount of tumor-bearing hepatic parenchyma should be resected while preserving a sufficient remnant liver volume. Considering these features of hepatic resection in cirrhotic liver, AR typically involves resection of the tumor-bearing portal pedicles, correspond to the liver parenchyma, without disrupting the blood flow through and the biliary drainage from the remnant segments. This approach may be ideal for limited liver resection. Although the benefits of this procedure on patient survival are still controversial, AR is often considered the gold standard procedure based not only on the pattern of intrahepatic spread of HCC but also because it preserves as much of the remnant liver tissue as possible, thereby reducing the risk of postoperative hepatic failure. It has been reported to offer several benefits in terms of achieving adequate surgical margins, reducing intraoperative blood loss, and reducing perioperative biliary complications by preserving the vascular and biliary structures in the remnant liver[15,22-29]. The advantages of AR are further magnified in patients with deep-seated tumors. Therefore, in most reports, AR was usually preferred in patients with HCC, whenever possible.

### *Long-term results*

Dozens of well-designed studies have compared the benefits of AR versus NAR using the systematic segmentectomy technique or the Glissonean pedicle transection method. Most of the prior studies were published between 1990 and 2005. All of the studies were conducted retrospectively, and most were case-series, including one large nationwide survey in Japan and three case-controlled studies with propensity score matching. The results of these studies are summarized in Table 1 for case-series and Table 2 for propensity score matching studies[7,12-15,18,22,25-38].

Of 18 studies evaluated, 8 reported that AR was beneficial while 8 revealed no benefits of AR. Meanwhile, one report described benefits of AR in

**Table 1 Summary of studies comparing the outcomes of anatomical and non-anatomical resection in patients with hepatocellular carcinoma**

| Ref. | Study period | Patient number total (AR:NAR) | Inclusion criteria | Method of AR D:Dye injection G:Glissonian | Cirrhosis (yes/no) ICG $R_{15}$ Difference B/W AR and NAR | Bleeding amount (mL, AR:NAR) | Survival benefit of AR for OS and RFS | Recurrence pattern (local or multicentric) | Others |
|---|---|---|---|---|---|---|---|---|---|
| Benefit | | | | | | | | | |
| Yamamoto *et al*[18] | 1990-1994 | *n* = 204 (90:114) | Solitary ≤ 5 cm | Glissonian | *P* = 0.02<br>NA | Not shown | Yes<br>OS (*P* = 0.0002) | NA | |
| Regimbeau *et al*[7] | 1990-1996 | *n* = 64 (30:34) | Solitary < 4 cm | Glissonean + US | NA<br>NA | Not shown | Yes<br>OS and RFS (*P* < 0.05) | NA | |
| Hasegawa *et al*[12] | 1994-2001 | *n* = 210 (156:54) | Solitary | Dye injection | 0.002<br>*P* < 0.0001 | *P* = 0.8 (574: 560) | Yes<br>OS (*P* = 0.01)<br>RFS (*P* = 0.006) | NA | |
| Cho *et al*[38] | 1998-2001 | *n* = 168 (99:69) | Solitary ≤ 5 cm | Not described | *P* = 0.026<br>NA | Not shown | Yes<br>OS (*P* = 0.032)<br>RFS (*P* = 0.003) | NA | |
| Wakai *et al*[15] | 1990-2004 | *n* = 158 (95:63) | Solitary pT1-T2 | Glissonean | *P* = 0.015 AR < NAR<br>*P* = 0.001 | *P* = 0.017 (813:590) | Yes<br>OS (*P* = 0.03) RFS (*P* = 0.008), for pT2 OS (*P* = 0.001) and RFS (*P* = 0.0004) | NA | |
| Yamashita *et al*[25] | 1985-2004 | *n* = 321 (201:120) | Solitary | Glissonean | *P* < 0.01<br>*P* < 0.01 | *P* < 0.01 (1353:993) | Yes<br>OS and RFS (*P* = 0.01) for liver damaged, No for less damaged | NA | |
| Ueno *et al*[37] | 1990-2004 | *n* = 116 (52:64) | ≤ 3 nodules, ≤ 3 cm | Dye injection | NA<br>*P* = 0.006 | *P* = 0.46 (1609:1224) | No for OS (*P* = 0.19)<br>Yes for RFS (*P* < 0.03) | NA | |
| Kobayashi *et al*[33] | 1990-2004 | *n* = 233 (106:127) | Solitary | Dye injection | *P* < 0.0001<br>*P* < 0.0001 | NA | Yes<br>RFS (*P* = 0.0002) | Different local recu: AR < NAR (*P* < 0.002) | |
| Eguchi *et al*[31] | 1994-2001 | *n* = 5781 (2267:3514) | Mixed | D&G mixed | NA | Not shown | Yes<br>OS (*P* = 0.0529)<br>RFS (*P* = 0.0089) | NA | Japanese nationwide survey |
| Yamazaki *et al*[26] | 1994-2007 | *n* = 209 (111:98) | Solitary ≤ 5 cm | Glissonean | *P* = 0.003<br>NA | *P* < 0.0001 (1266:842) | Yes<br>OS (*P* = 0.004), RFS (*P* = 0.023) | NA | |
| No benefit | | | | | | | | | |
| Capussotti *et al*[30] | 1985-2001 | *n* = 216 (156:60) | No limitation | Not clear | NA | Not shown | No OS (*P* = 0.9) | NA | |
| Portolani *et al*[36] | 1986-2003 | *n* = 213 (131:82) | NA | Not described | NA<br>NA | Not shown | No | NA | |
| Tanaka *et al*[14] | 1992-2005 | *n* = 125 (83:42) | Solitary | Not clear | *P* = 0.035<br>NA | *P* = 0.23 (1000:1200) | No<br>OS (*P* = 0.34) | No diff (*P* = 0.39) | |
| Kaibori *et al*[13] | 1992-2003 | *n* = 237 (34:217) | HepC(+) | Dye injection | *P* = 0.006 | 0.27 (1779:1414) | No<br>(OS = 0.7 and DFS 0.76) | No diff (*P* = 0.12) | |
| Tomimaru *et al*[27] | 1990-2008 | *n* = 92 (30:62) | Solitary ≤ 3 cm | Not clear | *P* = 0.4<br>*P* = 0.7 | *P* = 0.03 (1112:756) | No<br>OS (*P* = 0.67)<br>RFS (*P* = 0.77) | No diff (*P* = 0.29) | |
| Ahn *et al*[22] | 2001-2011 | *n* = 140 (65:75) | Solitary | Dye injection | *P* = 0.008<br>*P* < 0.001 | *P* = 0.05 (410:559) | No<br>OS (*P* = 0.08) | NA | Segmentectomy *vs* NAR |
| Marubashi *et al*[34] | 2001-2012 | *n* = 424 (243:181) | No limitation | Dye injection | NA<br>*P* < 0.001 | *P* < 0.001 (1237:640) | No<br>RFS *P* = 0.3 | No diff (*P* = 0.23) | No difference in recurrence pattern |
| Yamamoto *et al*[29] | 2003-2013 | *n* = 44 (16:28) | Solitary | Dye injection | 0.005<br>*P* = 0.029 | *P* = 0.002 (711:222) | No<br>OS (*P* = 0.6), DFS (0.58)<br>Yes for HBsAg(+) (*P* = 0.008) | NA | |

AR: Anatomical resection; NAR: Non-anatomical resection; D: Dye injection method; G: Glissonean pedicle method; ICG R15: Indocyanine green retention rate at 15 min; B/W: Between; OS: Overall survival; RFS: Recurrence-free survival; NA: Not applicable; US: Ultrasound; DFS: Disease-free survival; diff: Difference; HBsAg: Hepatitis B surface antigen.

**Table 2 Summary of case-control studies using the propensity score matching method to compare the outcomes of anatomical and non-anatomical resection for hepatocellular carcinoma**

| Ref. | Study period | Patient total number and after propensity score matched (AR:NAR) | Inclusion criteria | Method of AR D:Dye injection G:Glissonian | ICG $R_{15}$ Difference B/W AR and NAR | Bleeding amount (mL, AR:NAR) | Survival benefit of AR |
|---|---|---|---|---|---|---|---|
| Okamura *et al*[35], 2014 | 2002-2013 | $n$ = 236 (139:97 and 64:64) | Solitary | Dye injection | $P$ = 0.07 | $P$ = 0.008 (551:465) | No RFS ($P$ = 0.52) |
| Ishii *et al*[32], 2014 | 2002-2010 | $n$ = 268 (110:158 and 44:44) | Solitary ≤ 5 cm | Not Clear | $P$ = 0.053 | 0.9 (400:355) | No OS ($P$ = 0.29) RFS ($P$ = 0.28) |
| Marubashi *et al*[28], 2015 | 1981-2012 | $n$ = 1102 (577:525 and 329:329) | No limitation | Not clear | NA | Not shown | No OS ($P$ = 0.7) and RFS ($P$ = 0.4) |

AR: Anatomical resection; NAR: Non-anatomical resection; D: Dye injection method; G: Glissonean pedicle method; ICG R15: Indocyanine green retention rate at 15 min; B/W: Between; OS: Overall survival; RFS: Recurrence-free survival; NA: Not applicable.

patients with non-cirrhotic HCC but not in patients with cirrhotic HCC[25]. The nationwide survey in Japan revealed that AR was only beneficial in HCC patients whose tumor size within the range of 2 to 5 cm. All three case-controlled studies using the propensity score matching method revealed no benefit of AR.

### *Critical review of prior studies*

The prognosis of HCC is affected by the degree of cirrhosis and tumor stage. It is difficult to assess the degree of cirrhosis and tumor stage, and these classifications have changed over time. Therefore, survival analysis is difficult when considering the degree of cirrhosis and tumor stage. The type of surgical resection (*i.e.,* AR or NAR) may not affect the survival outcomes in cirrhotic patients. Furthermore, it is very difficult to determine whether disease recurrences are due to local recurrence, intrahepatic metastasis, or de novo multicentric recurrence. To determine the impact of AR, it is important to assess the pattern of recurrence, and local recurrence may be influenced by the surgical method. Therefore, in order to determine whether a specific surgical resection method has advantages on patient outcomes over another method, the endpoint should be recurrence-free survival rather than overall survival. However, many of the studies analyzed overall survival. It is also important to consider that there is a lot of bias, especially selection bias, in studies examining the benefits of AR.

First, the background liver functions of patients in both groups were significantly different in most of the studies shown in Table 1. Patients who underwent AR had better liver function in terms of cirrhotic status and/or the indocyanine green retention rate at 15 min, which closely reflects the degree of cirrhosis. Five studies assessed whether the recurrence pattern was local recurrence, intrahepatic metastasis, or multicentric recurrence. Only one study showed a significantly higher local recurrence rate in NAR than in AR; the other four studies did not find any differences in the recurrence pattern between the two groups[13,14,27,33,34]. If the background liver function was better in the AR group and the local recurrence rate was similar in the AR and NAR groups, how do we know that the survival benefit of AR was due to superiority of this resection rather than an effect of cirrhosis?

Second, all of the studies were conducted retrospectively, and the tumor size and T stage were not standardized between AR and NAR in most of the studies, although many studies were limited to patients with a solitary mass of less than 3-5 cm. In the nationwide survey in Japan, which comprised 5781 patients with a single HCC lesion, the overall and disease-free survival rates were significantly better for AR than for NAR. When the patients were stratified according to tumor size (< 2 cm, 2-5 cm, or > 5 cm), the disease-free survival rate was better in patients who underwent AR, but only in those with a tumor size of 2-5 cm. There was no benefit of AR in patients with tumors of < 2 cm or > 5 cm[31]. It seems reasonable that, in patients with small tumors (*i.e.,* < 2 cm), any type of surgery, even ablative therapy, is associated with favorable survival. By contrast, for larger tumors (*i.e.,* > 5 cm), survival is more likely to be affected by advanced tumor stage rather than the resection method. Therefore, the importance of AR should be emphasized in patients with a tumor of 2-5 cm in size. Most of the studies did not consider other factors likely to influence the short-term and long-term outcomes.

Finally, many other factors, including an anterior approach and perioperative transfusion, can influence the long-term survival after liver resection. During mobilization of the liver, the surgeon's or assistant's left hand may compress the liver, including the tumor. The degree of compression may affect intrahepatic metastasis differently, and its impact may also differ according to tumor location. This problem can be overcome by using an anterior approach or the hanging maneuver for standard right hepatectomy[39-41]. Unfortunately, this factor was not mentioned in most studies. Regarding transfusion, although the effects of perioperative transfusion on survival after hepatic resection for HCC are controversial, a meta-analysis of 22 studies revealed

that perioperative blood transfusion was associated with adverse clinical outcomes, including increased mortality, recurrence, and complication rates, but opposite findings were reported in other articles[42,43]. Extensive blood loss in cirrhotic patients, who were more likely to undergo NAR, might be associated with poor prognosis and this factor could represent a bias towards poor prognosis of NAR. A technique comprising low central venous pressure (LCVP) management combined with extrahepatic control of venous outflow enables the surgeon to easily control the hepatic veins before and during parenchymal transection. This LCVP technique combined with the Pringle maneuver reduced bleeding and blood transfusion, and improved the surgical outcomes. The hanging maneuver and LCVP approach was introduced in the late 1990s and its use widened in the early 2000s, a similar period of time over which most of the AR studies were conducted. As indicated in Table 1, although the amount of bleeding was not recorded in all of the studies, the available data are fairly high in the amount of bleeding. This may be possible because the study was performed under development of new surgical and anesthetic methods. Therefore, the changes over time in the surgical techniques, such as use of the hanging maneuver, and management by an anesthesiologist using a low CVP technique, may affect the recurrence rate after hepatectomy.

## CONCLUSION

AR in patients with HCC has a theoretical benefit in terms of improving recurrence-free survival, and this is partly observed in clinical practice. However, three recently published, well-designed, case-controlled studies using the propensity score matching method did not show an improvement in recurrence-free survival following AR. Studies examining the benefits of AR displayed considerable bias, including liver function, surgical techniques, anatomical variability, tumor size, tumor location, pathologic heterogeneity and chronology. Because prospective randomized studies are not possible for ethical reasons, it is difficult to reach a conclusion on the benefit of AR in HCC. However, the results of previous studies suggest that AR is associated with favorable perioperative and long-term outcomes in some conditions, including in patients with a tumor of 2-5 cm in size that is located in a deep region of the parenchyma.

## REFERENCES


1 **Petrick JL**, Braunlin M, Laversanne M, Valery PC, Bray F, McGlynn KA. International trends in liver cancer incidence, overall and by histologic subtype, 1978-2007. *Int J Cancer* 2016; **139**: 1534-1545 [PMID: 27244487 DOI: 10.1002/ijc.30211]

2 **Forner A**, Llovet JM, Bruix J. Hepatocellular carcinoma. *Lancet* 2012; **379**: 1245-1255 [PMID: 22353262 DOI: 10.1016/s0140-6736(11)61347-0]

3 **Belghiti J**, Kianmanesh R. Surgical treatment of hepatocellular carcinoma. *HPB* 2005; **7**: 42-49

4 **Clavien PA**, Selzner M, Rüdiger HA, Graf R, Kadry Z, Rousson V, Jochum W. A prospective randomized study in 100 consecutive patients undergoing major liver resection with versus without ischemic preconditioning. *Ann Surg* 2003; **238**: 843-850; discussion 851-852 [PMID: 14631221 DOI: 10.1097/01.sla.0000098620.27623.7d]

5 **Rüdiger HA**, Kang KJ, Sindram D, Riehle HM, Clavien PA. Comparison of ischemic preconditioning and intermittent and continuous inflow occlusion in the murine liver. *Ann Surg* 2002; **235**: 400-407 [PMID: 11882762]

6 **Yadav SS**, Sindram D, Perry DK, Clavien PA. Ischemic preconditioning protects the mouse liver by inhibition of apoptosis through a caspase-dependent pathway. *Hepatology* 1999; **30**: 1223-1231 [PMID: 10534344 DOI: 10.1002/hep.510300513]

7 **Regimbeau JM**, Kianmanesh R, Farges O, Dondero F, Sauvanet A, Belghiti J. Extent of liver resection influences the outcome in patients with cirrhosis and small hepatocellular carcinoma. *Surgery* 2002; **131**: 311-317 [PMID: 11894036]

8 **Makuuchi M**, Hasegawa H, Yamazaki S. Ultrasonically guided subsegmentectomy. *Surg Gynecol Obstet* 1985; **161**: 346-350 [PMID: 2996162]

9 **Makuuchi M**. Surgical treatment for HCC--special reference to anatomical resection. *Int J Surg* 2013; **11** Suppl 1: S47-S49 [PMID: 24380552 DOI: 10.1016/s1743-919160015-1]

10 **Makuuchi M**, Mori T, Gunvén P, Yamazaki S, Hasegawa H. Safety of hemihepatic vascular occlusion during resection of the liver. *Surg Gynecol Obstet* 1987; **164**: 155-158 [PMID: 3810429]

11 **Takasaki K**. Glissonean pedicle transection method for hepatic resection: a new concept of liver segmentation. *J Hepatobiliary Pancreat Surg* 1998; **5**: 286-291 [PMID: 9880776]

12 **Hasegawa K**, Kokudo N, Imamura H, Matsuyama Y, Aoki T, Minagawa M, Sano K, Sugawara Y, Takayama T, Makuuchi M. Prognostic impact of anatomic resection for hepatocellular carcinoma. *Ann Surg* 2005; **242**: 252-259 [PMID: 16041216]

13 **Kaibori M**, Matsui Y, Hijikawa T, Uchida Y, Kwon AH, Kamiyama Y. Comparison of limited and anatomic hepatic resection for hepatocellular carcinoma with hepatitis C. *Surgery* 2006; **139**: 385-394 [PMID: 16546504 DOI: 10.1016/j.surg.2005.08.035]

14 **Tanaka K**, Shimada H, Matsumoto C, Matsuo K, Nagano Y, Endo I, Togo S. Anatomic versus limited nonanatomic resection for solitary hepatocellular carcinoma. *Surgery* 2008; **143**: 607-615 [PMID: 18436008 DOI: 10.1016/j.surg.2008.01.006]

15 **Wakai T**, Shirai Y, Sakata J, Kaneko K, Cruz PV, Akazawa K, Hatakeyama K. Anatomic resection independently improves long-term survival in patients with T1-T2 hepatocellular carcinoma. *Ann Surg Oncol* 2007; **14**: 1356-1365 [PMID: 17252289 DOI: 10.1245/s10434-006-9318-z]

16 **Tajima T**, Honda H, Taguchi K, Asayama Y, Kuroiwa T, Yoshimitsu K, Irie H, Aibe H, Shimada M, Masuda K. Sequential hemodynamic change in hepatocellular carcinoma and dysplastic nodules: CT angiography and pathologic correlation. *AJR Am J Roentgenol* 2002; **178**: 885-897 [PMID: 11906868 DOI: 10.2214/ajr.178.4.1780885]

17 **IHPBA TCot**. Terminology of liver anatomy and resections. *HPB Surg* 2000; **2**: 333-339

18 **Yamamoto M**, Takasaki K, Ohtsubo T, Katsuragawa H, Fukuda C, Katagiri S. Effectiveness of systematized hepatectomy with Glisson's pedicle transection at the hepatic hilus for small nodular hepatocellular carcinoma: retrospective analysis. *Surgery* 2001; **130**: 443-448 [PMID: 11562668 DOI: 10.1067/msy.2001.116406]

19 **Xu W**, Wang HJ, Kim BW, Park YK, Li G. Anatomical Variation of the Glissonean Pedicle of the Right Liver. *Korean J Hepatobiliary Pancreat Surg* 2011; **15**: 101-106 [PMID: 26421024 DOI: 10.14701/kjhbps.2011.15.2.101]

20 **Cho A**, Okazumi S, Takayama W, Takeda A, Iwasaki K, Sasagawa S, Natsume T, Kono T, Kondo S, Ochiai T, Ryu M. Anatomy of the right anterosuperior area (segment 8) of the liver: evaluation with helical CT during arterial portography. *Radiology* 2000; **214**: 491-495 [PMID: 10671598 DOI: 10.1148/radiology.214.2.r00fe38

491]
21 **Kogure K**, Kuwano H, Fujimaki N, Ishikawa H, Takada K. Reproposal for Hjortsjo's segmental anatomy on the anterior segment in human liver. *Arch Surg* 2002; **137**: 1118-1124 [PMID: 12361415]
22 **Ahn KS**, Kang KJ, Park TJ, Kim YH, Lim TJ, Kwon JH. Benefit of systematic segmentectomy of the hepatocellular carcinoma: revisiting the dye injection method for various portal vein branches. *Ann Surg* 2013; **258**: 1014-1021 [PMID: 23478518 DOI: 10.1097/SLA.0b013e318281eda3]
23 **Makuuchi M**, Imamura H, Sugawara Y, Takayama T. Progress in surgical treatment of hepatocellular carcinoma. *Oncology* 2002; **62** Suppl 1: 74-81 [PMID: 11868790]
24 **Liau KH**, Blumgart LH, DeMatteo RP. Segment-oriented approach to liver resection. *Surg Clin North Am* 2004; **84**: 543-561 [PMID: 15062661 DOI: 10.1016/j.suc.2003.12.003]
25 **Yamashita Y**, Taketomi A, Itoh S, Kitagawa D, Kayashima H, Harimoto N, Tsujita E, Kuroda Y, Maehara Y. Longterm favorable results of limited hepatic resections for patients with hepatocellular carcinoma: 20 years of experience. *J Am Coll Surg* 2007; **205**: 19-26 [PMID: 17617328 DOI: 10.1016/j.jamcollsurg.2007.01.069]
26 **Yamazaki O**, Matsuyama M, Horii K, Kanazawa A, Shimizu S, Uenishi T, Ogawa M, Tamamori Y, Kawai S, Nakazawa K, Otani H, Murase J, Mikami S, Higaki I, Arimoto Y, Hanba H. Comparison of the outcomes between anatomical resection and limited resection for single hepatocellular carcinomas no larger than 5 cm in diameter: a single-center study. *J Hepatobiliary Pancreat Sci* 2010; **17**: 349-358 [PMID: 20464566 DOI: 10.1007/s00534-009-0253-9]
27 **Tomimaru Y**, Eguchi H, Marubashi S, Wada H, Kobayashi S, Tanemura M, Umeshita K, Doki Y, Mori M, Nagano H. Equivalent outcomes after anatomical and non-anatomical resection of small hepatocellular carcinoma in patients with preserved liver function. *Dig Dis Sci* 2012; **57**: 1942-1948 [PMID: 22407377 DOI: 10.1007/s10620-012-2114-7]
28 **Marubashi S**, Gotoh K, Akita H, Takahashi H, Ito Y, Yano M, Ishikawa O, Sakon M. Anatomical versus non-anatomical resection for hepatocellular carcinoma. *Br J Surg* 2015; **102**: 776-784 [PMID: 25847111 DOI: 10.1002/bjs.9815]
29 **Yamamoto T**, Yagi S, Kita R, Masui H, Kinoshita H, Sakamoto Y, Okada K, Miki A, Kondo M, Hashida H, Kobayashi H, Uryuhara K, Kaihara S, Hosotani R. Comparison between anatomical subsegmentectomy and nonanatomical partial resection for hepatocellular carcinoma located within a single subsegment: a single-center retrospective analysis. *Hepatogastroenterology* 2015; **62**: 363-367 [PMID: 25916064]
30 **Capussotti L**, Muratore A, Amisano M, Polastri R, Bouzari H, Massucco P. Liver resection for hepatocellular carcinoma on cirrhosis: analysis of mortality, morbidity and survival--a European single center experience. *Eur J Surg Oncol* 2005; **31**: 986-993 [PMID: 15936169 DOI: 10.1016/j.ejso.2005.04.002]
31 **Eguchi S**, Kanematsu T, Arii S, Okazaki M, Okita K, Omata M, Ikai I, Kudo M, Kojiro M, Makuuchi M, Monden M, Matsuyama Y, Nakanuma Y, Takayasu K. Comparison of the outcomes between an anatomical subsegmentectomy and a non-anatomical minor hepatectomy for single hepatocellular carcinomas based on a Japanese nationwide survey. *Surgery* 2008; **143**: 469-475 [PMID: 18374043 DOI: 10.1016/j.surg.2007.12.003]
32 **Ishii M**, Mizuguchi T, Kawamoto M, Meguro M, Ota S, Nishidate T, Okita K, Kimura Y, Hui TT, Hirata K. Propensity score analysis demonstrated the prognostic advantage of anatomical liver resection in hepatocellular carcinoma. *World J Gastroenterol* 2014; **20**: 3335-3342 [PMID: 24696614 DOI: 10.3748/wjg.v20.i12.3335]
33 **Kobayashi A**, Miyagawa S, Miwa S, Nakata T. Prognostic impact of anatomical resection on early and late intrahepatic recurrence in patients with hepatocellular carcinoma. *J Hepatobiliary Pancreat Surg* 2008; **15**: 515-521 [PMID: 18836806 DOI: 10.1007/s00534-007-1293-7]
34 **Marubashi S**, Gotoh K, Akita H, Takahashi H, Sugimura K, Miyoshi N, Motoori M, Kishi K, Noura S, Fujiwara Y, Ohue M, Nakazawa T, Nakanishi K, Ito Y, Yano M, Ishikawa O, Sakon M. Analysis of Recurrence Patterns After Anatomical or Non-anatomical Resection for Hepatocellular Carcinoma. *Ann Surg Oncol* 2015; **22**: 2243-2252 [PMID: 25373536 DOI: 10.1245/s10434-014-4214-4]
35 **Okamura Y**, Ito T, Sugiura T, Mori K, Uesaka K. Anatomic versus nonanatomic hepatectomy for a solitary hepatocellular carcinoma: a case-controlled study with propensity score matching. *J Gastrointest Surg* 2014; **18**: 1994-2002 [PMID: 25213582 DOI: 10.1007/s11605-014-2646-6]
36 **Portolani N**, Coniglio A, Ghidoni S, Giovanelli M, Benetti A, Tiberio GA, Giulini SM. Early and late recurrence after liver resection for hepatocellular carcinoma: prognostic and therapeutic implications. *Ann Surg* 2006; **243**: 229-235 [PMID: 16432356 DOI: 10.1097/01.sla.0000197706.21803.a1]
37 **Ueno S**, Kubo F, Sakoda M, Hiwatashi K, Tateno T, Mataki Y, Maemura K, Shinchi H, Natsugoe S, Aikou T. Efficacy of anatomic resection vs nonanatomic resection for small nodular hepatocellular carcinoma based on gross classification. *J Hepatobiliary Pancreat Surg* 2008; **15**: 493-500 [PMID: 18836803 DOI: 10.1007/s00534-007-1312-8]
38 **Cho YB**, Lee KU, Lee HW, Cho EH, Yang SH, Cho JY, Yi NJ, Suh KS. Anatomic versus non-anatomic resection for small single hepatocellular carcinomas. *Hepatogastroenterology* 2007; **54**: 1766-1769 [PMID: 18019714]
39 **Lai EC**, Fan ST, Lo CM, Chu KM, Liu CL. Anterior approach for difficult major right hepatectomy. *World J Surg* 1996; **20**: 314-317; discussion 318 [PMID: 8661837]
40 **Liu CL**, Fan ST, Lo CM, Tung-Ping Poon R, Wong J. Anterior approach for major right hepatic resection for large hepatocellular carcinoma. *Ann Surg* 2000; **232**: 25-31 [PMID: 10862191]
41 **Belghiti J**, Guevara OA, Noun R, Saldinger PF, Kianmanesh R. Liver hanging maneuver: a safe approach to right hepatectomy without liver mobilization. *J Am Coll Surg* 2001; **193**: 109-111 [PMID: 11442247]
42 **Yang T**, Lu JH, Lau WY, Zhang TY, Zhang H, Shen YN, Alshebeeb K, Wu MC, Schwartz M, Shen F. Perioperative blood transfusion does not influence recurrence-free and overall survivals after curative resection for hepatocellular carcinoma: A Propensity Score Matching Analysis. *J Hepatol* 2016; **64**: 583-593 [PMID: 26596543 DOI: 10.1016/j.jhep.2015.10.012]
43 **Wei AC**, Tung-Ping Poon R, Fan ST, Wong J. Risk factors for perioperative morbidity and mortality after extended hepatectomy for hepatocellular carcinoma. *Br J Surg* 2003; **90**: 33-41 [PMID: 12520572 DOI: 10.1002/bjs.4018]

**P- Reviewer**: Lam V, Perini MV, Santambrogio R
**S- Editor**: Gong ZM **L- Editor**: O'Neill M **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/ *World J Gastroenterol* 2017 February 21; 23(7): 1147-1162

DOI: 10.3748/wjg.v23.i7.1147 ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

# Investigation of mitigating effect of colon-specific prodrugs of boswellic acid on 2,4,6-trinitrobenzene sulfonic acid-induced colitis in Wistar rats: Design, kinetics and biological evaluation

Ajinkya Sarkate, Suneela S Dhaneshwar

Ajinkya Sarkate, Suneela S Dhaneshwar, Department of Pharmaceutical Chemistry, Poona College of Pharmacy, Bharati Vidyapeeth University, Maharashtra 411038, India

Author contributions: Sarkate A and Dhaneshwar SS substantially contributed to the conception and design of the study, acquisition, analysis and interpretation of data; both 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.

Institutional review board statement: No human subjects were used in this study.

Institutional animal care and use committee statement: All animal experimentation was approved by the Institutional Animal Ethics Committee (IAEC-approval number: CPCSEA/PCH/10/2014-15) of Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Erandwane, Pune- 411038, India.

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

Data sharing statement: There are no additional data available in relation to this manuscript.



Manuscript source: Invited manuscript

Correspondence to: Suneela S Dhaneshwar, PhD, Professor, Department of Pharmaceutical Chemistry, Poona College of Pharmacy, Bharati Vidyapeeth University, Erandwane, Pune, Maharashtra 411038, India. suneeladhaneshwar@rediffmail.com
Telephone: +91-20-25437237
Fax: +91-20-25439382

Received: October 14, 2016
Peer-review started: October 14, 2016
First decision: December 1, 2016
Revised: December 16, 2016
Accepted: January 4, 2017
Article in press: January 4, 2017
Published online: February 21, 2017

## Abstract

### *AIM*

To develop a colon-targeting bioreversible delivery system for β-boswellic acid (BBA) and explore utility of its prodrugs in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats.

### *METHODS*

Synthesis of 4 co-drugs of BBA with essential amino acids was achieved by CDI coupling, followed by their spectral characterization. *In vitro* kinetics were studied by HPLC in aqueous buffers, homogenates of gastrointestinal tract and fecal matter. *In vivo* kinetic studies were performed in Wistar rat plasma, urine and feces. The prodrugs were screened in TNBS-induced colitis modeled Wistar rats. Statistical significance was assumed at $P < 0.05$, $P < 0.01$, $P < 0.001$ when compared with disease controls using one-way and

two-way ANOVAs.

***RESULTS***

Prodrugs were stable in 0.05 mol/L HCl buffer (pH 1.2) and stomach homogenates. Negligible hydrolysis was observed in phosphate buffer and intestinal homogenates. Substantial release (55%-72% and 68%-86%) of BBA was achieved in rat fecal matter and homogenates of colon. *In vivo* studies of BBA with L-tryptophan (BT) authenticated colon-specific release of BBA. But, surprisingly substantial concentration of BBA was seen to reach the systemic circulation due to probable absorption through colonic mucosa. Site-specifically enhanced bioavailability of BBA could be achieved in colon, which resulted in demonstration of significant mitigating effect on TNBS-induced colitis in rats without inducing any adverse effects on stomach, liver and pancreas. Prodrug of BT was found to be 1.7% ($P < 0.001$) superior than sulfasalazine in reducing the inflammation to colon among all prodrugs tested.

***CONCLUSION***

The outcome of this study strongly suggests that these prodrugs might have dual applicability to inflammatory bowel disease and chronotherapy of rheumatoid arthritis.

**Key words:** Inflammatory bowel disease; Boswellic acid; Complementary and alternative medicine; Colon-targeting; Mutual prodrugs; Amino acids; TNBS-induced colitis



**Core tip:** Boswellic acids (BAs) are traditionally used in the treatment of inflammatory diseases and are effective in the treatment of inflammatory bowel disease (IBD) in particular, but they undergo extensive metabolism that results in low oral bioavailability. Colon-targeted delivery of BA was achieved by designing prodrugs that deliver BA site-specifically. The synthesized prodrugs were designed by semi-synthetic approach, wherein β-boswellic acid (BBA) was derivatized into a bioreversible delivery system by incorporation of amino acids as promoities for targeted delivery to inflamed colon in IBD. Prodrug of BBA with L-tryptophan (BT) was 1.7-times more effective than sulfasalazine (SLZ) in 2,4,6-trinitrobenzene sulfonic acid-induced colitis in Wistar rats. *In vivo* behavior of prodrug BT was very interesting and similar to SLZ, which is known to treat local inflammation in IBD as well as in rheumatoid arthritis (RA). The outcome of this study strongly suggests that these prodrugs might have dual applicability to IBD and chronotherapy of RA.

Sarkate A, Dhaneshwar SS. Investigation of mitigating effect of colon-specific prodrugs of boswellic acid on 2,4,6-trinitrobenzene sulfonic acid-induced colitis in Wistar rats: Design, kinetics and biological evaluation. *World J Gastroenterol* 2017; 23(7): 1147-1162 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1147.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1147

## INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are together known as inflammatory bowel disease (IBD). IBD is a chronic inflammatory state, comprised of relapsing inflammation of the gastrointestinal tract (GIT) with unknown etiology and which lasts throughout the lifetime[1-3]. It is estimated that about 1.4 million people in the United States, as well as 2.2 million people in Europe, are affected by IBD[4]. In CD, the inflammation is characteristically discontinuous, transmural and often granulomatous, and can occur anywhere in the GIT, from the mouth to the anus[3,5]. UC is limited to the colon and usually affects the superficial layers of the intestinal wall. The onset of IBD may be a result of complex and elusive interactions between genetic alterations in intestinal barrier function, inherent apoptosis, signal transduction, and immunological and environmental factors[1,6].

5-aminosalicylate (5-ASA) and corticosteroids are used as first-line therapy of IBD[7]. Azathioprine, 6-mercaptopurine, methotrexate, calcineurin inhibitors and anti-TNF-α-antibodies have an important role in the treatment of severe disease stages[8]. Minimizing drug-induced side effects and mortality are the main challenges during management of IBD. Sulfasalazine (SLZ) was the first colon-targeting prodrug of 5-ASA and sulfapyridine, representing a cornerstone of IBD therapy. Thirty percent of patients taking SLZ suffer from serious adverse effects, such as hypersensitivity, nephritis, pancreatitis, hepatitis, pneumonitis, drug-induced blood disorders and male infertility. Corticosteroid therapy induces side effects such as hypertension, hyperglycemia, osteoporosis, glaucoma, depression and development of cataracts[9]. Treatment with immunosuppressive agents is linked with an increased susceptibility to infections, malignoma, increased vulnerability for opportunistic infections and GI complaints[2,10-12]. It has been observed that use of complementary and alternative medicine is on the rise generally, and particularly for herbal medicine for IBD management.

The gum resin of *Boswellia serrata* (*Bs*) Roxb. Ex Colebr. (Family *Burseraceae, Syn. B. glabra*) is a traditional Ayurvedic remedy with anti-inflammatory properties and has become popular in Western countries for its usefulness in treatment of IBD[13-15]. It was in the latter part of the 20th century that the resin received scientific interest as an anti-inflammatory phytomedicine, and extracts of the resin have been applied to treat a variety of chronic inflammatory and autoimmune diseases[16]. Boswellic acids (BAs) are

chemically a mixture of triterpenic acids obtained from the oleo gum resin of BS and consist of β-boswellic acid (BBA), acetyl-β-boswellic acid (ABA), 11-keto-β-boswellic acid (KBA) and acetyl-11-keto-β-boswellic acid (AKBA). BAs have been studied extensively for anti-inflammatory, immunomodulatory and anti-tumor activities. They help to preserve the structural integrity of joint cartilage, promote gastrointestinal health and maintain a healthy immune mediator cascade at the cellular level[17-28].

BAs are non-redox, non-competitive inhibitors of 5-lipoxygenase (5-LOX), human leukocyte elastase (HLE) and the nuclear factor-κB pathway, without exerting the adverse effects known for steroids[17,19,29]. The inhibition of leukotrienes is the primary and the most scientifically proven mechanism for anti-inflammatory and anti-arthritic activity of BAs[17,30]. The leukotrienes are also involved in the pathogenesis of IBD. Gerhardt *et al*[31] reported that alcoholic extract of BS oleo gum resin improved the quantifying parameters of UC in 34 patients. In several clinical studies, extracts of oleo gum resins of BS appeared to be effective in the treatment of chronic bowel diseases and the effects were comparable with the conventional treatment. The non-steroidal anti-inflammatory drug-induced GIT side effects were not observed with *BS*. The oleo gum resin has proven to be effective in dextran-induced and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis models in rodents. The *in vitro* assays, animal studies and numerous clinical trials have established the efficacy of *BS* and formulations containing BAs to be effective in the treatment of IBD[16,32]. The results obtained from the studies of BA were found to be comparable with that of standard marketed drugs SLZ and mesalazine for the treatment of CD and UC, with risk-benefit analysis findings being in favor of BAs[24,31]. BAs are reported to inhibit the intestinal motility and reduce chemically-induced edema and inflammation in intestine in rodents[33].

It has been reported that BAs improve the clinical well-being of patients with IBD. Treatment with this herbal drug is associated with improvement in a number of parameters, such as stool properties, microscopic findings of rectal biopsies, hemoglobin and other blood parameters, serum iron, calcium, phosphorus, proteins, total leukocytes and eosinophils, as well as the CD activity index[24,31,34]. BAs reduce lipid peroxidation and increase the levels of superoxide dismutase, thus ameliorating the oxidative stress associated with intestinal inflammation[17,18,35]. A clinical study conducted on patients with IBD has shown that BA reduces mucosal injury by inhibiting activity of activated leucocytes as well as their adherence to intestinal mucosal cells[36,37].

Borrelli *et al*[38] evaluated the effect of a *Bs* gum resin extract (BSE) on intestinal motility and diarrhea in a clinical study in rodents. BSE depressed electrically acetylcholine and barium chloride-induced contractions in the isolated guinea-pig ileum. BSE also prevented diarrhea and normalized intestinal motility in pathophysiological states without slowing the rate of transit in control animals. These results explain the clinical efficacy of BSE in reducing diarrhea in IBD patients.

In a study using BA in chronic UC patients, its efficacy on UC with minimal side effects was confirmed[24] and it produced an 82% remission rate, compared to 75% of those in the SLZ group. Thus, BA offered improvement of UC symptoms similar to that of SLZ[24,39]. Similar findings were reported in patients with CD[31]. As a result of the clinically established symptomatic improvement of IBD symptoms by treatment with BA, it is now considered as a potential therapeutic agent for IBD therapy[38]. Gupta *et al*[39] have investigated the treatment options for UC. In grade Ⅱ and grade Ⅲ colitis patients, the positive effect of BS has also been observed.

Bioavailability is a major hurdle in the conversion of the pre-clinical potential of many botanical extracts into remedial effects, especially for those whose active ingredients show poor water solubility and strong affinity towards self-aggregation. This is observed for many polyphenolics and triterpenoid acids[14]. Pharmacokinetic studies have demonstrated that the systemic absorption of BAs is very low, both in animals and in humans, and extensive metabolism could play a vital role in limiting their systemic availability[14,19]. A United States' patent has utilized the extract of the gum resin of BS in a phytonutrient formulation for the relief of chronic pain resulting from inflammation[40]. A semi-synthetic form of AKBA significantly reduced the disease activity in experimental murine colitis induced by dextran sodium sulfate (DSS)[32].

For the present work, BBA was selected as the drug candidate for developing its colon-targeting prodrugs because it is present in the highest percentage in the oleo gum resin and shows prominent anti-inflammatory activity[15,16,20-25]. Bioavailability of BAs at the site of action, *i.e.* colon, can be improved by enhancing hydrophilicity through designing colon-targeting prodrugs where the selected carriers will impart more hydrophilicity to the BAs so that their systemic absorption is minimized and they reach an effective concentration level in the colon. A mutual prodrug strategy was explored by conjugating BBA with various amino acids into amide co-drugs that would undergo colon-specific activation by N-acyl amidases[41]. This would ensure attainment of effective concentration of BA in the colon for its local mitigating effect on colonic inflammation.

The selection of 4 amino acids, namely L-tryptophan, L-histidine, D-phenylalanine and L-tyrosine, as carriers for designing mutual prodrugs of BBA was based on the recent evidence that has indicated that amino

acids may play an important role in maintaining gut health, modulate intestinal immune functions and influence inflammatory responses, and may be useful as alternative or ancillary treatment of IBD. The role of amino acids in reducing inflammation, oxidative stress and apoptosis in the gut is well documented in the literature[42,43]. L-tryptophan has been shown to reduce oxidative stress and immune suppression. It was also reported to decrease pro-inflammatory cytokine expression, thereby inhibiting Th1-mediated inflammation in DSS-induced porcine IBD[44]. Histidine significantly inhibited both hydrogen peroxide- and TNF-α-induced IL-8 secretion and mRNA expression in Caco-2 cells and HT-29 cells. Dysregulation of these cytokines' balance plays a key role in the pathogenesis of IBD[45,46]. D-phenylalanine restores gut immune homeostasis by attenuating inflammatory responses[47,48]. D-phenylalanine, L-tryptophan and L-tyrosine were chosen as promoieties due to their marked anti-inflammatory activity[43,46,49,50]. Being the natural components of our body, these amino acids would be nontoxic and free from any side effects. N-aromatic acyl amino acid conjugates reported in the literature are stable in upper intestine and hydrolyzed when incubated with mammalian cecal content[51]. Introduction of amide linkage in the prodrug would increase aqueous solubility, thus transcellular absorption by lipid membrane permeation might be limited in the upper GIT. This would be expected to facilitate delivery of intact prodrug to the colon.

## MATERIALS AND METHODS

### Materials

BA was purchased from Herbal Remedies Corp. (Bangalore, India). All reagents and chemicals used were of analytical reagent grade. Microwave (MW)-assisted synthesis was performed on the MW synthesizer (Discover System; CEM Corp., Matthews, NC, United States). Infra-red (FTIR) spectra were recorded using KBr on a Jasco V-530 FT/IR-4100 spectrophotometer. $^{1}$H-NMR and $^{13}$C-NMR spectra were recorded on a Bruker Avance II 400 instrument at 400 MHz. Chemical shifts (δ) are given in ppm using TMS as an internal reference. Mass spectra were recorded on an Agilent 1260 Infinity HPLC-MASS Analyzer 6460 Triple Quad LC/MS. Elemental analysis was performed using Vario Micro Cube (Elementar, Germany). The HPLC system consisted of Jasco PU model 2080, with UV detector. Thermo Scientific Syncronis C18 column (250 mm × 4.6 mm) was used for estimation of prodrugs and their active metabolites.

### Synthesis of title compounds

The title prodrugs were synthesized by CDI coupling[52] and purified by column chromatography or preparative TLC. The products were characterized by spectral and elemental analyses.

### MW-assisted synthesis of amino acid methyl ester hydrochloride

A mixture of amino acid (0.1 mol) and freshly distilled thionyl chloride (0.05 mol) was placed in a MW-safe glass vial. To this, methanol (5 mL) was added and the resulting solution was irradiated with 40 W, 150 psi at 65 ℃ for 9-15 min in the MW synthesizer. The reaction mixture was cooled at room temperature to give a clear solution and then concentrated on a rotary evaporator to yield amino acid methyl ester hydrochloride (AAME. HCl) and recrystallized with methanol[46]. TEA (0.05 mol) was added to a suspension of AAME. HCl (0.025 mol) in 30 mL chloroform at 0 ℃ and stirred for 30 min. The solvent was distilled off under vacuum and the dry residue of ester (AAME) was recrystallized with methanol.

### Synthesis of mutual amide prodrugs of BBA with amino acids by CDI coupling

BBA (0.001 mol) was dissolved in DCM (10 mL), and to this solution CDI (0.0015 mol) was added at room temperature with stirring for 2-4 h. AAME (0.001 mol) in DCM (10 mL) was then added to the above solution and refluxed at 45 ℃ for 16-20 h. The completion of reaction was monitored by TLC using DCM:n-hexane: TEA (0.8:0.2:0.05 v/v/v). The reaction mixture was washed with distilled water (3 × 10 mL) and saturated solution of sodium bicarbonate (2 × 10 mL). The organic layer was separated and dried over anhydrous sodium sulfate. The residue obtained upon evaporation of the organic layer was recrystallized with ethanol. Purification of prodrugs of BBA with L-tryptophan, D-phenylalanine and L-tyrosine was achieved by column chromatography silica gel (mesh size: 60-120, for column chromatography packed in n-hexane; Merck) using ethyl acetate:hexane (80:20 v/v), while for prodrug of BBA with L-histidine preparative TLC was used (DCM:n-hexane:TEA; 80:15:5 v/v/v) for purification.

### Amide prodrug of BBA and L-histidine

Methyl 2-(3-hydroxy-4,6b,8a,11,12,14a,14b heptamethyl 1, 2, 3, 4, 4a, 5, 6, 6a, 6b, 7, 8, 8a, 9, 10, 11, 12, 12a, 14, 14a, 14b-icosahydropicene-4-carboxamido)-3-(4*H*-imidazol-4-yl)propanoate yield: 61% (solid); M.P 84-88 ℃ FTIR (KBr) cm$^{-1}$: 3554 (OH str), 3327 (NH str), 3102 (aliphatic CH), 1737 (C=O str. ester), 1666 (C=O str. amide); 1H NMR (400 MHz, DMSO, δ ppm): 8.2 [s, 1H] NH, 7.3 [d, 2H] imidazole ring, 5.1 [t, 1H] CH=C, 4.4 [t, 1H] CH, 3.9 [s, 1H] OH, 3.6 [s, 3H] $CH_3$, 3.3 [t, 2H] $CH_2$, 2.2-1.1 [m, 25H] methylenes and methines of BA, 1.1-0.6 [m, 21H] methyls of BA. $^{13}$C NMR (400 MHz, $CDCl_3$, δ ppm): 15.5, 17.0, 18.2, 19.9, 20.1, 21.2, 23.4, 23.7, 26.8, 26.9, 27.0, 27.2, 29.0, 32.6, 33.0, 33.9, 37.8, 39.2, 39.3, 39.4, 40.1, 40.4, 41.3, 42.3, 44.0, 47.9, 51.4, 51.8, 52.0, 59.5, 76.7, 116.8, 144.4, 153.8, 163.8, 172.2, 172.8 Mass: m/z 608.87 (M + 1). Elemental analysis: Calculated for $C_{37}H_{57}N_3O_4$: C, 73.11; H,

9.45; N, 6.91. Found: C, 73.23; H, 9.52; N, 6.98. Aq. Solubility: 29.45 mg/mL; log $P_{oct}$: 5.1

### *Amide prodrug of BBA and D-phenylalanine*

Methyl 2-(3-hydroxy-4, 6b, 8a,11, 12, 14a, 14b-heptamethyl 1, 2, 3, 4, 4a, 5, 6, 6a, 6b, 7, 8, 8a, 9, 10, 11, 12, 12a, 14, 14a, 14b-icosahydropicene-4-carboxamido)-3-phenylpropanoate Yield: 65% (solid); M.P 72-76 ℃. FTIR (KBr) $cm^{-1}$: 3554 (OH str), 3337 (NH str), 3034 (aliphatic CH), 1748 (C=O str. ester), 1613 (C=O str. amide); 1H NMR (400 MHz, DMSO, δ ppm): 8.2 [1H] NH, 7.3 [d, 2H] Ar. CH, 7.2 [d, 2H] Ar. CH, 7.02 [t, 1H], Ar. CH, 5.19 [t, 1H] CH=C, 4.5 [t, 1H] CH, 4.0 [s, 1H] OH, 3.6 [s, 3H] $CH_3$, 3.3 [t, 1H] CH, 3.2 [d, 2H] $CH_2$, 2.2-1.2 [m, 23H] methylenes and methines of BA, 1.2-0.7 [m, 21H] methyls of BA. $^{13}C$ NMR (400 MHz, $CDCl_3$, δ ppm): 15.5, 17.0, 18.2, 19.9, 20.1, 21.2, 23.4, 23.7, 26.8, 26.9, 27.0, 27.3, 29.0, 32.6, 33.9, 36.8, 37.8, 39.2, 39.3, 39.5, 40.1, 40.4, 41.3, 42.4, 44.0, 47.9, 51.9, 57.3, 59.2, 76.8, 116.5, 125.9, 127.7, 127.9, 128.6, 128.7, 136.6, 144.1, 171.6, 172.1; Mass: m/z 617.90 ($M^+$ ion). Elemental analysis: Calculated for $C_{40}H_{59}NO_4$: C, 77.75; H, 9.62; N, 2.27. Found: C, 77.82; H, 9.66; N, 2.28. Aq. Solubility: 21.08 mg/mL; log $P_{oct}$: 5.7.

### *Amide prodrug of BBA and L-tryptophan*

Methyl 2-(3-hydroxy-4, 6b, 8a, 11, 12, 14a, 14b-heptamethyl-1, 2, 3, 4, 4a, 5, 6, 6a, 6b, 7, 8, 8a, 9, 10, 11, 12, 12a, 14, 14a, 14b-icosahydropicene-4-carboxamido)-3-(1*H*-indol-3-yl)propanoate. Yield: 68% (solid); M.P 90-94 ℃. FTIR (KBr) $cm^{-1}$: 3543 (OH str), 3330 (NH str), 2866 (aliphatic CH), 1735 (C=O str. ester), 1653 (C=O str. amide); 1H NMR (400 MHz, DMSO, δ ppm): 8.2 [s, 1H] NH, 7.4 [d, 1H] Ar. CH, 7.3 [d, 1H] Ar. CH, 7.08 [s, 1H] Ar. CH, 7.02 [t, 1H] Ar. CH, 6.9 [t, 1H] Ar. CH, 5.2 [t, 1H] CH=C, 4.6 [t, 1H] CH, 4.1 [s, 1H] OH, 3.6 [s, 3H] $CH_3$, 3.32 [t, 1H] CH, 3.03 [d, 2H] $CH_2$, 2.1-1.3 [m, 23H] methylenes and methines of BA, 1.3-0.7 [m, 21H] methyls of BA. $^{13}C$ NMR (400 MHz, $CDCl_3$, δ ppm): 15.5, 17.0, 18.2, 19.9, 20.1, 21.2, 23.4, 23.7, 26.8, 26.9, 27.0, 27.2, 28.5, 29.0, 32.6, 33.9, 37.8, 39.2, 39.3, 39.4, 40.1, 40.4, 41.3, 42.3, 44.0, 47.9, 51.9, 58.4, 59.2, 76.8, 109.8, 111.1, 116.5, 118.8, 119.9, 121.8, 123.0, 127.5, 136.6, 144.2, 171.6, 172.2; Mass: m/z 656.94 ($M^+$ ion); Elemental analysis: Calculated for $C_{42}H_{60}N_2O_4$: C, 76.79; H, 9.21; N, 4.26. Found: C, 76.84; H, 9.25; N, 4.31. Aq. Solubility: 25.22 mg/mL; log $P_{oct}$: 4.1.

### *Amide prodrug of BBA and L-tyrosine*

Methyl 2-(3-hydroxy-4, 6b, 8a, 11, 12, 14a, 14b-heptamethyl-1, 2, 3, 4, 4a, 5, 6, 6a, 6b, 7, 8, 8a, 9, 10, 11, 12, 12a, 14, 14a, 14b-icosahydropicene-4-carboxamido)-3-(4-hydroxyphenyl)propanoate. Yield: 45% (solid); M.P 82-86 ℃. FTIR (KBr) $cm^{-1}$: 3547 (OH str), 3334 (NH str), 2856 (aliphatic CH), 1745 (C=O str. ester), 1691 (C=O str. amide); 1H NMR (400 MHz, DMSO, δ ppm): 8.2 [s, 1H] NH, 6.9 [d, 2H] CH, 6.7 [d, 2H] CH, 5.1 [t, 1H] CH=C, 4.5 [t, 1H] CH, 4.0 [s, 1H] OH, 3.6 [s, 3H] $CH_3$, 3.3 [t, 1H] CH, 3.1 [d, 2H] $CH_2$, 2.2-1.2 [m, 23H] methylenes and methines of BA, 1.2-0.6 [m, 21H] methyls of BA. $^{13}C$ NMR (400 MHz, $CDCl_3$, δ ppm): 15.5, 17.0, 18.2, 19.9, 20.1, 21.2, 23.4, 23.7, 26.8, 26.9, 27.0, 27.1, 29.0, 32.6, 33.9, 36.8, 37.8, 39.2, 39.3, 39.4, 40.1, 40.4, 41.3, 42.3, 44.0, 47.9, 51.9, 57.3, 59.2, 76.8, 115.7, 115.8, 116.5, 129.2, 130.1, 130.2, 144.1, 155.7, 171.5, 172.2; Mass: m/z 633.91 ($M^+$ ion); Elemental analysis: Calculated for $C_{40}H_{59}NO_5$: C, 75.79; H, 9.38; N, 2.21. Found: C, 75.83; H, 9.40; N, 2.24. Aq. Solubility: 15.22 mg/mL; log $P_{oct}$: 6.3.

### *Partition coefficient and aqueous solubility*

Partition coefficients of BBA and its prodrugs were determined in n-octanol/water system using shake flask method, whereas the aqueous solubility was determined in distilled water at 25 ± 1 ℃. Estimation was carried out on UV-visible double beam spectrophotometer at predetermined $\lambda_{max}$ values.

### *Stability studies of prodrugs in aqueous buffers*

Stability studies of prodrugs were carried out in 0.05 M HCl and phosphate buffers prepared as per IP 2007[53] (pH 1.2 and 7.4 respectively). Prodrugs in the presence of their hydrolyzed products were simultaneously estimated by RP-HPLC method using acetonitrile: water:methanol (70:25:5; v/v/v) at a flow rate of 0.5 mL/min with UV estimation wavelength of 215 nm. All-glass double-distilled water was used throughout the kinetic studies. Before analysis, the mobile phase was degassed using sonicator and filtered through a 0.45 μm membrane filter. Sample solutions were also filtered through the same. Calibration curves of prodrugs and BBA were constructed in HCl and phosphate buffers in the range of 10-100 μg/mL. Prodrug (10 mg) was introduced in 100 mL of HCl or phosphate buffer in a beaker kept in a constant temperature bath at 37 ± 1 ℃, with occasional stirring. Aliquots (5 mL) were withdrawn and replaced with the fresh aqueous buffer at regular intervals of 15 min at the 1st hour and after every 30 min up to 3 h for HCl buffer and 4 h for phosphate buffer. Samples (20 μL) reconstituted with mobile phase were injected in the column. The equations generated from calibration curves were used to calculate the concentration of BBA and prodrugs. Samples were analyzed in triplicate and methods were validated as per International Conference on 1 Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines.

### *In vitro kinetics in tissue homogenates of Wistar rats*

Wistar rats were sacrificed to remove stomach, small intestine and colon samples, which were homogenized in HCl or phosphate buffer. A stock solution of prodrug

(100 μg/mL) was prepared in respective buffers. To each test tube, 0.8 mL of prodrug solution was placed and 0.2 g of stomach or small intestinal homogenate was added. In the case of colon homogenates, 0.9 mL of prodrug solution and 0.1 mL of homogenate were added. The tubes containing prodrugs and homogenates of stomach, small intestine and colon were incubated at 37 ± 1 ℃ for 3, 4 and 12 h respectively. The samples were removed from the incubator at predetermined time intervals, centrifuged at 10000 rpm, filtered through 0.45 μm membrane filter and analyzed by HPLC using the same mobile phase, flow rate and detection wavelength as mentioned in the above section.

### *In vitro kinetics in fecal matter*

Fresh rat fecal matter suspended in phosphate buffer (0.1 mL) and prodrug solution (0.9 mL) were placed in test tubes and incubated at 37 ± 1 ℃. The samples were removed from the incubator at predetermined time intervals over a period of 12 h and the same procedure was followed for analysis as mentioned in the above section.

### *In vivo studies*

BBA and L-tryptophan (BT) was selected as a representative of the four synthesized prodrugs to investigate the *in vivo* behavior, which was then compared with standard BBA. Male Wistar rats (200-250 g; $n$ = 6) were housed in metabolic cages, individually, under normal conditions (at 27 ± 0.5 ℃ and relative humidity of 70% ± 0.5% under natural light/dark conditions). The same HPLC system, column and mobile phase were used for this purpose as mentioned in the above section. All the kinetic studies were carried out in triplicate. Rats were starved for 24 h prior to experimentation and administered water *ad libitum*. Blood (0.5 mL) was withdrawn by retro-orbital puncture and the reading was considered as the 0 min reading. BT and BBA (377.5 mg and 270 mg respectively) were suspended separately in sodium CMC solution prepared in physiological saline (1 mL) and were orally administered to the animals. Blood samples were collected in EDTA-coated tubes at an interval of 15 min for the first 1 h. Then, subsequent blood collection was made on a bi-hourly basis up to the 12$^{th}$ h and finally at the 24$^{th}$ h. Blood samples were centrifuged (2 cycles of 15 min each at 5000 rpm, 0-4 ℃). Supernatant was filtered through 0.45 μm membrane filter, reconstituted with methanol (0.5 mL), centrifuged and filtered again. Calibration curves generated in 80% human plasma were used to calculate the concentration of released BBA and intact prodrugs in blood, and the samples were then analyzed by HPLC. Urine and feces of the prodrug-treated rats were collected from the metabolic cages at different time intervals over a period of 24 h and then pooled together, diluted with HCl buffer (pH 1.2) and phosphate buffer (pH 7.4) by 10-fold and 100-fold respectively, and then centrifuged at 5000 rpm at 0-5 ℃ for 10 min. Supernatant of the centrifuged solutions of urine and feces (0.1 mL) were added to eppendorf tube (1 mL capacity) and 0.9 mL methanol was added. All the solutions were then vortexed for 2 min, re-centrifuged at 5000 rpm for 10 min at 0-5 ℃ (in order to precipitate other impurities), and analyzed by HPLC using the procedure described above to study the excretion pattern of prodrugs. The equations generated from the calibration curves in HCl buffer (pH 1.2) and phosphate buffers (pH 7.4) were used to calculate the concentrations of released components in urine and feces respectively.

### *Biological evaluation*

**Materials:** The Institutional Animal Ethical Committee approved the experimental protocols (CPCSEA/PCH/10/2014-15), and all protocols were followed for pharmacological screening of synthesized prodrugs. The activity was performed at the CPCSEA-approved animal facilities of Poona College of Pharmacy (Pune, India). Wistar rats (males, 200-230 g) were procured from the National Institute of Biosciences (Pune, India). Animals were divided into 16 groups ($n$ = 6 each). TNBS was purchased from Sigma Chemicals (United States). Gastric ulcers were analyzed using Adobe Photoshop and ImageJ software. Histopathological evaluation was carried out at SAI Pathology Labs (Pune, India). The tissue sections were stained with hematoxylin-eosin (H/E) and images were captured with the camera-equipped Nikon Optical Microscope Eclipse E-at 200 resolution × 40.

### *Statistical analysis*

An average of six readings was calculated and data were expressed as mean ± SEM. Statistical evaluation was performed using one-way ANOVA followed by Dunnett's multiple comparison test for colon-to-body weight ratio, myeloperoxidase (MPO) assay and ulcerogenic activity, and by two-way ANOVA followed by Bonferroni's test for clinical activity score rate. Statistical significance was considered at $P < 0.05$, $P < 0.01$, $P < 0.001$ when compared to the disease control.

### *TNBS-induced experimental colitis*

Colitis was induced by intrarectal administration of 0.25 mL of TNBS in ethanol, following a reported procedure (dose of TNBS was 100 mg/kg of body weight in 50% v/v ethanol solution)[54]. The doses of prodrugs were calculated on an equimolar basis to BBA and are presented in Table 1. The standard and the test compounds were administered orally as a suspension in 1% sodium CMC. Throughout the 11-d study, the animals were monitored for three parameters, *viz* weight loss, stool consistency and

**Table 1 Doses of test and standard drugs**

| Compound | Dose[1] in mg/kg | Compound | Dose[1] in mg/kg |
|---|---|---|---|
| HC | 0.9 % saline (1 mL) | P | 759 |
| BBA | 2159 | T | 938 |
| SLZ | 300 | TY | 832 |
| BH | 2795 | BBA + H | 2871 |
| BP | 2841 | BBA + P | 2918 |
| BT | 3020 | BBA + T | 3097 |
| BTY | 2914 | BBA + TY | 2991 |
| H | 712 | | |

[1]All doses are calculated on an equimolar basis to the dose of BBA. HC: Healthy control; BBA: β-boswellic acid; SLZ: Sulfasalazine; BH: Prodrug of BBA with L-histidine; BP: Prodrug of BBA with D-phenylalanine; BT: Prodrug of BBA with L-tryptophan; BTY: Prodrug of BBA with L-tyrosine; H: L-histidine; P: D-phenylalanine; T: L-tryptophan; TY: L-tyrosine; BBA + H: Physical mixture of BBA + L-histidine; BBA + P: Physical mixture of BBA + D-phenylalanine; BBA + T: Physical mixture of BBA + L-tryptophan; BBA + TY: Physical mixture of BBA + L-tyrosine.

**Table 2 Scoring rate of clinical activity**

| Sr. No. | Weight loss, % | Stool consistency | Rectal bleeding | Score rate |
|---|---|---|---|---|
| 1 | No loss | Well-formed pellets | No blood | 0 |
| 2 | 1-5 | - | - | 1 |
| 3 | 5-10 | Pasty and semi-formed stools, not sticking to anus | Positive finding | 2 |
| 4 | 10-20 | - | - | 3 |
| 5 | > 20 | Liquid stools, sticking to anus | Gross bleeding | 4 |

Adapted from Hartmann *et al*[55].

rectal bleeding. Colitis activity was quantified with a clinical activity score assessing these parameters as previously applied by Hartmann *et al*[55] (Table 2). The clinical activity score was determined by calculating the average of the above three parameters for each day, for each group and ranged from 0 (healthy) to 4 (maximal activity of colitis). On the 11$^{th}$ day, the animals were sacrificed. Colon-to-body weight ratio and MPO activity[56] were determined on the dissected sections of colon. Anti-colitic activity of prodrugs was compared with the standard drugs SLZ and BBA. Rat stomach, colon, liver and pancreas were removed. Gastric ulcers were scanned and ulcer index was calculated by scoring the ulcers as per the method reported by Cioli *et al*[57]. Specimens of colon, liver and pancreas were fixed in formalin and sent for histopathological evaluation.

## RESULTS

### *Synthesis and characterization*

Synthesis of novel amide conjugates of BBA and

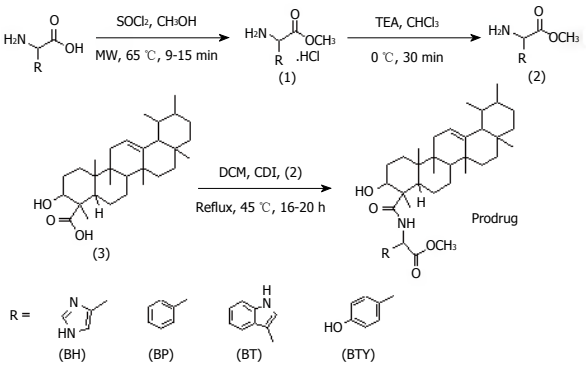



**Figure 1 Scheme of synthesis for the amide prodrugs.**

L-histidine (BH), BBA and D-phenylalanine (BP), BT and BBA and L-tyrosine (BTY) was accomplished successfully using the CDI coupling method (Figure 1). MW-assisted technology was specially developed and optimized for conversion of amino acids into their methyl ester hydrochlorides[58]. Methyl esters were further coupled with BBA by refluxing with CDI at 45 ℃ for 16-20 h. Reactions were monitored by TLC (DCM:n-hexane: TEA, 0.8:0.2:0.01 v/v/v). Synthesized prodrugs were characterized by FTIR, $^{1}$H-NMR, $^{13}$C-NMR, mass spectroscopy and elemental analysis. Formation of amide linkage was confirmed through IR, where the characteristic amide C=O stretch and NH-stretch were observed in the range of 1653-1666 cm$^{-1}$ and 3327-3439 cm$^{-1}$ respectively. The other characteristic bands, such as O-H stretch of BBA between 3542 to 3554 cm$^{-1}$ and C=O stretch of methyl ester of amino acid in the range of 1735-1740 cm$^{-1}$, further confirmed the structures of anticipated prodrugs. Formation of amide linkage was also confirmed by $^{1}$H-NMR spectra, where the chemical shift of proton of secondary amide appeared between δ 8.1 to 8.3 as a singlet. Chemical shifts for protons of the BBA backbone: CH-OH (δ 3.9), 23 protons methylenes and methines between δ 1.1-2.2 and 21 protons of 7 methyl groups between δ 0.6-1.1, as well as relevant chemical shifts for amino acid backbone were also observed. Number of carbon atoms was confirmed by $^{13}$C-NMR. Elemental analysis and molecular ion peaks documented by mass spectroscopy for respective conjugates matched with their anticipated molecular weights.

### *Aqueous solubility and partition coefficient*

BBA was found to be practically insoluble in water, with log$P_{oct}$ of 9.3 when determined experimentally. For a successful colon-targeted delivery, it is essential to restrict absorption from upper GIT so that maximum concentration is achieved at the site of action (colon). Conjugation of BBA with amino acids significantly enhanced the aqueous solubility of prodrugs (15-29 mg/mL), which was in accordance with the observed log$P_{oct}$ of prodrugs (4.1-6.3).

**Table 3** ***In vitro*** **kinetics of prodrugs in aqueous buffers, gastrointestinal tract homogenates and fecal matter**

| Prodrug | Incubation medium | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | HCl buffer (pH 1.2) and stomach homogenates | Phosphate buffer (pH 7.4) and intestinal homogenates | Colon homogenate | | | | Fecal matter | | | |
| | | | K ± SD ($min^{-1}$)[1] | $t_{1/2}$ (min) | Prodrug hydrolyzed | BBA released | K ± SD ($min^{-1}$)[1] | $t_{1/2}$ (min) | Prodrug hydrolyzed | BBA released |
| BH | Stable | Negligible hydrolysis | 0.0013 | 514.1 | 78.10% | 73.60% | 0.0009 | 723.1 | 62.29% | 57.37% |
| BP | Stable | Negligible hydrolysis | 0.0014 | 488.3 | 75.08% | 71.79% | 0.0010 | 636.7 | 57.38% | 56.67% |
| BT | Stable | Negligible hydrolysis | 0.0018 | 379.4 | 86.67% | 86.39% | 0.0010 | 662.5 | 69.75% | 72.52% |
| BTY | Stable | Negligible hydrolysis | 0.0014 | 484.4 | 70.33% | 68.57% | 0.0010 | 677.6 | 55.95% | 55.13% |

[1]Average of three readings; follows first-order kinetics.

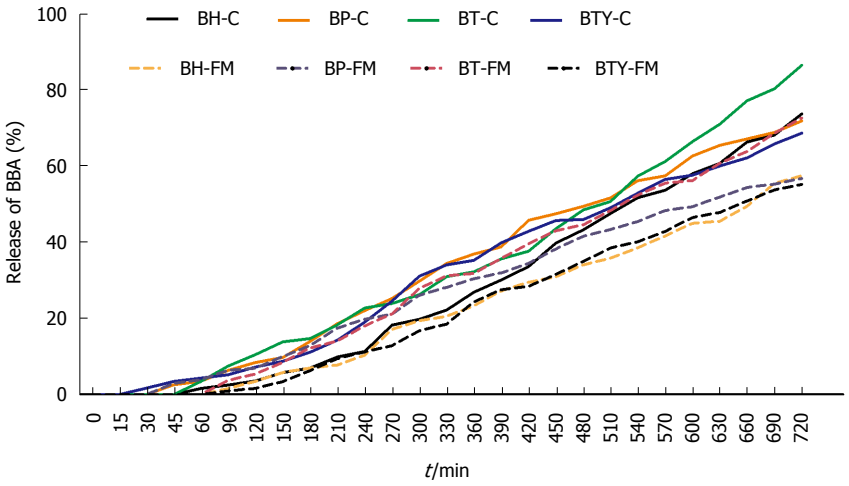



**Figure 2** ***In vitro*** **release kinetics of β-boswellic acid from prodrugs in colon homogenates and fecal matter.** FM: Fecal matter; C: Colon homogenates.

### *Stability studies of prodrugs in aqueous buffers*

Chemical stability of colon-targeted prodrugs at physiological pH values of upper GIT is the essence of their successful design, otherwise the prodrug might revert back to the active drug prematurely. Colon-specific prodrugs face the challenge of surviving during their passage through upper GIT in an intact form, so as to reach the colon, where they are expected to become degraded (hydrolyzed) by colonic microbial enzymes. Therefore, to explore the behavior of prodrugs in acidic and basic environments, their stability was studied in HCl buffer (pH 1.2) and phosphate buffer (pH 7.4). *In vitro* studies confirmed the stability of prodrugs in HCl buffer and negligible hydrolysis in phosphate buffer (pH 7.4) after 4 h (Table 3).

### *In vitro kinetics in tissue homogenates of Wistar rats*

To quantify the sensitivity of the synthesized prodrugs to the enzymatic environment of the GIT, release of BBA from prodrugs was studied in homogenates of stomach, small intestine and colon of Wistar rats. Prodrugs were incubated at 37 ± 1 ℃ in stomach, small intestine and colon homogenates for 3 h, 4 h and 12 h respectively. All prodrugs were stable in stomach homogenate, which was in accordance with their similar behavior in HCl buffer (pH 1.2). However, negligible hydrolysis of prodrugs was observed in intestinal homogenates at the end of 4 h. In colon homogenates, 68%-86% release (Figure 2) of BBA was achieved in 12 h, following first-order kinetics that suggests anticipated colon-targeted release of BBA with minimum loss in the upper GIT. The half-lives of the prodrugs were found to be in the range of 379-514 min, whereas rate constants (K) were in the range of 0.0013-0.0018 $min^{-1}$ (Table 3).

### *In vitro kinetics in fecal matter*

The prodrugs were incubated in fecal matter for a period of 12 h, furnishing 55%-72% BBA. The half-lives and rate constants were in the range of 636-723 min and 0.0009-0.0010 $min^{-1}$ respectively. Significant release in colon homogenates and fecal matter indicates colon-specific, hydrolytic activation of prodrugs into their active metabolites, which could be mediated by N-acyl amidases secreted by the endogenous colonic microorganisms. The proposed mechanism of activation is depicted in Figure 3.

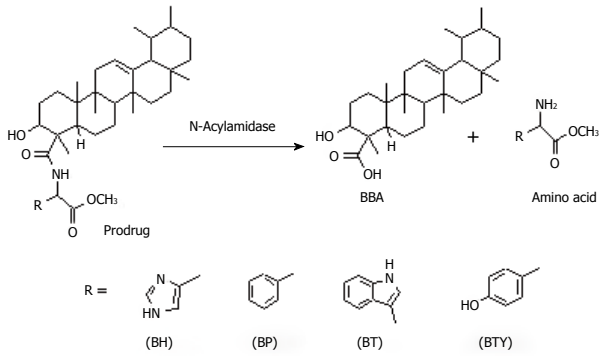



**Figure 3 Proposed activation mechanism.**

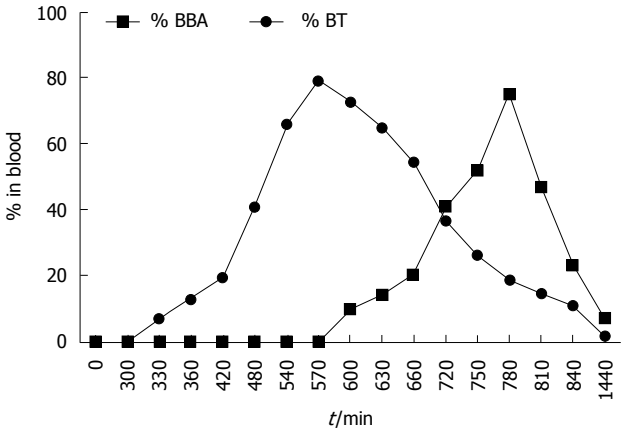



**Figure 4 *In vivo* pharmacokinetics after oral administration of prodrug of β-boswellic acid with L-tryptophan (blood).**

### *In vivo pharmacokinetics in plasma*

The prodrugs of BBA were designed to increase its bioavailability in colon by enhancing the hydrophilicity, so that systemic absorption of prodrugs is minimized, helping to ensure reaching the colon in intact form followed by colon-specific release of BBA for its local mitigating effect on colonic inflammation. BT was selected as a representative of the four synthesized prodrugs to study *in vivo* behavior, and was compared with orally administered BBA. BT and plain BBA were orally administered to the rats and their plasma concentrations were compared at pre-determined time intervals.

Appearance of BBA in blood started at 30 min, reaching a maximum at 240 min (4 h) and then declining gradually with disappearance at 24 h (Figure 4). This behavior indicated ready absorption of BBA in the upper GIT. On the contrary, when BT was administered orally, neither BT nor BBA was observed in blood until 300 min (5 h), indicating that prodrug did not absorb from the stomach and remained intact there. BT started appearing in blood at 330 min (5.5 h), indicating minimal absorption through the small intestine. The concentration of BT consistently increased in blood, reaching a maximum of 79% at 570 min (9.5 h), indicating its absorption from large intestine (colonic mucosa) into systemic circulation.

Interestingly, appearance of BBA was observed in blood at 600 min (10 h), indicating colon-specific hydrolysis of BT into BA in the large intestine, which - due to its high lipophilicity - might have traversed through colonic mucosa into systemic circulation. The BBA concentration reached a maximum of 75% at 780 min (13 h). The concentration of BT and BBA started declining, reaching negligible level at 24 h. Prodrug BT could restrict release of BBA throughout its passage in the upper GIT - which might be due to the introduction of an amide linkage in the prodrug - ensuring efficient delivery to the large intestine in contrast to plain BBA, which was promptly absorbed from the upper GIT with negligible fraction of administered dose available in the colon.

Incidentally, the *in vivo* behavior of BT seemed quite similar to that of SLZ, which has dual applicability in IBD as well as RA. Therefore, from the outcome of these *in vivo* studies, we hypothesize that although BT was designed for IBD, it might prove to be worthy for the treatment of inflammatory diseases affected by circadian rhythm, such as RA, due to systemic availability of BBA in high concentration at 13 h[59-62].

### *Biological evaluation*

The synthesized prodrugs were evaluated for clinical activity score rate, colon-to-body weight ratio and MPO activity in TNBS-induced experimental colitis modeled Wistar rats[63-65]. The anti-colitic activity of prodrugs was compared with SLZ and BBA. Before induction of colitis, the animals were starved for 48 h. On the 3rd day, colitis was induced by intrarectal administration of TNBS and by 5th day colitis was fully developed. The clinical activity score increased rapidly and consistently for all TNBS-treated groups (Table 4). From day 6 to 10, standards SLZ and BBA, prodrugs, carriers and physical mixtures were orally administered to animals. All prodrugs significantly minimized the clinical activity score rate (85%-101%) as compared to BBA (79%), revealing efficient delivery of BBA to colon by the developed prodrugs. BT was 1.7% superior to SLZ in lowering the clinical activity, which might be due to significant inhibition of leukotrienes by BBA[17,30] and the inhibitory effect of L-tryptophan on Th1-mediated inflammation in the gut, thus providing a synergistic effect[44]. Overall, the positive contribution of amino acids[42-50] was obvious from the gross difference between the lowering effect of all the synthesized prodrugs and plain BBA.

On the 11th day, all animals were sacrificed and colon-to-body weight ratio was calculated to quantify inflammation. Prodrug-treated groups showed a distinct decrease in the colon-to-body weight ratio, compared to the colitis control group (Figure 5). The extent of decrease in colon-to-body weight ratio shown by BT was highest among all prodrug-treated groups,

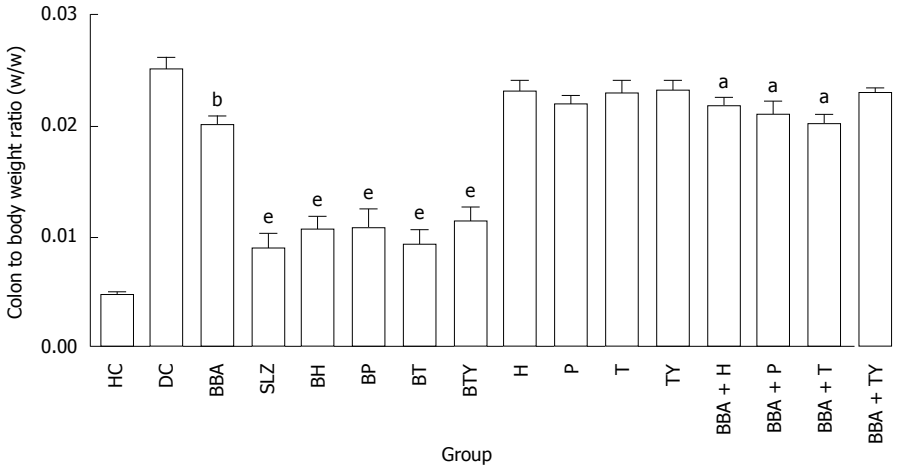



**Figure 5 Colon-to-body weight ratio.** Average of six readings is presented; One-way ANOVA followed by Dunnett's multiple comparison Test, and statistical significance was considered at $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* disease control. HC: Healthy control; DC: Disease control; BBA: β-boswellic acid; SLZ: Sulfasalazine; BH: Prodrug of BBA with L-histidine; BP: Prodrug of BBA with D-phenylalanine; BT: Prodrug of BBA with L-tryptophan; BTY: Prodrug of BBA with L-tyrosine; H: L-histidine; P: D-phenylalanine; T: L-tryptophan; TY: L-tyrosine; BBA + H: Physical mixture of BBA + L-histidine; BBA + P: Physical mixture of BBA + D-phenylalanine; BBA + T: Physical mixture of BBA + L-tryptophan; BBA + TY: Physical mixture of BBA + L-tyrosine.

**Table 4 Clinical activity score rate**[1]

| Intervention | 1 d | 2 d | 3 d | 4 d | 5 d | 6 d | 7 d | 8 d | 9 d | 10 d | 11 d |
|---|---|---|---|---|---|---|---|---|---|---|---|
| HC | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| DC | 0 ± 0 | 0.77 ± 1.34 | 1.00 ± 1.73 | 1.66 ± 1.52 | 1.66 ± 1.52 | 1.88 ± 1.71 | 3.11 ± 1.01 | 3.22 ± 1.07 | 3.33 ± 1.15 | 3.33 ± 1.15 | 3.33 ± 1.15 |
| BBA | 0 ± 0 | 0.66 ± 1.15 | 1.00 ± 1.73 | 1.66 ± 1.52 | 2.00 ± 1.73 | 2.77 ± 1.34 | 3.00 ± 1.00 | 2.33 ± 0.57 | 1.99 ± 0.66 | 1.33 ± 1.15 | 0.99 ± 1.10$^{e}$ |
| SLZ | 0 ± 0 | 0.38 ± 0.67 | 0.83 ± 0.92 | 1.88 ± 0.83 | 2.72 ± 0.63 | 2.88 ± 0.83 | 2.44 ± 0.50 | 1.61 ± 1.13 | 1.11 ± 1.01 | 0.72 ± 0.75 | 0.38 ± 0.60$^{e}$ |
| BH | 0 ± 0 | 0.66 ± 1.15 | 1.55 ± 1.50 | 2.11 ± 0.83 | 2.77 ± 1.01 | 3.00 ± 1.00 | 2.66 ± 0.57 | 2.11 ± 0.50 | 1.55 ± 1.07 | 1.31 ± 0.83 | 0.70 ± 1.10$^{e}$ |
| BP | 0 ± 0 | 0.77 ± 1.34 | 1.00 ± 1.73 | 1.50 ± 1.58 | 2.16 ± 1.09 | 2.66 ± 0.88 | 2.55 ± 0.76 | 1.88 ± 1.01 | 1.22 ± 1.17 | 0.97 ± 1.07 | 0.61 ± 0.70$^{e}$ |
| BT | 0 ± 0 | 0.72 ± 1.25 | 1.05 ± 1.82 | 2.16 ± 0.86 | 2.38 ± 0.67 | 2.38 ± 0.67 | 2.16 ± 0.92 | 1.44 ± 1.17 | 0.94 ± 1.10 | 0.50 ± 0.86 | 0.33 ± 0.50$^{e}$ |
| BTY | 0 ± 0 | 0.66 ± 1.15 | 1.44 ± 1.50 | 2.00 ± 0.88 | 2.77 ± 0.69 | 3.05 ± 1.00 | 2.88 ± 1.07 | 2.00 ± 1.20 | 1.61 ± 1.25 | 1.22 ± 1.17 | 0.83 ± 0.90$^{e}$ |
| H | 0 ± 0 | 0.66 ± 1.15 | 1.11 ± 1.16 | 1.33 ± 1.52 | 1.88 ± 1.01 | 2.33 ± 0.67 | 2.55 ± 0.50 | 2.88 ± 0.83 | 2.77 ± 0.69 | 2.60 ± 0.82 | 2.00 ± 1.00$^{NS}$ |
| P | 0 ± 0 | 0.50 ± 0.86 | 0.88 ± 1.53 | 1.22 ± 1.57 | 1.99 ± 0.88 | 2.22 ± 0.83 | 2.55 ± 0.50 | 3.16 ± 1.04 | 3.33 ± 1.15 | 2.66 ± 1.44 | 2.27 ± 1.13$^{NS}$ |
| T | 0 ± 0 | 0.61 ± 1.05 | 1.00 ± 1.73 | 1.66 ± 1.52 | 2.11 ± 0.83 | 2.44 ± 0.69 | 2.60 ± 0.58 | 2.94 ± 0.82 | 2.66 ± 0.66 | 2.16 ± 1.08 | 1.88 ± 1.17$^{a}$ |
| TY | 0 ± 0 | 0.55 ± 0.95 | 1.00 ± 1.73 | 1.00 ± 1.73 | 1.99 ± 0.88 | 2.11 ± 0.84 | 2.55 ± 0.50 | 3.22 ± 1.07 | 3.33 ± 1.15 | 2.72 ± 1.49 | 2.33 ± 1.45$^{NS}$ |
| BBA + H | 0 ± 0 | 0.44 ± 0.76 | 1.00 ± 1.73 | 1.88 ± 0.96 | 1.99 ± 0.88 | 2.33 ± 0.67 | 3.22 ± 1.07 | 3.33 ± 1.15 | 2.88 ± 1.92 | 2.72 ± 1.80 | 1.77 ± 1.34$^{a}$ |
| BBA + P | 0 ± 0 | 0.66 ± 1.15 | 1.00 ± 1.73 | 1.44 ± 1.50 | 1.99 ± 0.88 | 2.33 ± 0.88 | 3.11 ± 1.01 | 3.22 ± 1.07 | 3.22 ± 1.07 | 2.55 ± 1.64 | 1.88 ± 1.17$^{a}$ |
| BBA + T | 0 ± 0 | 0.38 ± 0.66 | 0.88 ± 1.53 | 1.66 ± 1.20 | 2.10 ± 0.77 | 2.44 ± 1.07 | 2.99 ± 0.87 | 3.33 ± 1.15 | 2.99 ± 1.45 | 2.55 ± 1.71 | 1.77 ± 1.34$^{b}$ |
| BBA + TY | 0 ± 0 | 0.55 ± 0.95 | 0.88 ± 1.53 | 1.33 ± 1.52 | 1.88 ± 1.01 | 2.55 ± 0.50 | 2.99 ± 0.87 | 3.22 ± 1.07 | 3.27 ± 1.10 | 2.44 ± 1.26 | 1.88 ± 1.17$^{a}$ |

[1]Average of six readings; Two-way ANOVA followed by Bonferroni's test, statistical significance considered at $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* disease control. HC: Healthy control; DC: Disease control; BBA: β-boswellic acid; SLZ: Sulfasalazine; BH: Prodrug of BBA with L-histidine; BP: Prodrug of BBA with D-phenylalanine; BT: Prodrug of BBA with L-tryptophan; BTY: Prodrug of BBA with L-tyrosine; H: L-histidine; P: D-phenylalanine; T: L-tryptophan; TY: L-tyrosine; BBA + H: Physical mixture of BBA + L-histidine; BBA + P: Physical mixture of BBA + D-phenylalanine; BBA + T: Physical mixture of BBA + L-tryptophan; BBA + TY: Physical mixture of BBA + L-tyrosine; NS: Non-significant.

which are in accordance with its superior lowering effect on the clinical activity score rate.

MPO activity is an important quantitative index for colonic inflammation and it was measured according to the technique described by Krawisz *et al*[56]. It is a peroxidase enzyme secreted by the activated neutrophils into the inflamed tissue, and is directly proportional to severity of inflammation. The results were expressed as MPO units per gram of wet tissue, and one unit of MPO activity was defined as that degrading 1 mmol $min^{-1}$ of hydrogen peroxide at 25 ℃. Maximum colonic MPO activity was shown by BP and BT (39.74 and 37.49 mU/100 mg tissue respectively), which was comparable to SLZ (36.21 mU/100 mg tissue), indicating significant anti-inflammatory effect on colonic inflammation (Figure 6). However, plain BBA showed significantly high MPO activity (60.71 mU/100 mg tissue), suggesting higher extent of neutrophil infiltrate in the inflamed colon, which might be due to ready absorption and considerably high concentration of BBA in upper GIT and not at the targeted site (colon).

The severity of colonic inflammation and the effect of prodrugs on the recovery from TNBS-induced colitis were evaluated by examining the H/E-stained colon sections. In healthy colon, the normal colonic architecture was observed with intact mucosal layer,

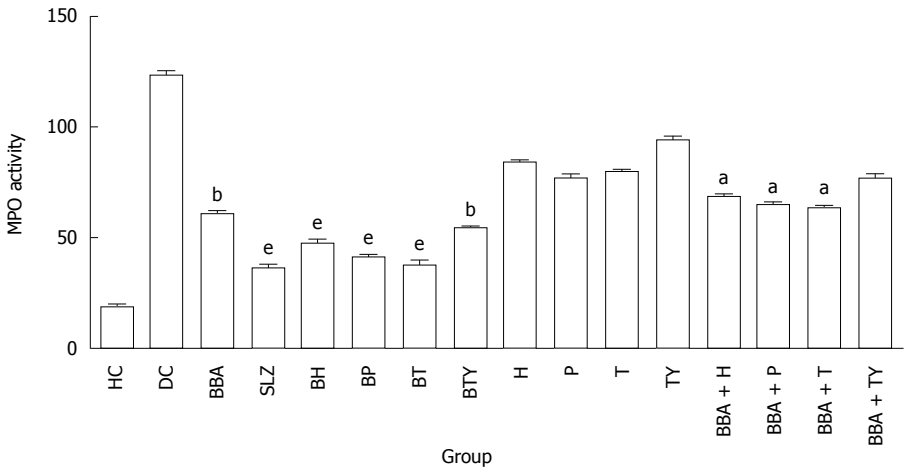



**Figure 6 MPO activity.** Average of six readings is presented; One-way ANOVA followed by Dunnett's multiple comparison test, and statistical significance was considered at $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* disease control. HC: Healthy control; DC: Disease control; BBA: β-boswellic acid; SLZ: Sulfasalazine; BH: Prodrug of BBA with L-histidine; BP: Prodrug of BBA with D-phenylalanine; BT: Prodrug of BBA with L-tryptophan; BTY: Prodrug of BBA with L-tyrosine; H: L-histidine; P: D-phenylalanine; T: L-tryptophan; TY: L-tyrosine; BBA + H: Physical mixture of BBA + L-histidine; BBA + P: Physical mixture of BBA + D-phenylalanine; BBA + T: Physical mixture of BBA + L-tryptophan; BBA + TY: Physical mixture of BBA + L-tyrosine.

**Table 5 Ulcerogenic activity**

| Code | Ulcer index ± SD[1] |
|---|---|
| HC | 1.16 ± 0.40 |
| BBA | 3.66 ± 0.51[e] |
| SLZ | 7.66 ± 1.21[e] |
| BH | 3.16 ± 0.40[e] |
| BP | 2.83 ± 0.40[e] |
| BT | 3.06 ± 0.89[e] |
| BTY | 3.33 ± 0.51[e] |

[1]Average of six readings; One-way ANOVA followed by Dunnett's multiple comparison test, statistical significance was considered at $^{e}P < 0.001$ *vs* healthy control. HC: Healthy control; DC: Disease control; BBA: β-boswellic acid; SLZ: Sulfasalazine; BH: Prodrug of BBA with L-histidine; BP: Prodrug of BBA with D-phenylalanine; BT: Prodrug of BBA with L-tryptophan; BTY: Prodrug of BBA with L-tyrosine.

crypt-architecture and presence of goblet cells. The colitis control showed severe erosion with absence of mucosal layer, goblet cells' depletion, distorted crypts' architecture, lymphocytic infiltration, and thickening of the muscularis mucosa. The lamina propria was also infiltrated with leukocytes. Due to destruction of the crypts, the normal mucosal architecture was lost completely.

*In vivo* treatment with the synthesized prodrugs significantly decreased the extent and severity of colonic damage. Histopathology slides of the colon samples revealed that the inflammation and crypt damage associated with the TNBS administration were corrected by treatment with prodrugs (Figure 7). These results were comparable with those obtained for the SLZ-treated group. The prodrugs were also assessed for their probable damaging effects on pancreas and liver with the help of histopathological analysis. No adverse effects on liver and pancreas were observed (Figures 8 and 9). Ulcer indices of BBA and prodrugs were profoundly lower (2.83-3.33) than for SLZ (7.66 ± 1.21) (Table 5).

In conclusions, the present work aimed at developing novel colon-specific prodrugs of BBA with essential amino acids involved in restoration of gut immune homeostasis by attenuating inflammatory response and modulating intestinal immune functions to maintain gut health. The synthesized prodrugs were designed innovatively by applying a semi-synthetic approach, wherein one natural active constituent of *BS*, namely BBA, was derivatized into a bioreversible delivery system by incorporation of amino acids as promoities for targeted delivery to inflamed colon in IBD. Site-specifically enhanced bioavailability of BBA could be achieved in colon, which resulted in demonstration of significant mitigating effect on TNBS-induced colitis in rats without any adverse effects on stomach, liver and pancreas.

Interestingly, *in vivo* pharmacokinetic studies of BT revealed that after release of BBA in colon, its substantial concentration reached the systemic circulation due to probable absorption through colonic mucosa. This behavior was similar to that of SLZ, which is known to release 5-ASA and sulfapyridine in the colon, the former being involved in treating local inflammation in IBD. However, 30% of released 5-ASA and 100% of sulfapyridine enter circulation due to their absorption from large intestine, accounting for their usefulness in the treatment of RA. Therefore, screening of the synthesized prodrugs in animal models of RA has been undertaken and is currently under progress. The outcome of this study strongly suggests that these prodrugs might find dual applicability in IBD and chronotherapy of RA.

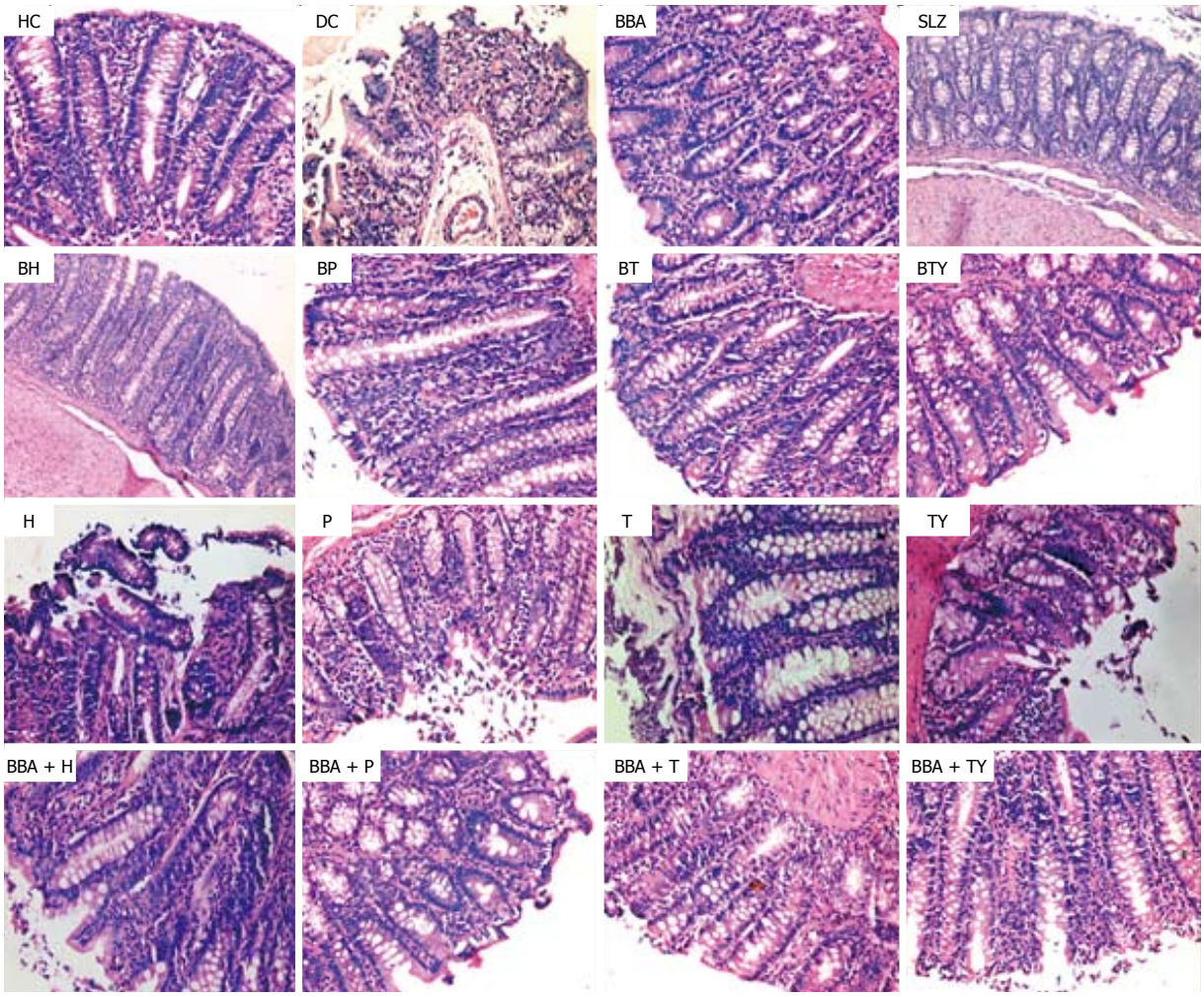
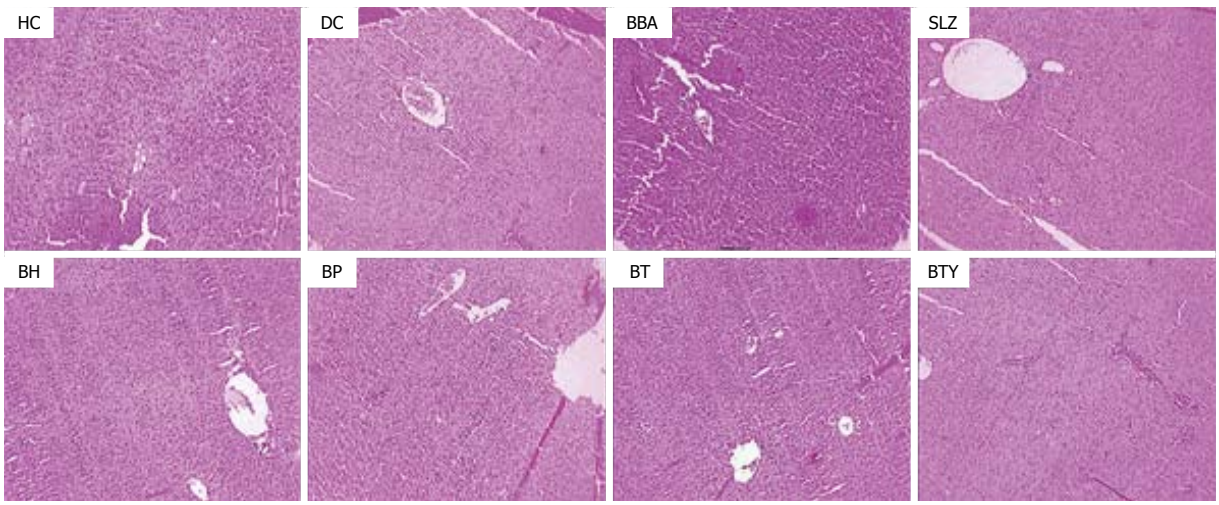



**Figure 7 Histology of colon of rats subjected to 2,4,6-trinitrobenzene sulfonic acid.** HC: Healthy control showing intact colonic cyto-architecture; DC: Disease control showing mucosal injury characterized by absence of epithelium, architectural distortion and a massive mucosal/submucosal infiltration of inflammatory cells; BBA: β-boswellic acid showing slight mucosal abscess and moderate protection against 2,4,6-trinitrobenzene sulfonic acid (TNBS); SLZ: Sulfasalazine showing corrected cyto-architecture of colon; BH: Prodrug of BBA with L-histidine; BP: Prodrug of BBA with D-phenylalanine; BT: Prodrug of BBA with L-tryptophan; BTY: Prodrug of BBA with L-tyrosine showing corrected morphology of colon; H: L-histidine; P: D-phenylalanine; T: L-tryptophan; TY: L-tyrosine; TY: Showing significant loss of epithelium without mucosal injury providing mild protection against TNBS; BBA + H: Physical mixture of BBA + L-histidine; BBA + P: Physical mixture of BBA + D-phenylalanine; BBA + T: Physical mixture of BBA + L-tryptophan; BBA + TY: Physical mixture of BBA + L-tyrosine showing some loss of epithelial layer while other layers seem reconstructed, indicating moderate protection of cyto-architecture.



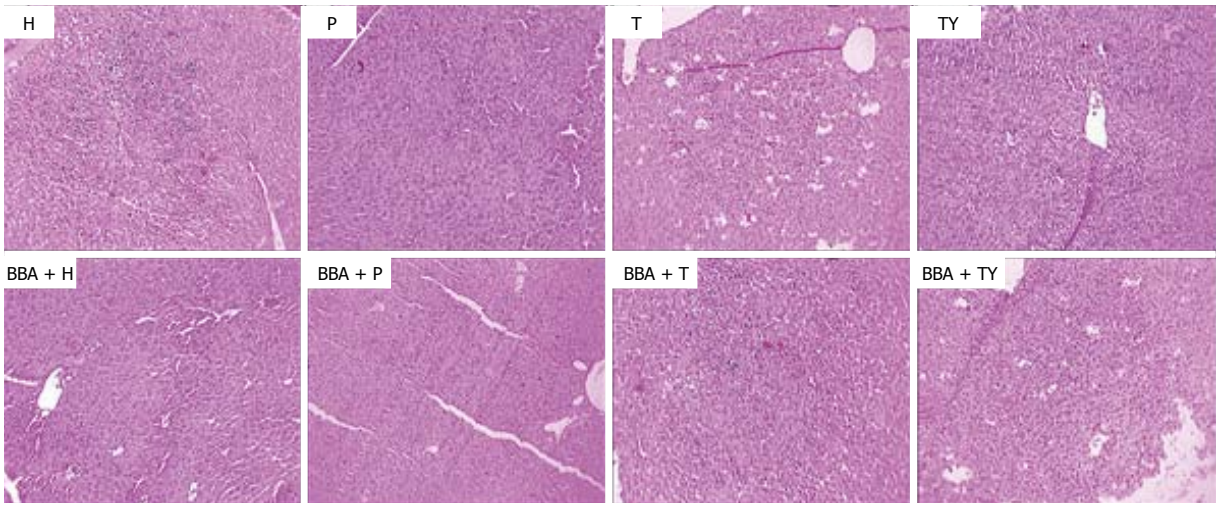



**Figure 8 Histology of liver of rats subjected to 2,4,6-trinitrobenzene sulfonic acid.** All groups showing normal liver architecture with no significant pathological changes nor adverse effects on liver. HC: Healthy control; DC: Disease control; BBA: β-boswellic acid; SLZ: Sulfasalazine; BH: Prodrug of BBA with L-histidine; BP: Prodrug of BBA with D-phenylalanine; BT: Prodrug of BBA with L-tryptophan; BTY: Prodrug of BBA with L-tyrosine; H: L-histidine; P: D-phenylalanine; T: L-tryptophan; TY: L-tyrosine; BBA + H: Physical mixture of BBA + L-histidine; BBA + P: Physical mixture of BBA + D-phenylalanine; BBA + T: Physical mixture of BBA + L-tryptophan; BBA + TY: Physical mixture of BBA + L-tyrosine.

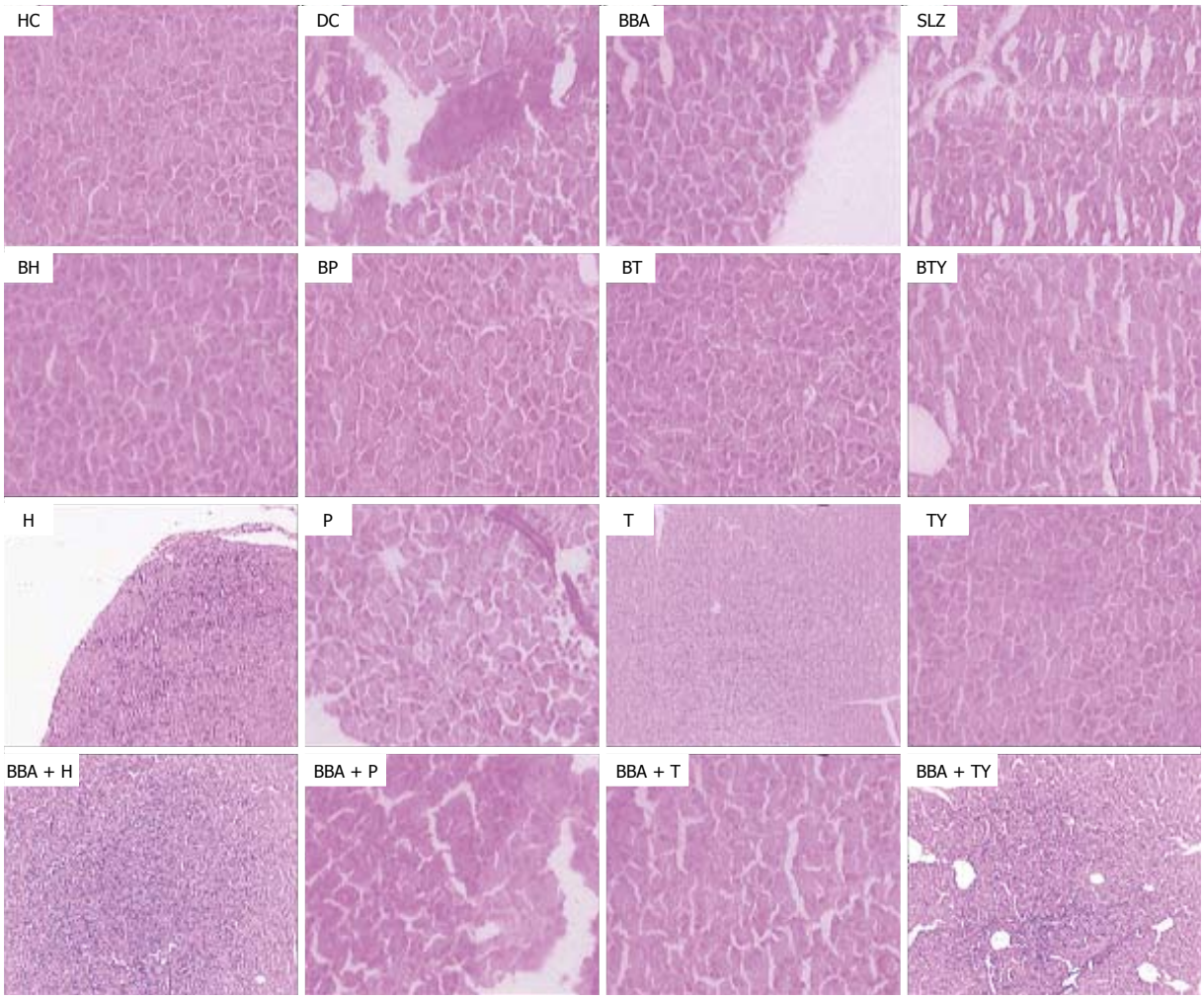



**Figure 9 Histology of pancreas of rats subjected to 2,4,6-trinitrobenzene sulfonic acid.** All groups showing normal pancreas architecture without any evidence of pancreatitis. HC: Healthy control; DC: Disease control; BBA: β-boswellic acid; SLZ: Sulfasalazine; BH: Prodrug of BBA with L-histidine; BP: Prodrug of BBA with D-phenylalanine; BT: Prodrug of BBA with L-tryptophan; BTY: Prodrug of BBA with L-tyrosine; H: L-histidine; P: D-phenylalanine; T: L-tryptophan; TY: L-tyrosine; BBA + H: Physical mixture of BBA + L-histidine; BBA + P: Physical mixture of BBA + D-phenylalanine; BBA + T: Physical mixture of BBA + L-tryptophan; BBA + TY: Physical mixture of BBA + L-tyrosine.

## COMMENTS

### Background

The need to discover effective treatment options for inflammatory bowel disease (IBD) is growing due to the incidence of treatment-related side-effects. The use of complementary and alternative medicine is also on the rise, particularly herbal medicine for IBD management. β-boswellic acid (BBA) was selected as the drug candidate for developing its colon-targeting prodrugs. 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis modeling of Wistar rats was used for investigation and to ensure attainment of an effective concentration of BBA in the colon for its local mitigating effect on colonic inflammation.

### Research frontiers

To our knowledge, this is the first study of its kind to identify the therapeutic effect of prodrugs of BBA in the TNBS-induced colitis model in Wistar rats for IBD.

### Innovations and breakthroughs

This is the first study examining the potential for prodrugs of BBA for restoration of gut immune homeostasis by attenuating inflammatory response and modulating intestinal immune functions to maintain gut health. The synthesized prodrugs were designed innovatively by applying a semi-synthetic approach, wherein one natural active constituent of *Boswellia serrata,* namely BBA, was derivatized into a bioreversible delivery system by incorporation of amino acids as promoities for targeted delivery to inflamed colon in IBD. The prodrug BBA + L-tryptophan (BT) was found to be 1.7% superior in reducing inflammation in the colon and showed similar behavior to standard drug sulfasalazine (SLZ). Interestingly, the *in vivo* pharmacokinetic behavior of BT was found to be similar to SLZ in treatment of IBD, as well as for rheumatoid arthritis (RA). Therefore, screening of the synthesized prodrugs on animal models of RA has been undertaken and is currently under progress. The outcome of this study strongly suggests that these prodrugs might find dual applicability in IBD and chronotherapy of RA.

### Applications

The promising findings presented in the current study indicate that the prodrugs exhibited an improvement in selected parameters of the TNBS-induced colitis model. Site-specifically enhanced bioavailability of BBA could be achieved in colon that resulted in demonstration of a significant mitigating effect on TNBS-induced colitis in rats, without any adverse effects on the stomach, liver and pancreas. These prodrugs might find dual applicability in IBD and chronotherapy of RA.

### Terminology

A prodrug is a pharmacologically-inactive, bioreversible derivative of a parent drug molecule that requires chemical or enzymatic activation in the biological environment to release the active drug. Physico-chemical properties can be tailored by means of changing the structure of the promoiety, and the intrinsic activity of the parent drug is assured through *in vivo* cleavage of the prodrugs. A mutual prodrug or co-drug strategy involves formation of a covalent linkage between drug and carrier in such a manner that upon oral administration the moiety remains intact in the stomach and small intestine but releases the active drug in the colon through enzymatic activation. This ensures attainment of effective concentration of the drug in the colon for its local mitigating effect on colonic inflammation of IBD.

### Peer-review

The authors demonstrated the efficacy of colon-specific prodrugs of BA on TNBS-induced colitis in mice. The present study was well organized and well investigated, and will give us important information, especially in the field of IBD.

## REFERENCES


1 **Khor B**, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature* 2011; **474**: 307-317 [PMID: 21677747 DOI: 10.1038/nature10209]

2 **Lautenschläger C**, Schmidt C, Fischer D, Stallmach A. Drug delivery strategies in the therapy of inflammatory bowel disease. *Adv Drug Deliv Rev* 2014; **71**: 58-76 [PMID: 24157534 DOI: 10.1016/j.addr.2013.10.001]

3 **Van Limbergen J**, Wilson DC, Satsangi J. The genetics of Crohn's disease. *Annu Rev Genomics Hum Genet* 2009; **10**: 89-116 [PMID: 19453248 DOI: 10.1146/annurev-genom-082908-150013]

4 **Loftus EV**. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. *Gastroenterology* 2004; **126**: 1504-1517 [PMID: 15168363 DOI: 10.1053/j.gastro.2004.01.063]

5 **Van Limbergen J**, Russell RK, Drummond HE, Aldhous MC, Round NK, Nimmo ER, Smith L, Gillett PM, McGrogan P, Weaver LT, Bisset WM, Mahdi G, Arnott ID, Satsangi J, Wilson DC. Definition of phenotypic characteristics of childhood-onset inflammatory bowel disease. *Gastroenterology* 2008; **135**: 1114-1122 [PMID: 18725221 DOI: 10.1053/j.gastro.2008.06.081]

6 **Danese S**, Sans M, Fiocchi C. Inflammatory bowel disease: the role of environmental factors. *Autoimmun Rev* 2004; **3**: 394-400 [PMID: 15288007 DOI: 10.1016/j.autrev.2004.03.002]

7 **Taylor KM**, Irving PM. Optimization of conventional therapy in patients with IBD. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 646-656 [PMID: 21970871 DOI: 10.1038/nrgastro.2011.172]

8 **Isaacs KL**, Lewis JD, Sandborn WJ, Sands BE, Targan SR. State of the art: IBD therapy and clinical trials in IBD. *Inflamm Bowel Dis* 2005; **11** Suppl 1: S3-S12 [PMID: 16254481 DOI: 10.1097/01.MIB.0000184852.84558.b2]

9 **Buchman AL**. Side effects of corticosteroid therapy. *J Clin Gastroenterol* 2001; **33**: 289-294 [PMID: 11588541 DOI: 10.1097/00004836-200110000-00006]

10 **Cunliffe RN**, Scott BB. Review article: monitoring for drug side-effects in inflammatory bowel disease. *Aliment Pharmacol Ther* 2002; **16**: 647-662 [PMID: 11929382 DOI: 10.1046/j.1365-2036.2002.01216.x]

11 **Mason M**, Siegel CA. Do inflammatory bowel disease therapies cause cancer? *Inflamm Bowel Dis* 2013; **19**: 1306-1321 [PMID: 23470503 DOI: 10.1097/MIB.0b013e3182807618]

12 **Guilliams TG**. Inflammatory bowel disease and irritable bowel syndrome, understanding, distinguishing and addressing. A review of natural and nutraceutical therapies for clinical practice. *Standard* 2010; **10**: 1-20

13 **Catanzaro D**, Rancan S, Orso G, Dall'Acqua S, Brun P, Giron MC, Carrara M, Castagliuolo I, Ragazzi E, Caparrotta L, Montopoli M. Boswellia serrata Preserves Intestinal Epithelial Barrier from Oxidative and Inflammatory Damage. *PLoS One* 2015; **10**: e0125375 [PMID: 25955295 DOI: 10.1371/journal]

14 **Hüsch J**, Bohnet J, Fricker G, Skarke C, Artaria C, Appendino G, Schubert-Zsilavecz M, Abdel-Tawab M. Enhanced absorption of boswellic acids by a lecithin delivery form (Phytosome(®)) of Boswellia extract. *Fitoterapia* 2013; **84**: 89-98 [PMID: 23092618 DOI: 10.1016/j.fitote.2012.10.002]

15 **Hüsch J**, Gerbeth K, Fricker G, Setzer C, Zirkel J, Rebmann H, Schubert-Zsilavecz M, Abdel-Tawab M. Effect of phospholipid-based formulations of Boswellia serrata extract on the solubility, permeability, and absorption of the individual boswellic acid constituents present. *J Nat Prod* 2012; **75**: 1675-1682 [PMID: 23013292 DOI: 10.1021/np300009w]

16 **Scior T**, Verhoff M, Gutierrez-Aztatzi I, Ammon HP, Laufer S, Werz O. Interference of boswellic acids with the ligand binding domain of the glucocorticoid receptor. *J Chem Inf Model* 2014; **54**: 978-986 [PMID: 24512031 DOI: 10.1021/ci400666a]

17 **Safayhi H**, Mack T, Sabieraj J, Anazodo MI, Subramanian LR, Ammon HP. Boswellic acids: novel, specific, nonredox inhibitors of 5-lipoxygenase. *J Pharmacol Exp Ther* 1992; **261**: 1143-1146 [PMID: 1602379]

18 **Sailer ER**, Subramanian LR, Rall B, Hoernlein RF, Ammon HP, Safayhi H. Acetyl-11-keto-beta-boswellic acid (AKBA): structure requirements for binding and 5-lipoxygenase inhibitory activity. *Br J Pharmacol* 1996; **117**: 615-618 [PMID: 8646405 DOI: 10.1111/

j.1476-5381.1996.tb15235.x]
19 **Krüger P**, Daneshfar R, Eckert GP, Klein J, Volmer DA, Bahr U, Müller WE, Karas M, Schubert-Zsilavecz M, Abdel-Tawab M. Metabolism of boswellic acids in vitro and in vivo. *Drug Metab Dispos* 2008; **36**: 1135-1142 [PMID: 18356270 DOI: 10.1124/dmd.107.018424]
20 **Singh GB**, Singh S, Bani S. Alcoholic extract of salai-guggal ex-Boswellia serrata, a new natural source NSAID. *Drugs Today* 1996; **32**: 109-112 [DOI: 10.1358/dot.1996.32.2.354254]
21 **Singh GB**, Singh S, Bani S. Anti-inflammatory actions of boswellic acids. *Phytomedicine* 1996; **3**: 81-85 [PMID: 23194868 DOI: 10.1016/S0944-7113(96)80017-1]
22 **Singh GB**, Bani S, Singh S. Toxicity and safety evaluation of boswellic acids. *Phytomedicine* 1996; **3**: 87-90 [PMID: 23194869 DOI: 10.1016/S0944-7113(96)80018-3]
23 **Hostanska K**, Daum G, Saller R. Cytostatic and apoptosis-inducing activity of boswellic acids toward malignant cell lines in vitro. *Anticancer Res* 2002; **22**: 2853-2862 [PMID: 12530009]
24 **Gupta I**, Parihar A, Malhotra P, Gupta S, Lüdtke R, Safayhi H, Ammon HP. Effects of gum resin of Boswellia serrata in patients with chronic colitis. *Planta Med* 2001; **67**: 391-395 [PMID: 11488449 DOI: 10.1055/s-2001-15802]
25 **Singh S**, Khajuria A, Taneja SC, Khajuria RK, Singh J, Johri RK, Qazi GN. The gastric ulcer protective effect of boswellic acids, a leukotriene inhibitor from Boswellia serrata, in rats. *Phytomedicine* 2008; **15**: 408-415 [PMID: 18424019 DOI: 10.1016/j.phymed.2008.02.017]
26 **Schweizer S**, von Brocke AF, Boden SE, Bayer E, Ammon HP, Safayhi H. Workup-dependent formation of 5-lipoxygenase inhibitory boswellic acid analogues. *J Nat Prod* 2000; **63**: 1058-1061 [PMID: 10978197 DOI: 10.1021/np000069k]
27 **Safayhi H**, Sailer ER, Ammon HP. Mechanism of 5-lipoxygenase inhibition by acetyl-11-keto-beta-boswellic acid. *Mol Pharmacol* 1995; **47**: 1212-1216 [PMID: 7603462]
28 **Shah BA**, Kumar A, Gupta P, Sharma M, Sethi VK, Saxena AK, Singh J, Qazi GN, Taneja SC. Cytotoxic and apoptotic activities of novel amino analogues of boswellic acids. *Bioorg Med Chem Lett* 2007; **17**: 6411-6416 [PMID: 17950603 DOI: 10.1016/j.bmcl.2007.10.011]
29 **Poeckel D**, Werz O. Boswellic acids: biological actions and molecular targets. *Curr Med Chem* 2006; **13**: 3359-3369 [PMID: 17168710 DOI: 10.2174/092986706779010333]
30 **Singh S**, Khajuria A, Taneja SC, Johri RK, Singh J, Qazi GN. Boswellic acids: A leukotriene inhibitor also effective through topical application in inflammatory disorders. *Phytomedicine* 2008; **15**: 400-407 [PMID: 18222672 DOI: 10.1016/j.phymed.2007.11.019]
31 **Gerhardt H**, Seifert F, Buvari P, Vogelsang H, Repges R. Therapy of active Crohn disease with Boswellia serrata extract H 15. *Z Gastroenterol* 2001; **39**: 11-17 [PMID: 11215357 DOI: 10.1055/s-2001-10708]
32 **Anthoni C**, Laukoetter MG, Rijcken E, Vowinkel T, Mennigen R, Müller S, Senninger N, Russell J, Jauch J, Bergmann J, Granger DN, Krieglstein CF. Mechanisms underlying the anti-inflammatory actions of boswellic acid derivatives in experimental colitis. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G1131-G1137 [PMID: 16423918 DOI: 10.1152/ajpgi.00562.2005]
33 **Qurishi Y**, Hamid A, Zargar MA, Singh SK, Saxena AK. Potential role of natural molecules in health and disease: Importance of boswellic acid. *J Med Plants Res* 2010; **4**: 2778-2785
34 **Rentea R**. Therapeutic Advantages of highly standardized Boswellia Extracts. Original article 2008 with a review and addendum2011. Available from: URL: http://www.koliskoinstitute.org/wp-content/uploads/2014/09/Therapeutic-Advantages-of-AKBAv2.pdf
35 **Algieri F**, Rodriguez-Nogales A, Rodriguez-Cabezas ME, Risco S, Ocete MA, Galvez J. Botanical Drugs as an Emerging Strategy in Inflammatory Bowel Disease: A Review. *Mediators Inflamm* 2015; **2015**: 179616 [PMID: 26576073 DOI: 10.1155/2015/179616]
36 **Krieglstein CF**, Anthoni C, Rijcken EJ, Laukötter M, Spiegel HU, Boden SE, Schweizer S, Safayhi H, Senninger N, Schürmann G. Acetyl-11-keto-beta-boswellic acid, a constituent of a herbal medicine from Boswellia serrata resin, attenuates experimental ileitis. *Int J Colorectal Dis* 2001; **16**: 88-95 [PMID: 11355324 DOI: 10.1007/s003840100292]
37 **Tabassum N**, Hamdani M, Najar IH. Natural treatment for inflammatory bowel disease. *Br Biomed Bull* 2014; **2**: 85-94
38 **Borrelli F**, Capasso F, Capasso R, Ascione V, Aviello G, Longo R, Izzo AA. Effect of Boswellia serrata on intestinal motility in rodents: inhibition of diarrhoea without constipation. *Br J Pharmacol* 2006; **148**: 553-560 [PMID: 16633355 DOI: 10.1038/sj.bjp.0706740]
39 **Gupta I**, Parihar A, Malhotra P, Singh GB, Lüdtke R, Safayhi H, Ammon HP. Effects of Boswellia serrata gum resin in patients with ulcerative colitis. *Eur J Med Res* 1997; **2**: 37-43 [PMID: 9049593]
40 **Lemmo E**, Schulman R. Phytonutrient formula for the relief of chronic pain resulting from inflammation. United States Patent 2004, US 20040037903
41 **Faigle JW**. Drug Metabolism in the Colon Wall and Lumen. Ed. NY: Marcel Dekker, Inc, 1993: 29-54
42 **Zhang H**, Hu CA, Kovacs-Nolan J, Mine Y. Bioactive dietary peptides and amino acids in inflammatory bowel disease. *Amino Acids* 2015; **47**: 2127-2141 [PMID: 25501277 DOI: 10.1007/s00726-014-1886-9]
43 **Dhaneshwar SS**, Chail M, Patil M, Naqvi S, Vadnerkar G. Colon-specific mutual amide prodrugs of 4-aminosalicylic acid for their mitigating effect on experimental colitis in rats. *Eur J Med Chem* 2009; **44**: 131-142 [PMID: 18472188 DOI: 10.1016/j.ejmech.2008.03.035]
44 **Kim CJ**, Kovacs-Nolan JA, Yang C, Archbold T, Fan MZ, Mine Y. l-Tryptophan exhibits therapeutic function in a porcine model of dextran sodium sulfate (DSS)-induced colitis. *J Nutr Biochem* 2010; **21**: 468-475 [PMID: 19428234 DOI: 10.1016/j.jnutbio.2009.01.019]
45 **Son DO**, Satsu H, Shimizu M. Histidine inhibits oxidative stress- and TNF-alpha-induced interleukin-8 secretion in intestinal epithelial cells. *FEBS Lett* 2005; **579**: 4671-4677 [PMID: 16107255 DOI: 10.1016/j.febslet.2005.07.038]
46 **Dhaneshwar SS**, Gairola N, Kandpal M, Vadnerkar G, Bhatt L, Rathi B, Kadam SS. Synthesis, kinetic studies and pharmacological evaluation of mutual azo prodrugs of 5-aminosalicylic acid for colon-specific drug delivery in inflammatory bowel disease. *Eur J Med Chem* 2009; **44**: 3922-3929 [PMID: 19442419 DOI: 10.1016/j.ejmech.2009.04.018]
47 **Walsh NE**, Ramamurthy S, Schoenfeld L, Hoffman J. Analgesic effectiveness of D-phenylalanine in chronic pain patients. *Arch Phys Med Rehabil* 1986; **67**: 436-439 [PMID: 3524509]
48 **Balagot RC**, Ehrenpreis S, Kubota K, Greenberg J. Analgesia in mice and humans by D-phenylalanine: Relation to inhibition of enkephalin degradation and enkephalin levels. *Adv Pain Res Ther* 1983; **5**: 289-293
49 **Meyers BE**, Moonka DK, Davis RH. The effect of selected amino acids on gelatin-induced inflammation in adult male mice. *Inflammation* 1979; **3**: 225-233 [PMID: 478594 DOI: 10.1007/BF00914179]
50 **Takagi K**, Okabe S. Prevention of gastric lesions. Available from: URL: http://www.patentimages.storage.googleapis.com/pdfs/US3988466.pdf
51 **Lee JS**, Jung YJ, Kim YM. Synthesis and evaluation of N-acyl-2-(5-fluorouracil-1-yl)-D,L-glycine as a colon-specific prodrug of 5-fluorouracil. *J Pharm Sci* 2001; **90**: 1787-1794 [PMID: 11745736 DOI: 10.1002/jps.1128]
52 **Kashmira P**, Dhaneshwar S, Shakuntala C, Poorvashree J. Design, Synthesis and In Vitro Release Studies of Co-Drugs for Rheumatoid Arthritis. *Inflamm Allergy Drug Targets* 2015; **14**: 47-52 [PMID: 26631094 DOI: 10.2174/1871528114666151201200157]
53 Indian Pharmacopoeia 2007; 1: 480-482. Available from: URL: http://www.ajprd.com/downloadebooks_pdf/8.pdf
54 **Dhaneshwar S**, Gautam H. Exploring novel colon-targeting

antihistaminic prodrug for colitis. *J Physiol Pharmacol* 2012; **63**: 327-337 [PMID: 23070081]
55 **Hartmann G**, Bidlingmaier C, Siegmund B, Albrich S, Schulze J, Tschoep K, Eigler A, Lehr HA, Endres S. Specific type IV phosphodiesterase inhibitor rolipram mitigates experimental colitis in mice. *J Pharmacol Exp Ther* 2000; **292**: 22-30 [PMID: 10604928]
56 **Krawisz JE**, Sharon P, Stenson WF. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models. *Gastroenterology* 1984; **87**: 1344-1350 [PMID: 6092199]
57 **Cioli V**, Putzolu S, Rossi V, Scorza Barcellona P, Corradino C. The role of direct tissue contact in the production of gastrointestinal ulcers by anti-inflammatory drugs in rats. *Toxicol Appl Pharmacol* 1979; **50**: 283-289 [PMID: 505458 DOI: 10.1016/0041-008X(79)90153-4]
58 **Sarkate A**, Dhaneshwar SS. Microwave- assisted one-pot synthesis of amino acid methyl ester hydrochlorides. *Int J Pharm Res* 2016; In Press
59 **Dhaneshwar SS**, Vadnerkar G. Rational design and development of colon-specific prodrugs. *Curr Top Med Chem* 2011; **11**: 2318-2345 [PMID: 21671865 DOI: 10.2174/156802611797183249]
60 **Taggart AJ**, Neumann VC, Hill J, Astbury C, Le Gallez P, Dixon JS. 5-Aminosalicylic acid or sulphapyridine. Which is the active moiety of sulphasalazine in rheumatoid arthritis? *Drugs* 1986; **32** Suppl 1: 27-34 [PMID: 2877851 DOI: 10.2165/00003495-198600321-00006]
61 **Neumann VC**, Taggart AJ, Le Gallez P, Astbury C, Hill J, Bird HA. A study to determine the active moiety of sulphasalazine in rheumatoid arthritis. *J Rheumatol* 1986; **13**: 285-287 [PMID: 2873245]
62 **Bird HA**. Sulphasalazine, sulphapyridine or 5-aminosalicylic acid--which is the active moiety in rheumatoid arthritis? *Br J Rheumatol* 1995; **34** Suppl 2: 16-19 [PMID: 8535642 DOI: 10.1093/rheumatology/XXXIV.suppl_4.16]
63 **Tian L**, Huang YX, Tian M, Gao W, Chang Q. Downregulation of electroacupuncture at ST36 on TNF-alpha in rats with ulcerative colitis. *World J Gastroenterol* 2003; **9**: 1028-1033 [PMID: 12717850 DOI: 10.3748/wjg.v9.i5.1028]
64 **Morris GP**, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL. Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology* 1989; **96**: 795-803 [PMID: 2914642 DOI: 10.1016/S0016-5085(89)80079-4]
65 **Zingarelli B**, Squadrito F, Graziani P, Camerini R, Caputi AP. Effects of zileuton, a new 5-lipoxygenase inhibitor, in experimentally induced colitis in rats. *Agents Actions* 1993; **39**: 150-156 [PMID: 7905704 DOI: 10.1007/BF01998968]

**P- Reviewer**: Naito Y **S- Editor**: Qi Y **L- Editor**: Filipodia
**E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/ *World J Gastroenterol* 2017 February 21; 23(7): 1163-1170

DOI: 10.3748/wjg.v23.i7.1163 ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

# Effect of biofilm formation by clinical isolates of *Helicobacter pylori* on the efflux-mediated resistance to commonly used antibiotics

Bahareh Attaran, Tahereh Falsafi, Nassim Ghorbanmehr

Bahareh Attaran, Tahereh Falsafi, Department of Microbiology, Faculty of Biological Sciences, Alzahra University, Tehran 1993893973, Iran

Nassim Ghorbanmehr, Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran 1993893973, Iran

Author contributions: Attaran B and Falsafi T designed the study, wrote the manuscript, and provided vital analytical tools; Attaran B performed all experiments; Ghorbanmehr N participated in providing analytical tools and reading of the manuscript.

Institutional review board statement: The *H. pylori* strains used for this study were isolated from the patients who were admitted to Medical Centers for their persistent upper gastrointestinal problems, and for this reason they underwent endoscopy for biopsy. All routine biopsy specimens from the patients were taken after informed consent and the protocols, under which the biopsies for culture were obtained, were in accordance with the Helsinki Declaration of 1975.

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

Data sharing statement: No additional data are available.



Manuscript source: Invited manuscript

Correspondence to: Dr. Tahereh Falsafi, Professor, Department of Microbiology, Faculty of Biological Sciences, Alzahra University, Vanak Village Street, Tehran 1993893973, Iran. falsafi.tahereh@yahoo.com
Telephone: +98-21-88044040

Received: September 23, 2016
Peer-review started: September 25, 2016
First decision: October 20, 2016
Revised: November 11, 2016
Accepted: January 4, 2017
Article in press: January 4, 2017
Published online: February 21, 2017

## Abstract

***AIM***

To evaluate the role of biofilm formation on the resistance of *Helicobacter pylori* (*H. pylori*) to commonly prescribed antibiotics, the expression rates of resistance genes in biofilm-forming and planktonic cells were compared.

***METHODS***

A collection of 33 *H. pylori* isolates from children and adult patients with chronic infection were taken for the present study. The isolates were screened for biofilm formation ability, as well as for polymerase chain reaction (PCR) reaction with *HP1165* and *hp1165* efflux pump genes. Susceptibilities of the selected strains to antibiotic and differences between susceptibilities of planktonic and biofilm-forming cell populations were determined. Quantitative real-time PCR (qPCR) analysis was performed using *16S rRNA* gene as a *H. pylori*-specific primer, and two efflux pumps-specific primers, *hp1165* and *hefA*.

***RESULTS***

The strains were resistant to amoxicillin, metronidazole, and erythromycin, except for one strain, but they were all susceptible to tetracycline. Minimum bactericidal

concentrations of antibiotics in the biofilm-forming cells were significantly higher than those of planktonic cells. qPCR demonstrated that the expression of efflux pump genes was significantly higher in the biofilm-forming cells as compared to the planktonic ones.

***CONCLUSION***

The present work demonstrated an association between *H. pylori* biofilm formation and decreased susceptibility to all the antibiotics tested. This decreased susceptibility to antibiotics was associated with enhanced functional activity of two efflux pumps: *hp1165* and *hefA*.

**Key words:** *Helicobacter pylori*; Biofilm; Antibiotic resistance; Efflux genes; *hp1165*; *hefA*



**Core tip:** The current study has addressed the co-incidence of biofilm formation by *Helicobacter pylori* (*H. pylori*) and decreased susceptibility to antibiotics. The results demonstrated a significantly higher expression of two efflux pump genes, *hp1165* and *hefA* (involved in the specific resistance to tetracycline and multidrug resistance, respectively), in the biofilm-forming cells as compared to the planktonic cells. There was also association between *H. pylori* biofilm formation and decreased susceptibility to antibiotics. This event would probably be involved in the failure of *H. pylori* eradication and might be beneficial for developing new therapeutic approaches for this infection.

Attaran B, Falsafi T, Ghorbanmehr N. Effect of biofilm formation by clinical isolates of *Helicobacter pylori* on the efflux-mediated resistance to commonly used antibiotics. *World J Gastroenterol* 2017; 23(7): 1163-1170 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1163.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1163

## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) colonizes the stomach of approximately half of the world's population[1]. Gastritis may be the first manifestation of stomach colonization by *H. pylori* that can progress to the fatal states such as peptic ulceration and gastric adenocarcinoma. Although a low number of *H. pylori*-infected individuals can develop the serious symptoms of infection, in all cases non-treatment with appropriate antibiotics may result in a persistent infection, which can favor progression to the fatal outcomes[2].

The current treatment regimen of *H. pylori* infection includes a proton pump inhibitor in combination with two out of the following antibiotics: amoxicillin (AMX), metronidazole (MTZ), clarithromycin and tetracycline (TET)[3]. However, data from various geographical areas indicate that resistance to these antibiotics debilitates the treatment of *H. pylori* infections[3,4].

Bacterial biofilms are communities of individual cells that form microcolonies, grow in the third dimension and develop the phenotypic and biochemical properties which may differ from those of the corresponding freely existing (planktonic) cells[5,6]. Gene expression profiles in biofilms may, therefore, also be different from those of the planktonic cells[7]. The investigators have observed that biofilm formation by numerous pathogenic bacteria may be involved in increased rate of antibiotic resistance[8].

In practice, biofilm-forming cells may be observed on the surfaces of tissues and biomaterials at the site of persistent infections, where they can be protected from the killing effects of antibiotics[9]. Hence, during long-term infection, biofilm formation by pathogenic bacteria may play an important role in the emergence of resistant bacteria and can increase the rates of antibiotic resistance.

To date, the mechanisms of higher resistance to antibiotics in biofilm-forming cells are not fully understood. However, poor antibiotic penetration, adaptive stress responses due to expression of specific genes in the biofilm, slow growth rate, and formation of persister cells (which form multi-layers) may be the causes of higher resistance rates in biofilms[10,11].

Resistance mechanisms of *H. pylori* to macrolides, nitroimidazole, β-lactams, and TET have been reported to occur *via* mutations in the peptidyltransferase region encoded in 23S rRNA, different mutations involving the *rdxA* gene, multiple mutations in the penicillin binding protein "*pbp* gene" as well as production of β-lactamase, and mutations in the *16S rRNA* gene, respectively[12]. Involvement of the efflux pumps may also be one of the important reasons for decreased susceptibility of *H. pylori* to various antibiotics[13-17]. Numerous and structurally different efflux pumps have been identified in the *H. pylori* 26695 genome, capable of acting either on the structurally non-related antibiotics or more specifically on one antibiotic[13-15]. For this purpose, investigators have studied the roles of a few putative efflux pumps in the resistance of *H. pylori* to antibiotics[16]. Liu *et al*[17], for example, have observed the role of *hefA* on the multidrug resistance of *H. pylori* and have demonstrated the role of Hp1165 in TET resistance.

To gain a better understanding of biofilm formation's role in the resistance of *H. pylori* to commonly prescribed antibiotics, in the present study, the resistance profiles of biofilm-forming cells and their planktonic counterparts were evaluated and the expression of the two efflux pumps genes, *hp1165* and *hefA*, were compared between biofilm-forming and planktonic cells.

## MATERIALS AND METHODS

### *H. pylori strains and culture condition*

A collection of 33 clinical *H. pylori* strains isolated from

children and adult patients with chronic infections were examined for biofilm formation ability. The protocols, under which the biopsies for culture had been obtained, were in accordance with the Helsinki Declaration of 1975. The isolated strains were cultured at 37 ℃ under microaerobic atmosphere on modified Campy blood agar (MCBA) plates containing Brucella agar base (Merck, Germany), supplemented with 7% defibrinated sheep blood and antibiotics (polymyxin B, amphotericin B, vancomycin) for 3-7 d. The resulting colonies were identified by Gram staining, biochemical tests and polymerase chain reaction (PCR) using *H. pylori*-specific primers for *glmM*, as previously described by Espinoza *et al*[18].

### Screening of the strains for biofilm formation ability

The strains were screened for biofilm formation ability according to our previously described protocol[19]. Briefly, bacteria were inoculated in Brucella broth (Biolife, Italy) supplemented with fetal calf serum (2%) and glucose (0.3%) (Merck). Suspensions were incubated at 37 ℃ under microaerobic atmosphere with shaking (100 rpm), to an optical density (OD) of 0.2 at 600 nm ($A_{600}$) equivalent to approximately 5-8 × $10^3$ CFU/mL. Portions of these cultures were inoculated into the wells of 96-well flat-bottomed tissue culture plates (BIOFIL, Jet Bio-Filtration Products Co., Ltd, China) and were incubated at 37 ℃ under microaerobic conditions for 6 d. The wells were vigorously washed (3 times) with sterile phosphate buffered saline (PBS). Tightly attached bacteria were fixed with 99% ethanol (200 μL per well) for 20 min, and air-dried. Plates were then stained with 1% crystal violet (200 μL per well) for 5 min and the excess of stain was rinsed off with running tap water. Dried plates were treated with 33% glacial acetic acid (160 μL per well) and the OD of the wells was measured at 505 nm using an ELISA reader (SCO GmbH, Germany). A culture medium (Brucella broth) without bacterial cells was used as negative control.

### Antibiotics

Four classes of the antibiotics were selected for present study: MTZ as a nitromidazole (Sigma, United States), ERY as a macrolide (Sigma), AMX as a β-lactam (Shafa Pharmaceutical Co., Iran), and TET as a tetracycline (Sigma).

The stock solutions of MTZ (128 μg/mL) and AMX (200 μg/mL) were prepared by addition of acetic acid and dimethyl sulfoxide, respectively; those of TET (128 μg/mL) and ERY (128 μg/mL) were obtained by addition of ethanol.

### Antibiotic susceptibility testing

The minimal inhibitory concentration (MIC) was determined using agar dilution method in accordance with the guidelines established by the Clinical and Laboratory Standards Institute[20]. For this purpose, Mueller-Hinton agar (MH) plates containing 7% fresh sheep blood were supplemented with increasing concentration of antibiotics. A bacterial suspension adjusted to number 2 of McFarland standard, corresponding to $10^8$-2 × $10^8$ CFU/mL was inoculated onto the agar dilution plates. After spreading the bacteria, the plates were incubated under microaerobic conditions for 72 h. Quality control for the antibiotics was ensured using *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 strains. MIC breakpoints for resistance were defined as follows: MTZ: 8 mg/mL; ERY: 1.0 mg/mL; AMX: > 0.5 mg/mL; TET: 8 mg/mL[21,22].

### Comparison of antibiotic susceptibilities between biofilm-forming and planktonic cell populations

The susceptibilities of biofilm-forming cells (group B) and planktonic cells (group P) to the antibiotics were evaluated by comparing the minimal bactericidal concentration (MBC). For this purpose, the 96-well culture plates were inoculated with 100 μL of bacterial suspension containing 5-8 × $10^3$ CFU/mL according to the protocol described for biofilm formation. After biofilm formation, the planktonic cells were removed for further MBC determination. The remaining biofilm forming cells were washed (3 times) and exposed to 2-fold increasing concentrations of the antibiotics (from 0.5 μg/mL to 64 μg/mL) for 24 h. After the incubation period, the cells were removed *via* an ultrasonic bath for 7 min (Elmasonic S 60/ (H); Elma Schmidbauer GmbH, Germany) using ultrasonic frequency of 37 kHz, and cultured on MCBA plates[10]. To determine the susceptibility of planktonic cells, similar concentrations of the antibiotics were added to the 96-well plates containing 5-8 × $10^3$ CFU/mL planktonic bacteria (previously removed from the plates after biofilm formation) and incubated for 24 h. Their antibiotic susceptibility was determined by culturing a small amount (10 μL) of bacterial suspension on the MCBA plates. The plates were then incubated at 37 ℃ under microaerobic conditions for > 72 h incubation. Test controls were similar culture plates containing bacteria but without antibiotics. The results presented for this analysis correspond to the mean of 3 independent tests.

### Determination of minimal biofilm inhibitory concentration

By this assay, minimal concentration of the antibiotics which inhibited formation of the biofilms on plates was determined. For this purpose, similar serial dilutions of the antibiotics noted above were made in the culture medium. The microplates were seeded with these serial dilutions of antibiotics and were inoculated with 100 μL of bacterial suspension containing $10^8$-2 × $10^8$ CFU/mL. After incubation, the plates were vigorously washed (3 times) with sterile PBS, in order to remove all non-adherent bacteria, and then incubated with Brucella broth supplemented with 0.05% (w/v) of

**Table 1 Polymerase chain reaction primers**

| Primer name | Sequence | Product size, bp |
|---|---|---|
| *glm-F* | 5' GGATAAGCTTTTAGGGGTGTTAGGGG 3' | 140 |
| *glm-R* | 5' GCATTCACAAACTTATCCCCAATC 3' | |
| *16s rRNA-F* | 5' GAAGATAATGACGGTATCTAAC 3' | 139 |
| *16s rRNA-R* | 5' ATTTCACACCTGACTGACTAT 3' | |
| *hp1165-F* | 5' F; TACAACCCCCACGCTAAAAG 3' | 117 |
| *hp1165-R* | 5' GGATTTGATGAGCCGAAAAA 3' | |
| *hefA-F* | 5' CTCGCTCGCATGATCGC 3' | 162 |
| *hefA-R* | 5' CGTATTCGCTCAAATTCCCT 3' | |

2,3,5-triphenyl tetrazolium chloride (TTC; Merck) at 37 ℃ under microaerobic conditions for 24 h. The control assay was performed with the same medium but without bacteria. After incubation, the broth was removed, the wells were air-dried and the bound TTC dyes were dissolved using 20% acetone/80% ethanol. To quantify the biofilm formation, absorbance (OD) of the wells was measured at 505 nm[23].

### *Detection of 16S rRNA, hp1165, and hefA genes by PCR*

*H. pylori* specific primers for *16S rRNA* and *glmM*, were designed according to the previously described protocol[24]. Efflux pump-specific primers for *hp1165* was designed according to *hp1165* gene sequences using Primer3 software online, and efflux-specific primers for *hefA* were designed according to Zhang *et al*[25]. All primer sequences, including that of *glmM*, that were used for bacterial identification process are listed in Table 1.

DNA was extracted using boiling method according to the previously described protocol[26]. PCR reactions were performed in a 25 mL mixture containing 0.5 μg of extracted DNA, 0.2 mmol (each) deoxyribonucleoside triphosphates, 0.2-0.4 μmol/L (each) primer, 1.5-2 mmol/L $MgCl_2$, and 5 U of *Taq* polymerase in PCR buffer (SinaClon BioScience, Iran). Following denaturation at 94 ℃ (2 min), the fragments were amplified through 35 cycles at 94 ℃ (30 s). Annealing for *16S rRNA* gene, *hp1165* and *hefA* was performed at 58 ℃, 59 ℃ and 59.5 ℃ respectively. Reactions were continued at 72 ℃ for 1 min and 72 ℃ for 10 min.

### *Quantitative real-time reverse transcription-PCR analysis*

Biofilms were removed *via* an ultrasonic bath for 7 min. RNA was extracted from the planktonic and biofilm-forming cell cultures. For this purpose and following bacterial wash with PBS (× 3), total RNA extraction was carried out using a commercial Cinna Pure RNA kit (SinaClon BioScience). The RNA samples were then treated with DNase I (Roche Diagnostics GmbH, Germany) according to the manufacturer's recommendations. Reverse transcription (RT) was carried out with an RT-PCR Kit (Vivantis Technologies, Malaysia) according to the company's recommendations for using 2 ng RNA sample. Real-time RT-PCR was performed for the cDNA samples using *16S rRNA*-specific primers and efflux pump-specific primers, including *hp1165* and *hefA*[25]. Real-time PCR was performed with the QuantiFast SYBR Green PCR Kit (Qiagen, Germany) in a StepOne Real-time PCR system (Applied Biosystems, United States). The PCR reaction was held at 95 ℃ for 5 min, followed by 2- step cycling consisting of 40 cycles at 95 ℃ for 10 s and 60 ℃ for 30 s.

The final results were expressed as the amount of expression for each efflux pump gene relative to that of the *16S rRNA* gene. We selected *16S rRNA* for control of the quantitative real-time RT-PCR (qPCR) analysis since its expression does not change in biofilm-forming and planktonic cells in the RNA samples.

Statistical analyses were carried out using the ANOVA one-way test with Minitab 17 statistical software, and probability levels of $< 0.05$ were considered as statistically significant.

## RESULTS

### *Selection of strains*

Screening of clinical isolates for biofilm production demonstrated their variable ability for biofilm formation, such that their biofilm formation abilities could be classified as high (one strain), moderate (23 strains), and low (six strains). The strains were also screened for detection of two efflux genes, *hp1165* and *hefA*, by PCR. The PCR results were then analyzed according to production of the sharp bands on gels. To select the most suitable strains for further experiments, beside higher biofilm formation ability and PCR result, their subculture states (none more than three laboratory passages) were also taken into account. PCR results from three selected strains (Hp141, Hp932 and Hp70) for *hp1165*, *hefA,* and *16Sr RNA* genes which demonstrated moderate biofilm formation ability are shown in Figure 1.

### *Determination of MIC by agar dilution*

All three selected isolates were resistant to AMX, MTZ and ERY, except one. All three were susceptible to TET. MICs of AMX for Hp932, Hp141 and Hp70 were 2, 2 and 1 μg/mL, respectively. The MIC of TET was 4 μg/mL for all the three isolates. MICs of ERY for Hp141, Hp932 and Hp70 were 2, 2 and 1 μg/mL, respectively. MICs of MTZ for Hp932, Hp141 and Hp70 were 16, 8 and 8 μg/mL, respectively.

### *Comparison of antibiotic resistance between biofilm-forming and planktonic cell populations*

For this comparison, MBCs were selected as the better criteria. The results of comparison between MBC of biofilm-forming cells and their planktonic counterparts are demonstrated in Table 2. We noted that the MBCs to all the antibiotics were increased significantly in biofilm-forming cells as compared to those of

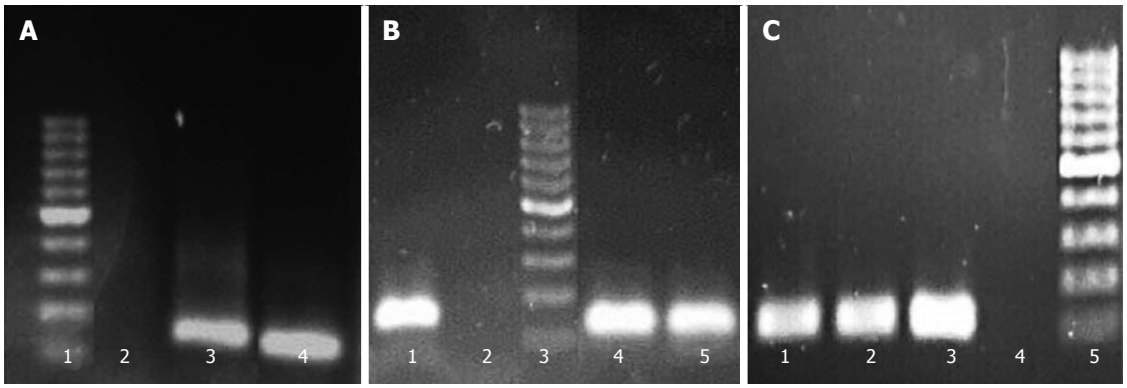



**Figure 1 Polymerase chain reaction products of *hefA* (162 bp), *hp1165* (117 bp) and *16S rRNA* genes on 1.5% agarose gel.** A: Detection of *hefA* gene: 1: 100 bp size marker, 2: Control, 3: Hp932 strain, 4: Hp141 strain; B: Detection of *hp1165* gene: 1: HP70 strain, 2: Control, 3: Size marker, 4: Hp932 strain, 5: Hp141 strain; C: Detection of *16S rRNA* gene: 1: Hp932 strain, 2: Hp141 strain, 3: Hp70 strain, 4: Control, 5: Size marker.

**Table 2 Minimal bactericidal concentration of selected antibiotics in biofilm-forming cells and their planktonic counterparts**

| Strain | MBC, μg/mL | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | AMX | | TET | | ERY | | MTZ | |
| | MBC-P | MBC-B | MBC-P | MBC-B | MBC-P | MBC-B | MBC-P | MBC-B |
| Hp932 | 2 | 8 | 8 | 16 | 4 | 4 | 16 | 32 |
| Hp141 | 2 | 4 | 4 | 16 | 2 | 4 | 16 | 32 |
| Hp70 | 1 | 4 | 4 | 8 | 2 | 4 | 8 | 16 |

MBC: Minimal bactericidal concentration; AMX: Amoxicillin; TET: Tetracycline; MTZ: Metronidazole; B: Biofilm-forming; P: Planktonic counterparts.

**Table 3 Minimal biofilm inhibitory concentration**

| Strain | MBIC, μg/mL | | | |
|---|---|---|---|---|
| | AMX | TET | ERY | MTZ |
| Hp932 | 2 | 2 | 1 | 8 |
| Hp141 | 2 | 2 | 1 | 4 |
| Hp70 | 1 | 1 | 0.5 | 4 |

MBIC: Minimal biofilm inhibitory concentration; AMX: Amoxicillin; TET: Tetracycline; MTZ: Metronidazole.

planktonic ones.

### *Determination of minimal biofilm inhibitory concentration*

We observed that the minimal biofilm inhibitory concentrations (MBICs) of AMX required for preventing biofilm formation was similar to that of the MICs for all three isolates. However, MBICs of TET, ERY and MTZ were at least 2-folds lower than the MICs of the isolates determined in agar dilution (Table 3).

### *Expression of hp1165 and hefA in biofilm-forming and planktonic cells*

The differences in transcription levels of *hefA* and *hp1165* efflux genes between biofilm-forming and planktonic cells, using *hp16S rRNA* as internal control, were compared. For this purpose, the quantities of cDNA corresponding to these genes were determined by real-time qPCR and their amounts were normalized using *16S rRNA* gene in each unique reaction. Each experiment was repeated 3 times with at least duplicate samples from independently isolated RNA preparations. Data are expressed as the means of all experiments ± standard error. The results of qPCR products for *hp1165* (117 bp), *hefA* (162 bp), and *16S rRNA* (136 bp) genes in biofilm-forming (group B) and planktonic (group P) populations on 1.5% agarose gel are demonstrated in Figure 2. The results displaying the amounts of expression for each efflux pump gene relative to that of the *16S rRNA* gene are demonstrated in Figure 3. We noted that expression of these genes was significantly higher in the biofilm-forming cells as compared to their planktonic counterparts.

## DISCUSSION

Long-term infection of humans by *H. pylori* may favor the possibilities of biofilm formation in the stomach, which might be a barrier for antibiotic capture, thereby increasing resistance to antibiotic[11].

Several investigators have reported that *H. pylori* can form the biofilm *in vitro* and most probably *in vivo*[27-31]. In light of these investigations, a few scientific observations, including that of Yonezawa *et al*[29], have reported *in vitro* biofilm formation by *H. pylori* strains.

In a previous study, we investigated the role of biofilm formation on *H. pylori* colonization of the mouse model[19]. In the present work, we investigated its role in the resistance to commonly used antibiotics by comparing the expression rates of two efflux pumps between the biofilm-forming cells and their planktonic

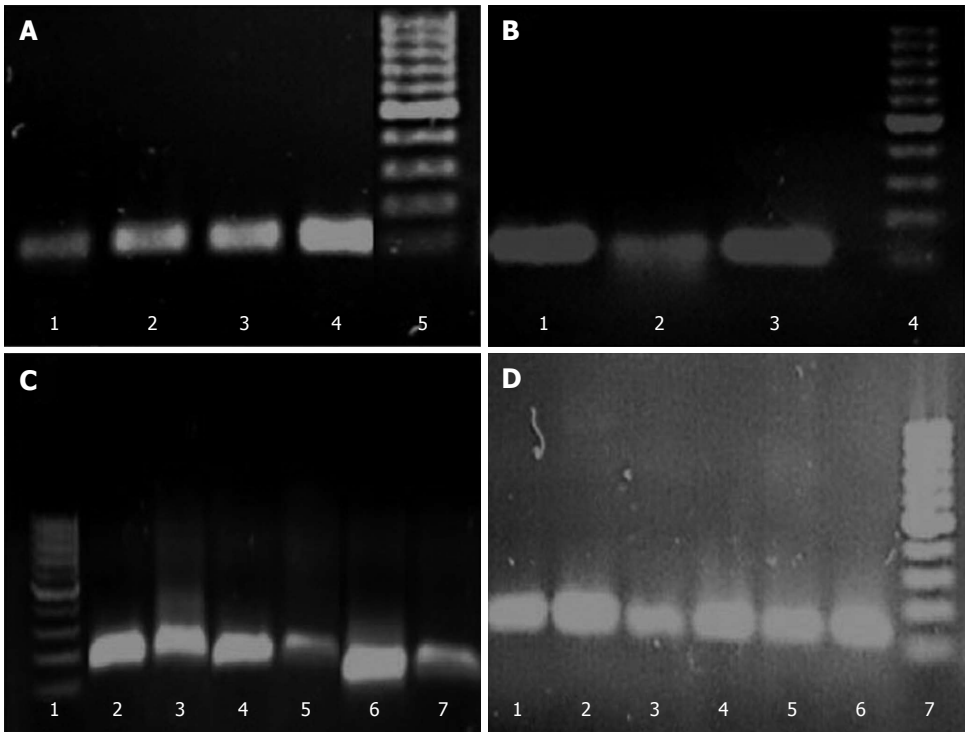



**Figure 2 Real-time reverse transcription-polymerase chain reaction products of *hp1165* (117 bp), *hefA* (162 bp), and *16S rRNA* (136 bp) genes in biofilm-forming (B) and planktonic (P) populations on 1.5% agarose gel.** A: Detection of *hp1165* gene: 1: Hp141 strain (B), 2: Hp141 strain (P), 3: Hp932 strain (B), 4: Hp932 strain (P), 5: 100 bp size marker; B: Detection of *hp1165* gene: 1-2: Hp70 strain (B), 3: Hp70 strain (P), 4: Size marker; C: Detection of *hefA* gene: 1: Size marker, 2: Hp932 strain (B), 3: Hp932 strain (P), 4: Hp141 strain (B), Hp141 strain (P), 5: Hp70 strain (B), 6: Hp70 strain (P); D: Detection of *16S rRNA* gene: 1: Hp932 strain (B), 2: Hp932 strain (P), 3: Hp141 strain (B), 4: Hp141 strain (P), 5: Hp70 strain (B), 6: Hp70 strain (P), 7: Size marker.

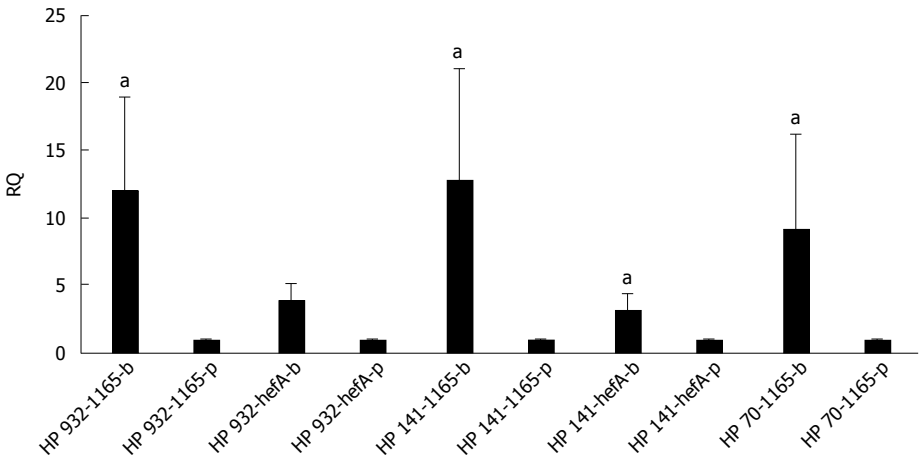



**Figure 3 Expression rates of *Helicobacter pylori* efflux pump genes, *hefA* and *hp1165*.** Data are expressed as the means of multiple experiments ± standard deviation. Significantly different ($^aP \leq 0.05$) relative to the mRNA expression level (planktonic *vs* biofilm-forming).

counterparts.

To observe the effectiveness of antibiotics against planktonic and biofilm-forming cells, we compared their MBCs, instead of MICs, since it was demonstrated that planktonic cells are more susceptible to antibiotics and the eventual presence of planktonic cells (even at low density) in established biofilms may not result in a fair MIC comparison between biofilm-forming and planktonic cells[32]. Furthermore, the possibility exists that in such an evaluation, instead of MIC comparison, the MIC of planktonic cells be compared with the MBIC of biofilm-forming cells. This comparison may result in an erroneous conclusion since MBIC and MIC may not be similar for the majority of the antibiotics. In agreement with this situation, we found that MBIC of selected *H. pylori* isolates for TET, ERY and MTZ were at least 2-folds lower than their MIC (Table 3). Using MBC as a criterion of comparison, the MBC of biofilm-forming cells were at least 2-folds higher than their planktonic counterparts (Table 2).

Expression analysis of mRNA for two efflux pump genes, *hp1165* and *hef*A, involved in the specific resistance to TET and multidrug resistance, respectively, showed that they were active in both the biofilm-forming and planktonic cell populations (Figure 2). By analyzing the amount of expression, we observed that both efflux genes had significantly increased expression in biofilm-forming cells, compared to their planktonic counterparts (Figure 3).

Impact of biofilm formation by *H. pylori* on antibiotic resistance was also studied by Yonezawa and coworkers[33], who found a decreased susceptibility to clarithromycin *via* increased frequency of mutations in the biofilm-forming cells.

Although the actual treatment regimen for eradication of *H. pylori* infection may be effective in some cases, the failure of this approach is well documented in many cases[34]. The main reasons for unsuccessful eradication appear to be the emergence of resistant strains or resistant populations to selected antibiotics. Biofilm-forming populations may develop multiple functional genes which favor more resistance to antibiotics, while planktonic cells do not. Involvement of the efflux pumps may be one of the important reasons for reduced susceptibility of these resistant populations to the various antibiotics. Situations triggering an overexpression of the efflux pump genes could also have several effects on biofilm formation through an increase in the interchange of quorum sensing molecules[35].

The present research provides a new finding about the roles of biofilm on the increased expression of two efflux pumps, *hefA* and *hp1165*, in the clinical isolates of *H. pylori*. Future investigations based upon these findings may reveal the nature of this mechanism and illuminate the role of biofilm formation in the antibiotic resistance of *H. pylori in vivo*.

As prevalence of *H. pylori* infection is high in West Asia, particularly in Iran[3], the need exists for improving our knowledge about the mechanism(s) of antibiotic resistance during chronic infection by *H. pylori*. Furthermore, investigations about the role and the nature of biofilms may help select better eradication regiments capable of acting on biofilm-forming cells as well.

The results of the present work demonstrate the existence of an association between *H. pylori* biofilm formation and decreased susceptibility to various antibiotics *via* increased functional activity of two efflux pumps, *hp1165* and *hefA*, in biofilm-forming cells.

## ACKNOWLEDGMENTS

We thank Alzahra University for supporting this work. We cordially thank Dr. Sara Gharavi and Miss Zahra Landarani from Alzahra University, Tehran, Iran for helping in this work.

## COMMENTS

### Background

In this study, the authors investigated the role of biofilm formation on the resistance of *Helicobacter pylori* (*H. pylori*) to commonly prescribed antibiotics and examined expression of two efflux pump genes, *hp1165* and *hefA*, involved in the specific resistance to tetracycline and multidrug resistance, respectively, in the biofilm-forming and planktonic cells. In addition, these studies can provide insights into the way for development and application of the new therapeutic approaches in the case of *H. pylori* infection.

### Research frontiers

The authors investigated the importance of biofilm formation in the antibiotic resistance of clinical isolates of *H. pylori*. This study will provide new approaches towards the treatment of *H. pylori*-related diseases.

### Innovations and breakthroughs

The current research showed significantly increased expression levels of *hefA* and *hp1165* efflux pumps genes in the biofilm-producing *H. pylori* cell populations. In addition, the results showed that the efflux pumps may be one of the important reasons for reduced susceptibility of these resistant populations to various antibiotics.

### Applications

One of the fatal outcomes of chronic *H. pylori* infection may be production of gastric cancer. So, the next step of this research would be to understand the mechanism and modality of antibiotic resistance in biofilm formation during chronic infection by *H. pylori*. Another application would be the development of novel therapeutic strategies to overcome biofilm formation *in vivo*.

### Terminology

In bacteria, the biofilm is a well-organized assembly of the cells clustered together to form microcolonies. These colonies attach to surfaces and take up different characteristics from the free-floating or planktonic bacteria. Gram-negative bacteria are able to exclude drugs by efflux pumps, which may represent the mechanism by which they can be protected from toxic effects of non-desirable compounds. In general, the efflux pumps of Gram-negative bacteria consist of an inner-membrane protein, a periplasmic adaptor protein and an outer-membrane protein in which the first one acts with the second and third ones. These efficiently structured complexes are involved in specific resistance as well as multi-resistance to antibiotics.

### Peer-review

This is an interesting manuscript about up-regulation of two efflux pumps in biofilm-forming cell populations. Also, the manuscript provides valuable and interesting information about *H. pylori*.

## REFERENCES

1 **Andersen LP**, Rasmussen L. Helicobacter pylori-coccoid forms and biofilm formation. *FEMS Immunol Med Microbiol* 2009; **56**: 112-115 [PMID: 19453756 DOI: 10.1111/j.1574-695X.2009.00556.x]

2 **Kusters JG**, van Vliet AH, Kuipers EJ. Pathogenesis of Helicobacter pylori infection. *Clin Microbiol Rev* 2006; **19**: 449-490 [PMID: 16847081 DOI: 10.1128/CMR.00054-05]

3 **Fakheri H**, Bari Z, Aarabi M, Malekzadeh R. Helicobacter pylori eradication in West Asia: a review. *World J Gastroenterol* 2014; **20**: 10355-10367 [PMID: 25132752 DOI: 10.3748/wjg.v20.i30.10355]

4 **De Francesco V**, Giorgio F, Hassan C, Manes G, Vannella L, Panella C, Ierardi E, Zullo A. Worldwide H. pylori antibiotic resistance: a systematic review. *J Gastrointestin Liver Dis* 2010; **19**: 409-414 [PMID: 21188333]

5 **Cole SP**, Harwood J, Lee R, She R, Guiney DG. Characterization of monospecies biofilm formation by Helicobacter pylori. *J Bacteriol* 2004; **186**: 3124-3132 [PMID: 15126474]

6 **Cellini L**, Grande R, Di Campli E, Di Bartolomeo S, Di Giulio M, Traini T, Trubiani O. Characterization of an Helicobacter pylori environmental strain. *J Appl Microbiol* 2008; **105**: 761-769 [PMID: 18410343 DOI: 10.1111/j.1365-2672.2008.03808.x]

7 **Resch A**, Rosenstein R, Nerz C, Götz F. Differential gene expression profiling of Staphylococcus aureus cultivated under biofilm and planktonic conditions. *Appl Environ Microbiol* 2005; **71**: 2663-2676 [PMID: 15870358 DOI: 10.1128/AEM.71.5.2663-2676.2005]

8 **Cammarota G**, Sanguinetti M, Gallo A, Posteraro B. Review article: biofilm formation by Helicobacter pylori as a target for eradication of resistant infection. *Aliment Pharmacol Ther* 2012; **36**: 222-230 [PMID: 22650647]

9 **Hall-Stoodley L**, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2004; **2**: 95-108 [PMID: 15040259]

10 **Beaudoin T**, Zhang L, Hinz AJ, Parr CJ, Mah TF. The biofilm-specific antibiotic resistance gene ndvB is important for expression of ethanol oxidation genes in Pseudomonas aeruginosa biofilms. *J Bacteriol* 2012; **194**: 3128-3136 [PMID: 22505683 DOI: 10.1128/JB.06178-11]

11 **Mah TF**. Biofilm-specific antibiotic resistance. *Future Microbiol* 2012; **7**: 1061-1072 [PMID: 22953707 DOI: 10.2217/fmb.12.76]

12 **Nishizawa T**, Suzuki H. Mechanisms of Helicobacter pylori antibiotic resistance and molecular testing. *Front Mol Biosci* 2014; **1**: 19 [PMID: 25988160 DOI: 10.3389/fmolb.2014.00019]

13 **Iwamoto A**, Tanahashi T, Okada R, Yoshida Y, Kikuchi K, Keida Y, Murakami Y, Yang L, Yamamoto K, Nishiumi S, Yoshida M, Azuma T. Whole-genome sequencing of clarithromycin resistant Helicobacter pylori characterizes unidentified variants of multidrug resistant efflux pump genes. *Gut Pathog* 2014; **6**: 27 [PMID: 24995043 DOI: 10.1186/1757-4749-6-27]

14 **Francesco VD**, Zullo A, Hassan C, Giorgio F, Rosania R, Ierardi E. Mechanisms of Helicobacter pylori antibiotic resistance: An updated appraisal. *World J Gastrointest Pathophysiol* 2011; **2**: 35-41 [PMID: 21860834 DOI: 10.4291/wjgp.v2.i3.35]

15 **Kutschke A**, de Jonge BL. Compound efflux in Helicobacter pylori. *Antimicrob Agents Chemother* 2005; **49**: 3009-3010 [PMID: 15980386 DOI: 10.1128/AAC.49.7.3009-3010.2005]

16 **Bina JE**, Alm RA, Uria-Nickelsen M, Thomas SR, Trust TJ, Hancock RE. Helicobacter pylori uptake and efflux: basis for intrinsic susceptibility to antibiotics in vitro. *Antimicrob Agents Chemother* 2000; **44**: 248-254 [PMID: 10639345]

17 **Liu ZQ**, Zheng PY, Yang PC. Efflux pump gene hefA of Helicobacter pylori plays an important role in multidrug resistance. *World J Gastroenterol* 2008; **14**: 5217-5222 [PMID: 18777600 DOI: 10.3748/wjg.14.5217]

18 **Espinoza MG**, Vazquez RG, Mendez IM, Vargas CR, Cerezo SG. Detection of the glmM gene in Helicobacter pylori isolates with a novel primer by PCR. *J Clin Microbiol* 2011; **49**: 1650-1652 [PMID: 21289140 DOI: 10.1128/JCM.00461-10]

19 **Attaran B**, Falsafi T, Moghaddam AN. Study of biofilm formation in C57Bl/6J mice by clinical isolates of Helicobacter pylori. *Saudi J Gastroenterol* 2016; **22**: 161-168 [PMID: 26997224]

20 **Franklin R**, Cockerill III M. Performance Standards for Antimicrobial Susceptibility Testing, Twenty-First Informational Supplement M100-S21. Clinical and Laboratory Standard Institute, 2011: 68-80

21 **Gerrits MM**, van Vliet AH, Kuipers EJ, Kusters JG. Helicobacter pylori and antimicrobial resistance: molecular mechanisms and clinical implications. *Lancet Infect Dis* 2006; **6**: 699-709 [PMID: 17067919 DOI: 10.1016/S1473-3099(06)70627-2]

22 **Grignon B**, Tankovic J, Mégraud F, Glupczynski Y, Husson MO, Conroy MC, Emond JP, Loulergue J, Raymond J, Fauchère JL. Validation of diffusion methods for macrolide susceptibility testing of Helicobacter pylori. *Microb Drug Resist* 2002; **8**: 61-66 [PMID: 12002651 DOI: 10.1089/10766290252913773]

23 **Brown HL**, van Vliet AH, Betts RP, Reuter M. Tetrazolium reduction allows assessment of biofilm formation by Campylobacter jejuni in a food matrix model. *J Appl Microbiol* 2013; **115**: 1212-1221 [PMID: 23910098]

24 **Rinttilä T**, Kassinen A, Malinen E, Krogius L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* 2004; **97**: 1166-1177 [PMID: 15546407 DOI: 10.1111/j.1365-2672.2004.02409.x]

25 **Zhang Z**, Liu ZQ, Zheng PY, Tang FA, Yang PC. Influence of efflux pump inhibitors on the multidrug resistance of Helicobacter pylori. *World J Gastroenterol* 2010; **16**: 1279-1284 [PMID: 20222174 DOI: 10.3748/wjg.v16.i10.1279]

26 **Queipo-Ortuño MI**, De Dios Colmenero J, Macias M, Bravo MJ, Morata P. Preparation of bacterial DNA template by boiling and effect of immunoglobulin G as an inhibitor in real-time PCR for serum samples from patients with brucellosis. *Clin Vaccine Immunol* 2008; **15**: 293-296 [PMID: 18077622 DOI: 10.1128/cvi.00270-07]

27 **Stark RM**, Gerwig GJ, Pitman RS, Potts LF, Williams NA, Greenman J, Weinzweig IP, Hirst TR, Millar MR. Biofilm formation by Helicobacter pylori. *Lett Appl Microbiol* 1999; **28**: 121-126 [PMID: 10063642 DOI: 10.1046/j.1365-2672.1999.00481.x]

28 **Cellini L**, Grande R, Traini T, Di Campli E, Di Bartolomeo S, Di Iorio D, Caputi S. Biofilm formation and modulation of luxS and rpoD expression by Helicobacter pylori. *Biofilms* 2005; **2**: 1-9

29 **Yonezawa H**, Osaki T, Kurata S, Zaman C, Hanawa T, Kamiya S. Assessment of in vitro biofilm formation by Helicobacter pylori. *J Gastroenterol Hepatol* 2010; **25** Suppl 1: S90-S94 [PMID: 20586874 DOI: 10.1111/j.1440-1746.2009.06213.x]

30 **Carron MA**, Tran VR, Sugawa C, Coticchia JM. Identification of Helicobacter pylori biofilms in human gastric mucosa. *J Gastrointest Surg* 2006; **10**: 712-717 [PMID: 16713544 DOI: 10.1016/j.gassur.2005.10.019]

31 **Coticchia JM**, Sugawa C, Tran VR, Gurrola J, Kowalski E, Carron MA. Presence and density of Helicobacter pylori biofilms in human gastric mucosa in patients with peptic ulcer disease. *J Gastrointest Surg* 2006; **10**: 883-889 [PMID: 16769546 DOI: 10.1016/j.gassur.2005.12.009]

32 **Cerca N**, Martins S, Cerca F, Jefferson KK, Pier GB, Oliveira R, Azeredo J. Comparative assessment of antibiotic susceptibility of coagulase-negative staphylococci in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry. *J Antimicrob Chemother* 2005; **56**: 331-336 [PMID: 15980094]

33 **Yonezawa H**, Osaki T, Hanawa T, Kurata S, Ochiai K, Kamiya S. Impact of Helicobacter pylori biofilm formation on clarithromycin susceptibility and generation of resistance mutations. *PLoS One* 2013; **8**: e73301 [PMID: 24039906 DOI: 10.1371/journal.pone.0073301]

34 **O"Morain C**, Smith S. Helicobacter pylori Treatment Failure: The Rationale for Alternative Antibiotics. *Digestion* 2016; **93**: 309-310 [PMID: 27246467 DOI: 10.1159/000444362]

35 **Soto SM**. Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. *Virulence* 2013; **4**: 223-229 [PMID: 23380871 DOI: 10.4161/viru.23724]


**P- Reviewer**: Chai FY, March-Rossello GA, Yakoob J, Yamaoka Y
**S- Editor**: Qi Y **L- Editor**: Filipodia **E- Editor**: Zhang FF

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/

*World J Gastroenterol* 2017 February 21; 23(7): 1171-1179

DOI: 10.3748/wjg.v23.i7.1171

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

ORIGINAL ARTICLE

Basic Study

# Thymoquinone suppresses migration of LoVo human colon cancer cells by reducing prostaglandin E2 induced COX-2 activation

Hsi-Hsien Hsu, Ming-Cheng Chen, Cecilia Hsuan Day, Yueh-Min Lin, Shin-Yi Li, Chuan-Chou Tu, Viswanadha Vijaya Padma, Hui-Nung Shih, Wei-Wen Kuo, Chih-Yang Huang

Hsi-Hsien Hsu, Division of Colorectal Surgery, Mackay Memorial Hospital, Taipei 10449, Taiwan

Hsi-Hsien Hsu, Mackay Medicine, Nursing and Management College, Taipei 10449, Taiwan

Ming-Cheng Chen, Division of Colorectal Surgery, Taichung Veterans General Hospital, Taichung 40705, Taiwan

Cecilia Hsuan Day, Department of Nursing, MeiHo University, Pingtung 912, Taiwan

Yueh-Min Lin, Department of Pathology, Changhua Christian Hospital, Changhua 500, Taiwan

Yueh-Min Lin, Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli 35664, Taiwan

Shin-Yi Li, Graduate Institute of Basic Medical Science, China Medical University, Taichung 40402, Taiwan

Chuan-Chou Tu, Division of Chest Medicine, Department of Internal Medicine, Armed Force Taichung General Hospital, Taichung 41152, Taiwan

Viswanadha Vijaya Padma, Department of Biotechnology, Bharathiar University, Coimbatore 641046, India

Hui-Nung Shih, Chih-Yang Huang, Graduate Institute of Chinese Medical Science, China Medical University, Taichung 41352, Taiwan

Wei-Wen Kuo, Department of Biological Science and Technology, China Medical University, Taichung 40402, Taiwan

Chih-Yang Huang, Department of Health and Nutrition Biotechnology, Asia University, Taichung 41352, Taiwan

Author contributions: Hsu HH, Kuo WW and Huang CY contributed equally to this work; Hsu HH, Chen MC, Day CH, Lin YM, Li SY, Tu CC, Padma VV, Shih HN, Kuo WW and Huang CY designed the research; Hsu HH, Chen MC, Day CH, Lin YM, Li SY, Tu CC, Padma VV, Kuo WW and Huang CY performed the research; Hsu HH and Shih HN wrote the paper.

Supported by (in part) the Taiwan Ministry of Health and Welfare Clinical Trial and Research Center of Excellence, No. MOHW105-TDU-B-212-133019.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of China Medical University, Taiwan.

Institutional animal care and use committee statement: All protocols were reviewed and approved by the Institutional Review Board (IRB, ethical clearance number 104-223-N), Animal Care and Use Committee of China Medical University, Taichung, China, and the study was conducted in accordance with the principles of laboratory animal care.

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: Chih-Yang Huang, PhD, Graduate Institute of Chinese Medical Science, China Medical University, No. 91, Hsueh-Shih Road, Taichung 40402,

Taiwan. cyhuang@mail.cmu.edu.tw
Telephone: +886-4-22053366-3313
Fax: +886-4-22032295

Received: September 6, 2016
Peer-review started: September 8, 2016
First decision: October 20, 2016
Revised: November 9, 2016
Accepted: December 16, 2016
Article in press: December 19, 2016
Published online: February 21, 2017

## Abstract

***AIM***

To identify potential anti-cancer constituents in natural extracts that inhibit cancer cell growth and migration.

***METHODS***

Our experiments used high dose thymoquinone (TQ) as an inhibitor to arrest LoVo (a human colon adenocarcinoma cell line) cancer cell growth, which was detected by cell proliferation assay and immunoblotting assay. Low dose TQ did not significantly reduce LoVo cancer cell growth. Cyclooxygenase 2 (COX-2) is an enzyme that is involved in the conversion of arachidonic acid into prostaglandin E2 (PGE2) in humans. PGE2 can promote COX-2 protein expression and tumor cell proliferation and was used as a control.

***RESULTS***

Our results showed that 20 μmol/L TQ significantly reduced human LoVo colon cancer cell proliferation. TQ treatment reduced the levels of p-PI3K, p-Akt, p-GSK3β, and β-catenin and thereby inhibited the downstream COX-2 expression. Results also showed that the reduction in COX-2 expression resulted in a reduction in PGE2 levels and the suppression of EP2 and EP4 activation. Further analysis showed that TG treatment inhibited the nuclear translocation of β-catenin in LoVo cancer cells. The levels of the cofactors LEF-1 and TCF-4 were also decreased in the nucleus following TQ treatment in a dose-dependent manner. Treatment with low dose TQ inhibited the COX-2 expression at the transcriptional level and the regulation of COX-2 expression efficiently reduced LoVo cell migration. The results were further verified *in vivo* by confirming the effects of TQ and/or PGE2 using tumor xenografts in nude mice.

***CONCLUSION***

TQ inhibits LoVo cancer cell growth and migration, and this result highlights the therapeutic advantage of using TQ in combination therapy against colorectal cancer.

**Key words:** Thymoquinone; LoVo cell; Cyclooxygenase 2; Prostaglandin E2; Migration



**Core tip:** Prostaglandin E2 (PGE2) induces migration of human LoVo colon cancer cells, and the major mechanism involves the activation of the p-Akt/p-PI3K/p-GSK3β/β-catenin/LEF-1/TCF-4 pathway that ultimately up-regulates cyclooxygenase 2 (COX-2) expression. Thymoquinone (TQ) suppresses cancer cell migration and represents a potential therapeutic target for colon adenocarcinoma metastasis. PGE2 activation of COX-2 and β-catenin to induce human LoVo colon cancer cell migration was blocked by TQ. Our study used cell proliferation assay, immunoblotting assay, immunofluorescence assay, nuclear extraction and *in vivo* experiments to examine the COX2 protein, which affects the metastasis of highly metastatic LoVo cancer cells treated with TQ.

Hsu HH, Chen MC, Day CH, Lin YM, Li SY, Tu CC, Padma VV, Shih HN, Kuo WW, Huang CY. Thymoquinone suppresses migration of LoVo human colon cancer cells by reducing prostaglandin E2 induced COX-2 activation. *World J Gastroenterol* 2017; 23(7): 1171-1179 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1171.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1171

## INTRODUCTION

Colorectal cancer is one of the most universally diagnosed gastrointestinal cancers and among the main causes of cancer-related death in western developed countries[1,2]. Despite advanced chemotherapeutic treatments, more than 130000 new cases of colon cancer are diagnosed each year[3], causing more than 56000 deaths/year in America[4]. Thymoquinone (TQ) is a phytochemical compound isolated from *Nigella sativa* that possesses anti-carcinogenic activity and induces apoptosis in tumor cells, and it can interfere with cancer cell survival through different mechanisms[5,6]. Available treatments for cancer include surgical removal, chemotherapy and adjuvant chemotherapy for patients who are strong enough to undergo it. To date, the surgical removal of cancer tissue is considered the most appropriate way to address colon cancer. Our present study investigated the use of phytochemical drugs as a supplementary chemotherapy approach. Laboratory studies have shown that TQ significantly inhibits oral cancer through the p38β MAPK family[7]. Among the hereditary colon cancers, hereditary non-polyposis colon cancer (HNPCC) patients present a particularly high risk for synchronous metastasis *via* the lymphatic system[8,9]. In this study, we used the colorectal cancer cell line LoVo, which was developed from a 56-year-old colon cancer patient. Many previous studies have verified that prostaglandin E2 (PGE2) promotes cancer development and have considered it a cancer marker; therefore, we used PGE2 as a control[10-12]. PGE2 seems to assist cell survival in colorectal cancer cells

by augmenting Ras-MAPK signaling[13].

Compared to normal intestinal tissues, COX-2 expression is 80%-90% higher in colorectal cancers. Cancers of the head, breast, cervix, bladder and gastrointestinal system have also shown high levels of COX-2 expression[14-16]. COX-2/PGE2 signaling affects cell physiology in multiple tumor types and maintains colorectal tumorigenesis[12,17]. PGE2 as a proangiogenic factor is associated with transformed vascular permeability and angiogenesis[18]. COX-2 expression is thought to contribute to the principal PGE2 metabolic product[19,20]. Some non-steroidal anti-inflammatory drugs (NSAIDs) and vegetables produce anti-tumor effects that reduce PGE2 synthesis or inhibit COX-2[21-24]. In our experiments, we sought to identify compounds similar to NSAIDs or adjunct drugs to increase the effectiveness of cancer chemotherapy. Our experimental drug, TQ, has promising anti-tumor effects, and it inhibited the incidence of fore-stomach tumors and fibrosarcoma tumors and increased cellular longevity[25,26]. We previously evaluated PGE2-induced migration in human LoVo cancer cells, and the major mechanism involves the activation of the p-Akt/p-PI3K/p-GSK3β/β-catenin pathway that ultimately up-regulates COX-2 expression (unpublished data). After the addition of TQ, the exact anticancer mechanism produced by PGE2 was determined. Previous studies have demonstrated that β-catenin translocation, which includes co-interaction with and activation of the promoters LEF-1 and TCF-4, subsequently modulates downstream gene expression[27]. The nuclear cofactors LEF and TCF were triggered to initiate the transcription and translation of COX-2[28]. Cell metastasis efficiency is a focus of our work because it correlates with COX-2 activity[29,30]. Moreover, cell migration is promoted due to COX-2 expression[31]. Numerous studies of animals treated with TQ have demonstrated that TQ is not toxic[32-34].

Our study used immunoblotting assays, immunofluorescence assays, nuclear extraction and *in vivo* experiments to examine the COX2 protein, which affects the metastasis of highly metastatic LoVo cancer cells treated with TQ.

## MATERIALS AND METHODS

### *Cells, antibodies, reagents, and enzymes*

The human colon cancer cell line LoVo was obtained from the American Tissue Culture Collection (ATCC) (Rockville, MD, United States). LoVo cells were established from metastatic nodules that were resected from a 56-year-old colon adenocarcinoma patient.

We utilized antibodies against the following proteins: phospho-PI3K, phospho-Akt, COX-2, phospho-GSK3β, β-catenin, LEF-1, HADAC-1 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, United States), and TCF-4 (Cell Signaling Technology, Inc. Beverly, MA, United States). α-tubulin and β-actin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, United States) were used as loading controls. The following horseradish peroxidase-conjugated antibodies were purchased from Santa Cruz Biotechnology, Inc. (CA, United States): goat anti-mouse IgG, goat anti-rabbit IgG, and rabbit anti-goat IgG. Nude mice were purchased from the National Laboratory Animal Center (NLAC).

### *Cell culture*

The colon cancer cell line LoVo was cultured in 10-cm$^2$ culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 μg/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine, 1 mmol/L HEPES buffer, and 10% fetal bovine serum (FBS) in humidified air (5% $CO_2$) at 37 ℃.

### *Cell proliferation assay*

LoVo cells were seeded at a density of 1.5 × 10$^4$ cells per well in 24-well plates, and after 24 h the cells were treated with different concentrations of TQ varying from 0 to 20 μmol/L (Sigma Aldrich, St Louis MO, United States) dissolved in DMSO. An MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine living cells 24 h after treatment. Culture supernatants were removed, and MTT (Sigma, United States) in phosphate-buffered saline (PBS, pH 7.4) was added to each well. After 4 h of incubation at 37 ℃, the MTT solution was removed, and DMSO was added to dissolve the resultant formazan crystals. Absorbance was read at 540 nm in a Flexstation 3 device (MDS Analytical Technologies, Canada). The percentage viability was calculated as (test-background)/(control-background) × 100.

### *Immunoblotting assay*

Cultured LoVo cells were washed with cold PBS and resuspended in lysis buffer [50 mmol/L Tris (pH 7.5), 0.5 mol/L NaCl, 1.0 mmol/L EDTA (pH 7.5), 10% glycerol, 1 mmol/L BME, 1% IGEPAL-630, and a proteinase inhibitor cocktail (Roche Molecular Biochemical)] to isolate total proteins. After incubation for 30 min on ice, the supernatant was collected by centrifugation at 12000 × *g* for 15 min at 4 ℃. Protein concentration was then determined using the Bradford method. Samples containing equal protein amounts (60 μg) were loaded and analyzed using immunoblotting analysis. Proteins were separated using 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, Belford, MA, United States). The membranes were blocked with blocking buffer (5% non-fat dry milk, 20 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, and 0.1% Tween 20) for at least 1 h at room temperature. The membranes were incubated with primary antibodies in the above solution on an orbital shaker at 4 ℃ overnight. Following the primary antibody incubations, the membranes were incubated with horseradish

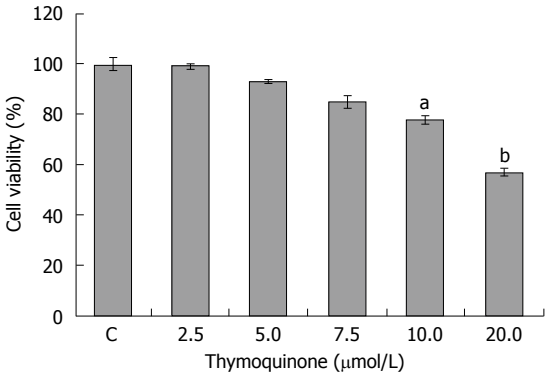



**Figure 1 Thymoquinone affects the viability of LoVo colon cancer cells.** To explore the effect of thymoquinone (TQ) on the viability of human LoVo colon cancer cells, we first treated LoVo cells with various concentrations (2.5, 5, 7.5, 10 and 20 μmol/L) of TQ for 24 h and subsequently measured cell viability by MTT assay. The results showed a significant reduction of cell viability of approximately 60% following treatment (20 μmol/L) for 24 h. Each value represents the mean ± SE. $^{a}P < 0.05$; $^{b}P < 0.01$.

peroxidase-linked secondary antibodies (anti-rabbit, anti-mouse, or anti-goat IgG). Detection was performed using ECL reagents and a digital imaging system.

### *Nuclear extraction*

Cytoplasmic and nuclear fractions were obtained using extraction reagents containing membrane lysis buffer [10 mmol/L HEPES (pH 8.0), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 1 mmol/L DDT, and proteinase inhibitor] and nuclear lysis buffer [20 mmol/L HEPES (pH 8.0), 1.5 mmol/L $MgCl_2$, 10 mmol/L NaCl, 1 mmol/L DDT, 0.2 mmol/L EDTA, 0.25 mol/L glycerol, and proteinase inhibitor]. Following treatment, the cells were resuspended in PBS, incubated with ice-cold membrane lysis buffer for 10 min, and centrifuged at 12000 rpm for 2 min to pellet nuclei. The supernatant was stored for use as a cytoplasmic fraction, and the nuclear pellet was lysed with nuclear lysis buffer to obtain a nuclear fraction.

### *Immunofluorescence assay*

LoVo cells were treated with increasing TQ dosages (5, 10, and 20 μmol/L) for 24 h, and PGE2 (5 μmol/L) was added for the last six hours. An immunofluorescence assay was performed on the LoVo cells using a β-catenin antibody (1:250) directed against a target of interest. A fluorophore-conjugated secondary antibody (green) that was directed against the primary antibody was used for detection, and a blue fluorescent DAPI nuclear acid counterstain that preferentially stains dsDNA was included. Anti-β-catenin (green) merged with DAPI (blue) is also shown in the resultant confocal microscopy images.

### *Migration assay*

A migration assay was performed using a 48-well Boyden chamber (Neuro Probe) plate with 8-μm pore size polycarbonate membrane filters[35]. The lower compartment was filled with DMEM containing 10% FBS. LoVo cells were placed in the upper part of the Boyden chamber, which contained serum-free medium, and incubated for 4-8 h. After incubation, cells on the membrane filter were fixed with methanol and stained with 0.05% Giemsa for 1 h. The cells on the upper filter surface were removed with a cotton swab. The filters were then rinsed in double-distilled water for additional stain leaching. The cells were then air-dried for 20 min. Migratory phenotypes were determined by counting the cells that migrated to the lower side of the filter using microscopy at 200 × and 400 × magnification. Ten fields were blindly selected and counted as the mean cell number in each filter. The sample was assayed in triplicate.

### *Study animals*

Twelve nude mice were bred in the animal care facility of the Chinese Medicine Library (Taichung, Taiwan) and were purchased from The National Laboratory Animal Center. The animals received subcutaneous injections of LoVo colorectal cancer cells ($1 \times 10^6$ cells per injection) in the back. Ambient temperature was maintained at 25 ℃, and the mice were kept on an artificial 12-h light-dark cycle, with the light period beginning at 7:00 am. All of the protocols were approved by the Institutional Animal Care and Use Committee of China Medical University, Taichung, Taiwan, ROC.

LoVo cells were treated with PGE2 (5 μmol/L) for 24 h and were then injected subcutaneously into the mice ($1 \times 10^6$ cells). After one week, various concentrations (0.5, 10 and 20 μmol/L) of TQ were administered for three weeks (3 times per week) by intraperitoneal injection. All protocols were reviewed and approved by the Institutional Review Board (IRB, ethical clearance number 104-223-N), Animal Care and Use Committee of China Medical University, Taichung, Republic of China, and the study was conducted in accordance with the principles of laboratory animal care[36].

### *Statistical analysis*

Each experiment was duplicated at least three times. The results are presented as the mean ± SE, and statistical comparisons were made using Student's *t*-test. Significance was defined at $P < 0.05$ or 0.01.

## RESULTS

### *Effect of TQ on viability of LoVo cells*

We first tested the effect of TQ on the viability of LoVo cells. Several TQ concentrations were used to evaluate cell viability after co-culture for 24 h. The results indicated that 20 μmol/L TQ can significantly reduce LoVo cell proliferation (Figure 1).

### *p-PI3K-, p-Akt-, p-GSK3β- and β-catenin-induced COX-2 expression is down-regulated by TQ*

Previous reports have demonstrated that PGE2

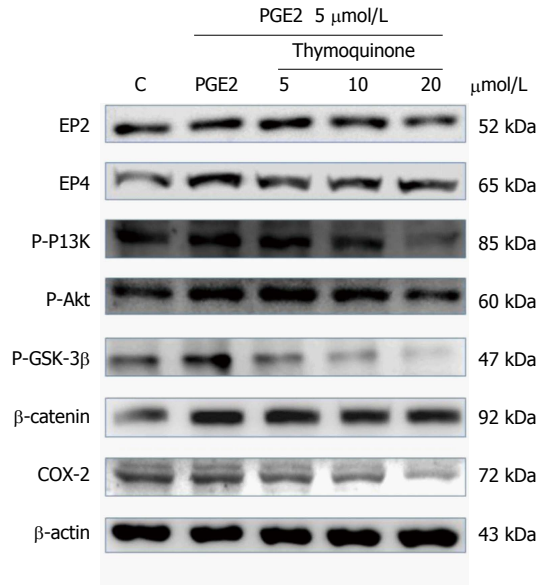



**Figure 2 Thymoquinone inhibits the expression of PI3K, Akt, p-GSK3β, β-catenin and COX-2 proteins in LoVo cells.** LoVo cells were cultured in medium that was treated with TQ (5, 10 and 20 μmol/L) for 24 h; PGE2 (5 μmol/L) was added for the last 6 h. Following this, the cells were immunoblotted with the indicated antibodies. The cells were lysed, and the extracts were separated by 10% SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibodies against p-PI3K, p-Akt, p-GSK3β, β-catenin and COX-2.

increases COX-2 expression *via* activation of the EP4/β-catenin pathway, suggesting that PGE2 regulates p-PI3K, p-AKT, and p-GSK-3β expression in LoVo cells. PGE2 has been reported to activate downstream signaling *via* EP2 and EP4 to elicit various biological responses. As shown in Figure 2, TQ treatment reduced the up-regulation of COX-2 expression by PGE2, and β-catenin had an obvious effect on EP2 and EP4 regulation by PGE2.

### *Effect of TQ on β-catenin translocation*

β-catenin is a key protein that affects nuclear COX-2 transcription. We used an immunofluorescence assay to evaluate β-catenin protein levels in the cytosol and nucleus. Confocal microscopy confirmed that TQ treatment led to the translocation of β-catenin into the LoVo cell nucleus (Figure 3).

### *Nuclear translocation inhibition of β-catenin in the LoVo cancer cell line*

Previous studies have demonstrated that the translocation of β-catenin involves co-interaction with and activation of the promoters LEF-1 and TCF-4, which subsequently modulate downstream gene expression. Evaluation of isolated LoVo cell nuclei showed that β-catenin translocation decreased in the nucleus, but its interactions with the cofactors LEF-1 and TCF-4 did not change. We observed a concentration-dependent decrease in the levels of β-catenin and the proteins LEF-1 and TCF-4 in the nucleus following TQ treatment (Figure 4).

### *TQ treatment inhibits LoVo cell migration*

Migration is an important step in LoVo colon cancer progression because this cancer metastasizes supraclavicular lymph nodes. We performed a Boyden chamber migration assay to determine the effect of TQ on LoVo cells and found that TQ inhibited LoVo cell migration in a dose-dependent manner (Figure 5).

### *Effect of TQ on tumor growth in nude mouse xenografts*

To verify previous results on the growth inhibition and anti-metastatic effects of TQ in human LoVo cells, the therapeutic potential of TQ in a pre-subcutaneous LoVo cell model in nude mice was determined. TQ was administered for 30 consecutive days. The drug did not induce death or weight loss of more than 15% to 20% of original weight, which is a very sensitive parameter for perniciousness in mice. The effects of TQ on PGE2-associated protein markers for inflammation and metastasis in the nude mouse xenograft model were then determined. COX-2 and β-catenin protein levels in different tissue groups in the nude mice were down-regulated by TQ treatment in a concentration-dependent manner (Figure 6).

## DISCUSSION

TQ was shown to be an anticancer agent by virtue of its anti-proliferative potential and capacity to induce cell cycle arrest[37]. In our previous studies, COX-2 protein was found to be regulated by PGE2, resulting in cell proliferation and migration. Here, a 20 μmol/L concentration of TQ, which is 50% of the non-cytotoxic concentration determined by MTT assay, arrested the migration of LoVo colorectal cancer cells. We tested TQ on LoVo colorectal cancer cells that were already induced by PGE2 (5 μmol/L); these cells exhibited significantly enhanced performance over the control group. Our experimental drug TQ inhibited the survival pathway proteins p-PI3K, p-Akt, p-GSK3β, β-catenin and COX-2.

When β-catenin is translocated from the cytosol into the nucleus, it plays a role in the transcription and translation of COX-2[28]. The translocation of β-catenin from the cytosol into the nucleus also plays a role in the transcription and translation of COX-2.

Nuclear isolation techniques and immunofluorescence were used to determine the localization of β-catenin and the COX-2 transcription cofactors LEF-1 and TCF-4[38]. Our experiments showed that TQ suppresses β-catenin translocation induced by PGE2, and the expression of both LEF-1 and TCF-4 was also suppressed. This result suggests that β-catenin loses its ability to translocate into the nucleus and bind to LEF-1 and TCF-4 following TQ treatment. These cofactors were also affected by TQ with a gradually declining, concentration-dependent trend. Immunofluorescence confocal microscopy showed

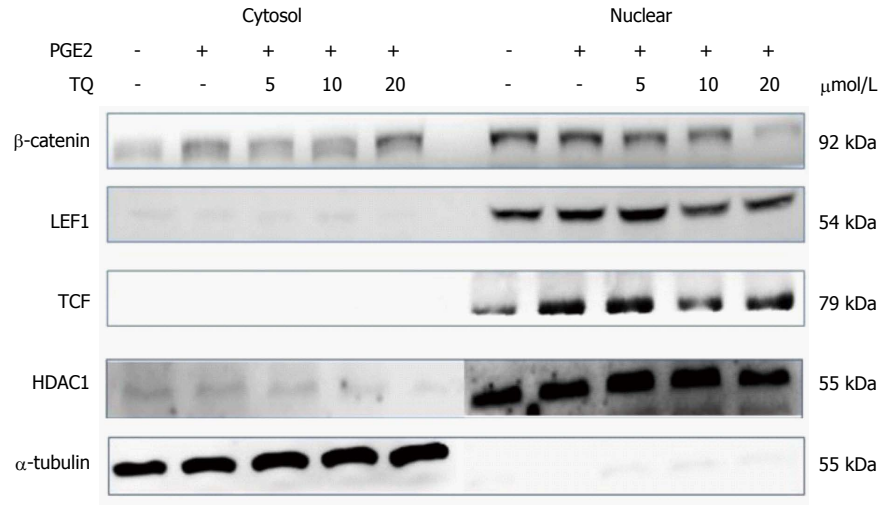



**Figure 3 Thymoquinone inhibits β-catenin nuclear translocation in the LoVo cancer cell line.** Nuclear isolation showed a decrease in β-catenin translocation into the nucleus. The cofactors LEF-1 and TCF-4 decreased in the nucleus following TQ treatment in a dose-dependent manner. TQ: Thymoquinone.

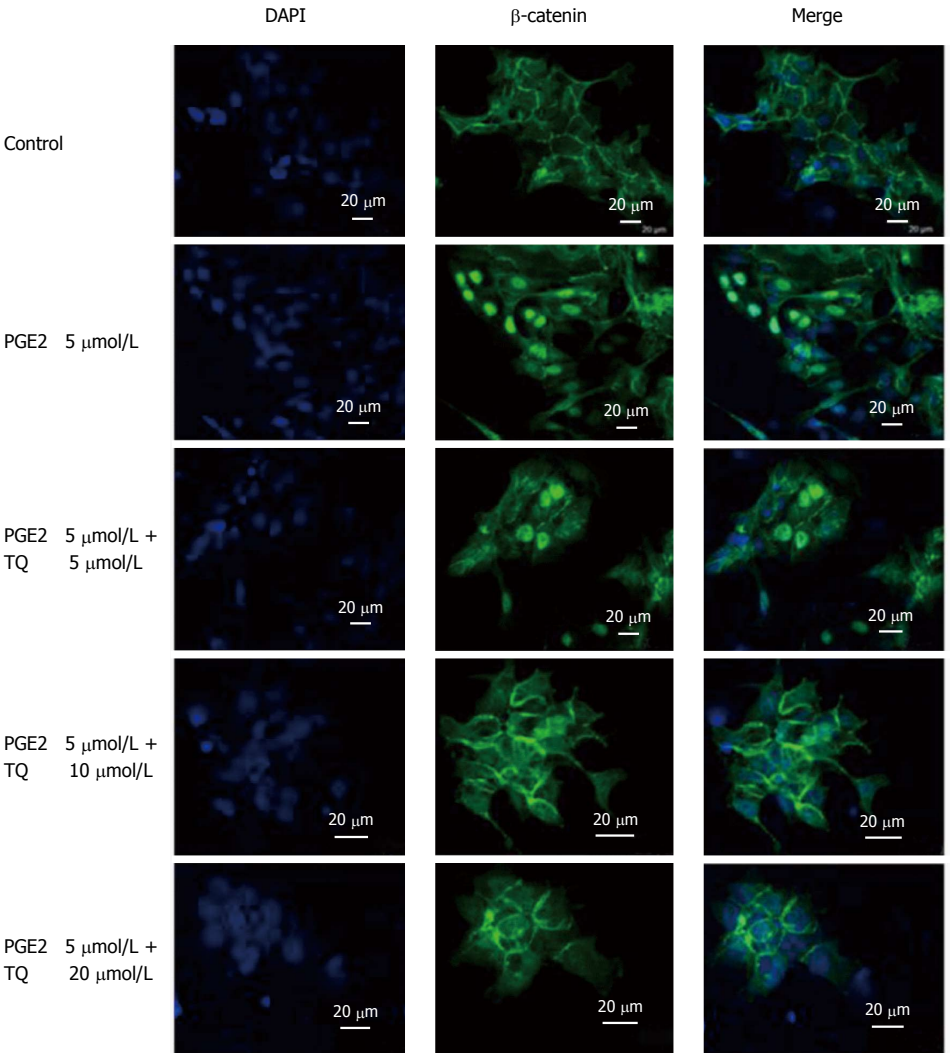



**Figure 4 Thymoquinone treatment inhibits β-catenin translocation into LoVo cell nuclei.** An immunofluorescence assay was performed on LoVo cells using a β-catenin primary antibody and a secondary antibody (1:250) producing green fluorescence; DAPI (blue fluorescence) was included to stain cell nuclei. Merged β-catenin and DAPI (green and blue, respectively) signals are shown. The indicated treatments were assessed. PGE2: Prostaglandin E2; TQ: Thymoquinone.

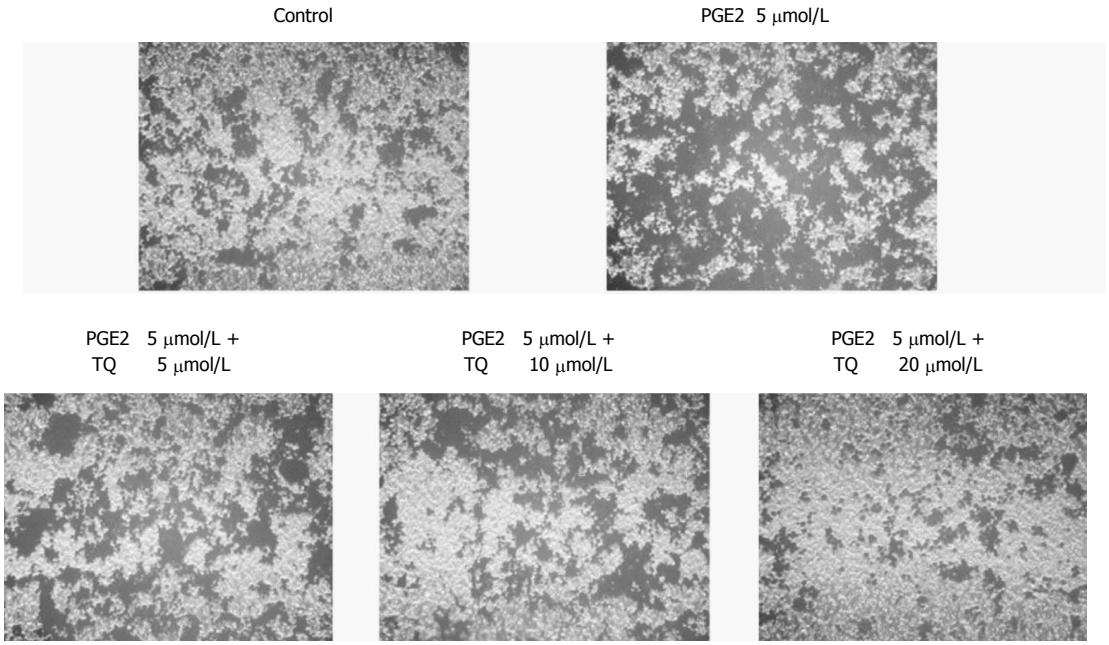



**Figure 5 Thymoquinone efficiently inhibits LoVo cell migration.** LoVo cells were pretreated with increasing dosages (5, 10, and 20 μmol/L) of TQ for 24 h. A Boyden chamber migration assay was performed to assess cell migration ability. The responses to different treatments were analyzed *via* microscopy. PGE2: Prostaglandin E2; TQ: Thymoquinone.

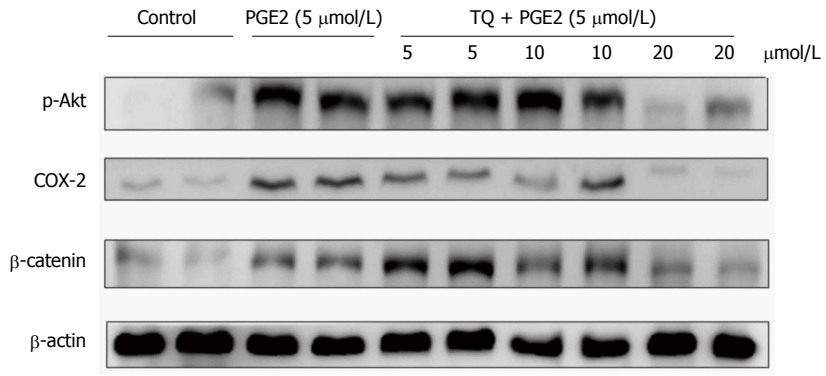



**Figure 6 Impact of thymoquinone and/or prostaglandin E2 administration on tumor growth in nude mice xenografts.** Tumor tissues were harvested from nude mice and lysed. Protein content was quantified and analyzed by immunoblotting. p-Akt, β-catenin and COX-2 levels in LoVo cells were detected. PGE2: Prostaglandin E2; TQ: Thymoquinone.

fluorescent images of anti-β-catenin (green) and DAPI staining (blue) in the panel representing the cell nucleus. These results indicate that TQ treatment inhibited β-catenin translocation into the nucleus and subsequently reduced COX-2 activation.

We can observe the impact of TQ on COX-2 by assessing these translation- and transcription-associated proteins. COX-2 plays a central role in cell migration. These results showed that decreasing COX-2 levels will correspondingly reduce cell migration. In this context, we demonstrated that TQ significantly reduces metastasis *in vivo*. However, we investigated the therapeutic potential of TQ using a highly aggressive human LOVO cancer cell xenograft nude mouse model. p-Akt, β-catenin and COX-2 expression substantially decreased. TQ inhibited LoVo colorectal cancer cell growth and inhibited migration. In the future, we will evaluate the therapeutic advantage of combining chemotherapeutic agents for colorectal cancer.

## COMMENTS

### Background

Several types of phytochemicals are used for cancer chemotherapy. The aim was to identify potential anti-cancer constituents in natural extracts that inhibit cancer cell growth and migration. Thymoquinone (TQ) is a phytochemical compound isolated from *Nigella sativa*. Previous data show that TQ suppresses the activation of AKT, inhibits cellular proliferation, and shows anti-oxidant/anti-inflammatory effects.

### Research frontiers

Prostaglandin E2 (PGE2) induces migration of human LoVo colon cancer cells, and the major mechanism involves the activation of the p-Akt/p-PI3K/p-GSK3β/β-catenin/LEF-1/TCF-4 pathway that ultimately up-regulates cyclooxygenase 2

(COX-2) expression.

### Innovations and breakthroughs

TQ suppresses cancer cell migration and represents a potential therapeutic target for colon adenocarcinoma metastasis. PGE2 activation of COX-2 and β-catenin to induce human LoVo colon cancer cell migration was blocked by thymoquinone.

### Applications

The results reveal that TQ can be considered a potential treatment strategy for advanced stage colon cancer treatment.

### Peer-review

The study described in this paper investigated the use of phytochemical drugs as a supplementary chemotherapy approach. For this purpose the authors used the high dose of TQ as an inhibitor to arrest LoVo (a human colon adenocarcinoma cell line) cell growth. The authors used immunoblotting assays, immunofluorescence assays, nuclear extraction and *in vivo* experiments to examine the COX2 protein, which affects the metastasis of highly metastatic LoVo cancer cells treated with TQ.

## REFERENCES


1 **Gali-Muhtasib H**, Ocker M, Kuester D, Krueger S, El-Hajj Z, Diestel A, Evert M, El-Najjar N, Peters B, Jurjus A, Roessner A, Schneider-Stock R. Thymoquinone reduces mouse colon tumor cell invasion and inhibits tumor growth in murine colon cancer models. *J Cell Mol Med* 2008; **12**: 330-342 [PMID: 18366456 DOI: 10.1111/j.1582-4934.2007.00095.x]

2 **Lin TY**, Fan CW, Maa MC, Leu TH. Lipopolysaccharide-promoted proliferation of Caco-2 cells is mediated by c-Src induction and ERK activation. *Biomedicine* (Taipei) 2015; **5**: 5 [PMID: 25705585 DOI: 10.7603/s40681-015-0005-x]

3 **Jemal A**, Thomas A, Murray T, Thun M. Cancer statistics, 2002. *CA Cancer J Clin* 2002; **52**: 23-47 [PMID: 11814064]

4 **Jemal A**, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, Feuer EJ, Thun MJ. Cancer statistics, 2004. *CA Cancer J Clin* 2004; **54**: 8-29 [PMID: 14974761]

5 **Woo CC**, Kumar AP, Sethi G, Tan KH. Thymoquinone: potential cure for inflammatory disorders and cancer. *Biochem Pharmacol* 2012; **83**: 443-451 [PMID: 22005518 DOI: 10.1016/j.bcp.2011.09.029]

6 **Towhid ST**, Schmidt EM, Schmid E, Münzer P, Qadri SM, Borst O, Lang F. Thymoquinone-induced platelet apoptosis. *J Cell Biochem* 2011; **112**: 3112-3121 [PMID: 21688304 DOI: 10.1002/jcb.23237]

7 **Abdelfadil E**, Cheng YH, Bau DT, Ting WJ, Chen LM, Hsu HH, Lin YM, Chen RJ, Tsai FJ, Tsai CH, Huang CY. Thymoquinone induces apoptosis in oral cancer cells through p38β inhibition. *Am J Chin Med* 2013; **41**: 683-696 [PMID: 23711149 DOI: 10.1142/S0192415X1350047X]

8 **Kalady MF**. Surgical management of hereditary nonpolyposis colorectal cancer. *Adv Surg* 2011; **45**: 265-274 [PMID: 21954693]

9 **Wu Z**, Zhang S, Aung LH, Ouyang J, Wei L. Lymph node harvested in laparoscopic versus open colorectal cancer approaches: a meta-analysis. *Surg Laparosc Endosc Percutan Tech* 2012; **22**: 5-11 [PMID: 22318051 DOI: 10.1097/SLE.0b013e3182432b49]

10 **Chang SH**, Liu CH, Conway R, Han DK, Nithipatikom K, Trifan OC, Lane TF, Hla T. Role of prostaglandin E2-dependent angiogenic switch in cyclooxygenase 2-induced breast cancer progression. *Proc Natl Acad Sci USA* 2004; **101**: 591-596 [PMID: 14688410 DOI: 10.1073/pnas.2535911100]

11 **Eruslanov E**, Kaliberov S, Daurkin I, Kaliberova L, Buchsbaum D, Vieweg J, Kusmartsev S. Altered expression of 15-hydroxyprostaglandin dehydrogenase in tumor-infiltrated CD11b myeloid cells: a mechanism for immune evasion in cancer. *J Immunol* 2009; **182**: 7548-7557 [PMID: 19494278 DOI: 10.4049/jimmunol.0802358]

12 **Greenhough A**, Smartt HJ, Moore AE, Roberts HR, Williams AC, Paraskeva C, Kaidi A. The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis* 2009; **30**: 377-386 [PMID: 19136477 DOI: 10.1093/carcin/bgp014]

13 **Kaidi A**, Qualtrough D, Williams AC, Paraskeva C. Direct transcriptional up-regulation of cyclooxygenase-2 by hypoxia-inducible factor (HIF)-1 promotes colorectal tumor cell survival and enhances HIF-1 transcriptional activity during hypoxia. *Cancer Res* 2006; **66**: 6683-6691 [PMID: 16818642 DOI: 10.1158/0008-5472.CAN-06-0425]

14 **Voutsadakis IA**. Pathogenesis of colorectal carcinoma and therapeutic implications: the roles of the ubiquitin-proteasome system and Cox-2. *J Cell Mol Med* 2007; **11**: 252-285 [PMID: 17488476 DOI: 10.1111/j.1582-4934.2007.00032.x]

15 **Sano H**, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K, Kimura S, Kato H, Kondo M, Hla T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer Res* 1995; **55**: 3785-3789 [PMID: 7641194]

16 **Eberhart CE**, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994; **107**: 1183-1188 [PMID: 7926468]

17 **Brown JR**, DuBois RN. COX-2: a molecular target for colorectal cancer prevention. *J Clin Oncol* 2005; **23**: 2840-2855 [PMID: 15837998 DOI: 10.1200/JCO.2005.09.051]

18 **Form DM**, Auerbach R. PGE2 and angiogenesis. *Proc Soc Exp Biol Med* 1983; **172**: 214-218 [PMID: 6572402]

19 **Pugh S**, Thomas GA. Patients with adenomatous polyps and carcinomas have increased colonic mucosal prostaglandin E2. *Gut* 1994; **35**: 675-678 [PMID: 8200564]

20 **Rigas B**, Goldman IS, Levine L. Altered eicosanoid levels in human colon cancer. *J Lab Clin Med* 1993; **122**: 518-523 [PMID: 8228569]

21 **Hanif R**, Pittas A, Feng Y, Koutsos MI, Qiao L, Staiano-Coico L, Shiff SI, Rigas B. Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochem Pharmacol* 1996; **52**: 237-245 [PMID: 8694848]

22 **Elder DJ**, Halton DE, Hague A, Paraskeva C. Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug: independence from COX-2 protein expression. *Clin Cancer Res* 1997; **3**: 1679-1683 [PMID: 9815550]

23 **Chan TA**, Morin PJ, Vogelstein B, Kinzler KW. Mechanisms underlying nonsteroidal antiinflammatory drug-mediated apoptosis. *Proc Natl Acad Sci USA* 1998; **95**: 681-686 [PMID: 9435252]

24 **Ciou SY**, Hsu CC, Kuo YH, Chao CY. Effect of wild bitter gourd treatment on inflammatory responses in BALB/c mice with sepsis. *Biomedicine* (Taipei) 2014; **4**: 17 [PMID: 25520930 DOI: 10.7603/s40681-014-0017-y]

25 **Badary OA**, Gamal El-Din AM. Inhibitory effects of thymoquinone against 20-methylcholanthrene-induced fibrosarcoma tumorigenesis. *Cancer Detect Prev* 2001; **25**: 362-368 [PMID: 11531013]

26 **Badary OA**, Al-Shabanah OA, Nagi MN, Al-Rikabi AC, Elmazar MM. Inhibition of benzo(a)pyrene-induced forestomach carcinogenesis in mice by thymoquinone. *Eur J Cancer Prev* 1999; **8**: 435-440 [PMID: 10548399]

27 **Kriegl L**, Horst D, Reiche JA, Engel J, Kirchner T, Jung A. LEF-1 and TCF4 expression correlate inversely with survival in colorectal cancer. *J Transl Med* 2010; **8**: 123 [PMID: 21092222 DOI: 10.1186/1479-5876-8-123]

28 **Nuñez F**, Bravo S, Cruzat F, Montecino M, De Ferrari GV. Wnt/β-catenin signaling enhances cyclooxygenase-2 (COX2) transcriptional activity in gastric cancer cells. *PLoS One* 2011; **6**: e18562 [PMID: 21494638 DOI: 10.1371/journal.pone.0018562]

29 **Xu L**, Stevens J, Hilton MB, Seaman S, Conrads TP, Veenstra TD, Logsdon D, Morris H, Swing DA, Patel NL, Kalen J, Haines DC, Zudaire E, St Croix B. COX-2 inhibition potentiates antiangiogenic cancer therapy and prevents metastasis in preclinical models. *Sci Transl Med* 2014; **6**: 242ra84 [PMID: 24964992 DOI: 10.1126/

scitranslmed.3008455]
30 **Emenaker NJ**, Zudaire E, St Croix B. Chemoprevention of metastasis. *Oncotarget* 2014; **5**: 6556-6557 [PMID: 25179303]
31 **Zhao L**, Wu Y, Xu Z, Wang H, Zhao Z, Li Y, Yang P, Wei X. Involvement of COX-2/PGE2 signalling in hypoxia-induced angiogenic response in endothelial cells. *J Cell Mol Med* 2012; **16**: 1840-1855 [PMID: 22050691 DOI: 10.1111/j.1582-4934.2011.01479.x]
32 **Kirui PK**, Cameron J, Benghuzzi HA, Tucci M, Patel R, Adah F, Russell G. Effects of sustained delivery of thymoqiunone on bone healing of male rats. *Biomed Sci Instrum* 2004; **40**: 111-116 [PMID: 15133944]
33 **Hosseinzadeh H**, Parvardeh S. Anticonvulsant effects of thymoquinone, the major constituent of Nigella sativa seeds, in mice. *Phytomedicine* 2004; **11**: 56-64 [PMID: 14971722 DOI: 10.1078/0944-7113-00376]
34 **Attoub S**, Sperandio O, Raza H, Arafat K, Al-Salam S, Al Sultan MA, Al Safi M, Takahashi T, Adem A. Thymoquinone as an anticancer agent: evidence from inhibition of cancer cells viability and invasion in vitro and tumor growth in vivo. *Fundam Clin Pharmacol* 2013; **27**: 557-569 [PMID: 22788741 DOI: 10.1111/j.1472-8206.2012.01056.x]
35 **Hsieh YC**, Hsieh SJ, Chang YS, Hsueh CM, Hsu SL. The lipoxygenase inhibitor, baicalein, modulates cell adhesion and migration by up-regulation of integrins and vinculin in rat heart endothelial cells. *Br J Pharmacol* 2007; **151**: 1235-1245 [PMID: 17592510 DOI: 10.1038/sj.bjp.0707345]
36 **U.S. National Institutes of Health**. Laboratory animal welfare; proposed U.S. government principles for the utilization and care of vertebrate animals used in testing, research and training. *Fed Regist* 1984; **49**: 29350-29351 [PMID: 11655723]
37 **Jrah-Harzallah H**, Ben-Hadj-Khalifa S, Almawi WY, Maaloul A, Houas Z, Mahjoub T. Effect of thymoquinone on 1,2-dimethyl-hydrazine-induced oxidative stress during initiation and promotion of colon carcinogenesis. *Eur J Cancer* 2013; **49**: 1127-1135 [PMID: 23131834 DOI: 10.1016/j.ejca.2012.10.007]
38 **Daniels DL**, Weis WI. Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. *Nat Struct Mol Biol* 2005; **12**: 364-371 [PMID: 15768032 DOI: 10.1038/nsmb912]

**P- Reviewer**: Kunac N **S- Editor**: Gong ZM
**L- Editor**: Wang TQ **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/

*World J Gastroenterol* 2017 February 21; 23(7): 1180-1188

DOI: 10.3748/wjg.v23.i7.1180

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

ORIGINAL ARTICLE

Basic Study

# Jianpi Qingchang decoction alleviates ulcerative colitis by inhibiting nuclear factor-κB activation

Lie Zheng, Ya-Li Zhang, Yan-Cheng Dai, Xuan Chen, De-Liang Chen, Yue-Ting Dai, Zhi-Peng Tang

Lie Zheng, Yan-Cheng Dai, Xuan Chen, De-Liang Chen, Yue-Ting Dai, Department of Gastroenterology, Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China

Lie Zheng, Ya-Li Zhang, Zhi-Peng Tang, Institute of Digestive Diseases, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China

Author contributions: Zheng L and Zhang YL contributed equally to this work, and both performed the majority of experiments and analysis of the data; Dai YC, Chen X and Dai YT participated in the treatment of animals; Chen DL conducted the ELISA; Zheng L and Tang ZP designed and coordinated the research; Zheng L wrote and revised the paper.

Supported by the National Natural Science Foundation of China, No. 81403355 and No. 81573892; and the Project of 3-Year Action Plan for Shanghai Municipal Chinese Medicine Development, No. ZY3-RCPY-2-2001.

Institutional review board statement: This study was reviewed and approved by the Institutional Review Board of the Institute of Digestive Disease, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China.

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 Shanghai University of Traditional Chinese Medicine (No. SZY 201510001).

Conflict-of-interest statement: The authors declare that there are no conflicts of interest related to this study.

Data sharing statement: No additional data are available.



Manuscript source: Unsolicited manuscript

Correspondence to: Zhi-Peng Tang, MD, PhD, Professor, Director, Institute of Digestive Diseases, Shanghai University of Traditional Chinese Medicine, 725 Wanping Road, Shanghai 200032, China. zhipengtang@sohu.com
Telephone: +86-21-64385700

Received: June 30, 2016
Peer-review started: July 1, 2016
First decision: August 29, 2016
Revised: September 13, 2016
Accepted: October 10, 2016
Article in press: October 10, 2016
Published online: February 21, 2017

## Abstract

***AIM***

To investigate the therapeutic effect of Jianpi Qingchang decoction (JPQCD) on dextran sulfate sodium (DSS)-induced ulcerative colitis (UC) in mice.

***METHODS***

C57BL/c mice were injected intragastrically with 5% DSS instead of drinking water for 7 d, and their body weight, diarrhea severity and fecal bleeding were monitored, while the mice in the control group were treated with standard drinking water, without DSS. After 7 d, the DSS drinking water was changed to normal water and the DSS group continued with DSS water. The control and DSS groups were given normal saline by intragastric injection. The 5-aminosalicylic acid (5-ASA) group was treated orally with 5-ASA at a dose of 100 mg/kg daily. The JPQCD group was treated orally with JPQCD at a dose of 17.1 g/kg daily. On day 14, the colon length was measured, the colorectal

histopathological damage score was assessed, and protein levels of interleukin (IL)-1β, IL-8 and tumor necrosis factor-alpha (TNF-α) in colon supernatants were measured by enzyme-linked immunosorbent assay. mRNA expression of IL-1β, IL-8, TNF-α and nuclear factor-kappa B (NF-κB) was detected by real-time quantitative polymerase chain reaction. Western blotting was used to detect the protein expression of NF-κB and inhibitor of kappa B.

***RESULTS***

Acute inflammation occurred in the mice administered DSS, including the symptoms of losing body weight, loose feces/watery diarrhea and presence of fecal blood; all these symptoms worsened at 7 d. The colons of mice treated with DSS were assessed by histological examination, and the results confirmed that acute inflammation had occurred, as evidenced by loss of colonic mucosa and chronic inflammatory cell infiltration, and these features extended into the deeper layer of the colon walls. The expression levels of IL-1β, IL-8 and TNF-α in the DSS group were higher than those in the control group ($P < 0.05$), and the expression levels of IL-1β, IL-8 and TNF-α in the JPQCD and 5-ASA groups were lower than those in the DSS group after treating with JPQCD and 5-ASA. Comparing with the DSS group, the mRNA level of IL-1β, IL-8, TNF-α and NF-κB was significantly reduced by 5-ASA and JPQCD. The difference between JPQCD and 5-ASA groups was not statistically significant ($P > 0.05$). Comparing with the DSS group, due to using JPQCD and 5-ASA, significant suppression of activation in DSS-induced NF-κB and increased phosphorylation of IκB in mice with experimental colitis occurred ($P < 0.05$). The difference between the JPQCD group and the 5-ASA group was not statistically significant ($P > 0.05$).

***CONCLUSION***

Activation of the NF-κB signaling pathway is inhibited by JPQCD, which shows the potential mechanism by which JPQCD treats UC.

**Key words:** Jianpi Qingchang decoction; Dextran sodium sulfate; Ulcerative colitis; Nuclear factor-κB; Inflammation



**Core tip:** Nuclear factor-kappa B (NF-κB) is a major transcription factor inducing the expression of inflammatory mediators, which plays a crucial role in the transcription in diverse immune responses, and its signaling has been confirmed to be a major pathway for ulcerative colitis. It is demonstrated in the current study that Jianpi Qingchang decoction (JPQCD), which can decrease levels of proinflammatory cytokines, plays a role in inhibiting nuclear translocation of NF-κB and phosphorylation of inhibitor of kappa B, thus indicating that JPQCD can suppress the activation of NF-κB signaling pathway.

Zheng L, Zhang YL, Dai YC, Chen X, Chen DL, Dai YT, Tang ZP. Jianpi Qingchang decoction alleviates ulcerative colitis by inhibiting nuclear factor-κB activation. *World J Gastroenterol* 2017; 23(7): 1180-1188 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1180.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1180

## INTRODUCTION

Ulcerative colitis (UC) is a chronic gastrointestinal disorder characterized by inflammation and mucosal damage in colon, the pathophysiology of which is multifactorial, with environmental, genetic and immunological factors being involved[1]. Chronic colonic inflammation in patients with UC may be attributed to activation of the immune system through intestinal flora. The main clinical manifestations of UC include recurrent abdominal pain and diarrhea, as well as mucous, bloody or purulent stools[2]. Currently, disease severity is an important factor in selecting treatment strategies, most of which concentrate on alleviation of disease symptoms, rather than disease treatment. In addition, a large number of ways for treating UC may result in systemic immunosuppression, thus leading to limited long-term application and an urgent need to develop a novel therapeutic. At present, many researchers are interested in traditional Chinese medicine (TCM), such as herbal therapy, because these complementary and alternative agents have few side effects in treating various diseases, including UC[3-6]. Multiple cellular signal transduction pathways highly regulate inflammatory responses, including the nuclear factor-kappa B (NF-κB) pathway, a nuclear factor that can be activated by a variety of pathogens.

NF-κB, a major transcription factor, which induces expression of a large array of inflammatory mediators, is crucial in transcription related to diverse immune responses, and the NF-κB signaling pathway is regarded as a major pathway for UC[7]. Abnormal expression of NF-κB leads to release of proinflammatory cytokines, for instance, interleukin (IL)-1β, IL-8, tumor necrosis factor-alpha (TNF-α) and adhesion molecules.

Jianpi Qingchang decoction (JPQCD) is a TCM prescription that consists of nine Chinese herbs, namely, Astragalus, Codonopsis pilosula, Portulaca oleracea, Sanguisorba officinalis, Notoginseng, Bletilla striata, Radix Aucklandiae, and Licorice. JPQCD can clear away heat and dampness, and strengthen the spleen to produce vitality, thanks to effects of these components. In the TCM system, pathogenesis of UC is formed in the case of accumulation of toxic dampness and heat, therefore the basic principle for JPQCD to treat inflammatory diseases is to clear away heat and eliminate dampness. In China, JPQCD has long been applied in the treatment of UC in clinic; however, its precise mechanism of action of anti-inflammatory activities remains unknown. In this study, C57BL mice

**Table 1 List of primers used in this study**

| Gene | | Primer sequence | Primer length (bp) |
|---|---|---|---|
| *IL-1β* | Forward | 5′-TAGACAACTGCACTACAGGCTCCGA-3′ | 25 |
| | Reverse | 5′-GGGTCCGACAGCACGAGGCT-3′ | 20 |
| *IL-8* | Forward | 5′-ATGCTGGTGACAACCACGGCC-3′ | 21 |
| | Reverse | 5′-CCTCTGTGAAGTCTCCTCTCCGGAC-3′ | 25 |
| *TNF-α* | Forward | 5′-TGGTGACCAGGCTGTCGCTACA-3′ | 22 |
| | Reverse | 5′-TACAGTCACGGCTCCCGTGGG-3′ | 21 |
| *NF-κB* | Forward | 5′-GTGGAGGCATGTTCGGTAGTG-3′ | 21 |
| | Reverse | 5′-TCTTGGCACAATCTTTAGGGC-3′ | 21 |
| *Actin* | Forward | 5′-GTGCCGCCTGGAGAAACC-3′ | 18 |
| | Reverse | 5′-GGTGGAAGAGTGGGAGTTGC-3′ | 20 |

with dextran sodium sulfate (DSS)-induced UC were used as models of human intestinal epithelium, so as to evaluate the anti-inflammatory effects of JPQCD and to investigate its molecular mechanism.

## MATERIALS AND METHODS

### Mice

C57BL/6 male mice weighing 18-22 g were purchased from the Laboratory Animal Center of Shanghai University of Traditional Chinese Medicine, and they were raised under the following conditions: a standard cycle of 12-h light/12-h dark, with the room temperature at 24 ± 2 ℃ and the relative humidity at 50% to 60%. Forty mice were randomly divided into four groups after 1 wk of acclimation, which were control group, DSS group, JPQCD group and 5-aminosalicylic acid (5-ASA) group, with 10 mice in each group.

### DSS-induced colitis

After acclimation, mice were fed with 5% (w/v) DSS (MW 36000-50000; MP Biomedicals, Santa Ana, CA, United States)[8] dissolved in sterile distilled water ad libitum for 7 d to induce acute UC. When DSS-induced acute UC models were successfully established 7 d later, the DSS drinking water was replaced by normal water but the DSS group continued to be fed with DSS drinking water. Control and DSS groups were given 0.3 mL normal saline by intragastric administration. The 5-ASA group was offered oral administration of 5-ASA at a dose of 100 mg/kg daily. The JPQCD group was treated orally with JPQCD at a dose of 17.1 g/kg daily. Body weights were monitored daily, and daily weight changes were calculated as the percentage of initial weights.

Parameters shown below were adopted to calculate the disease activity index (DAI) in combination with a scale involving the grading from 0-4: weight loss (0: normal; 1: 0%-5%; 2: 5%-10%; 3: 10%-15%; and 4: > 15%); stool consistency (0: normal; 2: loose stools; and 4: watery diarrhea); and fecal occult blood (0: negative; 2: positive; and 4: gross bleeding)[9,10]. Mice were sacrificed under anesthesia 14 d later. Colons were removed, cut open longitudinally along the main axis, and washed with phosphate-buffered saline (pH 7.4). After gross examination, colons were fixed in 10% neutral-buffered formalin for histological examination, and the remaining colons were used for enzyme-linked immunosorbent assay (ELISA), mRNA and western blot analyses.

### ELISA

Protein expressions of IL-1β, IL-8 and TNF-α (BioLegend, San Diego, CA, United States) in supernatants from the colon were measured by commercially available ELISA kits.

### Histological analysis

Paraffin-embedded samples were cut into 5-μm sections and examined under light microscope after being stained with hematoxylin and eosin (HE). Samples were analyzed and scored as described previously[11,12].

### RNA isolation and quantitative PCR

The tissue samples were frozen and dissociated mechanically in RNA buffer, and total RNA was extracted using RNAprep Pure Tissue Kit (Tiangen, Shanghai, China). Real-time (RT)-PCR was carried out with the help of an Eppendorf PCR system; meanwhile, QuantiFast SYBR Green PCR Master Mix (TOYOBO, Osaka, Japan), 1 mmol/L primers (Table 1), and 1 μL cDNA in 20 μL reaction mixture were also used. Activation was performed for 30 s at 95 ℃ to initiate the thermal cycling, followed by 40 cycles of 10 min at 95 ℃, 15 s at 95 ℃ and 1 min at 60 ℃. Immediately after amplification, melt curve protocol was utilized to guarantee minimization in primer dimers and other non-specific products. Expression of target genes was analyzed by the $^{\Delta\Delta}$Ct method.

### Protein extraction and western blot analysis

Western blot analysis described in previous studies was used[13]. Colonic tissue was cut into pieces and homogenized in 5-fold volumes of ice-cold homogenizing buffer (0.1 mmol/L NaCl, 0.1 mol/L Tris-HCl, and 0.001 mol/L EDTA) containing 1 mmol/L phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin, and 0.1 mmol/L leupeptin at 3000 g and 4 ℃ for 1

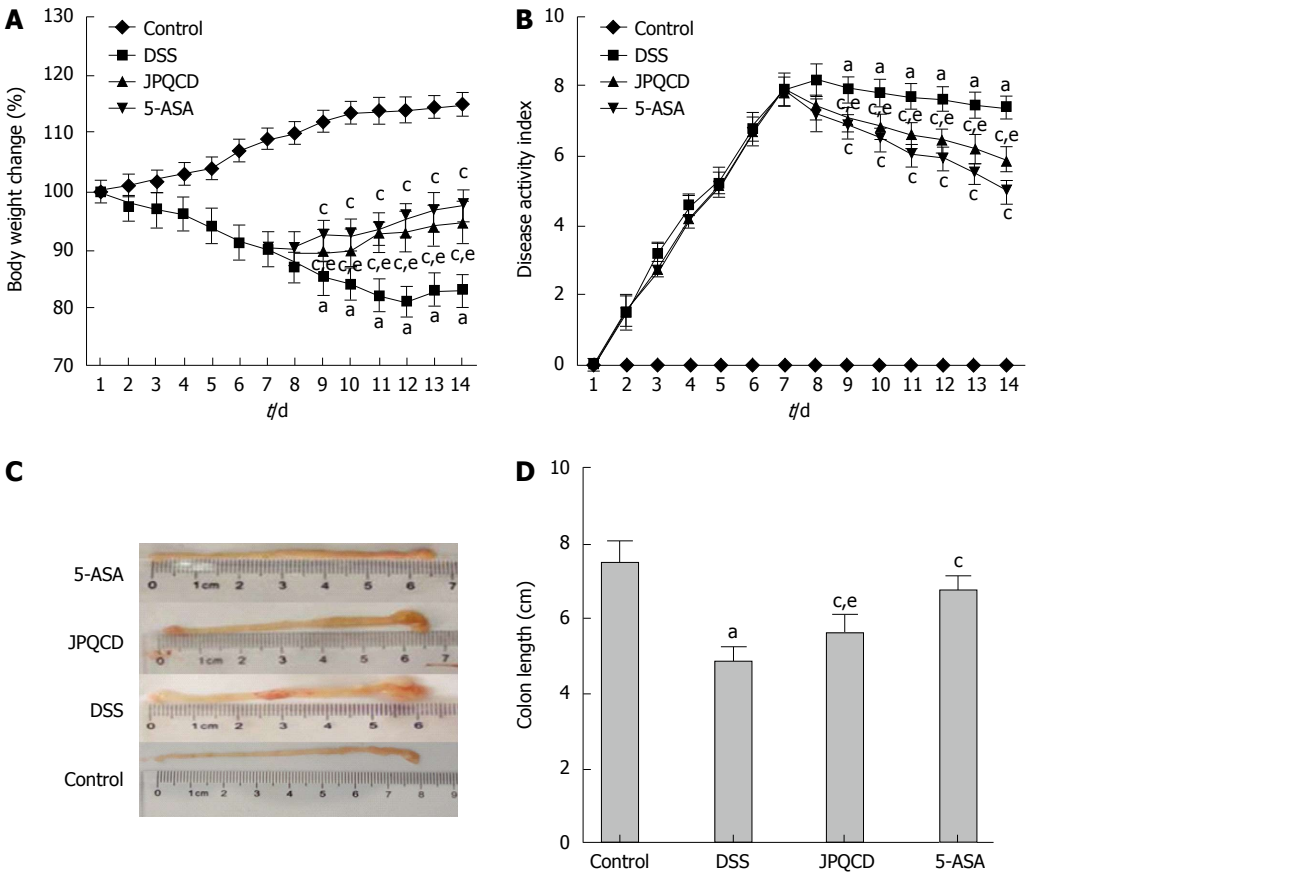



**Figure 1 Jianpi Qingchang decoction alleviated dextran sodium sulfate-induced experimental colitis.** A: Body weight changes after induction of colitis by dextran sodium sulfate (DSS); B: Disease activity index; C: Macroscopic appearance; and D: Length of colon. Data are presented as mean ± SEM of 10 mice in each group. $^{a}P < 0.01$ *vs* control group; $^{c}P < 0.05$ *vs* DSS group; $^{e}P > 0.05$ *vs* 5-aminosalicylic acid (5-ASA) group. JPQCD: Jianpi Qingchang decoction.

h. Bovine serum albumin was used to estimate the protein content in supernatants. The protein samples (40 μg in each sample) were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes using a transfer apparatus (Bio-Rad, Hercules, CA, United States). The membranes were blocked for 1 h before NF-κB and inhibitor of kappa B (IκB) (Cell Signaling Technology, Danvers, MA, United States) were adopted as the primary antibodies to incubate under the proper dilution at 4 ℃ overnight, and the corresponding horseradish-peroxidase-conjugated secondary antibody (Cell Signaling Technology) was used to incubate for another 1 h. Protein–antibody complexes were detected by Clarity Western ECL Substrate (Bio-Rad), and results were qualified with the ImageJ software (Gene Company Limited, China).

### *Statistical analysis*

Research results were analyzed by SPSS 21.0 (SPSS, Chicago, IL, United States); analyses were performed by adopting statistical methods, such as student's *t*-test and one-way analysis of variance (ANOVA), and histograms were generated by GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, United States). Difference with the *P* value < 0.05 was deemed as statistically significant.

## RESULTS

### *General observations*

Compared with the control group, the characteristic weight loss in mice with DSS-induced acute colitis developed on day 3 (Figure 1A), and significant differences ($P < 0.05$) could be seen from day 4. However, administration of JPQCD and 5-ASA resulted in increased body weights, with significant difference ($P < 0.05$) being seen on day 9. Results in previous studies had indicated that length of colon was negatively correlated with severity of experimental colitis[14,15]. Observation results in the DSS-treated mice showed that colon was significantly shortened, and the suppressed results derived from treatment with JPQCD and 5-ASA (Figure 1B-D).

### *JPQCD inhibits DSS-induced inflammatory response in acute colitis mice*

Secretion of IL-1β, IL-8 and TNF-α was measured to evaluate effects of JPQCD on DSS-induced experimental mice with acute colitis. Cytokines were released, which was regarded as an indicator of inflammatory response. JPQCD and 5-ASA groups were administered gavage once daily for 1 wk, and levels of IL-1β, IL-8 and TNF-α in supernatants from

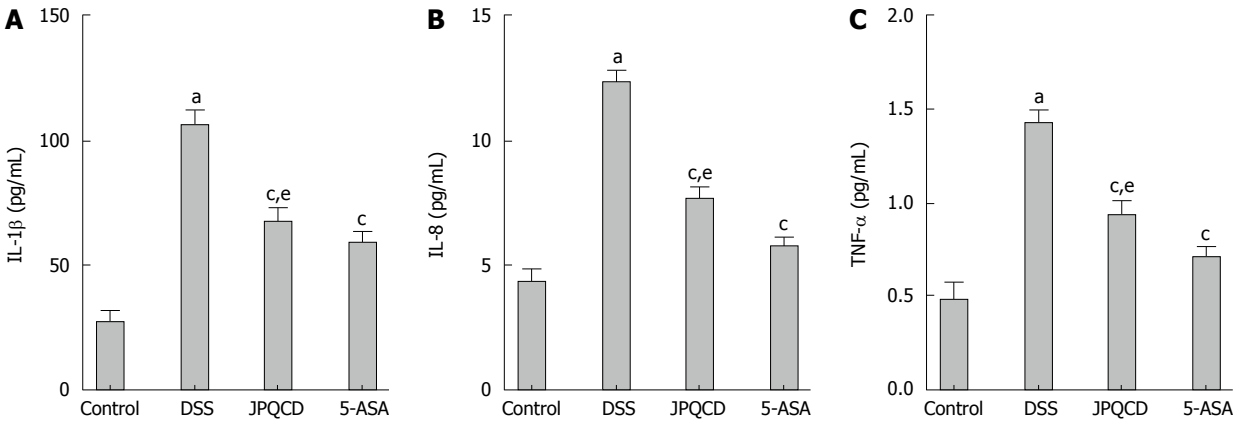



**Figure 2 Jianpi Qingchang decoction reduced proinflammatory mediator production in mice with dextran sodium sulfate-induced colitis.** IL-1β (A), IL-8 (B) and TNF-α (C) in colon were determined by ELISA. Data are presented as mean ± SEM. $^{a}P < 0.01$ *vs* control group; $^{c}P < 0.05$ *vs* dextran sodium sulfate (DSS) group; $^{e}P > 0.05$ *vs* 5-aminosalicylic acid (5-ASA) group. JPQCD: Jianpi Qingchang decoction.

colonic tissue were assessed by ELISA, the results of which indicated that JPQCD and 5-ASA significantly decreased the release of IL-1β, IL-8 and TNF-α in mice with experimental colitis (Figure 2).

### *JPQCD decreases microscopic colon damage in experimental colitis*

Histological and morphological characteristics of colon were assessed after HE staining, and representative results as well as the microscopic scores are shown in Figure 3A and B. Colons in the control group presented with normal crypt morphology, abundant goblet cells, no signs of mucosal thickening, and complete absence of ulceration. However, severe epithelial damage occurred in mice with induced colitis, giving rise to a higher score for microscopic damage (Figure 2A). On the contrary, scores for microscopic damage were lower in mice treated with JPQCD and 5-ASA than those in mice treated with DSS.

### *JPQCD reduces mRNA levels of IL-1β, IL-8, TNF-α and NF-κB*

Expression levels of IL-1β, IL-8, TNF-α and NF-κB in control and experimental mice were measured by RT-PCR (Figure 4). Expression of IL-1β, IL-8, TNF-α and NF-κB was elevated during UC[16], as had been shown in numerous studies. Compared with control mice, increased expression of IL-1β, IL-8, TNF-α and NF-κB could be detected in experimental mice in this study, which was consistent with previous findings. However, oral administration of JPQCD and 5-ASA contributed to reducing expression levels of IL-1β, IL-8, TNF-α and NF-κB ($P < 0.05$).

### *JPQCD suppresses NF-κB activation in experimental colitis*

As one of the core transcription factors in inflammation, activation of the NF-κB pathway involved several key processes, such as phosphorylation, IκB depredating and nuclear translocation of NF-κB. Effects of JPQCD on DSS-induced activation of NF-κB pathway in mice with experimental colitis were studied, with an aim to examine the mechanism of anti-inflammatory activity. Mice in the JPQCD group were pretreated with JPQCD once daily for 1 wk, and IκB was detected by western blot. Expression of IκB was significantly increased after treatment with JPQCD, and such increase was markedly alleviated by JPQCD (Figure 5).

## DISCUSSION

UC, a major form of inflammatory bowel disease (IBD), results from chronic intestinal inflammatory response[17,18], the exact cause of which remains undiscovered, thus giving rise to ineffective treatment results. Additionally, a majority of treatments applied at present are associated with occurrence of systemic immunosuppression. As a consequence, there is in an urgent need to develop new medicines targeting UC. Natural products with few side effects[19], such as in TCM, have been studied and applied in treating various diseases and for replacing some remedies. It has been revealed in previous studies that the NF-κB pathway regulates UC in a close way, so it may be an efficient strategy for treating UC. JPQCD, a TCM prescription, has been applied in treating UC in clinic for many years; nevertheless, its anti-inflammatory properties remain unknown. In the current study, effects of JPQCD and its molecular mechanism were evaluated in C57BL mice with DSS-induced UC, which were treated as experimental models of colitis[20-22].

It was successfully demonstrated in this study that clinical features of UC reoccur in models of acute colitis. Typical features of UC, such as diarrhea, bloody feces, and weight loss occur on d 3 and are markedly expressed by day 7. Results in mice could also be seen in human, suggesting that weight loss and colon shortening, the stable markers of colitis, are correlated with histopathological changes.

It is noted in our research that body weight and

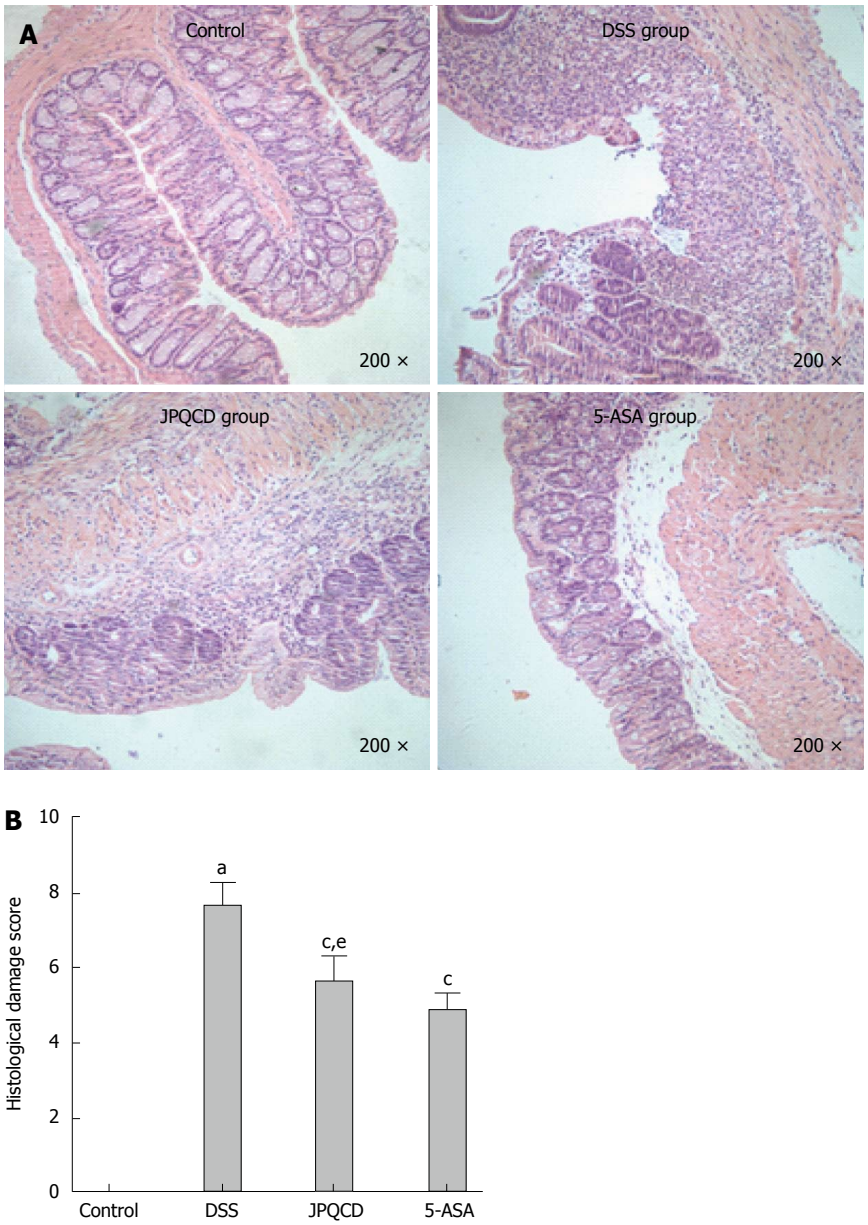



**Figure 3 Jianpi Qingchang decoction treatment protected mice against dextran sodium sulfate-induced colon damage.** A: Colon tissues were stained with HE; B: Histopathological scores were analyzed from slides. Data are presented as mean ± SEM. $^{a}P < 0.01$ *vs* control group; $^{c}P < 0.05$ *vs* dextran sodium sulfate (DSS) group; $^{e}P > 0.05$ *vs* 5-aminosalicylic acid (5-ASA) group. JPQCD: Jianpi Qingchang decoction.

colon length are outstandingly increased, while DAI is reduced after treating with JPQCD and 5-ASA for 7 d. Day 9 (2 d after completing the treatment) witnesses the beginning of inflammation reduction, which is consistent with results of research indicating that it takes a long time for the human body to respond to treatment. As an important nuclear transcription factor, NF-κB controls several important physiological processes, as well as immune and inflammatory responses. IκB kinase will phosphorylate IκB protein in the presence of pathologically stimulated cells. IκB proteins promote ubiquitination and degradation as a result of its phosphorylation, which leads to subsequent release of sequestered NF-κB, and further renders nuclear translocation, thus inducing expression of a variety of proinflammatory cytokines[23-25].

In the intestine, proinflammatory cytokines are produced, which play vital roles in pathogenesis of IBD. Hence, it is considered that release of proinflammatory cytokines is an indicator of inflammatory response[26]. TNF-α is crucial in recruiting immune cells at the sites of damaged tissues and in the pathogenesis of IBD. As a result, anti-TNF-α antibodies, including infliximab, are applied in treating UC and skin rash, extra-intestinal manifestations. However, it is indicated in the current study that anti-TNF antibodies, effective medicine in treating psoriasis, can result in psoriasiform skin lesion in IBD patients[27,28]. Furthermore, elevated levels of cytokines in UC, such as IL-8, IL-17 and IL-21 are reported[29,30].

It is observed that JPQCD has a remarkable effect on reducing DSS-induced secretion of proinflammatory

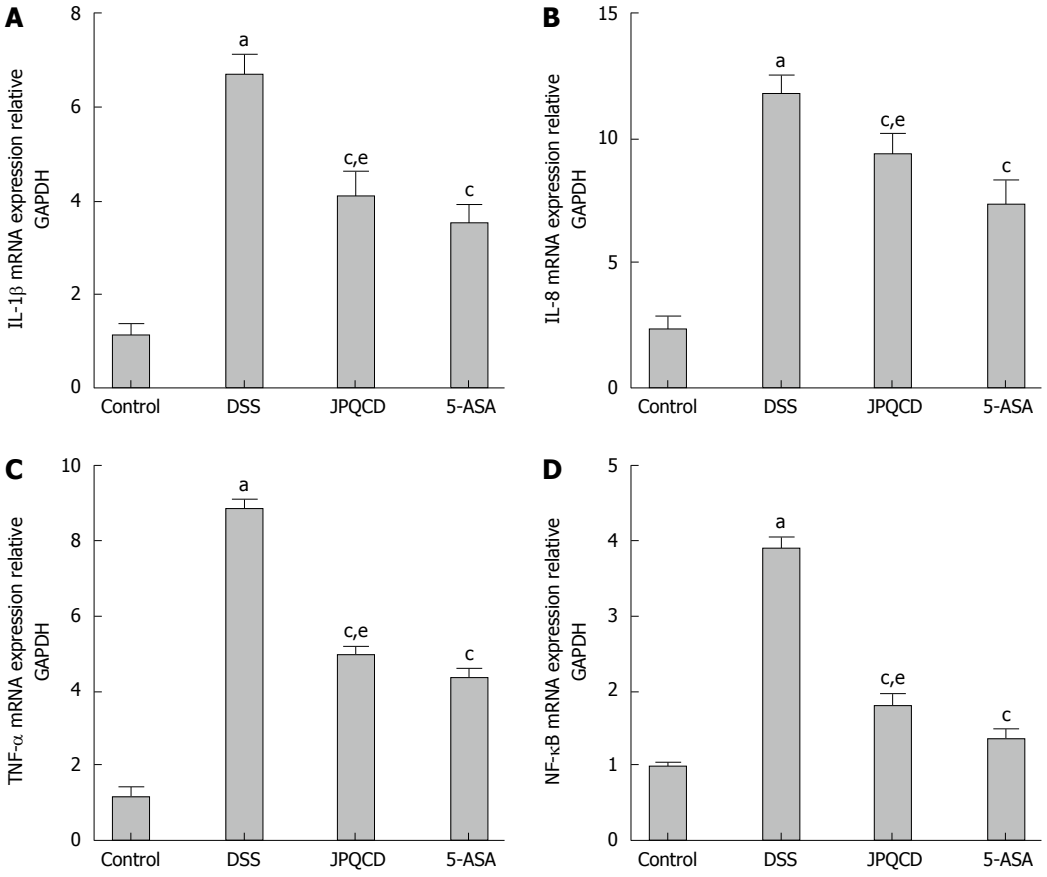



**Figure 4 Jianpi Qingchang decoction reduced mRNA levels in mice with dextran sodium sulfate-induced colitis.** IL-1β (A), IL-8 (B), TNF-α (C) and NF-κB (D) in colon were determined by RT-PCR. Data are presented as mean ± SEM. [a]*P* < 0.01 *vs* control group; [c]*P* < 0.05 *vs* dextran sodium sulfate (DSS)-group; [e]*P* > 0.05 *vs* 5-aminosalicylic acid (5-ASA) group. JPQCD: Jianpi Qingchang decoction.

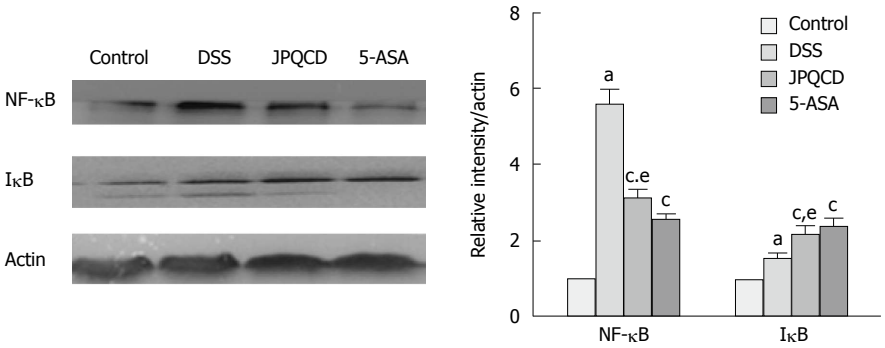



**Figure 5 Effects of Jianpi Qingchang decoction on dextran sodium sulfate-induced NF-κB nuclear translocation in mice with experimental colitis.** Jianpi Qingchang decoction (JPQCD) and 5-aminosalicylic acid (5-ASA) suppressed expression of NF-κB while increased that of IκB, as was indicated in western blot. β-actin was used as the internal control. Images are representative of three independent experiments. The dextran sodium sulfate (DSS) group had increased expression of NF-κB, which was reduced by subsequent administration of JPQCD and 5-ASA. Data are presented as mean ± SEM. [a]*P* < 0.01 *vs* control group; [c]*P* < 0.05 *vs* DSS group; [e]*P* > 0.05 *vs* 5-ASA group.

cytokines in mice with colitis, so as to inhibit inflammatory responses in intestinal epithelial cells. It can be seen from our findings that mRNA levels of IL-1β, IL-8, TNF-α and NF-κB in mice with DSS-induced colitis are increased, while those are decreased in the presence of oral administration of JPQCD and 5-ASA, suggesting that JPQCD and 5-ASA have similar effects, but there is no significant difference between them. Consequently, JPQCD contributes to decreasing levels of proinflammatory cytokines.

Furthermore, NF-κB is considered to be an important factor to activate IBD in human and colitis in animals[31-33]. Actually, antisense oligonucleotides can inhibit disease activity in mice with colitis, indicating that NF-κB plays a key role in mediating inflammatory response[34]. Results in our current study reveal that compared with mice in the control group, expression of NF-κB is increased, while that of IκB is decreased.

In the acute colitis model[35], mucosal inflammation can be controlled by using an agent to block the NF-κB pathway, which marks a successful attempt. Similar experimental results can also be obtained, which are that JPQCD treatment can inhibit phosphorylation of IκB and nuclear translocation of NF-κB, demonstrating that JPQCD inhibits the NF-κB signal pathway. In summary, results in our study show that JPQCD is promising in treating UC.

## ACKNOWLEDGMENTS

We would like to thank our colleagues in the Institute of Digestive Disease affiliated to Shanghai University of Traditional Chinese Medicine for their help and support in this research.

## COMMENTS

### Background

Multiple cellular signal transduction pathways, including the nuclear factor-kappa B (NF-κB) pathway, regulate the inflammatory response. A variety of pathogens can activate the NF-κB pathway, while the treatment is not effective in the long term. Currently, disease severity is an important factor when considering treatment strategies, and the attenuation of illness symptoms, rather than the treatment of illness is the focus in most of the strategies. As a result, development of a new treatment agent is urgently needed. Now, many researchers are interested in traditional Chinese medicine (TCM), such as herbal therapy, because these complementary and alternative agents have few side effects in treating various illnesses, including ulcerative colitis (UC).

### Research frontiers

The NF-κB signaling pathway plays a pivotal role in experimental UC in mice. This pathway is currently a hot topic in UC studies.

### Innovations and breakthroughs

NF-κB is thought to be vital in the activation and progression of inflammatory bowel disease in humans and colitis in animals. Abnormal expression of NF-κB leads to release of proinflammatory cytokines, adhesion molecules and growth factors, and overexpression of cell proliferation-related genes and survival-related genes. The present study demonstrated that Jianpi Qingchang decoction (JPQCD) can decrease the levels of proinflammatory cytokines, and inhibit phosphorylation of inhibitor of kappa B and nuclear translocation of NF-κB in experimental colitis, which indicates that JPQCD suppresses activation of the NF-κB signaling pathway.

### Applications

The present study provides evidence that JPQCD, a TCM decoction, inhibits activation of the NF-κB signaling pathway, suggesting that the potential mechanism by which JPQCD ameliorates dextran sulfate sodium (DSS)-induced UC is associated with this pathway. Thus, the findings of this study indicate a new potential mechanism by which JPQCD treats UC.

### Terminology

NF-κB is a transcription factor that can induce the expression of a large array of inflammatory mediators and plays a role as a core transcription factor in diverse immune responses.

### Peer-review

The authors demonstrated that JPQCD has potential therapeutic effect on the treatment of UC through the inhibition of NF-κB activation. This is an important mechanistic advancement in our understanding of the herbal product JPQCD and its role in preventing UC. The anti-inflammatory effects of JPQCD and its underlying molecular mechanisms have been correlated with the reduction in the level of pro-inflammatory cytokines (IL-1β, IL-8 and TNF-α). Using real-time PCR, ELISA and H&E staining of the colon tissue clearly shows that JPQCD has protective effects against DSS-induced colon damage in mice.

## REFERENCES

1 **Niu X**, Fan T, Li W, Huang H, Zhang Y, Xing W. Protective effect of sanguinarine against acetic acid-induced ulcerative colitis in mice. *Toxicol Appl Pharmacol* 2013; **267**: 256-265 [PMID: 23352506 DOI: 10.1016/j.taap.2013.01.009]

2 **Bressler B**, Marshall JK, Bernstein CN, Bitton A, Jones J, Leontiadis GI, Panaccione R, Steinhart AH, Tse F, Feagan B. Clinical practice guidelines for the medical management of nonhospitalized ulcerative colitis: the Toronto consensus. *Gastroenterology* 2015; **148**: 1035-1058.e3 [PMID: 25747596 DOI: 10.1053/j.gastro.2015.03.001]

3 **Yang H**, Li Y, Wu W, Sun Q, Zhang Y, Zhao W, Lv H, Xia Q, Hu P, Li H, Qian J. The incidence of inflammatory bowel disease in Northern China: a prospective population-based study. *PLoS One* 2014; **9**: e101296 [PMID: 25029440 DOI: 10.1371/journal.pone.0101296]

4 **Ahn BM**. [Herbal preparation-induced liver injury]. *Korean J Gastroenterol* 2004; **44**: 113-125 [PMID: 15385719]

5 **Langmead L**, Rampton DS: Review article: complementary and alternative therapies for inflammatory bowel disease. *Aliment Pharmacol Ther* 2006; **23**: 341-349

6 **Bensoussan M**, Jovenin N, Garcia B, Vandromme L, Jolly D, Bouché O, Thiéfin G, Cadiot G. Complementary and alternative medicine use by patients with inflammatory bowel disease: results from a postal survey. *Gastroenterol Clin Biol* 2006; **30**: 14-23 [PMID: 16514377]

7 **Shin EK**, Kwon HS, Kim YH, Shin HK, Kim JK. Chrysin, a natural flavone, improves murine inflammatory bowel diseases. *Biochem Biophys Res Commun* 2009; **381**: 502-507 [PMID: 19233127 DOI: 10.1016/j.bbrc.2009.02.071]

8 **Wirtz S**, Neufert C, Weigmann B, Neurath MF. Chemically induced mouse models of intestinal inflammation. *Nat Protoc* 2007; **2**: 541-546 [PMID: 17406617 DOI: 10.1038/nprot.2007.41]

9 **Goto H**, Takemura N, Ogasawara T, Sasajima N, Watanabe J, Ito H, Morita T, Sonoyama K. Effects of fructo-oligosaccharide on DSS-induced colitis differ in mice fed nonpurified and purified diets. *J Nutr* 2010; **140**: 2121-2127 [PMID: 20943955 DOI: 10.3945/jn.110.125948]

10 **Agollah GD**, Wu G, Peng HL, Kwon S. Dextran sulfate sodium-induced acute colitis impairs dermal lymphatic function in mice. *World J Gastroenterol* 2015; **21**: 12767-12777 [PMID: 26668501 DOI: 10.3748/wjg.v21.i45.12767]

11 **Rath HC**, Herfarth H, Ikeda JS, Grenther WB, Hamm TE, Jr, Balish E, Taurog JD, Hammer RE, Wilson KH, Sartor RB. "Normal luminal bacteria, especially bacteroides species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/humanβ 2 microglobulin transgenic rats," *J Clin Invest* 1996; **4**: 945-953

12 **Van der Sluis M**, De Koning BA, De Bruijn AC, Velcich A, Meijerink JP, Van Goudoever JB, Büller HA, Dekker J, Van Seuningen I, Renes IB, Einerhand AW. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology* 2006; **131**: 117-129 [PMID: 16831596 DOI: 10.1053/j.gastro.2006.04.020]

13 **Pandurangan AK**, Saadatdoust Z, Esa NM, Hamzah H, Ismail A. Dietary cocoa protects against colitis-associated cancer by activating the Nrf2/Keap1 pathway. *Biofactors* 2015; **41**: 1-14 [PMID: 25545372 DOI: 10.1002/biof.1195]

14 **Bento AF**, Marcon R, Dutra RC, Claudino RF, Cola M, Leite DF, Calixto JB. β-Caryophyllene inhibits dextran sulfate sodium-induced colitis in mice through CB2 receptor activation and PPARγ pathway. *Am J Pathol* 2011; **178**: 1153-1166 [PMID: 21356367 DOI: 10.1016/j.ajpath.2010.11.052]

15 **Pandurangan AK**, Ismail S, Saadatdoust Z, Esa NM. Allicin Alleviates Dextran Sodium Sulfate- (DSS-) Induced Ulcerative Colitis in BALB/c Mice. *Oxid Med Cell Longev* 2015; **2015**: 605208 [PMID: 26075036 DOI: 10.1155/2015/605208]

16 **Sanchez-Munoz F**, Dominguez-Lopez A, Yamamoto-Furusho JK. Role of cytokines in inflammatory bowel disease. *World J Gastroenterol* 2008; **14**: 4280-4288 [PMID: 18666314 DOI: 10.3748/wjg.14.4280]

17 **Nielsen OH**. New strategies for treatment of inflammatory bowel disease. *Front Med* (Lausanne) 2014; **1**: 3 [PMID: 25685754 DOI: 10.3389/fmed.2014.00003]

18 **Ananthakrishnan AN**. Epidemiology and risk factors for IBD. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 205-217 [PMID: 25732745 DOI: 10.1038/nrgastro.2015.34]

19 **Amiot A**, Peyrin-Biroulet L. Current, new and future biological agents on the horizon for the treatment of inflammatory bowel diseases. *Therap Adv Gastroenterol* 2015; **8**: 66-82 [PMID: 25729432 DOI: 10.1177/1756283X14558193]

20 **Stickel F**, Schuppan D. Herbal medicine in the treatment of liver diseases. *Dig Liver Dis* 2007; **39**: 293-304 [PMID: 17331820 DOI: 10.1016/j.dld.2006.11.004]

21 **Langmead L**, Rampton DS. Review article: complementary and alternative therapies for inflammatory bowel disease. *Aliment Pharmacol Ther* 2006; **23**: 341-349 [PMID: 16422993 DOI: 10.1111/j.1365-2036.2006.02761.x]

22 **Hilsden RJ**, Verhoef MJ, Best A, Pocobelli G. Complementary and alternative medicine use by Canadian patients with inflammatory bowel disease: results from a national survey. *Am J Gastroenterol* 2003; **98**: 1563-1568 [PMID: 12873578 DOI: 10.1111/j.1572-0241.2003.07519.x]

23 **Neuman MG**. Immune dysfunction in inflammatory bowel disease. *Transl Res* 2007; **149**: 173-186 [PMID: 17383591 DOI: 10.1016/j.trsl.2006.11.009]

24 **Wang D**, Dubois RN, Richmond A. The role of chemokines in intestinal inflammation and cancer. *Curr Opin Pharmacol* 2009; **9**: 688-696 [PMID: 19734090 DOI: 10.1016/j.coph.2009.08.003]

25 **Brynskov J**, Foegh P, Pedersen G, Ellervik C, Kirkegaard T, Bingham A, Saermark T. Tumour necrosis factor alpha converting enzyme (TACE) activity in the colonic mucosa of patients with inflammatory bowel disease. *Gut* 2002; **51**: 37-43 [PMID: 12077089]

26 **Strober W**, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology* 2011; **140**: 1756-1767 [PMID: 21530742 DOI: 10.1053/j.gastro.2011.02.016]

27 **Tillack C**, Ehmann LM, Friedrich M, Laubender RP, Papay P, Vogelsang H, Stallhofer J, Beigel F, Bedynek A, Wetzke M, Maier H, Koburger M, Wagner J, Glas J, Diegelmann J, Koglin S, Dombrowski Y, Schauber J, Wollenberg A, Brand S. Anti-TNF antibody-induced psoriasiform skin lesions in patients with inflammatory bowel disease are characterised by interferon-γ-expressing Th1 cells and IL-17A/IL-22-expressing Th17 cells and respond to anti-IL-12/IL-23 antibody treatment. *Gut* 2014; **63**: 567-577 [PMID: 23468464 DOI: 10.1136/gutjnl-2012-302853]

28 **Aldrich MB**, Sevick-Muraca EM. Cytokines are systemic effectors of lymphatic function in acute inflammation. *Cytokine* 2013; **64**: 362-369 [PMID: 23764549 DOI: 10.1016/j.cyto.2013.05.015]

29 **Araki A**, Nara H, Rahman M, Onoda T, Li J, Juliana FM, Jin L, Murata K, Takeda Y, Asao H. Role of interleukin-21 isoform in dextran sulfate sodium (DSS)-induced colitis. *Cytokine* 2013; **62**: 262-271 [PMID: 23557800 DOI: 10.1016/j.cyto.2013.03.006]

30 **Shin JH**, Ryu JH, Kang MJ, Hwang CR, Han J, Kang D. Short-term heating reduces the anti-inflammatory effects of fresh raw garlic extracts on the LPS-induced production of NO and pro-inflammatory cytokines by downregulating allicin activity in RAW 264.7 macrophages. *Food Chem Toxicol* 2013; **58**: 545-551 [PMID: 23583806 DOI: 10.1016/j.fct.2013.04.002]

31 **Atreya I**, Atreya R, Neurath MF. NF-kappaB in inflammatory bowel disease. *J Intern Med* 2008; **263**: 591-596 [PMID: 18479258 DOI: 10.1111/j.1365-2796.2008.01953.x]

32 **Wang KP**, Zhang C, Zhang SG, Liu ED, Dong L, Kong XZ, Cao P, Hu CP, Zhao K, Zhan YQ, Dong XM, Ge CH, Yu M, Chen H, Wang L, Yang XM, Li CY. 3-(3-pyridylmethylidene)-2-indolinone reduces the severity of colonic injury in a murine model of experimental colitis. *Oxid Med Cell Longev* 2015; **2015**: 959253 [PMID: 25874026 DOI: 10.1155/2015/959253]

33 **Spiik AK**, Ridderstad A, Axelsson LG, Midtvedt T, Björk L, Pettersson S. Abrogated lymphocyte infiltration and lowered CD14 in dextran sulfate induced colitis in mice treated with p65 antisense oligonucleotides. *Int J Colorectal Dis* 2002; **17**: 223-232 [PMID: 12073070 DOI: 10.1007/s00384-001-0366-3]

34 **Islam MS**, Murata T, Fujisawa M, Nagasaka R, Ushio H, Bari AM, Hori M, Ozaki H. Anti-inflammatory effects of phytosteryl ferulates in colitis induced by dextran sulphate sodium in mice. *Br J Pharmacol* 2008; **154**: 812-824 [PMID: 18536734 DOI: 10.1038/bjp.2008.137]

35 **Bruck R**, Aeed H, Brazovsky E, Noor T, Hershkoviz R. Allicin, the active component of garlic, prevents immune-mediated, concanavalin A-induced hepatic injury in mice. *Liver Int* 2005; **25**: 613-621 [PMID: 15910499 DOI: 10.1111/j.1478-3231.2005.01050.x]


**P- Reviewer**: Nihalani D **S- Editor**: Qi Y **L- Editor**: Filipodia
**E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/ *World J Gastroenterol* 2017 February 21; 23(7): 1189-1202

DOI: 10.3748/wjg.v23.i7.1189 ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

# Dysregulation of mRNA profile in cisplatin-resistant gastric cancer cell line SGC7901

Xiao-Que Xie, Qi-Hong Zhao, Hua Wang, Kang-Sheng Gu

Xiao-Que Xie, Hua Wang, Kang-Sheng Gu, Department of Oncology, the First Affiliated Hospital of Anhui Medical University, Hefei 230032, Anhui Province, China

Qi-Hong Zhao, Department of Food and Nutrition Hygiene, School of Public Health, Anhui Medical University, Hefei 230032, Anhui Province, China

Author contributions: Xie XQ performed experiments, prepared the manuscript and analyzed data; Zhao QH performed select experiments; Zhao QH and Wang H read the manuscript and gave important intellectual suggestion; Wang H and Gu KS designed and supervised the project.

Supported by Projects of Foreign Science and Technology Cooperation of Anhui Province of Anhui Province, No. 1604b0602027; New Century Excellent Talents in University, Ministry of Education of China, No. NCET-13-0644; and Wanjiang Scholars Program of Anhui Province of China.

Institutional review board statement: The study was reviewed and approved by the First Affiliated Hospital of Anhui Medical University Institutional Review Board.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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

Data sharing statement: Technical appendix and dataset available from the corresponding author at 13805692145@163.com. Participants gave informed consent for data sharing.



Manuscript source: Unsolicited manuscript

Correspondence to: Kang-Sheng Gu, PhD, Professor, Department of Oncology, the First Affiliated Hospital of Anhui Medical University, 218 Jixi Road, Hefei 230032, Anhui Province, China. 13805692145@163.com
Telephone: +86-551-62923504

Received: October 9, 2016
Peer-review started: October 10, 2016
First decision: November 9, 2016
Revised: November 24, 2016
Accepted: December 16, 2016
Article in press: December 19, 2016
Published online: February 21, 2017

## Abstract

***AIM***

To explore novel therapeutic target of cisplatin resistance in human gastric cancer.

***METHODS***

The sensitivity of SGC7901 cells and cisplatin-resistant SGC7901 cells (SGC7901/DDP) for cisplatin were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. High-quality total RNA which isolated from SGC7901/DDP cells and SGC7901 cells were used for mRNA microarray analysis. Results were analyzed bioinformatically to predict their roles in the development of cisplatin resistance and the expression of 13 dysregulated mRNAs we selected were validated by quantitative real-time polymerase chain reaction (qRT-PCR).

***RESULTS***

SGC7901/DDP cells highly resistant to cisplatin demonstrated by MTT assay. A total of 1308 mRNAs (578 upregulated and 730 downregulated) were

differentially expressed (fold change ⩾ 2 and *P*-value < 0.05) in the SGC7901/DDP cells compared with SGC7901 cells. The expression of mRNAs detected by qRT-PCR were consistent with the microarray results. Gene Ontology, Kyoto Encyclopedia of Genes and Genomes pathway and protein-protein interaction analysis demonstrated that the differentially expressed mRNAs were enriched in *PI3K-Akt*, *Notch*, *MAPK*, *ErbB*, *Jak-STAT*, *NF-kappaB* signaling pathways which may be involved in cisplatin resistance. Several genes such as *PDE3B*, *VEGFC*, *IGFBP3*, *TLR4*, *HIPK2* and *EGF* may associated with drug resistance of gastric cancer cells to cisplatin.

***CONCLUSION***

Exploration of those altered mRNAs may provide more promising strategy in diagnosis and therapy for gastric cancer with cisplatin resistance.

**Key words:** Gastric cancer; Dysregulate; Cisplatin resistance; Microarray; Biology



**Core tip:** We tested the sensitivity of human gastric cancer cells SGC7901/DDP and SGC7901 for cisplatin and compared their mRNA expression profile using a human mRNA microarray, and then performed bioinformatics analysis to depict comprehensively the properties of the differentially expressed mRNAs. Results demonstrated that the dysregulated mRNA were enriched in functions and pathways that may be involved in cisplatin resistance. Exploration of the dysregulated genes could suggest a promising strategy in diagnosis and therapy of gastric cancer with cisplatin resistance.

Xie XQ, Zhao QH, Wang H, Gu KS. Dysregulation of mRNA profile in cisplatin-resistant gastric cancer cell line SGC7901. *World J Gastroenterol* 2017; 23(7): 1189-1202 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1189.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1189

## INTRODUCTION

Gastric cancer is the fourth most common cancer and the second leading cause of cancer death globally[1], and more than two thirds of patients when diagnosed with unresectable disease[2]. The 5-year overall survival rate of patients with advanced gastric cancer approximately 25%[3]. Currently, platinum-based chemotherapy regimen is the standard first-line chemotherapy frequently used for advanced gastric cancer[4,5], and median overall survival and progression free survival was significantly longer in cisplatin-containing combination therapy compared to non-cisplatin containing regimens[6,7]. However, cisplatin-based chemotherapeutic agents are often limited in chemotherapy due to drug resistance[8,9].

Cisplatin resistance of gastric cancer is multi-factorial, accumulating evidence have suggested that the aberrant expression of proteins which associated with decreased cellular accumulation, increased DNA repair capacity, increased drug inactivation[10] play important role in the acquisition of cisplatin resistance. Previous researches have shown that abnormal expression of copper transporter 1 (CTR1) and MRP2 lead to cisplatin resistance by reducing the concentration of cisplatin in cells[11-13]. Moreover, the upregulation of excision repair cross complementing 1 (*ERCC1*)[14], X-ray repair cross complementing 1 (*XRCC1*)[15] and breast cancer 1 (*BRCA1*)[16] have shown to be involved in cisplatin resistance by removal of Pt-DNA adducts[17,18]. Other studies have shown that downregulation of the human epidermal growth factor receptor II (ErbB2) can significantly enhanced the apoptosis-inducing effects of cisplatin in gastric cancer[19,20].

The mechanisms of cisplatin resistance are quite complex and have not been fully revealed till now, so investigation of the molecular mechanisms and biomarkers is urgently needed. This study aims to analyze mRNA expression profiles in SGC7901/DDP cells to explore more chemotherapeutic molecular targets and to guide appropriate chemotherapy for gastric cancer with cisplatin resistance.

## MATERIALS AND METHODS

### *Cell lines and culture*

The human cisplatin-resistant gastric cancer cell line SGC7901/DDP and its parental cells SGC7901 were purchased from KeyGEN Biotechnology Company (Nanjing, Jiangsu, China). Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, United States) containing 10% fetal calf serum (Gibco, NY, United States) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were cultured in a humidified atmosphere with 5% $CO_2$ at 37 ℃. Cisplatin (Sigma, CA, United States) with final concentration of 800 ng/mL was added to the culture media for SGC7901/DDP cells to maintain the cisplatin-resistant phenotype.

### *MTT method assay for SGC7901/DDP and SGC7901 cells viability*

SGC7901/DDP and SGC7901 cells were suspended at a density of $1 \times 10^5$ cells/mL and planted into 96-well culture plate. After 24 hours, the cells were treated with freshly prepared DDP. The final concentrations were 133.34 μmol /L, 66.67 μmol/L, 6.67 μmol/L, 0.67 μmol/L and 0.067 μmol/L, because the human peak plasma concentration for DDP has been reported

as 6.67 μmol/L[21]. Cell viability was examined after 48 h and was determined by adding 20 μL MTT (5 mg/mL) to each well and incubated for a further 4 h. The resulting formazan crystal was dissolved by addition of 150 μL dimethyl sulfoxide (DMSO) (sigma, Germany) each well, and then plates were shaken for 10 minutes. The absorbance at 490 nm was measured by spectrophotometer (ELx 800; BioTek; Winooski, VT, United States). The inhibition of growth (IC50) for DDP was calculated by the cells relative viability. Each experiment was performed in triplicate.

### Total RNA extraction and mRNA microarray

Cells were harvested when they had grown to 80%-90% confluency and were still in logarithmic phase. Total RNA was extracted from the three matched pairs of SGC7901/DDP and SGC7901 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. The quality of total RNA was measured by NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Waltham, MA, United States). Total RNA from three paired samples were amplified and transcribed into fluorescent cDNA, and then the fluorescent labeled samples were hybridized to the Agilent LncRNA-mRNA Human Gene Expression Microarray V4.0 (Capital Bio Corp, Beijing, China) which contains 25069 human mRNA according to the manufacturer's recommendations. The microarray was scanned by an Agilent Microarray Scanner. Image processing was conducted using Agilent Feature Extraction software and raw microarray signals normalized using Agilent Gene-Spring software. The normalized mRNA expression profiles data output was received in Excel spreadsheets. The two group of samples data were analyzed by *t*-test to get the *P*-values. FC values representing the differently expressed mRNAs between SGC7901/DDP and their parental cells. Cluster 3.0 software was performed to show differential expression patterns of mRNAs.

### Bioinformatics analysis

Bioinformatics analysis were generated using KOBAS software and STRING 9.1 software. KOBAS software was used to analyze Ontology, Disease and pathways of the dysregulated mRNAs. KOBAS associated with 1 ontology database (Gene Ontology), 5 disease databases (OMIM, KEGG DISEASE, PID Reactome, FunDO, GAD, NHGRI) and 7 pathway databases (KEGG PATHWAY, PID Curated, PID BioCarta, BioCyc, eactome, Panther). The entire analysis process includes two steps: first, bring the input gene ID map to the gene in the databases, and then annotate pathways, disease and function of these genes involved in. Second step, compare the first step results with background (usually the entire genome of the gene, or the entire probe on the chip), and unearth statistically significant enrichment pathways, disease or function. Fisher's exact test and $\chi^2$ test were used as statistical tests and the FDR was performed to correct the *P*-value[22]. Additionally, we used STRING 9.1 software to decipher the protein-protein interaction (PPI) network of the differentially expressed proteins. The PPI network may help in understanding the molecular mechanism of cisplatin resistance. All mRNA microarray data were given by Capital Bio Corp.

### Quantitative real-time PCR validation of microarray results

To validate the reliability of microarray analysis, we performed quantitative real-time PCR (qRT-PCR). The reverse transcription production cDNA was synthesized using oligo-dT primers and Superscript II reverse transcriptase. PCR was performed with SYBR® Premix Ex Taq™ (TaKaRa Bio; Japan) by a Light Cycler PCR system (Agilent Technologies, Palo Alto, CA, United States) according to the manufacturer's instructions. After amplification, melting curves were analyzed. Beta-actin snRNA used as endogenous control, each sample was done in triplicate. The relative expression levels of target mRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method (where ΔΔCt is the difference in threshold cycles for the ΔCt of SGC7901/DDP sample and SGC7901 sample, and Ct is the difference between the target gene and endogenous control beta-actin). Sequences of primers for qRT-PCR are provided in supporting Table 1.

### Statistical analysis

MTT test and qRT-PCR statistical analysis was performed using GraphPad Prism software (v. 5.0a; GraphPad Software, La Jolla, CA, United States). We used one-way analysis of variance (ANOVA) followed by Student's *t*-test to assess the statistical significance of differences between different cell groups. The threshold for statistical significance was *P*-values < 0.05. Fold changes of mRNAs validated by qRT-PCR in SGC7901/DDP cells compared with SGC7901 cells are shown as mean ± SD.

## RESULTS

### Sensitivity of SGC7901/DDP and SGC7901 cells to DDP

To determine the chemotherapy sensitivity of SGC7901/DDP and SGC7901 cell line to cisplatin, varying concentrations of cisplatin were added into the 96-well plates and incubated for 48 h. From these data, half maximal inhibitory concentration (IC50) cisplatin dose was calculated. IC50 cisplatin doses for SGC7901/DDP and SGC7901 (after 48 h in DDP-containing media) were 43.47 ± 0.21 μmol/L and 1.24 ± 0.02 μmol/L, respectively, and the resistance index for SGC7901/DDP cell lines was 35.12, confirming that these cells are refractory to cisplatin. Cell viability was checked by MTT assay (Figure 1).

**Table 1 Primer of quantitative real-time polymerase chain reaction**

| ID | Primer | Sequence (5'to3') | Base (bp) | Tm (℃) | GC |
|---|---|---|---|---|---|
| HIPK2 | Forward | CCCCGTGTACGAAGGTATGG | 20 | 59.90 | 60% |
| | Reverse | GGGATGTTCTTGCTCTGGCT | 20 | 60.03 | 55% |
| PDE3B | Forward | TGAGAGTTATGGCTGCCTGT | 20 | 58.72 | 50% |
| | Reverse | CTGAGGGGCATTTGTAGCCA | 20 | 60.30 | 55% |
| FGF2 | Forward | TCCACCTATAATTGGTCAAAGTGGT | 25 | 59.99 | 40% |
| | Reverse | CATCAGTTACCAGCTCCCCC | 20 | 59.82 | 60% |
| TWIST1 | Forward | ATTCAAAGAAACAGGGCGTGG | 21 | 59.39 | 47.6% |
| | Reverse | CAGAGGTGTGAGGATGGTGC | 20 | 60.39 | 40% |
| ZEB2 | Forward | GCCTCTGTAGATGGTCCAGTGA | 22 | 61.21 | 54.6% |
| | Reverse | ATCGCGTTCCTCCAGTTTTCT | 21 | 60.00 | 47.6% |
| VEGFC | Forward | CCCGCCTCTCCAAAAAGCTA | 20 | 60.04 | 55% |
| | Reverse | CGGGTGTCAGGTAAAAGCCT | 20 | 59.96 | 55% |
| SPHK1 | Forward | GCTGCGAAGTTGAGCGAAAA | 20 | 60.04 | 50% |
| | Reverse | CGTTCCCTACAGTGGCCTG | 19 | 60.08 | 63.2% |
| BAX | Forward | GCCCTTTTGCTTCAGGGTTT | 20 | 59.24 | 50% |
| | Reverse | CATCCTCTGCAGCTCCATGT | 20 | 59.82 | 55% |
| PTEN | Forward | CAGGATACGCGCTCGGC | 17 | 60.73 | 70.6% |
| | Reverse | ACAGCGGCTCAACTCTCAAA | 20 | 57.89 | 50% |
| HTRA1 | Forward | AGCCAAAGAGCTGAAGGACC | 20 | 59.96 | 55% |
| | Reverse | GACATCATTGGCGGAGACCA | 20 | 60.11 | 55% |
| CCL5 | Forward | TGCTGCTTTGCCTACATTGC | 20 | 59.76 | 50% |
| | Reverse | CTTGTTCAGCCGGGAGTCAT | 20 | 60.04 | 55% |
| TGM2 | Forward | CCTCTGTCTCTCCGGGAACC | 20 | 61.32 | 65% |
| | Reverse | TGGCAACCAGGGGTCCTAT | 19 | 60.23 | 57.9% |
| TLR4 | Forward | CTCGGTCAGACGGTGATAGC | 20 | 59.97 | 60% |
| | Reverse | TTTAGGGCCAAGTCTCCACG | 20 | 59.68 | 55% |
| ACTB | Forward | CTCACCATGGATGATGATATCGC | 23 | 59.13 | 47.8% |
| | Reverse | AGGAATCCTTCTGACCCATGC | 21 | 59.79 | 52.4% |

GC: Gastric cancer.

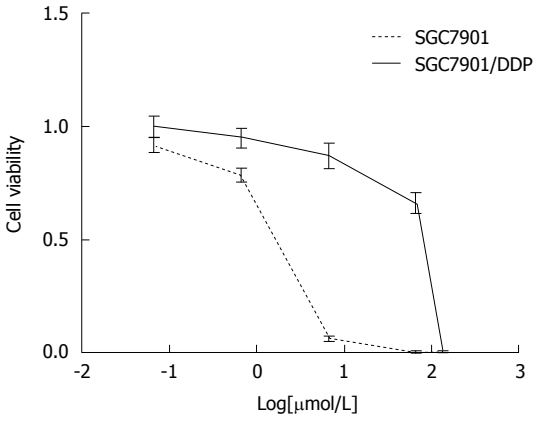



**Figure 1 Cell viability treated with different concentrations of cisplatin for 48 h.** MTT assay for SGC7901 cells and SGC7901/DDP cells treated with cisplatin (133.34, 66.67, 6.67, 0.67 and 0.067 μmol/L, respectively).

### *Expression profile of mRNAs in SGC7901/DDP cells*

To show mRNA expression profile in cisplatin-resistant SGC7901/DDP cells, we used a stringency cutoff to identify significantly differently mRNAs ($P < 0.05$, FC $\geqslant 2$) and two-dimensional hierarchical clustering 3.0 to represent expression profiles between samples (Figure 2). The results indicated that 1308 mRNAs were significantly differentially expressed in SGC7901/DDP cells compared with SGC7901 cells. Among these transcripts, 578 mRNAs were upregulated, and 730 mRNAs were downregulated.

### *Validation of microarray results by qRT-PCR of 13 mRNAs*

First, we concentrated on validating the microarray results. From the abnormally expressed ($P < 0.05$) mRNAs obtained from the microarray analyses, we selected 8 upregulated mRNAs (*HIPK2, PDE3B, FGF2, TWIST1, ZEB2, VEGFC, SPHK1, BAX*) and 5 downregulated (*PTEN, HTRA1, CCL5, TGM2, TLR4*) mRNAs for qRT-PCR validation. The relative fold-changes (SGC7901/DDP *vs* SGC7901) detected by qRT-PCR were consistent with the microarray results (Figure 3), indicating the dependability of our microarray platform.

### *Statistical analysis*

To depict comprehensively the properties of the differentially expressed mRNA in SGC7901/DDP cells, GO annotation and enrichment analysis was performed to evaluate which cellular components, molecular functions and biological processes may be are affected by this dysregulation. The GO enrichment analysis showed that the differentially expressed genes were involved in a variety of functions, including locomotion, chemotaxis, cell adhesion, regulation of cell migration, extracellular matrix disassembly, response to xenobiotic chemotaxis, localization of cell adhesion and blood vessel morphogenesis (Figure 4A).

Additionally, 59 human diseases were significant enriched ($P < 0.05$) in five human disease databases

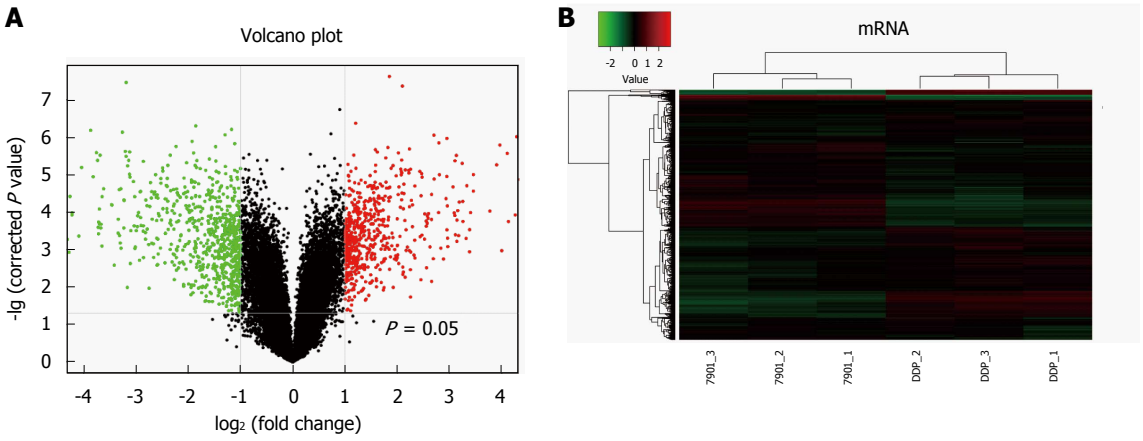



**Figure 2 mRNA expression levels from microarray.** A: The volcano plot image showed the mRNA expression levels of microarray in SGC7901/DDP cells compared with SGC7901 cells. Black dots: equally expressed mRNAs between SGC7901/DDP cells and SGC7901 cells (FC ⩽ 2); red dots: mRNAs were over-expressed in SGC7901/DDP cells compared with SGC7901 cells (FC ⩾ 2); green dots: mRNAs in SGC7901/DDP cells were down-expressed compared to SGC7901 cells (*P*-values < 0.05, FC ⩾ 2). Fold changes of these mRNAs in SGC7901/DDP cells compared with SGC7901 cells are shown as mean ± SD; B: Two-dimensional hierarchical clustering image of the 1308 dysregulated mRNAs in the SGC7901/DDP cells compared with the SGC7901 cells, each row represents an mRNA, each column represents a sample. 7901-1, 7901-2 and 7901-3 represent the three samples of SGC7901 cells, DDP-1, DDP-2 and DDP-3 represent the three samples of SGC7901/DDP cells. Red: Higher expression levels; green: Lower expression levels.

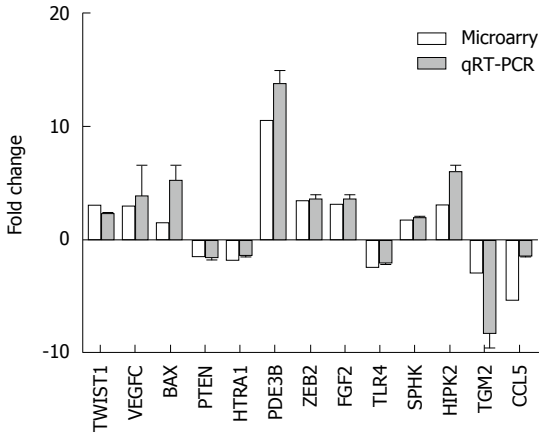



**Figure 3 Quantitative real-time polymerase chain reaction validation of the microarray results of the 13 mRNAs.** Relative fold changes in expression between SGC7901/DDP cells and SGC7901 cells were in agreement with microarray.

(KEGG DISEASE, FunDO, GAD, NHGRI GWAS Catalog and OMIM) (Figure 4B, Table 2). Furthermore, it is worth noting that in KEGG disease database, gastric cancer is the most highly enriched disease, and the input genes include *DCC, CD44, CDH1, VEGFC, EGF, TGFA.*

To determine which pathway might be involved in drug resistance formation, KEGG pathway analysis was used to authenticate pathways and understand biological functions of significantly differentially expressed genes. The result indicated that the differentially expressed mRNAs were enriched for 233 pathways, including the *Rap1* signaling pathway, *PI3K-Akt* signaling pathway, *ECM*-receptor interaction, *TNF* signaling pathway, and pathways in cancer, among others (Figure 5, Table 3). Cluster 3.0 software were performed the heat-map.This finding identified many candidate pathways and input genes that may play an important role in resistance mechanism.

### *Interaction network analysis*

The STRING 9.1 software (Search Tool for the Retrieval of Interacting Genes) was used to perceive functional relations and generate networks of differential expression of proteins (Figure 6). For all of the 1002 differentially expressed proteins, we extracted a network containing 443 upregulated and 559 downregulated proteins which functionally associated with each other. We found that interacting proteins which participate in angiogenesis, toll-like receptor signaling pathway and cell adhesion had a high level of co-expression.

## DISCUSSION

Cisplatin is widely used against a variety of solid neoplasms, including testicular, ovarian, colorectal, bladder, head and neck cancers and gastric cancer[23]. However, the repeated clinical expose to cisplatin often results in the tumor cells evading the apoptosis program initiated by cisplatin. Therefore, there is a need to explore the molecular mechanisms of cisplatin resistance, in order to overcome drug resistance in tumor therapy. Recently, several studies have indicated that many proteins are involved in the recognition of Pt-DNA adducts and cisplatin-induced apoptosis program[24,25]. In this study, we used microarray, GO, KEGG pathway and protein-protein interaction (PPI) analysis to explore the roles of differentially expressed mRNAs in cisplatin resistance and to support other studies.

Many genes which shown differentially expression in the microarray analysis have been demonstrated to be associated with cisplatin resistance in human cancer

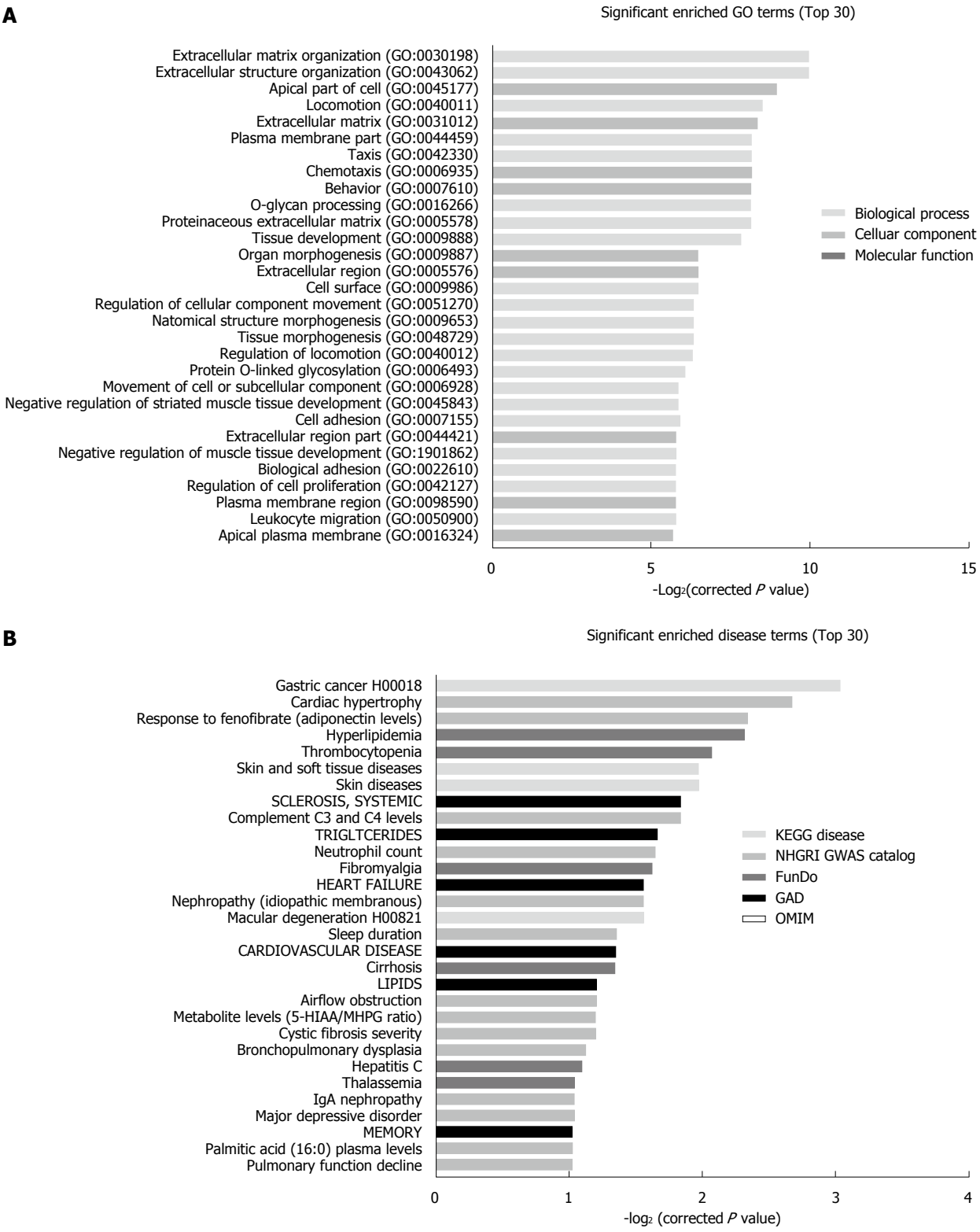



**Figure 4 Bioinformatic analysis of differentially expressed mRNAs.** Gene ontology analysis of mRNAs dysregulated in SGC7901/DDP cells compared with SGC7901 cells. A: Top 30 molecular functions of the dysregulated mRNAs may associated with. Gene ontology analysis include biological processes, cellular components and molecular function; B: Gene ontology enriched diseases. Top 30 diseases annotations of dysregulated mRNAs may involve in. The disease enrich system include 5 disease databases: OMIM, KEGG disease, FunDO, GAD and NHGRI GWAS Catalog.

(Table 4), such as *PDE3B*, which was substantially upregulated (*P* value = 0.00029, Fold Chang (FC) = 10.45) in SGC7901/DDP cells. Treatment with a combination of a *PDE3B* inhibitor and DDP can significantly increase the number of apoptotic and cell growth-suppressive cancer cells in cisplatin resistant

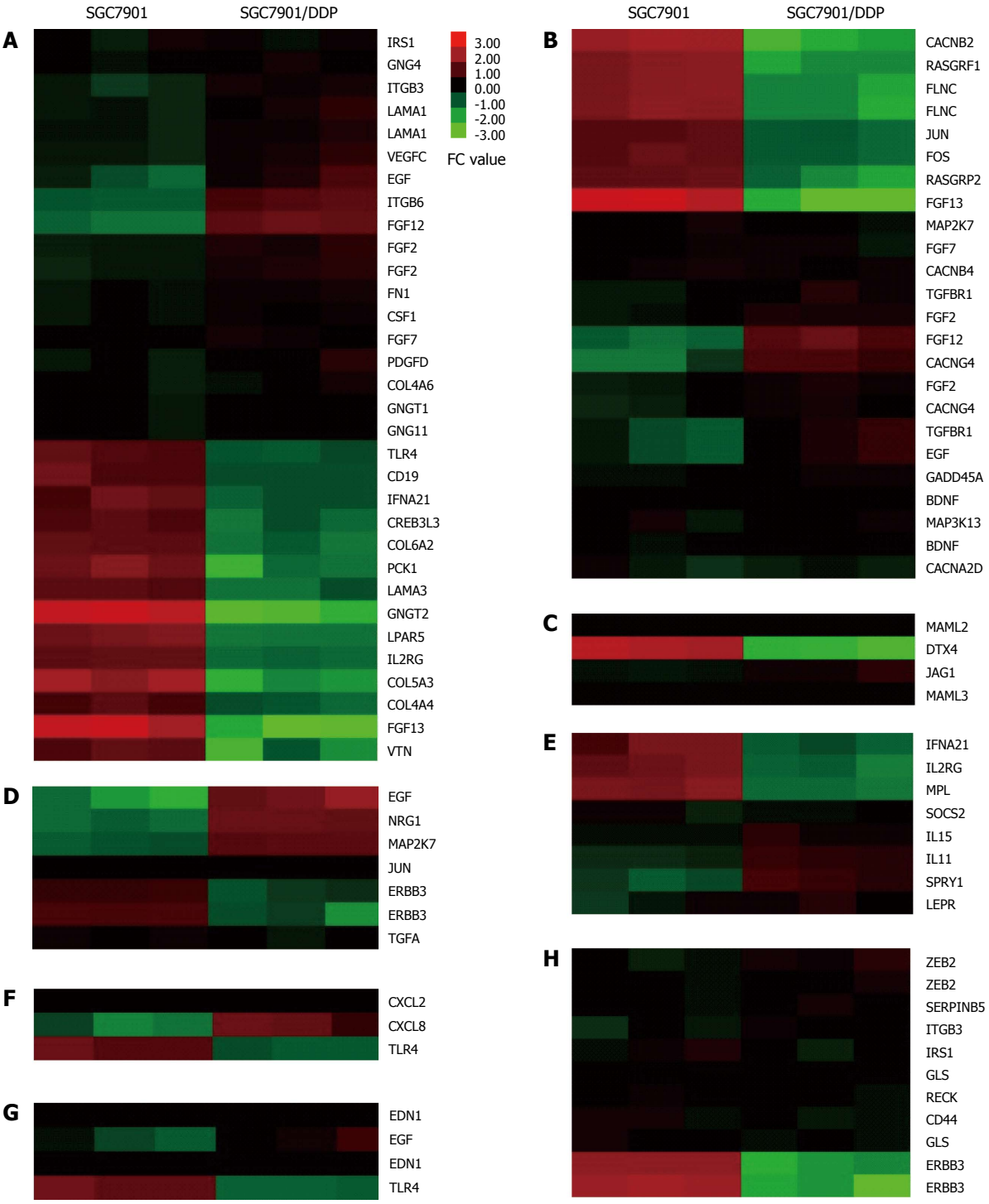



**Figure 5 Heat-map of gene ontology enriched cisplatin resistance pathways and input mRNAs which significantly altered in SGC7901/DDP cells compared with SGC7901 cells.** A: PI3K-Akt signaling pathway and input genes; B: MAPK signaling pathway and input genes; C: Notch signaling pathway and input genes; D: ErbB signaling pathway and input genes; E: Jak-STAT signaling pathway and input genes; F: NF-kappa B signaling pathway and input genes; G: HIF-1 signaling pathway and input genes; H: MicroRNAs in cancer and input genes. Each row represents an mRNA, and each column represents a sample. The intensity of the color indicates the relative levels of mRNAs. Red: Higher expression levels; green: Lower expression levels. The name of the input mRNAs which significantly altered ($P < 0.05$, FC $\geqslant 2$) is present at the right of the figure.

squamous cell carcinoma (SCC) and Hela cells[26]. Research shows that *VEGFC*, which is upregulated in our data (*P* value = 0.00013 FC = 2.93), enhanced cell invasion and cisplatin resistance in gastric cancer[27]. In non-small cell lung cancer, loss of *IGFBP-3* expression may activate the *PI3K/AKT* pathway and induce resistance to cisplatin[28]. In support of this association, our results showed that this mRNA is downregulated (*P* = 0.00007, FC = 2.93) in SGC7901/DDP cells.

GO enrichment analysis exhibits many functions which the differently expressed mRNAs are involved in, including locomotion, chemotaxis, cell adhesion,

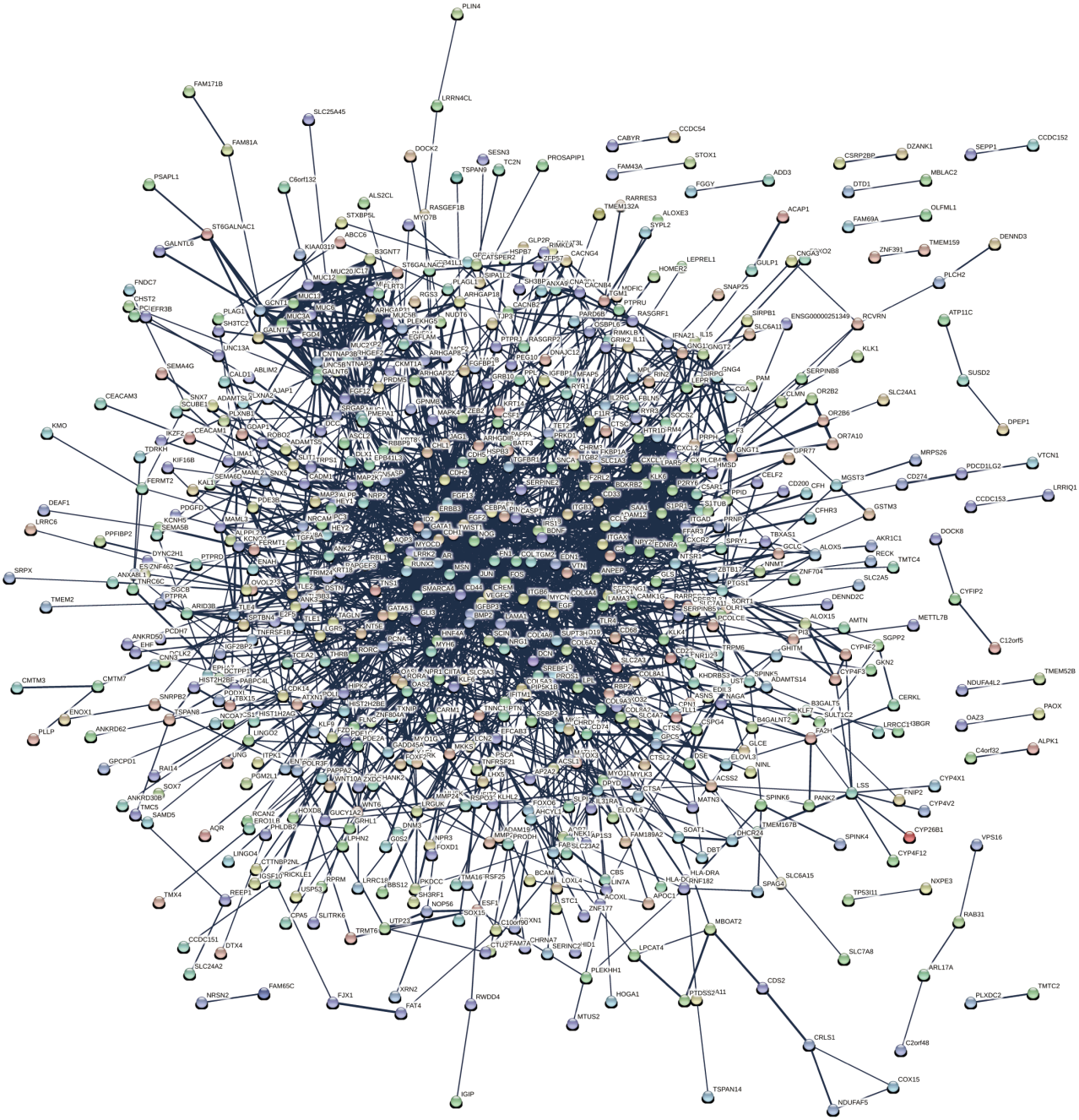


**Figure 6 Interaction network analyses of differentially express proteins.** In the network, nodes represents proteins, lines as functional associations between the abnormal expressed proteins and the thickness of the lines indicates the level of confidence in association reported.

regulation of cell migration, extracellular matrix disassembly, response to xenobiotic chemotaxis, localization of cell adhesion and blood vessel morphogenesis. Functional annotation showed that the differently expressed mRNAs mainly regulate cellular biological behaviors in the progress of regulation of transcription. How the underlying targets of each GO term are implicated in the cisplatin resistance needs further investigation in the future.

Our KEGG pathway analysis showed that the differently expressed mRNAs are enriched in pathways of *ECM*-receptor interaction, *PI3K-Akt, Rap1, MAPK, Notch1, ErbB, ABC* transporters, *Jak-STAT, NF-κB, HIF-1* and *TGF-β*. All of those pathways have been confirmed to be involved in cisplatin resistance in different experiments described previously. For example, the inhibition of *PI3K-Ak*t signaling pathway may increase the sensitivity of gastric cancer cells to cisplatin chemotherapy[29]. Another study found that Janus kinase 2 (*JAK2*) signal transducer and activator of transcription 3 (*STAT3*) signaling pathways were activated by overexpressed *AKT* in cisplatin resistant human gastric cancer cells[30]. A study revealed that the canonical *NF-κB* signaling pathway was involved in APRIL-mediated cisplatin resistance in gastric cancer[31]. Our data are consistent with these previous

**Table 2 Different expressed mRNAs enriched by KOBAS**

| Term | Database | *P* value | Input gene symbols |
|---|---|---|---|
| Gastric cancer | KEGG DISEASE | 0.0016 | *DCC, CD44, CDH1, VEGFC, EGF, TGFA* |
| Skin diseases | KEGG DISEASE | 0.0078 | *DSP, TGM1, CCL5, IL31RA, SPINK5, HLA, FERMT1, KRT14, CTSC, COL17A1, LAMA3, REEP1, RIN2, ALOXE3, ABCC6, WNT10A, FBLN5* |
| Skin and soft tissue diseases | KEGG DISEASE | 0.0078 | *DSP, TGM1, CCL5, IL31RA, SPINK5, HLA, FERMT1, KRT14, CTSC, COL17A1, LAMA3, REEP1, RIN2, ALOXE3, ABCC6, WNT10A, FBLN5* |
| Macular degeneration | KEGG DISEASE | 0.0140 | *C3, FBLN5, CFH, TLR4* |
| Cancers of the digestive system | KEGG DISEASE | 0.0439 | *DCC, CD44, CDH1, VEGFC, EGF, TGFA* |
| Familial thoracic aortic aneurysm and dissection (TAAD) | KEGG DISEASE | 0.0459 | *MYLK, TGFBR1* |
| Hypomagnesemia | KEGG DISEASE | 0.0459 | *TRPM6, EGF* |
| Multiple epiphyseal dysplasia (MED) | KEGG DISEASE | 0.0459 | *COL9A3, MATN3* |
| Transient neonatal diabetes mellitus (TNDM) | KEGG DISEASE | 0.0459 | *PLAGL1, ZFP57* |
| Non-syndromic autosomal dominant mental retardation | KEGG DISEASE | 0.0461 | *EPB41L1, DOCK8, PACS1, SMARCA4* |
| Cardiac hypertrophy | NHGRI GWAS Catalog | 0.0028 | *PLXNA2, GRIK2, COL17A1, JAG1, SNAP25, BTBD3, SLX4IP* |
| Response to fenofibrate (adiponectin levels) | NHGRI GWAS Catalog | 0.0046 | *OAS2, PMEPA1, SHANK2, SCUBE1, SLC30A4, PCK1* |
| Complement C3 and C4 levels | NHGRI GWAS Catalog | 0.0094 | *HLA, CFHR3, CFH, C3* |
| Neutrophil count | NHGRI GWAS Catalog | 0.0119 | *PLCB4, TGFA, FGGY, PDGFD, PSD3* |
| Nephropathy(idiopathic membranous) | NHGRI GWAS Catalog | 0.0137 | *HLA, ITGB6, PLA2R1* |
| Sleep duration | NHGRI GWAS Catalog | 0.0195 | *PLLP, TMC5, ADAMTS14* |
| Airflow obstruction | NHGRI GWAS Catalog | 0.0259 | *HYKK, LEF1, SERPINB8, GPR126, MAP3K13, PTPRD* |
| Cystic fibrosis severity | NHGRI GWAS Catalog | 0.0265 | *HLA, EHF, AHRR* |
| Metabolite levels (5-HIAA/ MHPG Ratio) | NHGRI GWAS Catalog | 0.0265 | *PIEZO2, ROBO2, ADAM12* |
| Bronchopulmonary dysplasia | NHGRI GWAS Catalog | 0.0296 | *PLXDC2, ZNF770, SPOCK1, TRPS1, RASGRF1, HIVEP3* |
| Major depressive disorder | NHGRI GWAS Catalog | 0.0346 | *PCLO, SLC6A15, ENOX1, SYPL2, IGFBP1, IGFBP3, C12orf5, ATXN1, PIEZO2, TRPS1, RASGEF1B, FGF12, KCNH5* |
| IgA nephropathy | NHGRI GWAS Catalog | 0.0346 | *HLA, ACOXL, TNFSF13* |
| Pulmonary function decline | NHGRI GWAS Catalog | 0.0368 | *MUSK, CSMD1, RORA, FLRT2* |
| Palmitic acid (16:0) plasma levels | NHGRI GWAS Catalog | 0.0368 | *SCD, CNN3, GRIK2, PTPRD* |
| Male-pattern baldness | NHGRI GWAS Catalog | 0.0439 | *AUTS2, EDA2R, AR* |
| Response to citalopram treatment | NHGRI GWAS Catalog | 0.0439 | *LAMA1, RORA, EGFLAM* |
| Hyperlipidemia | FunDO | 0.0050 | *IRS1, CCL5, C3, PAPPA, TXNIP, APOC1, F3, SCD* |
| Thrombocytopenia | FunDO | 0.0068 | *GATA1, CCL5, ITGB3, IL11, CXCL8, MPL* |
| Fibromyalgia | FunDO | 0.0126 | *MAOB, CXCL8, BDNF, IGFBP3* |
| Cirrhosis | FunDO | 0.0209 | *RBP4, KRT18, IGFBP3, KRT8, EGF, F3, FGF2IGFBP1* |
| Hepatitis C | FunDO | 0.0321 | *CD274, CCL5, RBP4, MKI67, CXCL8, KRT18, TLR4, KRT8, FGF2* |
| Thalassemia | FunDO | 0.0345 | *LCN2, CXCL8, ANK2, KIR3DL1, MUC1* |
| Gingival overgrowth | FunDO | 0.0417 | *EDN1, IL15, FGF7* |
| Pulmonary fibrosis | FunDO | 0.0474 | *CSF1, BDNF, MMP7, EDN1, CCL5, ERBB3* |
| Ovary cancer | FunDO | 0.0477 | *LCN2, IL15, CXCL8, FGF7, CASP1* |
| Esophageal tumor | FunDO | 0.0477 | *CD274, TSPAN8, FRAT1, PDCD1LG2, FGF7* |
| Hyperlipidemia | GAD | 0.0093 | *CCL5, HLA, CXCL8, CD22, TNFRSF1B, CD19* |
| Thrombocytopenia | GAD | 0.0114 | *CSMD1, DOCK4, GALNTL6, SOBP, PLXDC2, SESN3, ADAMTS5, EHF, TMC5, LPL, CD109, FAM117B, PDE1C, TAGLN, PTN, FGD4, DYNC2H1, GNG4, MUSK, FBLN5, CCDC54, TTC9, PMEPA1, TLR4, ANK3, EDA2R, APOC1, BMP2, TOX3, NRG1, ITPK1, PTPRD, KLF6, PAM, PTPRU, LEPR, IKZF2, LHX5, MCTP2, ANKRD50, SEMA6D, PLXNA2, DPYD, GRIK2, SRGAP3, ACOXL, TDRKH, FAM135B, VEGFC, CHST2* |
| Fibromyalgia | GAD | 0.0136 | *GLI3, CELF2, VWA3B, PLXDC2, EDNRA, EDN1, JUN, DOCK8, DCLK2, BTBD3, DCN, CD74, EGFLAM, TLL1, TLR4, BMP2, PTPRD, ANK2, PTPRU, JADE2, IGF2BP2, PAPPA, DOCK2, KLK4, FAM49A, RGS3, AATK, FN1, IGSF10, NCOA7, SCIN, TNS1, FAM135B, MUC16, ADAM19, ATXN1, MTUS2, NXNL2, KCNQ3, ANPEP, CDH2* |
| Cirrhosis | GAD | 0.0204 | *IRS1, CCL5, ITGB3, NPR1, NPR3, APOC1, LPL* |
| Hepatitis C | GAD | 0.0258 | *DPYD, CELF4, CELF2, FAM117B, TDRKH, LPCAT4, FBLN5, SOBP, PMEPA1, CSMD1, STOX1, CACNB2, CADM1, VEGFC, SLC7A11, LPL, CD109, MCTP2, SLC24A2, PTPRD, ITPK1* |
| Thalassemia | GAD | 0.0362 | *MCTP2, PSD3, CCDC54, ROBO2, ELOVL6* |
| Gingival overgrowth | GAD | 0.0419 | *PLXNA2, ATXN1, IGF2BP2, ABCA13, FN1* |
| Pulmonary fibrosis | GAD | 0.0420 | *CREG2, GALNTL6, LINC01550, KIF16B, SH3BGR, TRPS1, PDE1C, NCKAP5, TNFRSF21, RYR3, MAGEC2, EDIL3, CXCL16, MCF2, DTD1, GPC5, KLF6, IKZF2, KCNH5, AJAP1, BTBD3, PHACTR2, ITPK1, IGSF10, SRGAP3, C12orf75, ABI3BP, FOS, SCUBE1* |

| | | | |
|---|---|---|---|
| Ovary cancer | GAD | 0.0426 | *CELF4, TRPS1, TWIST1, PQLC2L, MAL2, PSD3, RCAN2, SUPT3H, TGFA, TMEM131L, HIVEP3, CSMD1, ROBO2, CCDC54, PRNP, APOC1, HRK, GPC5, AR, FN1, ABCA13, F2RL2, KLF6, IGF2BP2, LEPREL1, GNG4, SNAP25, MCTP2, FAM49A, ANKRD50, CACNA2D1, PLXNA2, ELOVL6, RUNX2, SCN8A, ATXN1, ID2, SLC24A2, CMTM7, LINGO2* |
| Esophageal tumor | GAD | 0.048 | *CACNA2D1, SLC46A3, CHST2, PKDCC, PPID, CDH2* |

**Table 3 Cisplatin resistance pathway and input gene ($P < 0.05$, FC $\geqslant$ 2.0)**

| Pathway | Input gene | Fold change | Regulation | Genomic coordinates | Cyto band |
|---|---|---|---|---|---|
| PI3K-Akt signaling pathway | *LAMA1* | 2.60826 | Up | Chr18:6958512-6956742 | hs \| 18p11.31 |
| | *LAMA1* | 2.75269 | Up | Chr18:6942035-6941976 | hs \| 18p11.31 |
| | *GNG4* | 2.09356 | Up | Chr1:235714443-235714384 | hs \| 1q42.3 |
| | *ITGB3* | 2.96629 | Up | Chr17:45389027-45389086 | hs \| 17q21.32 |
| | *ITGB6* | 7.72783 | Up | Chr2:160964233-160958330 | hs \| 2q24.2 |
| | *VEGFC* | 2.92538 | Up | Chr4:177604882-177604823 | hs \| 4q34.3 |
| | *PDGFD* | 2.42861 | Up | Chr11:103778445-103778386 | hs \| 11q22.3 |
| | *IRS1* | 2.00967 | Up | Chr2:227596677-227596618 | hs \| 2q36.3 |
| | *GNGT1* | 2.04779 | Up | Chr7:93536149-93540155 | hs \| 7q21.3 |
| | *CSF1* | 2.25620 | Up | Chr1:110466137-110466196 | hs \| 1p13.3 |
| | *EGF* | 4.76437 | Up | Chr4:110932689-110932748 | hs \| 4q25 |
| | *FGF2* | 3.02437 | Up | Chr4:123819331-123819390 | hs \| 4q28.1 |
| | *FGF2* | 2.99240 | Up | Chr4:123819317-123819376 | hs \| 4q28.1 |
| | *FN1* | 2.31254 | Up | Chr2:216288895-216288217 | hs \| 2q35 |
| | *COL4A6* | 2.08497 | Up | Chrx:107399109-107399050 | hs \| Xq22.3 |
| | *FGF12* | 10.99211 | Up | Chr3:191860574-191860515 | hs \| 3q28 |
| | *GNG11* | 2.01984 | Up | Chr7:93555764-93555823 | hs \| 7q21.3 |
| | *FGF7* | 2.19252 | Up | Chr15:49776810-49776869 | hs \| 15q21.2 |
| | *LAMA3* | 2.56116 | Down | Chr18:21534735-21534794 | hs \| 18q11.2 |
| | *IFNA21* | 2.30808 | Down | Chr9:21166331-21166272 | hs \| 9p21.3 |
| | *CREB3L3* | 2.40183 | Down | Chr19:4172219-4172278 | hs \| 19p13.3 |
| | *TLR4* | 2.13271 | Down | Chr9:120476856-120476915 | hs \| 9q33.1 |
| | *COL6A2* | 2.89458 | Down | Chr21:47546086-47546145 | hs \| 21q22.3 |
| | *CD19* | 2.09302 | Down | Chr16:28950600-28950659 | hs \| 16p11.2 |
| | *LPAR5* | 3.83177 | Down | Chr12:6728794-6728735 | hs \| 12p13.31 |
| | *COL4A4* | 2.11177 | Down | Chr2:227867523-227867464 | hs \| 2q36.3 |
| | *PCK1* | 4.49558 | Down | Chr20:56141030-56141089 | hs \| 20q13.31 |
| | *VTN* | 3.82587 | Down | Chr17:26694806-26694747 | hs \| 17q11.2 |
| | *GNGT2* | 16.48365 | Down | Chr17:47284034-47283975 | hs \| 17q21.32 |
| | *IL2RG* | 2.87954 | Down | Chrx:70328539-70328480 | hs \| Xq13.1 |
| | *COL5A3* | 7.53410 | Down | Chr19:10070602-10070543 | hs \| 19p13.2 |
| | *FGF13* | 17.08866 | Down | Chrx:137713947-137713888 | hs \| Xq26.3 |
| MAPK signaling pathway | *FLNC* | 4.57879 | Down | Chr7:128498538-128498597 | hs \| 7q32.1 |
| | *FLNC* | 4.81302 | Down | Chr7:128498476-128498535 | hs \| 7q32.1 |
| | *CACNB2* | 7.83293 | Down | Chr10:18787305-18787364 | hs \| 10p12.31 |
| | *RASGRF1* | 4.87152 | Down | Chr15:79254554-79254495 | hs \| 15q25.1 |
| | *FOS* | 2.17501 | Down | Chr14:75748214-75748273 | hs \| 14q24.3 |
| | *JUN* | 2.04000 | Down | Chr1:59246570-59246511 | hs \| 1p32.1 |
| | *RASGRP2* | 3.10358 | Down | Chr11:64508971-64508912 | hs \| 11q13.1 |
| | *FGF13* | 17.08866 | Down | Chrx:137713947-137713888 | hs \| Xq26.3 |
| | *TGFBR1* | 2.93035 | Up | Chr9:101916322-101916381 | hs \| 9q22.33 |
| | *TGFBR1* | 4.76437 | Up | Chr4:110932689-110932748 | hs \| 4q25 |
| | *EGF* | 4.76437 | Up | Chr4:110932689-110932748 | hs \| 4q25 |
| | *FGF12* | 10.99211 | Up | Chr3:191860574-191860515 | hs \| 3q28 |
| | *MAP3K13* | 2.25019 | Up | Chr3:185161379-185165590 | hs \| 3q27.2 |
| | *FGF2* | 3.02437 | Up | Chr4:123819331-123819390 | hs \| 4q28.1 |
| | *FGF2* | 2.99240 | Up | Chr4:123819317-123819376 | hs \| 4q28.1 |
| | *MAP2K7* | 2.08267 | Up | Chr19:7979302-7979361 | hs \| 19p13.2 |
| | *FGF7* | 2.19252 | Up | Chr15:49776810-49776869 | hs \| 15q21.2 |
| | *CACNG4* | 8.83585 | Up | Chr17:65028139-65028198 | hs \| 17q24.2 |
| | *CACNG4* | 2.94145 | Up | Chr17:65029115-65029174 | hs \| 17q24.2 |
| | *CACNB4* | 2.14311 | Up | Chr2:152694239-152694180 | hs \| 2q23.3 |
| | *GADD45A* | 2.56659 | Up | Chr1:68153371-68153430 | hs \| 1p31.3 |
| | *BDNF* | 2.32411 | Up | Chr11:27679959-27679900 | hs \| 11p14.1 |
| | *BDNF* | 2.30323 | Up | Chr11:27677072-27677013 | hs \| 11p14.1 |
| | *CACNA2D1* | 2.09452 | Up | Chr7:81579504-81579445 | hs \| 7q21.11 |

| | | | | | |
|---|---|---|---|---|---|
| Notch signaling pathway | *MAML2* | 2.03379 | Up | Chr11:95712434-95712375 | hs\|11q21 |
| | *JAG1* | 3.20086 | Up | Chr20:10619120-10619061 | hs\|20p12.2 |
| | *MAML3* | 2.57919 | Up | Chr4:140810806-140810747 | hs\|4q31.1 |
| | *DTX4* | 9.99859 | Down | Chr11:58975615-58975674 | hs\|11q12.1 |
| ErbB signaling pathway | *EGF* | 4.76437 | Up | Chr4:110932689-110932748 | hs\|4q25 |
| | *NRG1* | 2.77996 | Up | Chr8:32474390-32585512 | hs\|8p12 |
| | *MAP2K7* | 2.08267 | Up | Chr19:7979302-7979361 | hs\|19p13.2 |
| | *JUN* | 2.04000 | Down | Chr1:59246570-59246511 | hs\|1p32.1 |
| | *ERBB3* | 5.29571 | Down | Chr12:56482380-56482439 | hs\|12q13.2 |
| | *ERBB3* | 8.12050 | Down | Chr12:56496160-56496219 | hs\|12q13.2 |
| | *TGFA* | 2.37427 | Down | Chr2:70675378-70675319 | hs\|2p13.3 |
| Jak-STAT signaling pathway | *IL11* | 4.21849 | Up | Chr19:55875847-55875788 | hs\|19q13.42 |
| | *IL15* | 2.92970 | Up | Chr4:142654431-142654490 | hs\|4q31.21 |
| | *SOCS2* | 2.00180 | Up | Chr12:93969799-93969858 | hs\|12q22 |
| | *SPRY1* | 5.95682 | Up | Chr4:124324494-124324553 | hs\|4q28.1 |
| | *LEPR* | 2.90187 | Up | Chr1:66102129-66102188 | hs\|1p31.3 |
| | *IL2RG* | 2.87954 | Down | Chrx:70328539-70328480 | hs\|Xq13.1 |
| | *MPL* | 3.41581 | Down | Chr1:43819826-43819885 | hs\|1p34.2 |
| NF-kappaB signaling pathway | *CXCL2* | 2.03846 | Up | Chr4:74963044-74962985 | hs\|4q13.3 |
| | *CXCL8* | 9.97781 | Up | Chr4:74609265-74609324 | hs\|4q13.3 |
| | *TLR4* | 2.13271 | Down | Chr9:120476856-120476915 | hs\|9q33.1 |
| HIF-1 signaling pathway | *EDN1* | 2.39081 | Up | Chr6:12296672-12296731 | hs\|6p24.1 |
| | *EDN1* | 2.46437 | Up | Chr6:12296218-12296277 | hs\|6p24.1 |
| | *EGF* | 4.76437 | Up | Chr4:110932689-110932748 | hs\|4q25 |
| | *TLR4* | 2.13271 | Down | Chr9:120476856-120476915 | hs\|9q33.1 |
| MicroRNAs in cancer | *IRS1* | 2.00967 | Up | Chr2:227596677-227596618 | hs\|2q36.3 |
| | *ZEB2* | 3.32563 | Up | Chr2:145146320-145146261 | hs\|2q22.3 |
| | *ZEB2* | 2.70558 | Up | Chr2:145182422-145182363 | hs\|2q22.3 |
| | *CD44* | 2.02409 | Up | Chr11:35253812-35253871 | hs\|11p13 |
| | *RECK* | 2.25018 | Up | Chr9:36124319-36124378 | hs\|9p13.3 |
| | *ITGB3* | 2.96629 | Up | Chr17:45389027-45389086 | hs\|17q21.32 |
| | *SERPINB5* | 2.61864 | Up | Chr18:61172218-61172277 | hs\|18q21.33 |
| | *GLS* | 2.36144 | Up | Chr2:191829716-191829775 | hs\|2q32.2 |
| | *GLS* | 2.07371 | Up | Chr2:191827822-191827881 | hs\|2q32.2 |
| | *ERBB3* | 5.29571 | Down | Chr12:56482380-56482439 | hs\|12q13.2 |
| | *ERBB3* | 8.12050 | Down | Chr12:56496160-56496219 | hs\|12q13.2 |

**Table 4 Dysregulated mRNAs ($P < 0.05$, FC $\geq$ 2.0) associated with cisplatin resistance**

| Gene symbol | *P* value | FC (abs) | Regulation | Genename | Ref. |
|---|---|---|---|---|---|
| *FGF7* | 0.00035 | 2.19252 | Up | Fibroblast growth factor 7 | PMID: 22990650 |
| *HIPK2* | 2.63E-06 | 4.06213 | Up | Homeodomain interacting protein kinase 2 | PMID: 24846322 |
| *EDN1* | 9.94E-05 | 2.46437 | Up | Endothelin 1 | PMID: 21220476 |
| *CBS* | 0.00108 | 2.29340 | Up | Cystathionine-beta-synthase | PMID: 24236104 |
| *PDE3B* | 0.00029 | 10.44998 | Up | Phosphodiesterase 3B, cgmp-inhibited | PMID: 24133626 |
| *E2F5* | 0.00041 | 2.42888 | Up | E2F transcription factor 5, p130-binding | PMID: 22193543 |
| *PIN1* | 0.00104 | 2.13293 | Up | Peptidylprolyl cis/trans isomerase, NIMA-interacting 1 | PMID: 26820938 |
| *EGF* | 0.00346 | 4.76437 | Up | Epidermal growth factor | PMID: 27086487 |
| *CSF1* | 0.00025 | 2.25620 | Up | Colony stimulating factor 1 (macrophage) | PMID: 22005523 |
| *PCNA* | 0.00103 | 2.17028 | Up | Proliferating cell nuclear antigen | PMID: 24474685 |
| *HIPK2* | 2.63E-06 | 4.06213 | Up | Homeodomain interacting protein kinase 2 | PMID: 24846322 |
| *ENTPD6* | 0.00011 | 2.43726 | Up | Ectonucleoside triphosphate diphosphohydrolase 6 (putative) | PMID: 21519793 |
| *AKR1C1* | 0.00097 | 2.29646 | Up | Aldo-keto reductase family 1, member C1 | PMID: 23165153, PMID: 17266043 |
| *ASNS* | 0.00172 | 2.19491 | Up | Asparagine synthetase (glutamine-hydrolyzing) | PMID: 23956056, PMID: 17409444 |
| *BDNF* | 0.00062 | 2.32411 | Up | Brain-derived neurotrophic factor | PMID: 22276165, PMID: 17044982 |
| *CABYR* | 0.01089 | 2.55664 | Up | Calcium binding tyrosine-(Y)-phosphorylation regulated | PMID: 24362251 |
| *FGF2* | 2.15E-06 | 2.99240 | Up | Fibroblast growth factor 2 (basic) | PMID: 12894531 |
| *SLC7A11* | 1.95E-05 | 2.93256 | Up | Solute carrier family 7 member 11 | PMID: 24516043 |
| *TUBB3* | 0.00046 | 2.00213 | Up | Tubulin, beta 3 class III | PMID: 25107571 |
| *TWIST1* | 0.00180 | 2.96340 | Up | Twist family bhlh transcription factor 1 | PMID: 22673193, PMID: 22245869 |
| *JAG1* | 9.41E-05 | 3.20086 | Up | Jagged 1 | PMID: 24659709 |
| *ANXA11* | 0.00031 | 2.36619 | Down | Annexin A11 | PMID: 19484149, PMID: 17982121 |

| | | | | | |
|---|---|---|---|---|---|
| *CCL5* | 2.67E-05 | 5.05630 | Down | Chemokine (C-C motif) ligand 5 | PMID: 26983899 |
| *FGF13* | 0.00044 | 17.08866 | Down | Fibroblast growth factor 13 | PMID: 24113164 |
| *IGFBP3* | 7.48E-05 | 2.92508 | Down | Insulin-like growth factor binding protein 3 | PMID: 20023704 |
| *KLK6* | 0.00066 | 2.24596 | Down | Kallikrein-related peptidase 6 | PMID: 23307575 |
| *SLC7A8* | 4.50E-05 | 5.36735 | Down | Solute carrier family 7 member 8 | PMID: 23462296 |
| *TGM2* | 2.88E-05 | 6.24520 | Down | Transglutaminase 2 | PMID: 21424127, PMID: 24828664 |
| *TLR4* | 0.00114 | 2.13271 | Down | Toll-like receptor 4 | PMID: 21616060, PMID: 22583829 |
| *XAF1* | 0.02405 | 3.20613 | Down | XIAP associated factor 1 | PMID: 25824780, PMID: 25240826 |
| *TCEA2* | 0.00061 | 3.65969 | Down | Transcription elongation factor A (SII), 2 | PMID: 16142353 |

studies, and these pathways and input genes deserve our attention in gastric cancer cisplatin resistance.

Although protein expression is generally stable when organs mature, under various pathological and physiological conditions, gene expression may change and ultimately result in aberrant protein levels. Therefore, research on proteomics is helpful to illustrate some biological mechanisms, including cisplatin resistance. Protein-protein interaction network analysis might uncover previously unknown molecular mechanisms of cisplatin resistance. Hub proteins of subnetworks which interact with many partners might associate with drug resistance. For example, studies have shown that dysregulation of the genes *PDE3B, TLR4*, and *HIPK2* is associated with cisplatin resistance in human SCC cells, ovarian granulosa tumor cells and bladder cancer cells, respectively[26,32,33]. Moreover, hub proteins and their partners may have similar biological functions. Since downregulation of EGF has been shown to substantially overcome resistance to cisplatin in ovarian cancer[34], we predict that the proteins EDN1 and DCN, whose hub protein is EGF, may contribute to cisplatin resistance in a similar fashion. We also found that ZEB2, which over-expressed in SGC7901/DDP compared with SGC7901 has a similar expression profile to TWIST1, suggesting that ZEB2 may play an important role in cisplatin resistance by regulating the expression of TWIST1. Nevertheless, more evidence and research is needed.

In conclusion, our study identified mRNAs differentially expressed between gastric cancer cell lines SGC7901/DDP and SGC7901. These results provide a global view of the function of the differentially expressed mRNAs. Several molecular and pathway abnormalities detected in our study have previously been reported to be associated with drug resistance in gastric cancer. The dysregulated mRNAs identified participate in cisplatin resistance through diverse mechanisms, and further investigation is required to confirm the role in drug resistance of these transcripts, pathways and the interaction networks of the proteins they code for.

## COMMENTS

### Background

Cisplatin-contained chemotherapy is one of the most frequently used for advanced gastric cancer; however, this chemotherapeutic agent is often limited due to drug resistance and result unsatisfactory prognosis. Research increasingly suggests that abnormal expression of biological pathway and proteins associated with cisplatin resistance. This demonstrated that more bioinformatics study is needed to predict targets for gastric cancer with cisplatin.

### Research frontiers

Bioinformatics analysis demonstrated that some mRNAs which related to the biological behavior abnormal expression in SGC7901/DDP cells. These mRNAs have already been shown to play important roles in the process of cisplatin resistance of various cancers, including gastric cancer.

### Innovations and breakthroughs

The authors performed bioinformatics analysis of mRNA expression profile in SGC7901/DDP cells compared with SGC7901 cells, and found that many mRNAs and pathways in SGC7901/DDP cells expressed abnormally, these may participate in and predict cisplatin resistance in gastric cancer.

### Applications

These results suggest that targeting the differently expression mRNA may provide more selective approaches to reverse cisplatin resistance of therapeutic targets.

### Terminology

The definition of cisplatin resistance: in the clinic, if a patient who have disease recurrence within the first months after the recent cisplatin dose, the patient is considered cisplatin resistance; in cells, generally, resistance index > 20 exhibited high resistance, resistance index 5-15 is moderate resistance, resistance index < 5 represent low or no resistance. Correct *P*: Using Benjamini Hochberg FDR method for correction of p values. Fold change (FC): gene expression in SGC7901 / DDP cells compared with SGC7901 cells.

### Peer-review

The paper is a good study on mRNAs expression profile in SGC7901/DDP cells. The investigators shown that many mRNAs was abnormal expressed in SGC7901/DDP cells and these mRNAs enriched in many biological process which have already been shown to play important roles in the process of cisplatin resistance in human cancer.

## REFERENCES


1 **Ferro A**, Peleteiro B, Malvezzi M, Bosetti C, Bertuccio P, Levi F, Negri E, La Vecchia C, Lunet N. Worldwide trends in gastric cancer mortality (1980-2011), with predictions to 2015, and incidence by subtype. *Eur J Cancer* 2014; **50**: 1330-1344 [PMID: 24650579 DOI: 10.1016/j.ejca.2014.01.029]

2 **Wöhrer SS**, Raderer M, Hejna M. Palliative chemotherapy for advanced gastric cancer. *Ann Oncol* 2004; **15**: 1585-1595 [PMID: 15520058 DOI: 10.1093/annonc/mdh422]

3 **Jemal A**, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin* 2010; **60**: 277-300 [PMID: 20610543 DOI: 10.3322/caac.20073]

4 **Al-Batran SE**, Hartmann JT, Probst S, Schmalenberg H,

Hollerbach S, Hofheinz R, Rethwisch V, Seipelt G, Homann N, Wilhelm G, Schuch G, Stoehlmacher J, Derigs HG, Hegewisch-Becker S, Grossmann J, Pauligk C, Atmaca A, Bokemeyer C, Knuth A, Jäger E. Phase III trial in metastatic gastroesophageal adenocarcinoma with fluorouracil, leucovorin plus either oxaliplatin or cisplatin: a study of the Arbeitsgemeinschaft Internistische Onkologie. *J Clin Oncol* 2008; **26**: 1435-1442 [PMID: 18349393 DOI: 10.1200/jco.2007.13.9378]

5 **Kang YK**, Kang WK, Shin DB, Chen J, Xiong J, Wang J, Lichinitser M, Guan Z, Khasanov R, Zheng L, Philco-Salas M, Suarez T, Santamaria J, Forster G, McCloud PI. Capecitabine/cisplatin versus 5-fluorouracil/cisplatin as first-line therapy in patients with advanced gastric cancer: a randomised phase III noninferiority trial. *Ann Oncol* 2009; **20**: 666-673 [PMID: 19153121 DOI: 10.1093/annonc/mdn717]

6 **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 DOI: 10.1016/s1470-2045(08)70035-4]

7 **Wagner AD**, Unverzagt S, Grothe W, Kleber G, Grothey A, Haerting J, Fleig WE. Chemotherapy for advanced gastric cancer. *Cochrane Database Syst Rev* 2010; **(3)**: CD004064 [PMID: 20238327 DOI: 10.1002/14651858.CD004064.pub3]

8 **Rabik CA**, Dolan ME. Molecular mechanisms of resistance and toxicity associated with platinating agents. *Cancer Treat Rev* 2007; **33**: 9-23 [PMID: 17084534 DOI: 10.1016/j.ctrv.2006.09.006]

9 **Garcia JA**, Dreicer R. Systemic chemotherapy for advanced bladder cancer: update and controversies. *J Clin Oncol* 2006; **24**: 5545-5551 [PMID: 17158540 DOI: 10.1200/jco.2006.08.0564]

10 **Wilson TR**, Longley DB, Johnston PG. Chemoresistance in solid tumours. *Ann Oncol* 2006; **17** Suppl 10: x315-x324 [PMID: 17018746 DOI: 10.1093/annonc/mdl280]

11 **Borst P**, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst* 2000; **92**: 1295-1302 [PMID: 10944550]

12 **Hioki M**, Gotohda N, Konishi M, Nakagohri T, Takahashi S, Kinoshita T. Predictive factors improving survival after gastrectomy in gastric cancer patients with peritoneal carcinomatosis. *World J Surg* 2010; **34**: 555-562 [PMID: 20082194 DOI: 10.1007/s00268-010-0396-5]

13 **Yang T**, Chen M, Chen T, Thakur A. Expression of the copper transporters hCtr1, ATP7A and ATP7B is associated with the response to chemotherapy and survival time in patients with resected non-small cell lung cancer. *Oncol Lett* 2015; **10**: 2584-2590 [PMID: 26622894 DOI: 10.3892/ol.2015.3531]

14 **Metzger R**, Leichman CG, Danenberg KD, Danenberg PV, Lenz HJ, Hayashi K, Groshen S, Salonga D, Cohen H, Laine L, Crookes P, Silberman H, Baranda J, Konda B, Leichman L. ERCC1 mRNA levels complement thymidylate synthase mRNA levels in predicting response and survival for gastric cancer patients receiving combination cisplatin and fluorouracil chemotherapy. *J Clin Oncol* 1998; **16**: 309-316 [PMID: 9440758]

15 **Xu W**, Chen Q, Wang Q, Sun Y, Wang S, Li A, Xu S, Røe OD, Wang M, Zhang R, Yang L, Zhou J. JWA reverses cisplatin resistance via the CK2-XRCC1 pathway in human gastric cancer cells. *Cell Death Dis* 2014; **5**: e1551 [PMID: 25476899 DOI: 10.1038/cddis.2014.517]

16 **Shim HJ**, Yun JY, Hwang JE, Bae WK, Cho SH, Lee JH, Kim HN, Shin MH, Kweon SS, Lee JH, Kim HJ, Chung IJ. BRCA1 and XRCC1 polymorphisms associated with survival in advanced gastric cancer treated with taxane and cisplatin. *Cancer Sci* 2010; **101**: 1247-1254 [PMID: 20331623 DOI: 10.1111/j.1349-7006.2010.01514.x]

17 **Liu J**, Deng N, Xu Q, Sun L, Tu H, Wang Z, Xing C, Yuan Y. Polymorphisms of multiple genes involved in NER pathway predict prognosis of gastric cancer. *Oncotarget* 2016; **7**: 48130-48142 [PMID: 27340861 DOI: 10.18632/oncotarget.10173]

18 **Sargent RG**, Meservy JL, Perkins BD, Kilburn AE, Intody Z, Adair GM, Nairn RS, Wilson JH. Role of the nucleotide excision repair gene ERCC1 in formation of recombination-dependent rearrangements in mammalian cells. *Nucleic Acids Res* 2000; **28**: 3771-3778 [PMID: 11000269]

19 **Funato T**, Kozawa K, Fujimaki S, Miura T, Kaku M. Increased sensitivity to cisplatin in gastric cancer by antisense inhibition of the her-2/neu (c-erbB-2) gene. *Chemotherapy* 2001; **47**: 297-303 [PMID: 11399867]

20 **Zhou N**, Qu Y, Xu C, Tang Y. Upregulation of microRNA-375 increases the cisplatin-sensitivity of human gastric cancer cells by regulating ERBB2. *Exp Ther Med* 2016; **11**: 625-630 [PMID: 26893657 DOI: 10.3892/etm.2015.2920]

21 **Sileni VC**, Fosser V, Maggian P, Padula E, Beltrame M, Nicolini M, Arslan P. Pharmacokinetics and tumor concentration of intraarterial and intravenous cisplatin in patients with head and neck squamous cancer. *Cancer Chemother Pharmacol* 1992; **30**: 221-225 [PMID: 1628371]

22 **Zhou C**, Zhang T, Liu F, Zhou J, Ni X, Huo R, Shi Z. The differential expression of mRNAs and long noncoding RNAs between ectopic and eutopic endometria provides new insights into adenomyosis. *Mol Biosyst* 2016; **12**: 362-370 [PMID: 26662114 DOI: 10.1039/c5mb00733j]

23 **Muggia FM**, Bonetti A, Hoeschele JD, Rozencweig M, Howell SB. Platinum Antitumor Complexes: 50 Years Since Barnett Rosenberg's Discovery. *J Clin Oncol* 2015; **33**: 4219-4226 [PMID: 26503202 DOI: 10.1200/jco.2015.60.7481]

24 **Ge X**, Liu X, Lin F, Li P, Liu K, Geng R, Dai C, Lin Y, Tang W, Wu Z, Chang J, Lu J, Li J. MicroRNA-421 regulated by HIF-1α promotes metastasis, inhibits apoptosis, and induces cisplatin resistance by targeting E-cadherin and caspase-3 in gastric cancer. *Oncotarget* 2016; **7**: 24466-24482 [PMID: 27016414 DOI: 10.18632/oncotarget.8228]

25 **Sakai W**, Swisher EM, Karlan BY, Agarwal MK, Higgins J, Friedman C, Villegas E, Jacquemont C, Farrugia DJ, Couch FJ, Urban N, Taniguchi T. Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. *Nature* 2008; **451**: 1116-1120 [PMID: 18264087 DOI: 10.1038/nature06633]

26 **Uzawa K**, Kasamatsu A, Baba T, Usukura K, Saito Y, Sakuma K, Iyoda M, Sakamoto Y, Ogawara K, Shiiba M, Tanzawa H. Targeting phosphodiesterase 3B enhances cisplatin sensitivity in human cancer cells. *Cancer Med* 2013; **2**: 40-49 [PMID: 24133626 DOI: 10.1002/cam4.56]

27 **Cho HJ**, Kim IK, Park SM, Baek KE, Nam IK, Park SH, Ryu KJ, Choi J, Ryu J, Hong SC, Jeong SH, Lee YJ, Ko GH, Kim J, Won Lee C, Soo Kang S, Yoo J. VEGF-C mediates RhoGDI2-induced gastric cancer cell metastasis and cisplatin resistance. *Int J Cancer* 2014; **135**: 1553-1563 [PMID: 24585459 DOI: 10.1002/ijc.28801]

28 **Cortés-Sempere M**, de Miguel MP, Pernía O, Rodriguez C, de Castro Carpeño J, Nistal M, Conde E, López-Ríos F, Belda-Iniesta C, Perona R, Ibanez de Caceres I. IGFBP-3 methylation-derived deficiency mediates the resistance to cisplatin through the activation of the IGFIR/Akt pathway in non-small cell lung cancer. *Oncogene* 2013; **32**: 1274-1283 [PMID: 22543588 DOI: 10.1038/onc.2012.146]

29 **Fu X**, Feng J, Zeng D, Ding Y, Yu C, Yang B. PAK4 confers cisplatin resistance in gastric cancer cells via PI3K/Akt- and MEK/Erk-dependent pathways. *Biosci Rep* 2014; Epub ahead of print [PMID: 24471762 DOI: 10.1042/bsr20130102]

30 **Zhang LL**, Zhang J, Shen L, Xu XM, Yu HG. Overexpression of AKT decreases the chemosensitivity of gastric cancer cells to cisplatin in vitro and in vivo. *Mol Med Rep* 2013; **7**: 1387-1390 [PMID: 23546174 DOI: 10.3892/mmr.2013.1400]

31 **Zhi X**, Tao J, Xiang G, Cao H, Liu Z, Yang K, Lv C, Ni S. APRIL induces cisplatin resistance in gastric cancer cells via activation of the NF-κB pathway. *Cell Physiol Biochem* 2015; **35**: 571-585 [PMID: 25612651 DOI: 10.1159/000369720]

32 **Lin J**, Zhang Q, Lu Y, Xue W, Xu Y, Zhu Y, Hu X. Downregulation of HIPK2 increases resistance of bladder cancer cell to cisplatin by regulating Wip1. *PLoS One* 2014; **9**: e98418 [PMID: 24846322

DOI: 10.1371/journal.pone.0098418]

33 **Woods DC**, White YA, Dau C, Johnson AL. TLR4 activates NF-κB in human ovarian granulosa tumor cells. *Biochem Biophys Res Commun* 2011; **409**: 675-680 [PMID: 21616060 DOI: 10.1016/j.bbrc.2011.05.063]

34 **Tang XH**, Li M, Deng S, Lu MS. Cross-reacting material 197, a heparin-binding EGF-like growth factor inhibitor, reverses the chemoresistance in human cisplatin-resistant ovarian cancer. *Anticancer Drugs* 2014; **25**: 1201-1210 [PMID: 25115341 DOI: 10.1097/cad.0000000000000155]

**P- Reviewer**: Ghosh RD **S- Editor**: Qi Y **L- Editor**: A
**E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/ *World J Gastroenterol* 2017 February 21; 23(7): 1203-1214

DOI: 10.3748/wjg.v23.i7.1203 ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

# Caffeic acid phenethyl ester up-regulates antioxidant levels in hepatic stellate cell line T6 *via* an Nrf2-mediated mitogen activated protein kinases pathway

Ning Yang, Juan-Juan Shi, Feng-Ping Wu, Mei Li, Xin Zhang, Ya-Ping Li, Song Zhai, Xiao-Li Jia, Shuang-Suo Dang

Ning Yang, Juan-Juan Shi, Feng-Ping Wu, Mei Li, Xin Zhang, Ya-Ping Li, Song Zhai, Xiao-Li Jia, Shuang-Suo Dang, Department of Infectious Diseases, the Second Affiliated Hospital of Medical School of Xi'an Jiaotong University, Xi'an 710004, Shannxi Province, China

Author contributions: Yang N and Dang SS designed the research; Yang N, Shi JJ, Wu FP, Li M, Zhang X and Li YP performed the research; Yang N, Zhai S and Jia XL analyzed the data; and Yang N and Shi JJ wrote the paper.

Supported by the Liver Fibrosis Foundation of Wang Bao-En of China, No. 20100033; and the Science and Technology Foundation of Shaanxi Province of China, No. 2010K01-199.

Institutional review board statement: No human or animal subjects were used in this study.

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

Data sharing statement: No additional data are available.



Manuscript source: Unsolicited manuscript

Correspondence to: Shuang-Suo Dang, PhD, MD, Department of Infectious Diseases, the Second Affiliated Hospital of Medical School of Xi'an Jiaotong University, No. 157 Xi'wu Road, Xi'an 710004, Shaanxi Province, China. dang212@126.com
Telephone: +86-29-87679688
Fax: +86-29-87679688

Received: November 15, 2016
Peer-review started: November 15, 2016
First decision: December 19, 2016
Revised: December 27, 2016
Accepted: January 11, 2017
Article in press: January 11, 2017
Published online: February 21, 2017

## Abstract

***AIM***

To investigate the antioxidant effect of caffeic acid phenethyl ester (CAPE) in hepatic stellate cell-T6 (HSC-T6) cells cultured *in vitro* and the potential mechanisms.

***METHODS***

HSC-T6 cells were cultured *in vitro* and treated with various concentrations of CAPE for 24, 48 and 72 h, respectively. Cell proliferation was investigated using the MTT assay, and cell ultrastructural alterations were observed by transmission electron microscopy. Flow cytometry was employed to investigate the effects of CAPE on apoptosis and the levels of reactive oxygen species in HSC-T6 cells cultured *in vitro*. An enzyme immunoassay instrument was used to evaluate antioxidant enzyme expression. The effect on α-smooth muscle actin was shown using immunofluorescence. Gene and protein levels of Nrf2, related factors, and mitogen activated protein kinases (MAPKs), in HSC-T6 cells were detected using RT-PCR and Western blot, respectively.

***RESULTS***

CAPE inhibited the proliferation and activation of HSC-T6 cells cultured *in vitro*. CAPE increased the antioxidant levels and the translocation of Nrf2 from

the cytoplasm to the nucleus in HSC-T6 cells. Moreover, the phosphorylation of MAPKs in cells decreased in response to CAPE. Interestingly, CAPE-induced oxidative stress in the cells was significantly attenuated by pretreatment with MAPKs inhibitors.

***CONCLUSION***

CAPE inhibits cell proliferation and up-regulates the antioxidant levels in HSC-T6 cells partly through the Nrf2-MAPKs signaling pathway.

**Key words:** Caffeic acid phenethyl ester; Liver fibrosis; Antioxidation; Nrf2; Mitogen activated protein kinases



**Core tip:** Liver fibrosis is a pathological response to hepatocyte injury, including oxidative stress, which is a primary mechanism of liver damage. Caffeic acid phenethyl ester (CAPE) is a phenolic compound extracted from honeybee propolis that has strong biological properties in liver protection and as an antioxidant and anti-fibrosis agent. It has been used in the treatment of several diseases. In this study, we investigated the antioxidant effect of CAPE in HSC-T6 cells and its potential mechanism. Our results demonstrated that CAPE inhibited cell proliferation and up-regulated the antioxidant levels in HSC-T6 cells partly through the Nrf2-MAPKs signaling pathway.

Yang N, Shi JJ, Wu FP, Li M, Zhang X, Li YP, Zhai S, Jia XL, Dang SS. Caffeic acid phenethyl ester up-regulates antioxidant levels in hepatic stellate cell line T6 *via* an Nrf2-mediated mitogen activated protein kinases pathway. *World J Gastroenterol* 2017; 23(7): 1203-1214 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1203.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1203

## INTRODUCTION

Liver fibrosis is a pathological response to hepatocyte injury, including injury caused by viral infection, chronic inflammation, and other factors such as oxidative stress, which is a primary mechanism of liver damage[1,2]. Oxidative stress can stimulate the activation of hepatic stellate cells (HSCs) through paracrine and autocrine mechanisms, leading to the formation of liver fibrosis[1,2]. HSC-T6 cells, a well-differentiated transformed cell line[3,4], were used in this study to observe the role of HSC in liver fibrosis.

Caffeic acid phenethyl ester (CAPE) is a phenolic compound extracted from honeybee propolis that has strong biological properties in liver protection and as an antioxidant and anti-fibrosis agent. It has been used in the treatment of several diseases[5-7]. Our previous studies have shown that CAPE inhibited liver fibrosis in rats due to its ability to suppress oxidative stress[8].

Antioxidants play a pivotal role in the development of liver fibrosis[1]. Several antioxidative stress factors have been identified, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, and nonenzymatic compounds [vitamin E, carotene, and glutathione-S-transferase (GSTs)], in liver tissue[9,10]. SOD, CAT, and GSTs are recognized enzymes that are closely related to nuclear factor-erythroid 2 related factor 2 (Nrf2)[11,12]. Nrf2 is an important transcription factor regulating the expression of antioxidative stress factors. Nrf2 binds to an antioxidant response element (ARE) in the promoter region of genes encoding several phase-II detoxifying/antioxidant enzymes and related stress-responsive proteins, including SOD, CAT and GSTs[12]. Several studies have confirmed that Nrf2 is involved in the signaling pathway of mitogen activated protein kinase (MAPKs) in nuclear translocation[13]. MAPKs, including the ERK1/2, JNK1/2, and p38 signaling pathways, play a critical role in regulating the oxidative stress response in various types of cells. However, it is still largely unknown whether CAPE can reduce the expression of these factors *via* the Nrf2-mediated MAPKs signaling pathway in HSCs.

In this study, we show that CAPE inhibits the proliferation and activation, but increases apoptosis, of HSC-T6 cells *in vitro*. CAPE up-regulates the antioxidant capacity in HSC-T6 cells through the Nrf2-mediated MAPKs signaling pathway.

## MATERIALS AND METHODS

### *Reagents*

The cell apoptosis kit and SB203580 were purchased from Joincare Pharmaceutical Industry Group Co., Ltd. (Nanjing, China). SP600125 and PD98059 were obtained from Sigma-Aldrich (St. Louis, United States) and Cell Signaling Technology (Boston, United States), respectively. CAPE (Sigma-Aldrich, St. Louis, MO, United States) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, United States) to 40 mmol/L as a stock solution and stored at -20 ℃. Control flasks or plates contained DMSO at an equivalent dilution in cultures containing CAPE.

### *Cell culture*

HSC-T6 cells were purchased from Joincare Pharmaceutical Industry Group Co., Ltd. (NanJing, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, Utah, United States) supplemented with 10% (v/v) fetal bovine serum (FBS, HyClone, Utah, United States), 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, United States), 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, United States), 2 mmol/mL L-glutamine (Sigma-Aldrich, St. Louis, United States), and 100 U/mL DNase I (Sigma-Aldrich, St. Louis, United States) at 37 ℃ in a 5% (v/v) $CO_2$ humidity atmosphere. Cells adhering to the culture flask in logarithmic phase were trypsinized and subjected to

**Table 1 Primers used for real-time PCR analysis**

| Gene | Primer sequence | Tm (℃) | Size (bp) | Source |
|---|---|---|---|---|
| *Nrf2* | | | | |
| F | 5′-CTGCCATTAGTCAGTCGCTCTC-3′ | 57.3 | 22 | Rat |
| R | 5′-TCGGCTGGGACTTGTGTTC-3′ | 57.7 | 19 | Rat |
| *SOD1* | | | | |
| F | 5′-GCTTCTGTCGTCTCCTTGCT-3′ | 55.7 | 20 | Rat |
| R | 5′-CTCGAAGTGAATGA CGCCCT-3′ | 55.8 | 20 | Rat |
| *CAT* | | | | |
| F | 5′-TGGCTATGGCTCACACACCTTC-3′ | 59.3 | 22 | Rat |
| R | 5′-GAGGCCA TAATCCGGGTCTTC-3′ | 57.7 | 21 | Rat |
| *GSTs* | | | | |
| F | 5′-GTGGAGATTGACGGGATGAA -3′ | 54.2 | 20 | Rat |
| R | 5′-CGGTCTTGGCTTCTCTTTGG -3′ | 56.0 | 20 | Rat |
| *β-actin* | | | | |
| F | 5′-GGAGATTACTGCCCTGGCTCCTA-3′ | 60.2 | 23 | Rat |
| R | 5′-GACTCATCGTACTCCTGCTTGCTG-3′ | 59.3 | 24 | Rat |

passage as usual for further experimentation.

### Cell viability assay

The tetrazolium dye colorimetric test [3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyl-tetrazolium bromide, MTT, Sigma-Aldrich, St. Louis, MO, United States] was used to determine the viability of HSC-T6 cells. The MTT assay is based on the ability of functional mitochondria to catalyze the reduction of MTT to insoluble purple formazan, the concentration of which can be measured spectrophotometrically, as described previously[14]. HSC-T6 cells were incubated on a 96-well plate at a density of $5 \times 10^5$ cells/well for 24 h and treated with different concentrations of CAPE (0, 5, 10, 15, 20, 40, 60, 80 and 100 μmol/L). MTT solution (0.5 mg/mL) was then added to each well. The MTT solution was replaced with DMSO to dissolve the blue formazan crystals. Four hours later, absorbance was measured at 490 nm using a microplate reader (BioTekInstruments, United States) and the percentage viability was calculated.

### Transmission electron microscopy

Cells in the logarithmic phase were treated with a range of CAPE (5, 10 and 15 μmol/L) for 24 h or established as controls. At the end of the treatment, cells were washed twice with phosphate buffered solution (PBS, 137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L $Na_2HPO_4$, 1.4 mmol/L $KH_2PO_4$, pH 7.4), fixed in 2.5% (v/v) glutaraldehyde followed by 1% (v/v) perosmic acid, and dehydrated in an ethanol series. Ultrathin sections were placed on 400-mesh grids and double-stained with uranyl acetate and lead citrate. The ultrastructures were observed using a transmission electron microscope (HITACHI-H7650, Tokyo, Japan).

### Apoptosis assay

Cell apoptosis was determined using Annexin V-FITC and PI double staining (KaiJi, Nanjing, China), as described previously[15]. Cells grown in logarithmic phase were treated with a range of CAPE (5, 10 and 15 μmol/L) for 24 h or established as controls. At the end of the treatment, cells were collected by centrifugation and washed twice with PBS. Cell pellets were resuspended in 0.5 mL PBS and fixed in 5 mL 70% (v/v) ice-cold ethanol at 4 ℃ for 24 h. After resuspension in 1 mL PBS, the cells were incubated with ribonuclease A (Rnase A, 20 mg/L, Sigma-Aldrich) and propidium iodide (PI, 50 mg/L, Sigma-Aldrich) for 1 h at 37 ℃ in the dark. The stained cells were analyzed using a FACscan flow cytometer in combination with BD lysis II software (CALIBUR, BD, United States).

### Detection of SOD and CAT activity and GSH content

SOD and CAT activity and GSH content were measured using a SOD protein reagent kit (Bio-Rad, Hercules, CA, United States), CAT protein reagent kit (Bio-Rad, Hercules, CA, United States), and GSH protein reagent kit (Bio-Rad, Hercules, CA, United States), respectively. Briefly, HSC-T6 cells grown in logarithmic phase were treated with a range of CAPE (5, 10 and 15 μmol/L) for 24 h. The cells were washed twice with PBS, collected by centrifugation, and lysed in cell disrupter buffer (Bio-Rad, Hercules, CA, United States). After centrifugation, the protein concentrations of the supernatant were determined using a protein reagent kit. The optical density (OD) of SOD, GSH, and CAT was measured using a spectrophotometer (ND-1000, Thermo Fisher, United States) at wavelengths of 450 nm, 405 nm and 420 nm to generate the standard curve and calculate the concentrations of SOD, GSH and CAT, respectively.

### Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, United States) and reverse transcription was carried out using an RT-PCR kit (Takara, Japan), as described previously[16]. Real-time PCR was performed using the SYBRE Script TM RT-PCR Kit (Takara, Japan) on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States) according to the manufacturer's protocols. The primers are shown in Table 1. The comparative CT method was used to quantify the target gene expression, with β-actin used as an internal control[17].

### Immunocytochemistry

The expression of α-SMA and Nrf2 in HSC-T6 cells was investigated using immunofluoresence staining[18]. Briefly, the cells were fixed in 4% (w/v) paraformaldehyde (Sigma-Aldrich) and permeabilized with 0.1% (v/v) Triton X-100 (Sigma-Aldrich). After blocking with 5% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) and 1% (v/v) normal donkey serum (Sigma-Aldrich), the cells were incubated with rabbit anti-α-SMA (1:500) or Nrf2 (1:500) primary antibody (Table 2) for 16 h at 4 ℃. The cells were washed and further incubated with peroxidase-conjugated anti-rabbit IgG (1:1000, Pierce Biotechnology, Rockford, United States) for 1 h. The cell nuclei were counter-

**Table 2 Antibodies used for Western blot analysis**

| Antibody (dilution) | Clone | Species | Specificity | Source |
|---|---|---|---|---|
| Nrf2 (16396-1-AP 1:500) | M | Rabbit | Immunogen: ag9489 | ProteinTech, United States |
| P38MAPK (ab32142, 1:500) | M | Rabbit | Synthetic peptide corresponding to residues near the C-terminus of human p38 MAP kinase was used as immunogen. Predicted to react with CSBP1 splice form based on sequence homology | EPITOMICS, United States |
| P-p38MAPK (#4511, 1:500) | M | Rabbit | Phospho-P38 MAPKinase (Thr180/Tyr182) (3D7) rabbit mAb detects endogenous levels of p38MAP kinase only when dually phosphorylated at Thr180 and Tyr182. This antibody does not cross-react with the phosphorylated forms of either p42/44 MAPK or SAPK/JNK | Cell Signaling, United States |
| Erk1/2 (#4695, 1:500) | M | Rabbit | P44/42 MAP kinase (137F5) rabbit mAb detects endogenous level of total P44/42 MAP kinase (Erk1/Erk2) protein. The antibody does not cross-react with JNK/SAPK or p38 MAP kinase | Cell Signaling, United States |
| Erk1phospho(pY204)/ Erk2phospho (pY187) (ab76299 1:500) | M | Rabbit | A phospho-specific peptide corresponding to residues surrounding tyrosine 204 of human Erk1. This antibody detects Erk1 phosphorylated at tyrosine 204 | EPITOMICS, United States |
| SAPK/JNK (#9258 1:500) | M | Rabbit | SAPK/JNK(56G8) rabbit mAb detects endogenous levels of total SAPK/JNK protein | Cell Signaling, United States |
| JNK1phospho(pT183) /JNK2phospho(pT221) (ab124956 1:500) | M | Rabbit | A phospho-specific peptide corresponding to residues surrounding threonine 221 of human JNK3 was used as immunogen. The antibody detects JNK1 (pT183), JNK2 (pT183) and JNK3 (pT221) | EPITOMICS, United States |
| α-SMA (ab124964,1: 1000) | M | Rabbit | A synthetic peptide corresponding to N-terminus of human actin was used as immunogen | EPITOMICS, United States |
| Collegen-1 (bs-0578R 1:500) | P | Rabbit | KLH conjugated synthetic peptide derived from human Collagen I C-terminal propeptide | Bioss, China |
| β-actin (sc-47778, 1:1000) | M | Mouse | β-actin is recommended for detection of β-actin of mouse, rat, human, chicken, dog, pig, rabbit | SANTA CRUZ, United States |

M: Monoclone; P: Polyclone.

stained using 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). After neutral gum mounting, fluorescence microscopy images were obtained using a confocal microscope (Ti-E, Nikon, Japan) with a laser at 405 nm and 535 nm excitation.

### *Western blot analysis*

The expression of Nrf2, related factors, and MAPKs in HSC-T6 cells was investigated using Western blot, as described previously[16]. Briefly, the cells were harvested and re-suspended in a Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA, United States) or RIPA lysis buffer [20 mmol/L Tris, 150 mmol/L NaCl, 1% (v/v) Triton X-100, 1% (w/v) digestive phosphatase inhibitors, 1% (w/v) protease inhibitors, 1% (w/v) phenylmethyl sulfonylfluoride (PMSF), pH 7.5] (Sigma-Aldrich). The protein content was determined using a commercial protein reagent kit (Bio-Rad, Hercules, CA, United States). Equal amounts of proteins in each sample were resolved by 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE, Sigma-Aldrich) electrophoresis and the proteins were transferred onto PVDF membranes (Sigma-Aldrich). After blocking with skim milk, the membranes were incubated with the specific antibodies (Table 2) for 24 h at 4 ℃. After washing, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Pierce Biotechnology, Rockford, United States) for 2 h at 37 ℃. Proteins were detected with an all-enhanced chemiluminescence detection system (Syngene, United Kingdom) and quantified using a Gel-Pro Analyzer v4.0 (Media Cybernetics, L.P., United States). β-actin was used as the loading control.

### *Statistical analysis*

The results are shown as the mean ± SD. Differences between groups were assessed by Student's *t*-test and one- or two-way ANOVA with post-hoc Duncan multiple comparisons using SPSS 13.0. A $P$-value < 0.05 was considered statistically significant.

## RESULTS

### *Effect of different concentrations of CAPE on biological characteristics of HSC-T6 cells*

The MTT assay results indicated that 5, 10 and 15 μmol/L CAPE treatment for 24 h did not decrease cell viability compared to the control group ($P$ > 0.05, Figure 1A). However, 20-100 μmol/L of CAPE was cytotoxic in HSC-T6 cells ($P$ < 0.05, Figure 1A). Similar results were obtained after 48 and 72 h (data not shown). Therefore, 5, 10 and 15 μmol/L CAPE were used for all subsequent experiments. After treatment for 24 h, the proportion of cell apoptosis increased in a concentration-dependent manner compared to the control group (Figure 1B). Transmission electron microscopy was then used to investigate the ultrastructure of apoptotic cells. In the control group,

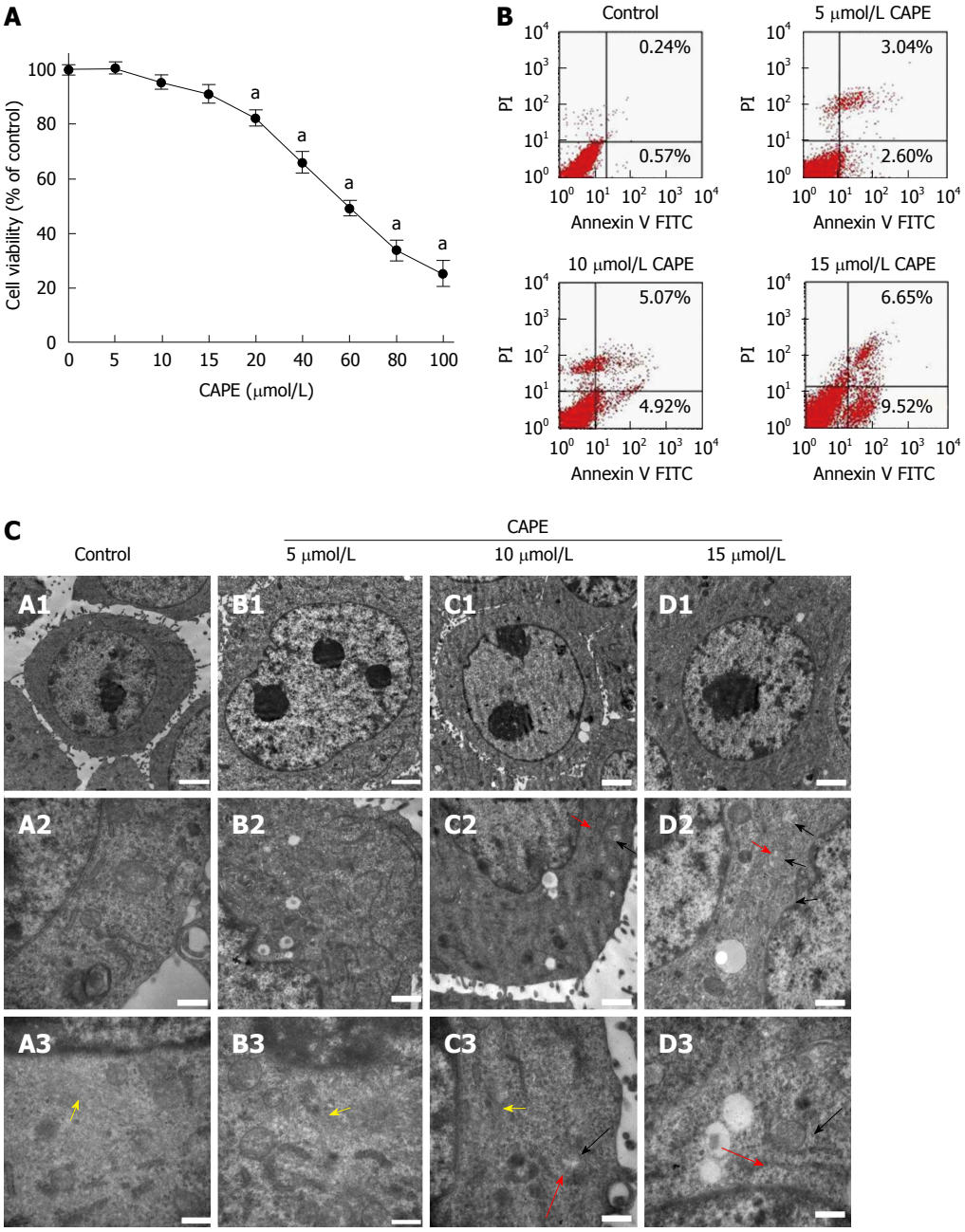



**Figure 1 Effect of different concentrations of caffeic acid phenethyl ester on biological characteristics of hepatic stellate cell-T6 cells.** After HSC-T6 cells were treated with CAPE(0, 5, 10, 15, 20, 40, 60, 80 and 100 μmol/L) for 24 h (A) the effect of CAPE on the viability of HSC-6 cells was detected by the MTT assay; B: Cell apoptosis was investigated using annexin V-FITC and PI and the proportion of cell apoptosis increased in a concentration-dependent manner; C: Ultrastructure of the HSC-T6 cells. The normal structure is shown in the control groups (group A). The treatment groups (groups B, C, and D) displayed prominent myofilament disarray and rupture, cytoplasmic vacuolization, and significant mitochondrial swelling (black bar: mitochondria; red bar: Endoplasmic reticulum; yellow bar: myofilament). The upper scale bar = 2 μm, the middle scale bar = 1 μm, and the lower scale bar = 0.5 μm. The data represent averages of the results of four independent experiments. [a]*P* < 0.05 *vs* control. CAPE: Caffeic acid phenethyl ester.

the cells were round with tiny villous projections observed on the cell membrane. Many plasmosomes were distributed in the nucleus; the structure of mitochondria was clear; the rough endoplasmic reticulum was streaky; and lipid droplets were found in the cytoplasm (Figure 1C-A$_{1-3}$). In the CAPE treatment groups, the growth of HSC-T6 cells was obviously inhibited; cell volume gradually declined; surface villous structure decreased or disappeared; there were fewer multiple nucleoli; there was mitochondrial swelling; the endoplasmic reticulum was slender; and a scattered distribution of lipid droplets was observed in the cytoplasm (Figure 1C-B/C/D).

### *α-SMA and collegen-1 protein expression in HSC-T6 cells treated with CAPE*

In the control group, HSC-T6 cells were spindle-shaped and fully stained with α-SMA (Figure 2A). After treatment with 5, 10 and 15 μmol/L of CAPE for 24 h, the cell volume was lower and the cell morphology

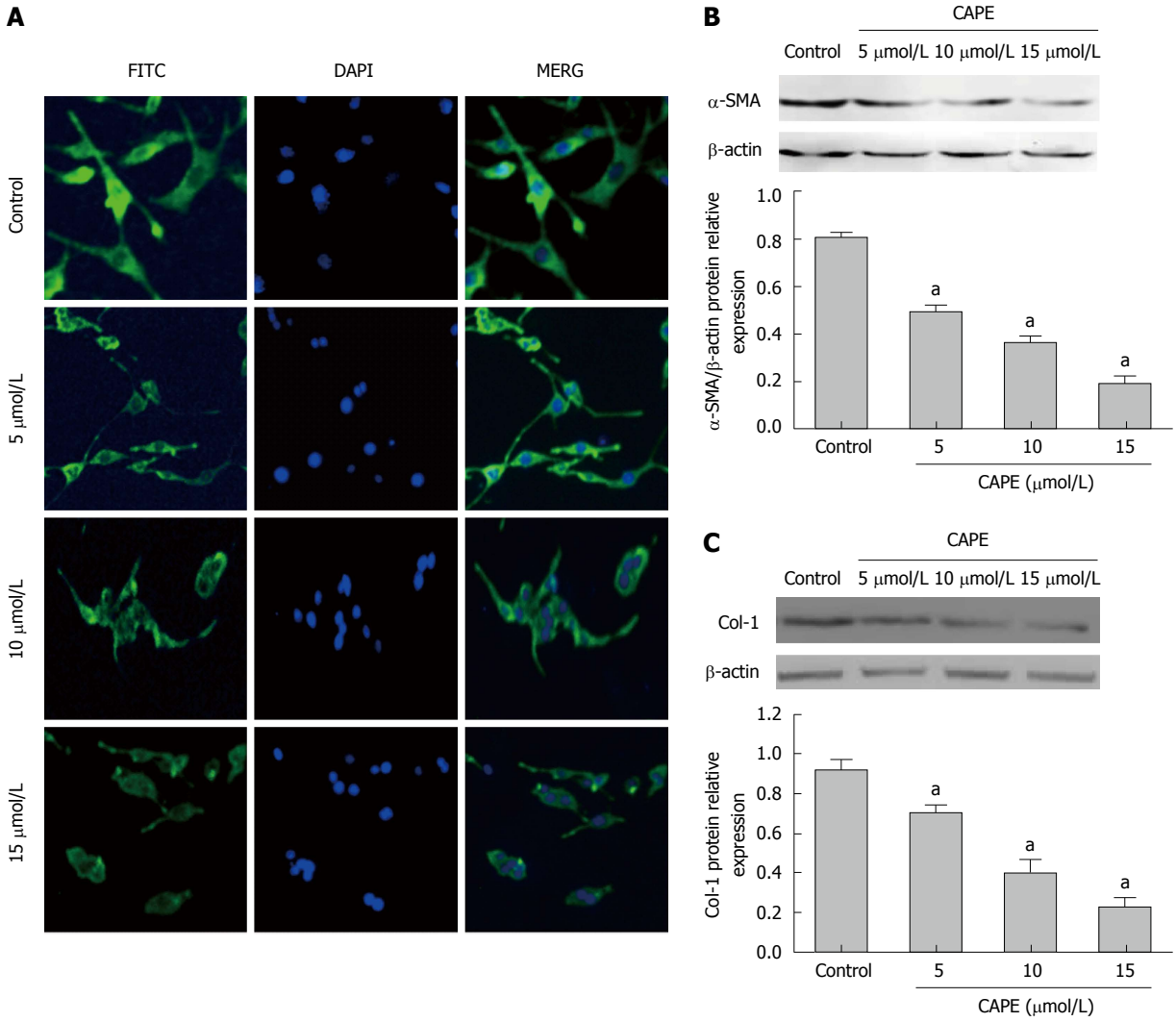



**Figure 2 α-SMA and collegen-1 protein expression in hepatic stellate cell-T6 cells.** After HSC-T6 cells were treated with 5 μmol/L, 10 μmol/L and 15 μmol/L CAPE for 24 h, indirect immunofluorescence (× 200) analysis of α-SMA protein expression (A) were undertaken. Western blot analysis of α-SMA and collegen-1 protein expression was also performed. Gray levels were normalized against those of the corresponding β-actin and the results are expressed relative to control (B and C). The data are the mean ± SD of three independent experiments. $^{a}P < 0.05$ *vs* control. CAPE: Caffeic acid phenethyl ester.

became round with reduced α-SMA fluorescent staining (Figure 2A). Western blot analysis showed that α-SMA and collegen-1 protein expression decreased in a dose-dependent manner in HSC-T6 cells compared to the control group ($P < 0.05$, Figure 2B and C).

### *Antioxidant-related indicator protein and mRNA expression in HSC-T6 cells*

After treatment with CAPE for 24 h, gene and protein expression of SOD, CAT, GSH and GSTs was significantly increased in HSC-T6 cells treated with 10 μmol/L or 15 μmol/L CAPE compared to the control group ($P < 0.05$, Figure 3A and B). However, 5 μmol/L of CAPE did not affect SOD, CAT, GSH, or GSTs ($P > 0.05$, Figure 3A and B).

### *Effect of CAPE on Nrf2 expression in HSC-T6 cells*

We observed that 10 μmol/L and 15 μmol/L of CAPE significantly increased Nrf2 gene expression in HSC-T6 cells ($P < 0.05$, Figure 4A). However, there was no alteration in Nrf2 gene expression in HSC-T6 cells in response to 5 μmol/L CAPE ($P > 0.05$, Figure 4A). Interestingly, Nrf2 protein expression in the cytosol was decreased in a dose-dependent manner ($P < 0.05$), whereas in the nucleus it was increased in a dose-dependent manner ($P < 0.05$) in HSC-T6 cells after treatment with CAPE (5, 10 and 15 μmol/L) for 24 h (Figure 4B). The nuclear/cytosol ratio of Nrf2 protein levels was significantly higher in CAPE-treated HSC-T6 cells than in the control group ($P < 0.05$, Figure 4B). Indirect immunofluorescence showed that Nrf2 protein translocated from the cytosol to the nucleus in HSC-T6 cells in the 15 μmol/L CAPE treatment group compared to the control group (Figure 4C). These results suggest that CAPE induces the activation and nuclear transcription of Nrf2.

### *CAPE up-regulates antioxidant levels through an Nrf2-mediated MAPKs signaling pathway in HSC-T6 cells*

The phosphorylation levels of ERK1/2, p38MARK

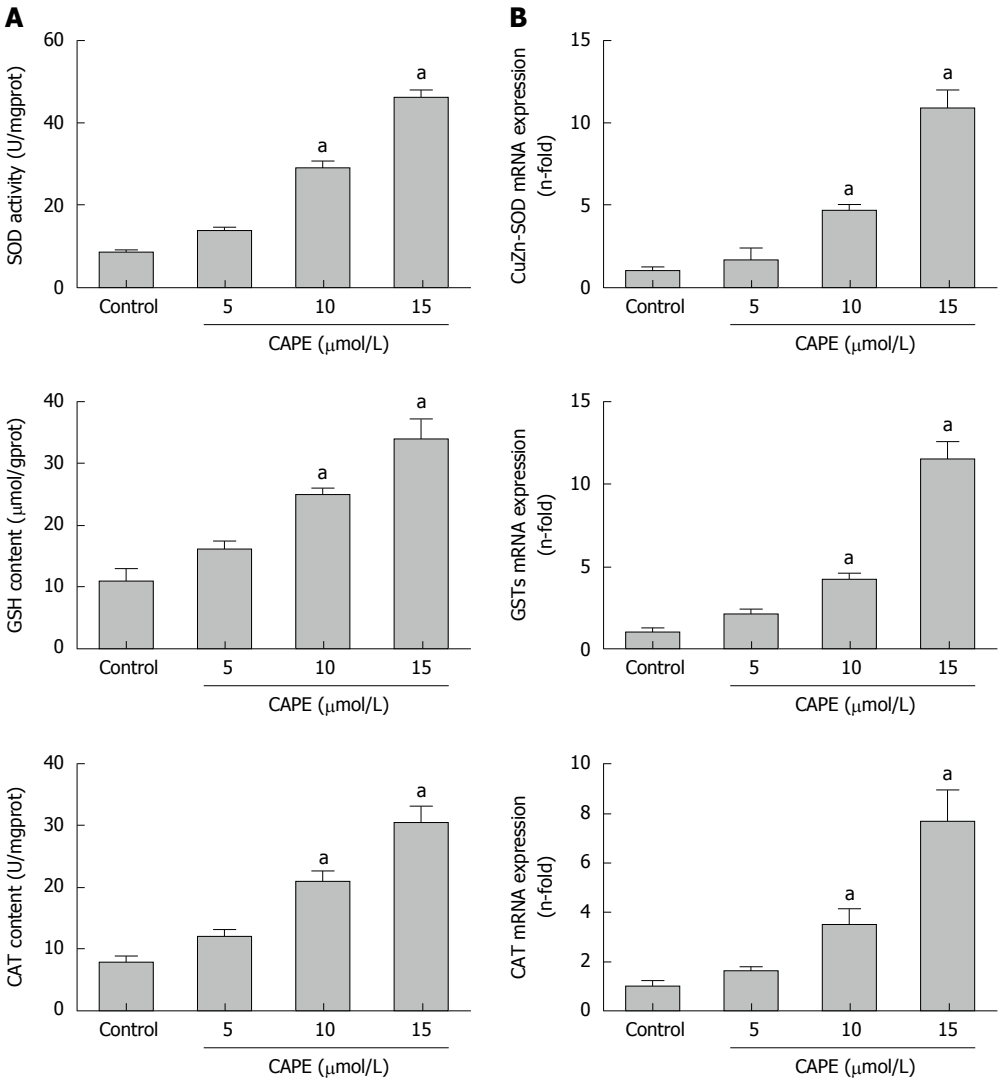



**Figure 3 Antioxidant-related indicator protein and mRNA expression in hepatic stellate cell-T6 cells.** After HSC-T6 cells were treated with 5 μmol/L, 10 μmol/L and 15 μmol/L CAPE for 24 h, the SOD activity, GSH and CAT content (A) and the mRNA expression of SOD, GSTs and CAT (B) were assessed. The data represent averages of the results of three independent experiments. [a]*P* < 0.05 *vs* control. CAPE: Caffeic acid phenethyl ester; SOD: Superoxide dismutase; CAT: Catalase; GST: Glutathione-S-transferase.

and JNK1/2 were significantly increased in a dose-dependent manner in HSC-T6 cells after treatment with CAPE (5, 10 and 15 μmol/L) for 24 h compared to the control group ($P < 0.05$, Figure 5A). To further investigate the connection between Nrf2 and MAPKs pathways in HSC-T6 cells, HSC-T6 cells were pre-treated with the ERK1/2 inhibitor PD98059, the p38MAPK inhibitor SB203580, and the JNK1/2 inhibitor SP600125 for 2 h, followed by treatment with CAPE (15 μmol/L) for 24 h. All of the inhibitors partially decreased Nrf2 mRNA expression and nuclear/cytosol protein levels in HSC-T6 cells ($P < 0.05$, Figure 5B), suggesting that inhibition of MAPKs suppresses the translocation of Nrf2 protein in HSC-T6 cells.

### *Inhibitors of MAPKs and CAPE alter antioxidant-related indicator protein and mRNA expression in HSC-T6 cells*

Gene and protein expression of SOD, CAT and GST was significantly decreased in HSC-T6 cells after incubation with the inhibitors of MAPKs and 15 μmol/L CAPE compared to the control group ($P < 0.05$, Figure 6A and B).

## DISCUSSION

Previous studies have confirmed that CAPE has active biological antioxidant and anti-fibrosis properties[5,6,19,20]. Considering the fact that HSC proliferation and activation are key during liver fibrosis[21-23], it is interesting to know whether CAPE treatment can influence HSC proliferation and activation. Unfortunately, only one study has shown that CAPE inhibited HSC cell proliferation *in vitro*[24]. In this study, our data showed that the proliferation and activation of HSC-T6 cells were significantly inhibited and apoptosis was induced by CAPE in a dose-dependent manner. In addition, the expression of α-SMA and collegen-1 proteins was significantly reduced in HSC-T6 cells in response to CAPE. Taken together, these results suggested that CAPE inhibited

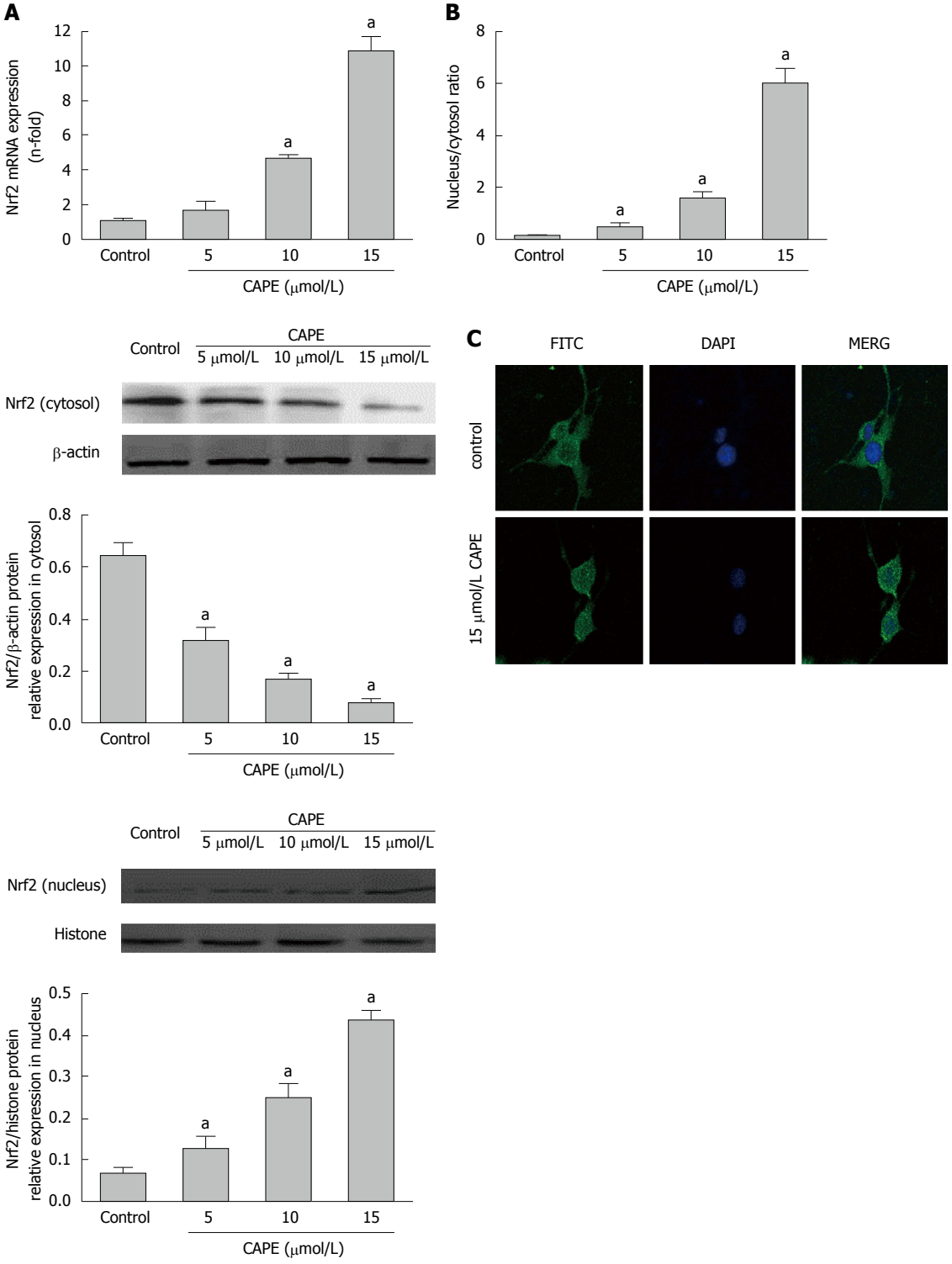



**Figure 4 Effect of caffeic acid phenethyl ester on Nrf2 expression in hepatic stellate cell-T6 cells.** A: HSC-T6 cells were treated with 5 μmol/L, 10 μmol/L and 15 μmol/L CAPE for 24 h. Nrf2 mRNA and cytosol and nuclear protein expression levels were investigated using real-time PCR and Western blot, respectively; B: The nucleus/cytosol ratio defines Nrf2 protein expression in the nucleus/Nrf2 protein expression in cytosol; C: Indirect immunofluorescence (× 200) analysis of Nrf2 protein expression in HSC-T6 cells. The upper: control group and the lower: 15 μmol/L CAPE group. The data presented are the mean ± SD ($n$ = 3). $^{a}P < 0.05$ *vs* control. CAPE: Caffeic acid phenethyl ester.

HSC-T6 cell proliferation and activation, which is one of the key events initiating the occurrence and development of liver fibrosis.

CAPE has been confirmed to inhibit liver fibrosis in rats[8], in this study the results showed that CAPE increased the expression levels of SOD, CAT and GSH activities in HSC-T6 cells. Therefore, it is reasonable to suspect that CAPE may inhibit HSC cell proliferation and activation through its antioxidant effect. To confirm our hypothesis, the effect of CAPE on the expression of Nrf2, an upstream transcription factor regulating the expression of anti-oxidative stress factors, in HSC-T6

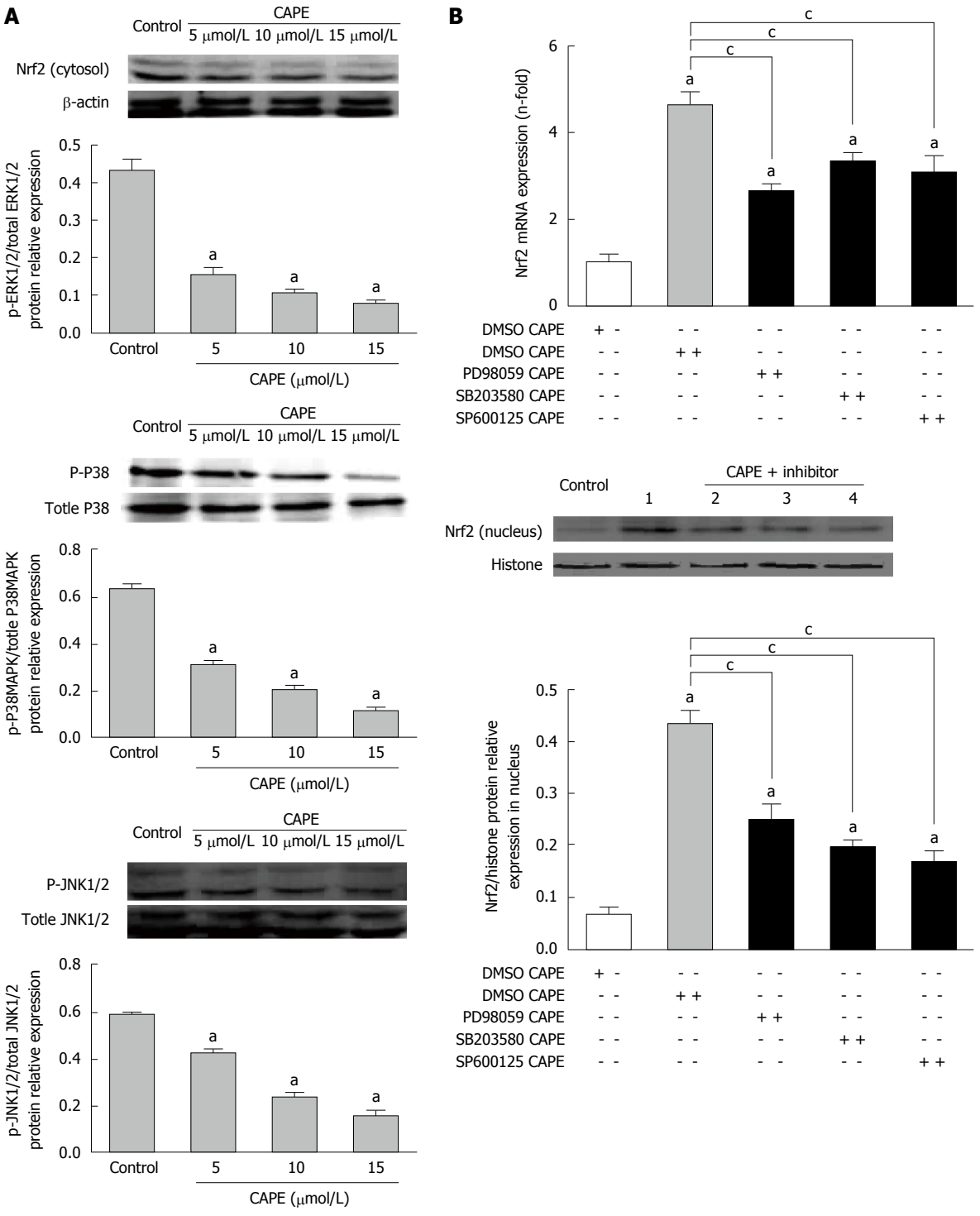



**Figure 5 Phosphorylation levels of ERK1/2, p38MARK, and JNK1/2 are significantly increased in a dose-dependent manner in hepatic stellate cell-T6 cells.** A: Effect of caffeic acid phenethyl ester on phosphorylation of ERK1/2, p38MAPK and JNK. Western blot analysis of total and phosphorylated protein levels of ERK1/2, p38MARK and JNK in HSC-T6 cells treated with CAPE (5 μmol/L, 10 μmol/L and 15 μmol/L) was performed. Total MAPKs were used as the internal control. The gray levels were normalized against those of the corresponding total MAPKs and the results are expressed, relative to control; B: Effect of inhibitors of MAPKs on the expression of Nrf2 in HSC-T6 cells. Cells were treated with ERK1/2 inhibitor PD98059 (30 μmol/L), p38 MAPK inhibitor SB203580 (20 μmol/L) or JNK inhibitor SP600125 (25 μmol/L) for 2 h, then incubated with CAPE (15 μmol/L) for 24 h, and protein expression was evaluated by Western blot. Lane 1: Control group; Lane 2: 15 μmol/L CAPE group; Lane 3: CAPE + PD98059 group; Lane 4: CAPE + SB203580 group; Lane 5: CAPE + SP600125 group. Histone was used as the internal control to reflect the expression of nuclear Nrf2. The gray levels were normalized against histone and the results are expressed, relative to control. The data are the mean ± SD of three independent experiments. $^{a}P < 0.05$ *vs* control; $^{c}P < 0.05$ *vs* CAPE group. CAPE: Caffeic acid phenethyl ester.

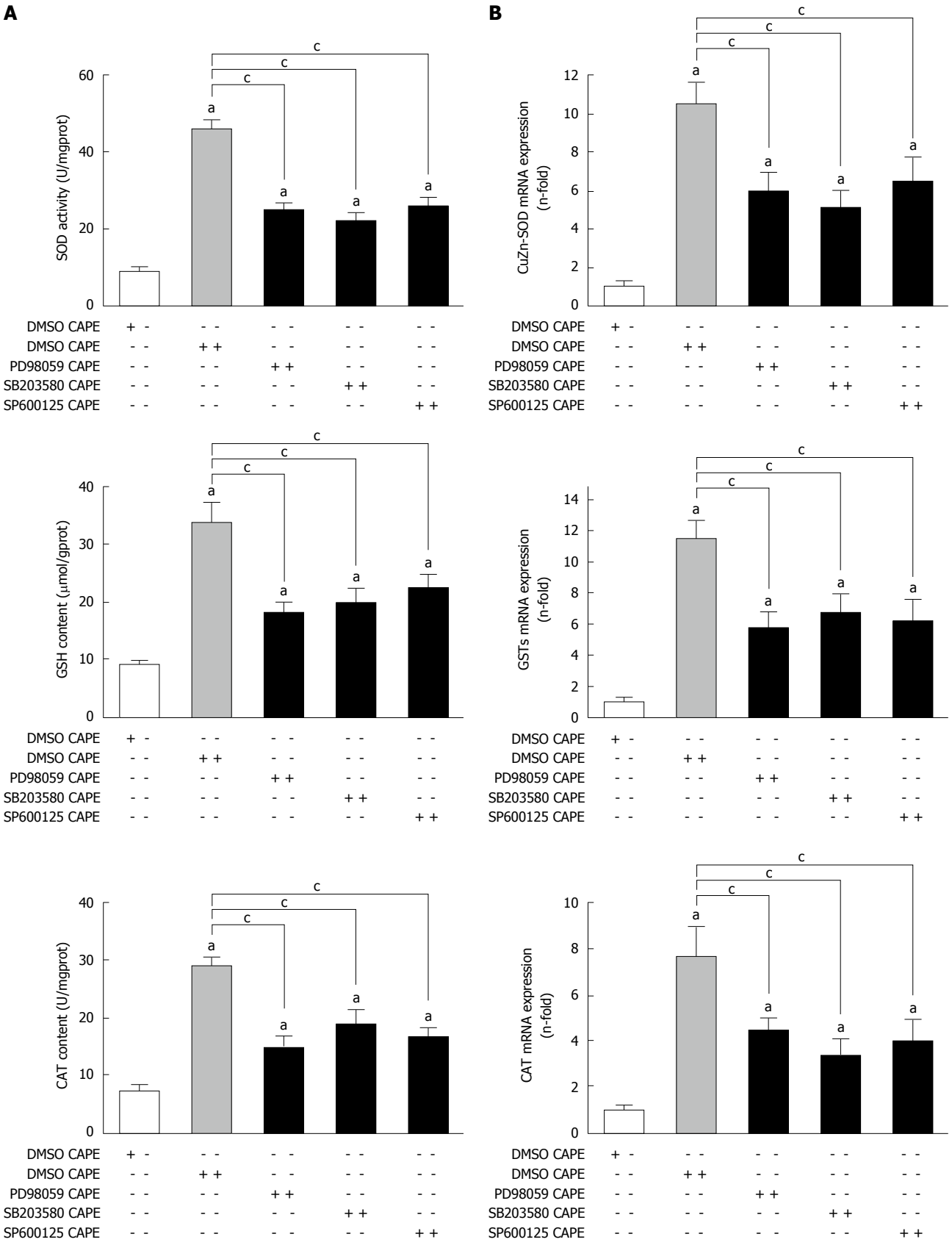



**Figure 6 Effect of inhibitors of mitogen activated protein kinases and caffeic acid phenethyl ester on the antioxidant-related indicator protein and mRNA expression.** A: Effect of inhibitors of MAPKs and CAPE on the SOD activity and GSH and CAT content in HSC-T6 cells. Cells were treated with the ERK1/2 inhibitor PD98059 (30 μmol/L), p38MAPK inhibitor SB203580 (20 μmol/L) or JNK inhibitor SP600125 (25 μmol/L) for 2 h, then incubated with CAPE (15 μmol/L) for 24 h; B: Effect of inhibitors of MAPKs and CAPE on antioxidant-related mRNA expression in HSC-T6 cells. The data represent averages of the results of three independent experiments. $^{a}P < 0.05$ *vs* control; $^{c}P < 0.05$ *vs* CAPE group. CAPE: Caffeic acid phenethyl ester; SOD: Superoxide dismutase; CAT: Catalase; GST: Glutathione-S-transferase.

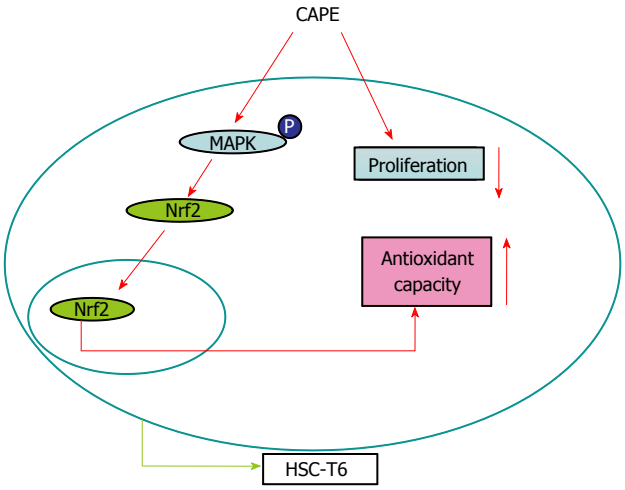



**Figure 7 Nrf2-mediated mitogen activated protein kinases signaling pathway contributes to antioxidant capacity of caffeic acid phenethyl ester in hepatic stellate cell-T6 cells.**

cells was investigated. Interestingly, we found that CAPE significantly increased *Nrf2* gene expression in HSC-T6 cells. Moreover, CAPE treatment significantly promoted Nrf2 protein translocation from the cytosol to the nucleus in HSC-T6 cells. These results indicated that CAPE promotes the synthesis and activation of Nrf2 in HSC cells.

The precise mechanism by which CAPE regulates Nrf2 expression and activation in HSC cells is still unknown. However, previous studies have shown that MAPKs, including ERK, JNK, and p38, play a role in regulating Nrf2 expression[25-30]. Therefore, we investigated the effects of CAPE on MAPKs signaling pathways in HSC-T6 cells. Our study showed that the phosphorylation levels of ERK1/2, p38MARK and JNK1/2 were significantly increased in a dose-dependent manner in HSC-T6 cells after treatment with CAPE. Meanwhile, pretreatment of HSC-T6 cells with inhibitors significantly attenuated antioxidant enzyme expression. Moreover, all of the inhibitors partially decreased Nrf2 mRNA expression and nuclear/cytosol protein levels in HSC-T6 cells, suggesting that inhibition of MAPKs suppresses the translocation of Nrf2 protein in HSC-T6 cells. Taken together, our results are the first report that MAPKs are the key signaling pathways involved in the CAPE-induced anti-oxidative responses in cultured HSC-T6 cells.

In conclusion, we demonstrated that CAPE inhibited cell proliferation, induced cell apoptosis, increased expression levels of SOD, CAT and GSH, and decreased α-SMA expression level in HSC-T6 cells by activation of the Nrf2-mediated MAPKs signaling pathways. We propose a working model of the antioxidative role and potential mechanism of CAPE in HSC-T6 cells (Figure 7). In this model, CAPE inhibits cell proliferation and activation and increases the expression levels of antioxidative enzymes in HSC-T6 cells, which might partly depend on an Nrf2-mediated MAPKs signaling pathway, and thereby is a potential anti-fibrosis agent for the treatment of human liver fibrosis.

## COMMENTS

### Background

Liver fibrosis is a pathological response to hepatocyte injury, including oxidative stress, which is a primary mechanism of liver damage. Oxidative stress can stimulate the activation of hepatic stellate cells (HSCs) through paracrine and autocrine, leading to the formation of liver fibrosis. Antioxidants play a pivotal role in the development of liver fibrosis. Caffeic acid phenethyl ester (CAPE) is a phenolic compound extracted from honeybee propolis that has strong biological properties in liver protection and as an antioxidant and anti-fibrosis agent. However, it is still largely unknown whether CAPE can reduce the expression of these factors *via* the Nrf2-mediated mitogen activated protein kinases (MAPKs) signaling pathway in HSC.

### Research frontiers

Previous studies proved that CAPE has active biological antioxidant and anti-fibrosis properties, also their previous studies have shown that CAPE inhibited liver fibrosis in rats.

### Innovations and breakthroughs

In this study, the authors demonstrated that CAPE inhibited cell proliferation, induced cell apoptosis, increased expression levels of SOD, CAT and GSH, and decreased α-SMA expression level in HSC-T6 cells by activation of the Nrf2-mediated MAPKs signaling pathways. They propose a working model of the anti-oxidative role and potential mechanism of CAPE in HSC-T6 cells.

### Applications

CAPE is a phenolic compound extracted from honeybee propolis that has strong biological properties in liver protection and as an antioxidant and anti-fibrosis agent. It has been used in the treatment of several diseases. These data also indicate that CAPE can up-regulate the antioxidant levels in HSC-T6 cells partly through the Nrf2-MAPKs signaling pathway and thereby is a potential anti-fibrosis agent for the treatment of human liver fibrosis in the future.

### Terminology

MAPK: a type of protein kinase that is specific to the amino acids serine, threonine, and tyrosine (*i.e.*, a serine/threonine-specific protein kinase). MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, heat shock, and proinflammatory cytokines. They regulate cell functions including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis. CAPE: a phenolic compound extracted from honeybee propolis.

### Peer-review

This is an interesting study showing that caffeic acid phenethyl ester up-regulates antioxidant levels in the hepatic stellate cell line. In this study, the authors investigated the antioxidation effect of caffeic acid phenethyl ester in hepatic stellate cells T6 cultured *in vitro* and the potential mechanisms of the effect. The effect on a-smooth muscle actin was shown using immunofluorescence. Gene and protein levels of Nrf2, including related factors and mitogen activated protein kinases, in HSC-T6 cells were detected using RT-PCR and Western blot, respectively. CAPE inhibited the proliferation and activation of HSC-T6 cells cultured in vitro. CAPE increased the antioxidant levels and the translocation of Nrf2 from the cytoplasm to the nucleus in HSC-T6 cells. Moreover, the phosphorylation of MAPKs in cells decreased in response to CAPE. Interestingly, CAPE-induced oxidative stress in the cells was significantly attenuated by pretreatment with MAPKs inhibitors. Overall, this study is well designed, and the results are interesting.

## REFERENCES

1 **Mormone E**, George J, Nieto N. Molecular pathogenesis of hepatic fibrosis and current therapeutic approaches. *Chem Biol*

*Interact* 2011; **193**: 225-231 [PMID: 21803030 DOI: 10.1016/j.cbi.2011.07.001]
2 **Bansal R**, Nagórniewicz B, Prakash J. Clinical Advancements in the Targeted Therapies against Liver Fibrosis. *Mediators Inflamm* 2016; **2016**: 7629724 [PMID: 27999454]
3 **Vogel S**, Piantedosi R, Frank J, Lalazar A, Rockey DC, Friedman SL, Blaner WS. An immortalized rat liver stellate cell line (HSC-T6): a new cell model for the study of retinoid metabolism in vitro. *J Lipid Res* 2000; **41**: 882-893 [PMID: 10828080]
4 **He L**, Li Z, Zhou D, Ding Y, Xu L, Chen Y, Fan J. Galanin receptor 2 mediates antifibrogenic effects of galanin on hepatic stellate cells. *Exp Ther Med* 2016; **12**: 3375-3380 [PMID: 27882166 DOI: 10.3892/etm.2016.3764]
5 **Márquez N**, Sancho R, Macho A, Calzado MA, Fiebich BL, Muñoz E. Caffeic acid phenethyl ester inhibits T-cell activation by targeting both nuclear factor of activated T-cells and NF-kappaB transcription factors. *J Pharmacol Exp Ther* 2004; **308**: 993-1001 [PMID: 14617683 DOI: 10.1124/jpet.103.060673]
6 **Altuğ ME**, Serarslan Y, Bal R, Kontaş T, Ekici F, Melek IM, Aslan H, Duman T. Caffeic acid phenethyl ester protects rabbit brains against permanent focal ischemia by antioxidant action: a biochemical and planimetric study. *Brain Res* 2008; **1201**: 135-142 [PMID: 18308295 DOI: 10.1016/j.brainres]
7 **Shi Y**, Guo L, Shi L, Yu J, Song M, Li Y. Caffeic Acid Phenethyl Ester inhibit Hepatic Fibrosis by Nitric Oxide Synthase and Cystathionine Gamma-Lyase in Rats. *Med Sci Monit* 2015; **21**: 2774-2780 [PMID: 26378818 DOI: 10.12659/MSM.895272]
8 **Li M**, Wang XF, Shi JJ, Li YP, Yang N, Zhai S, Dang SS. Caffeic acid phenethyl ester inhibits liver fibrosis in rats. *World J Gastroenterol* 2015; **21**: 3893-3903 [PMID: 25852274 DOI: 10.3748/wjg.v21.i13.3893]
9 **Ho YS**, Xiong Y, Ma W, Spector A, Ho DS. Mice lacking catalase develop normally but show differential sensitivity to oxidant tissue injury. *J Biol Chem* 2004; **279**: 32804-32812 [PMID: 15178682 DOI: 10.1074/jbc.M404800200]
10 **Eggler AL**, Gay KA, Mesecar AD. Molecular mechanisms of natural products in chemoprevention: induction of cytoprotective enzymes by Nrf2. *Mol Nutr Food Res* 2008; **52** Suppl 1: S84-S94 [PMID: 18435489 DOI: 10.1002/mnfr.200700249]
11 **Edeas M**, Attaf D, Mailfert AS, Nasu M, Joubet R. Maillard reaction, mitochondria and oxidative stress: potential role of antioxidants. *Pathol Biol* (Paris) 2010; **58**: 220-225 [PMID: 20031340 DOI: 10.1016/j.patbio.2009.09.011]
12 **Kim J**, Cha YN, Surh YJ. A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders. *Mutat Res* 2010; **690**: 12-23 [PMID: 19799917 DOI: 10.1016/j.mrfmmm.2009.09.007]
13 **Jain AK**, Bloom DA, Jaiswal AK. Nuclear import and export signals in control of Nrf2. *J Biol Chem* 2005; **280**: 29158-29168 [PMID: 15901726 DOI: 10.1074/jbc]
14 **Shi JJ**, Jia XL, Li M, Yang N, Li YP, Zhang X, Gao N, Dang SS. Guggulsterone induces apoptosis of human hepatocellular carcinoma cells through intrinsic mitochondrial pathway. *World J Gastroenterol* 2015; **21**: 13277-13287 [PMID: 26715810 DOI: 10.3748/wjg.v21.i47.13277]
15 **Sun MZ**, Dang SS, Wang WJ, Jia XL, Zhai S, Zhang X, Li M, Li YP, Xun M. Cytokeratin 8 is increased in hepatitis C virus cells and its ectopic expression induces apoptosis of SMMC7721 cells. *World J Gastroenterol* 2013; **19**: 6178-6187 [PMID: 24115814 DOI: 10.3748/wjg.v19.i37.6178]
16 **Dang SS**, Sun MZ, Yang E, Xun M, Ma L, Jia ZS, Wang WJ, Jia XL. Prohibitin is overexpressed in Huh-7-HCV and Huh-7.5-HCV cells harboring in vitro transcribed full-length hepatitis C virus RNA. *Virol J* 2011; **8**: 424 [PMID: 21896168 DOI: 10.1186/1743-422X-8-424]
17 **Schmittgen TD**, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008; **3**: 1101-1108 [PMID: 18546601]
18 **Bradford MM**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248-254 [PMID: 942051]
19 **Zhao WX**, Zhao J, Liang CL, Zhao B, Pang RQ, Pan XH. Effect of caffeic acid phenethyl ester on proliferation and apoptosis of hepatic stellate cells in vitro. *World J Gastroenterol* 2003; **9**: 1278-1281 [PMID: 12800240 DOI: 10.3748/wjg.v9.i6.1278]
20 **Jia CH**, Wang XY, Qi JF, Hong ST, Lee KT. Antioxidant Properties of Caffeic acid Phenethyl Ester and 4-Vinylcatechol in Stripped Soybean Oil. *J Food Sci* 2016; **81**: C35-C41 [PMID: 26641978 DOI: 10.1111/1750-3841.13160]
21 **Rockey DC**. The cell and molecular biology of hepatic fibrogenesis. Clinical and therapeutic implications. *Clin Liver Dis* 2000; **4**: 319-355 [PMID: 11232195 DOI: 10.1016/S1089-3261(05)]
22 **Fagone P**, Mangano K, Mammana S, Pesce A, Pesce A, Caltabiano R, Giorlandino A, Portale TR, Cavalli E, Lombardo GA, Coco M, Puleo S, Nicoletti F. Identification of novel targets for the diagnosis and treatment of liver fibrosis. *Int J Mol Med* 2015; **36**: 747-752 [PMID: 26135677]
23 **Romanelli RG**, Stasi C. Recent Advancements in Diagnosis and Therapy of Liver Cirrhosis. *Curr Drug Targets* 2016; **17**: 1804-1817 [PMID: 27296314 DOI: 10.2174/1389450117666160613101413]
24 **Roos TU**, Heiss EH, Schwaiberger AV, Schachner D, Sroka IM, Oberan T, Vollmar AM, Dirsch VM. Caffeic acid phenethyl ester inhibits PDGF-induced proliferation of vascular smooth muscle cells via activation of p38 MAPK, HIF-1α, and heme oxygenase-1. *J Nat Prod* 2011; **74**: 352-356 [PMID: 21265554 DOI: 10.1021/np100724f]
25 **Kang ES**, Woo IS, Kim HJ, Eun SY, Paek KS, Kim HJ, Chang KC, Lee JH, Lee HT, Kim JH, Nishinaka T, Yabe-Nishimura C, Seo HG. Up-regulation of aldose reductase expression mediated by phosphatidylinositol 3-kinase/Akt and Nrf2 is involved in the protective effect of curcumin against oxidative damage. *Free Radic Biol Med* 2007; **43**: 535-545 [PMID: 17640564 DOI: 10.1016/j.freeradbiomed.2007.05.006]
26 **Lubet RA**, Yao R, Grubbs CJ, You M, Wang Y. Induced expression of drug metabolizing enzymes by preventive agents: role of the antioxidant response element. *Chem Biol Interact* 2009; **182**: 22-28 [PMID: 19695238 DOI: 10.1016/j.cbi.2009.08.011]
27 **Chen CY**, Jang JH, Li MH, Surh YJ. Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells. *Biochem Biophys Res Commun* 2005; **331**: 993-1000 [PMID: 15882976]
28 **Hyung JH**, Ahn CB, Il Kim B, Kim K, Je JY. Involvement of Nrf2-mediated heme oxygenase-1 expression in anti-inflammatory action of chitosan oligosaccharides through MAPK activation in murine macrophages. *Eur J Pharmacol* 2016; **793**: 43-48 [PMID: 27826077 DOI: 10.1016/j.ejphar]
29 **Kadam PD**, Chuan HH. Erratum to: Rectocutaneous fistula with transmigration of the suture: a rare delayed complication of vault fixation with the sacrospinous ligament. *Int Urogynecol J* 2016; **27**: 505 [PMID: 26811110]
30 **Ahn CB**, Je JY, Kim YS, Park SJ, Kim BI. Induction of Nrf2-mediated phase II detoxifying/antioxidant enzymes in vitro by chitosan-caffeic acid against hydrogen peroxide-induced hepatotoxicity through JNK/ERK pathway. *Mol Cell Biochem* 2017; **424**: 79-86 [PMID: 27743232 DOI: 10.1007/s11010-016-2845-4]

**P- Reviewer**: Arredondo M, Kesavadevi J **S- Editor**: Qi Y
**L- Editor**: Wang TQ **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/ *World J Gastroenterol* 2017 February 21; 23(7): 1215-1223

DOI: 10.3748/wjg.v23.i7.1215 ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

# Surgery for gastric cancer patients of age 85 and older: Multicenter survey

Hirotaka Konishi, Daisuke Ichikawa, Hiroshi Itoh, Kenichiro Fukuda, Naoki Kakihara, Manabu Takemura, Kaori Okugawa, Kiyoshi Uchiyama, Masashi Nakata, Hiroshi Nishi, Toshiyuki Kosuga, Shuhei Komatsu, Kazuma Okamoto, Eigo Otsuji

Hirotaka Konishi, Daisuke Ichikawa, Toshiyuki Kosuga, Shuhei Komatsu, Kazuma Okamoto, Eigo Otsuji, Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan

Hiroshi Itoh, Department of Surgery, North Medical Center, Kyoto Prefectural University of Medicine, Yosa-gun, Kyoto 629-2261, Japan

Kenichiro Fukuda, Department of Surgery, Saiseikai Shigaken Hospital, Ritto, Shiga 520-3046, Japan

Naoki Kakihara, Department of Surgery, Japanese Red Cross Kyoto Daini Hospital, Kamigyo-ku, Kyoto 602-8026, Japan

Manabu Takemura, Department of Surgery, Osaka General Hospital of West Japan Railway Company, Abeno-ku, Osaka 545-0053, Japan

Kaori Okugawa, Department of Surgery, Omihachiman Community Medical Center, Omihachiman, Shiga 523-0082, Japan

Kiyoshi Uchiyama, Department of Surgery, Kyoto Okamoto Memorial Hospital, Kumiyama-cho, Kyoto 613-0034, Japan

Masashi Nakata, Department of Surgery, Kyoto Yamashiro General Medical Center, Kizugawa, Kyoto 619-0214, Japan

Hiroshi Nishi, Department of Surgery, Saiseikai Kyoto Hospital, Nagaokakyo, Kyoto 617-0814, Japan

**Author contributions:** Konishi H and Itoh H contributed equally to this study; Konishi H, Ichikawa D, Itoh H and Otsuji E designed the research; Konishi H, Ichikawa D, Itoh H, Fukuda K, Kakihara N, Takemura M, Okugawa K, Uchiyama K, Nakata M, Nishi H, Kosuga T, Komatsu S and Okamoto K treated the patients and collected the data; Konishi H, Ichikawa D and Itoh H analyzed the data; Konishi H, Ichikawa D, Itoh H and Otsuji E wrote the manuscript.

**Institutional review board statement:** The study was reviewed and approved by the Faculty of Science Ethics Committee at Kyoto Prefectural University of Medicine (RBMR-C-179).

**Informed consent statement:** All study participants provided informed written consent prior to their treatments and study enrollment.

**Conflict-of-interest statement:** All authors declare no conflict of interest related to this study or its publication.

**Data sharing statement:** The technical appendix, statistical code and dataset are available from Hirotaka Konishi at h-koni7@koto.kpu-m.ac.jp.



**Manuscript source:** Unsolicited manuscript

**Correspondence to: Daisuke Ichikawa, MD, PhD, Assistant Professor,** Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, Kajii-cho 465, Kamigyo-ku, Kyoto 602-8566, Japan. ichikawa@koto.kpu-m.ac.jp
**Telephone:** +81-75-2515527
**Fax:** +81-75-2515522

**Received:** October 19, 2016
**Peer-review started:** October 20, 2016
**First decision:** November 9, 2016
**Revised:** November 26, 2016
**Accepted:** January 11, 2017
**Article in press:** January 11, 2017
**Published online:** February 21, 2017

## Abstract

***AIM***

To investigate the surgical therapies for gastric cancer (GC) patients of age 85 or older in a multicenter survey.

***METHODS***

Therapeutic opportunities for elderly GC patients have expanded in conjunction with extended life expectancy. However, the number of cases encountered in a single institution is usually very small and surgical therapies for elderly GC patients have not yet been standardized completely. In the present study, a total of 134 GC patients of age 85 or older who underwent surgery in 9 related facilities were retrospectively investigated. The relationships between surgical therapies and clinicopathological or prognostic features were analyzed.

***RESULTS***

Eighty-nine of the patients (66%) presented with a comorbidity, and 26 (19% overall) presented with more than two comorbidities. Radical lymphadenectomy was performed in 59 patients (44%), and no patient received pre- or post-operative chemotherapy. Forty of the patients (30%) experienced perioperative complications, but no surgical or perioperative mortality occurred. Laparoscopic surgery was performed in only 12 of the patients (9.0%). Univariate and multivariate analyses of the 113 patients who underwent R0 or R1 resection identified the factors of pT3/4 and limited lymphadenectomy as predictive of worse prognosis (HR = 4.68, $P$ = 0.02 and HR =2.19, $P$ = 0.05, respectively). Non-cancer-specific death was more common in cStage Ⅰ patients than in cStage Ⅱ or Ⅲ patients. Limited lymphadenectomy correlated with worse cancer-specific survival ($P$ = 0.01), particularly in cStage Ⅱ patients ($P$ < 0.01). There were no relationships between limited lymphadenectomy and any comorbidities, except for cerebrovascular disease ($P$ = 0.07).

***CONCLUSION***

Non-cancer-specific death was not negligible, particularly in cStage Ⅰ, and gastrectomy with radical lymphadenectomy appears to be an effective treatment for cStage Ⅱ elderly GC patients.


**Key words:** Gastric cancer; Elderly more than 85; Surgery; Limited lymphadenectomy; Multicenter survey



**Core tip:** Therapeutic opportunities for elderly gastric cancer (GC) patients have expanded. This multicenter study investigated surgical therapies for GC patients of age 85 or older. Cancer-specific and overall survival rates were 100% and 56% in cStage Ⅰ. The factors of pT3/4 and limited lymphadenectomy were predictive of worse prognosis. Cancer-specific survival in cStage Ⅱ with radical lymphadenectomy was significantly better, but did not significantly benefit cStage Ⅲ. Only cerebrovascular disease was related with limited lymphadenectomy. Non-cancer-specific death was not negligible, particularly in cStage Ⅰ, and gastrectomy with radical lymphadenectomy appeared to be an effective treatment for cStage Ⅱ elderly patients.

Konishi H, Ichikawa D, Itoh H, Fukuda K, Kakihara N, Takemura M, Okugawa K, Uchiyama K, Nakata M, Nishi H, Kosuga T, Komatsu S, Okamoto K, Otsuji E. Surgery for gastric cancer patients of age 85 and older: Multicenter survey. *World J Gastroenterol* 2017; 23(7): 1215-1223 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1215.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1215

## INTRODUCTION

The elderly population is increasing worldwide, and life expectancy has also consistently increased in most countries[1]. In Japan, the average lifetime of women is 87 years while that of men is 81 years, and the life expectancies of 85-year-old women and men are 8.4 and 6.2 years, respectively[2]. Therefore, gastric cancer (GC) patients of age 85 or older may undergo radical gastrectomy with the aim of achieving 5-year survival. On the other hand, elderly patients may present with existing functional decline in major organs and comorbidities[1,3]. The rate of non-cancer-specific death in elderly patients has generally been increasing, and post-operative disorders following gastrectomy may indirectly influence the cause of death[4-6]. Therefore, the decision to perform surgery on elderly GC patients needs to be made carefully.

Standard therapeutic strategies for GC patients in Japan are selected according to the Japanese Gastric Cancer Treatment Guidelines[7]; however, these guidelines are not standardized for elderly GC patients, particularly those aged 85 or older. Although some retrospective studies of GC patients in single institutions have evaluated gastrectomy for its feasibility and safety among the elderly patient population[8-10], few have investigated patients of age 85 or older[11,12].

We previously reported the surgical outcomes of gastrectomy for elderly GC patients and concluded that the outcomes were similar to those in non-elderly patients[13]. However, it remains unclear as to whether radical gastrectomy has prognostic significance in elderly GC patients. There are some limitations in the selection of therapeutic strategies for elderly GC patients, due to the small number of cases that a single institution usually encounters. Therefore, we collected data on GC patients of age 85 or older who underwent surgery in our related hospitals, and

herein report our findings on the surgical therapies, clinicopathological features and survival.

## MATERIALS AND METHODS

### Patients

A total of 134 GC patients of age 85 or older who underwent surgery in any of our related hospitals (9 facilities) between 2000 and 2014 were retrospectively registered. Thirty-six patients were treated at hospital 1, 21 at hospital 2, 20 at hospital 3, 12 at hospital 4, 11 at hospital 5, 9 at hospital 6, 10 at hospital 7, 4 at hospital 8, and 11 at hospital 9. Clinical and pathological stages were determined based on the Japanese Classification of Gastric Carcinoma 3rd edition[14]. No patients in the present study received neo-adjuvant or adjuvant chemotherapy. Some clinicopathological and prognostic data were not usable, as they were outdated. The median length of follow-up for censored cases was 19.5 mo (range: 1-88 mo).

Written informed consent for surgery was obtained from all patients in each institution; however, it was confirmed that written informed consent for participation in the present study was not always necessary because this was a retrospective non-interventional study.

### Surgical therapy

Standard operability for each case was decided according to the Japanese Gastric Cancer Treatment Guidelines[7]. The operative procedure and extent of resection or lymphadenectomy were ultimately selected by each institution based on the clinical stage and location of the cancer. The extent of lymphadenectomy was re-evaluated using data obtained from the dissected lymph nodes and pre-operative clinical staging that was based on the Japanese Classification of Gastric Carcinoma 3rd edition[14]; briefly, radical lymphadenectomy was adapted to cT1N0 patients who underwent D1 or more extended lymphadenectomy and to cN+ or cT2-4 patients who underwent D2 lymphadenectomy. In the present study, splenectomy in total gastrectomy was not related to the extent of lymphadenectomy because significance of splenectomy due to No.10 or 11d lymph node dissection was controversial.

The status of residual tumors after surgery was also described as the R status, according to the Japanese Classification of Gastric Carcinoma 3rd edition[14]. R0 denoted curative resection, R1 denoted resection with a microscopic residual tumor (positive in the resection margin, or CY+), and R2 denoted resection with a macroscopic residual tumor. In the present study, patients with bypass or un-resected surgery were included among the R2 cases.

### Statistical analysis

All statistical analyses were performed using StatView 5.0 J software (SAS Institute Inc., Cary, NC, United States). Survival curves for overall survival, cancer-specific survival and non-original disease-specific death were derived using the Kaplan-Meier method and compared by the stratified Log-rank test. A multivariate survival analysis was performed using Cox's proportional hazard regression model. A *P*-value less than 0.05 was considered significant.

**Table 1 Patient characteristics and perisurgical outcomes *n* (%)**

| | Total patients (*n* = 134) |
|---|---|
| Age (yr) | 87.4 (85-97) |
| Sex (male/female) | 77/57 |
| Post-operative hospital stay (d) | 29 (9-305) |
| Comorbidity | 89 (66.0) |
| Hypertension | 52 (39.0) |
| Cardiovascular disease | 36 (27) |
| Respiratory disease | 13 (9.7) |
| Cerebrovascular disease | 9 (6.7) |
| Diabetes mellitus | 13 (9.7) |
| Renal dysfunction | 4 (3.0) |
| Other | 4 (3.0) |
| Number of comorbidities | |
| 0 | 45 |
| 1/2 | 63/18 |
| 3/4 | 6/2 |
| Operation | |
| Distal | 84 |
| Proximal | 5 |
| Total | 34 |
| Partial | 4 |
| Bypass | 4 |
| Unresectable | 3 |
| Procedure | |
| Open | 122 |
| Laparoscopy | 12 |
| Lymphadenectomy | |
| D0/D1 | 17/52 |
| D1$^+$/D2 | 40/25 |
| Lymphadenectomy | |
| Radical/limited | 59/75 |
| Number of resected LN | 23 (0-67) |
| Operative time (min) | 206 (62-426) |
| Bleeding (g) | 263 (10-1855) |
| Residual tumor | |
| R0/1/2 | 103/10/21 |

LN: Lymph node.

## RESULTS

### Patient characteristics

The characteristics and perisurgical outcomes of the total 134 patients analyzed in this study are shown in Table 1. All patients were diagnosed with adenocarcinoma, and 57 patients (43%) were female. Comorbidities were present in 89 of the patients (66%), and 26 of the patients (19%) had more than one comorbidity. Hypertension was the most frequent comorbidity (52/134, 39%), followed by cardiovascular disease (36/134, 27%), respiratory disease (13/134, 10%) and diabetes mellitus (13/134, 10%). The mean post-operative hospital stay was 29 d (range: 9-305 d),

**Table 2 Pathological features and complications *n* (%)**

| | Total patients, *n* = 134 | |
|---|---|---|
| Location | L/M/U | 59/51/24 |
| Macroscopic type | 0/1/2/3/4/5/unknown | 35/8/35/25/10/3/18 |
| cT | 1-2/3-4 | 64/70 |
| cN | -/+ | 70/64 |
| cStage | Ⅰ/Ⅱ/Ⅲ/Ⅳ | 54/36/32/12 |
| pT | 1-2/3-4 | 62/72 |
| pN | -/+/unknown | 59/68/7 |
| pStage | Ⅰ/Ⅱ/Ⅲ/Ⅳ | 49/29/39/17 |
| Differentiation | Well/moderate/poor/unknown | 46/17/55/16 |
| Lymphatic invasion | -+/unknown | 38/79/17 |
| Venous invasion | -/+/unknown | 55/62/17 |
| Complications | -/+ | 94/40 |
| | Surgical site infection | 11 (8.2) |
| | Anastomotic leakage | 12 (9.0) |
| | Anastomotic stenosis | 4 (3.0) |
| | Pneumonia | 7 (5.2) |
| | Pancreatic fistula | 3 (2.2) |
| | Intestinal hypoperistalsis | 2 (1.5) |
| | Cardiac complication | 1 (0.7) |
| | Brain complication | 1 (0.7) |
| | Other | 2 (1.5) |

L/M/U: Lower third/middle third/upper third.

and no operative or perioperative mortalities occurred, excluding gastric cancer-specific death.

### *Therapeutic outcomes*

Distal gastrectomy was performed on 84 patients, proximal gastrectomy on 5, total gastrectomy on 34, and partial gastrectomy on 4 (Table 1). Neither pre- nor post-operative chemotherapy was performed on any patient. The original lesions were un-resectable in 7 patients, and bypass surgery was performed on 4 of these patients. Laparoscopic surgery was performed on only 12 patients (12/134, 9.0%), including 9 distal and 3 total gastrectomies. The rates of R0+1 resection, radical lymphadenectomy and post-operative complications in laparoscopic surgery were 100% (12/12), 75% (9/12) and 17% (2/12), respectively.

### *Clinicopathological features and complications*

Table 2 shows the clinicopathological features and post-operative complications, with the Clavien-Dindo classification of more than grade Ⅱ[15,16]. Complications were present in 40 of the total 134 patients (30%). Although the frequencies of anastomotic leakage (12/134, 9.0%) and pneumonia (7/134, 5.2%) were slightly high, no lethal complications or re-operations occurred.

### *Survival analysis in each stage*

Overall survival and cancer-specific survival in cStage Ⅰ/Ⅱ/Ⅲ patients are shown in Figure 1A. In cStage Ⅰ patients, the 5-year cancer-specific survival rate was 100%, whereas the overall survival rate was remarkably worse (at 56%) because of non-cancer-specific deaths. The cStage Ⅱ or Ⅲ patients showed similar overall and cancer-specific survival rates, again with the rate of cancer-specific deaths higher than that of non-cancer-specific deaths. Cancer-specific survival was significantly different in each pStage ($P < 0.01$; Figure 1B).

### *Relationships between survival and clinicopathological features*

Table 3 shows the relationships between survival and clinicopathological features for the 113 patients applicable for these analyses, after exclusion of those patients who underwent R2 resection. Limited lymphadenectomy ($P = 0.01$), cT3-4 ($P < 0.01$), pT3-4 ($P < 0.01$), pN+ ($P < 0.01$), pStage Ⅲ-Ⅵ ($P < 0.01$), and positive venous invasion ($P < 0.01$) were identified as worse prognostic factors in the univariate analysis, whereas only an advanced pT factor and limited lymphadenectomy appeared to be prognostic factors in the multivariate analysis (HR = 4.68, 95%CI: 1.29-20.7, $P = 0.02$ and HR = 2.19, 95%CI: 1.00-4.97, $P = 0.05$, respectively).

Cancer-specific survival in the patients who underwent radical lymphadenectomy was significantly better than in those who underwent limited lymphadenectomy ($P = 0.01$; Figure 2A). In a subgroup analysis, the cancer-specific survival in cStage Ⅱ patients who underwent radical lymphadenectomy was also significantly better than in those who underwent limited lymphadenectomy ($P < 0.01$; Figure 2B); however, the difference was not significant in the cStage Ⅲ patients ($P = 0.08$; Figure 2C).

### *Preoperative factors affecting limited lymphadenectomy*

Most comorbidities were not associated with limited lymphadenectomy, and only the presence of cerebrovascular disease was found to be associated ($P = 0.07$; Table 4).

## DISCUSSION

Life-span has been extended for the elderly by advances in medical treatments[1,2]. In conjunction, therapeutic opportunities for elderly cancer patients, including those with GC, have also increased[1]. Therapeutic strategies for GC in Japan are standardized by the Japanese Gastric Cancer Association Guidelines[7]; however, strategies for elderly patients are not clearly stated. An important issue is that systemic conditions and previous histories generally vary among elderly patients[3-6,17], and generally the number of elderly patients is still smaller than of young patients treated in a single Japanese institution. Therefore, comprehensive data on GC patients, particularly those aged 85 or older, have not been reported in detail[11,12,18-20].

In the present study, we retrospectively collected

**Table 3 Univariate and multivariate analyses for survival**

| | *n* = 113 | 5-yr survival | Univariate | Multivariate | | |
|---|---|---|---|---|---|---|
| | | | *P* value | HR | 95%CI | *P* value |
| Sex | | | | | | |
| Male | 65 | 33 | 0.17 | - | | |
| Female | 48 | 48 | | | | |
| Comorbidities | | | | | | |
| < 2 | 93 | 35 | 0.35 | - | | |
| ⩾ 2 | 20 | 63 | | | | |
| Operation | | | | | | |
| Total | 29 | 36 | 0.13 | - | | |
| Others | 84 | 41 | | | | |
| Procedure | | | | | | |
| Open | 101 | 37 | 0.10 | - | | |
| Laparoscopy | 12 | 62 | | | | |
| Lymphadenectomy | | | | | | |
| Radical | 59 | 43 | 0.01 | 1.00 | 1.00-4.97 | 0.05 |
| Limited | 54 | 35 | | 2.19 | | |
| Operative time (min) | | | | | | |
| < 240 | 76 | 32 | 0.34 | - | | |
| ⩾ 240 | 33 | 58 | | | | |
| Unknown | 4 | | | | | |
| Bleeding (g) | | | | | | |
| < 400 | 91 | 40 | 0.12 | - | | |
| ⩾ 400 | 18 | 27 | | | | |
| Unknown | 4 | | | | | |
| cT | | | | | | |
| 1-2 | 63 | 49 | < 0.01 | 1.16 | 0.41-3.23 | 0.78 |
| 3-4 | 50 | 27 | | 1.00 | | |
| cN | | | | | | |
| Absent | 67 | 45 | 0.43 | - | | |
| Present | 46 | 35 | | | | |
| cStage | | | | | | |
| Ⅰ-Ⅱ | 87 | 40 | 0.59 | - | | |
| Ⅲ-Ⅳ | 26 | 37 | | | | |
| pT | | | | | | |
| 1-2 | 62 | 58 | < 0.01 | 1.00 | 1.29-20.7 | 0.02 |
| 3-4 | 51 | 16 | | 4.68 | | |
| pN | | | | | | |
| Absent | 58 | 56 | < 0.01 | 1.00 | 0.68-5.62 | 0.24 |
| Present | 55 | 23 | | 1.84 | | |
| pStage | | | | | | |
| Ⅰ-Ⅱ | 78 | 55 | < 0.01 | - | | |
| Ⅲ-Ⅳ | 35 | 9 | | | | |
| Residual tumor | | | | | | |
| R0 | 103 | 41 | 0.06 | 1.14 | 0.39-3.87 | 0.81 |
| R1 | 10 | 31 | | 1.00 | | |
| Lymphatic invasion | | | | | | |
| Absent | 37 | 55 | 0.09 | 1.49 | 0.43-5.10 | 0.53 |
| Present | 67 | 29 | | 1.00 | | |
| Unknown | 9 | | | | | |
| Venous invasion | | | | | | |
| Absent | 53 | 58 | < 0.01 | 1.00 | 0.75-5.80 | 0.17 |
| Present | 51 | 15 | | 2.00 | | |
| Unknown | 9 | | | | | |
| Complications | | | | | | |
| Absent | 81 | 34 | 0.31 | - | | |
| Present | 32 | 51 | | | | |

information on GC patients of age 85 or older who underwent surgery in our related hospitals in order to investigate therapeutic strategies for elderly GC patients. We were unable to confirm information on the frequency of patients treated by non-surgical therapies or untreated due to their general condition. Therefore, although this set of data may be slightly biased due to regional characteristics or therapeutic strategies in each institution, the average frequency of GC patients aged 85 or older treated by surgery was almost similar among all the institutions (at 2.6%, range: 2.2%-3.8%).

There were some distinct characteristics noted for the treatment of elderly GC patients compared

**Table 4 Relationships between lymphadenectomy and clinicopathlogical features**

| | *n* = 113 | Lymphadenectomy | | *P* value |
|---|---|---|---|---|
| | | Radical | Limited | |
| Comorbidity | | | | |
| Present | 75 | 45 | 30 | 0.02 |
| Absent | 38 | 14 | 24 | |
| Hypertension | | | | |
| Present | 43 | 28 | 15 | 0.03 |
| Absent | 70 | 31 | 39 | |
| Cardiovascular disease | | | | |
| Present | 31 | 14 | 17 | 0.36 |
| Absent | 82 | 45 | 37 | |
| Respiratory disease | | | | |
| Present | 11 | 7 | 4 | 0.42 |
| Absent | 102 | 52 | 50 | |
| Cerebrovascular disease | | | | |
| Present | 6 | 1 | 5 | 0.07 |
| Absent | 107 | 58 | 49 | |
| Diabetes mellitus | | | | |
| Present | 12 | 7 | 5 | 0.65 |
| Absent | 101 | 52 | 49 | |
| Renal dysfunction | | | | |
| Present | 3 | 3 | 0 | 0.09 |
| Absent | 110 | 56 | 54 | |
| Number of comorbidities | | | | |
| < 2 | 93 | 47 | 46 | 0.44 |
| ⩾ 2 | 20 | 12 | 8 | |
| cStage | | | | |
| Ⅰ-Ⅱ | 87 | 45 | 42 | 0.85 |
| Ⅲ-Ⅳ | 26 | 14 | 12 | |
| Operation | | | | |
| Total | 29 | 14 | 15 | 0.62 |
| Other | 84 | 45 | 39 | |

with younger GC patients. Neo-adjuvant or adjuvant chemotherapy was not performed in any of the elderly GC patients, and the frequencies of laparoscopic gastrectomy and radical lymphadenectomy were slightly low for the elderly GC patients as well. Although lymphadenectomy was limited in some patients, cancer-specific survival in cStage Ⅰ was remarkably favorable and the leading cause of death was non-cancer-specific death, specifically due to pneumonia or cardiovascular event. These non-cancer-specific events occurred equally among the elderly patients. Therefore, gastrectomy with limited lymphadenectomy or less-invasive laparoscopic surgery is permissible, at least for elderly patients with cStage Ⅰ [21]. On the other hand, among the elderly patients with cStage Ⅱ or Ⅲ, non-cancer-specific death was not of greater clinical importance than cancer-specific death. Cancer-specific survival in patients who underwent radical lymphadenectomy was significantly better than in those who underwent limited lymphadenectomy. In subgroup analysis, this tendency was more significant in cStage Ⅱ patients than in cStage Ⅲ patients.

There was no positive relationship found between the limited lymphadenectomy and the presence or number of comorbidities or the extent of resection; however, the

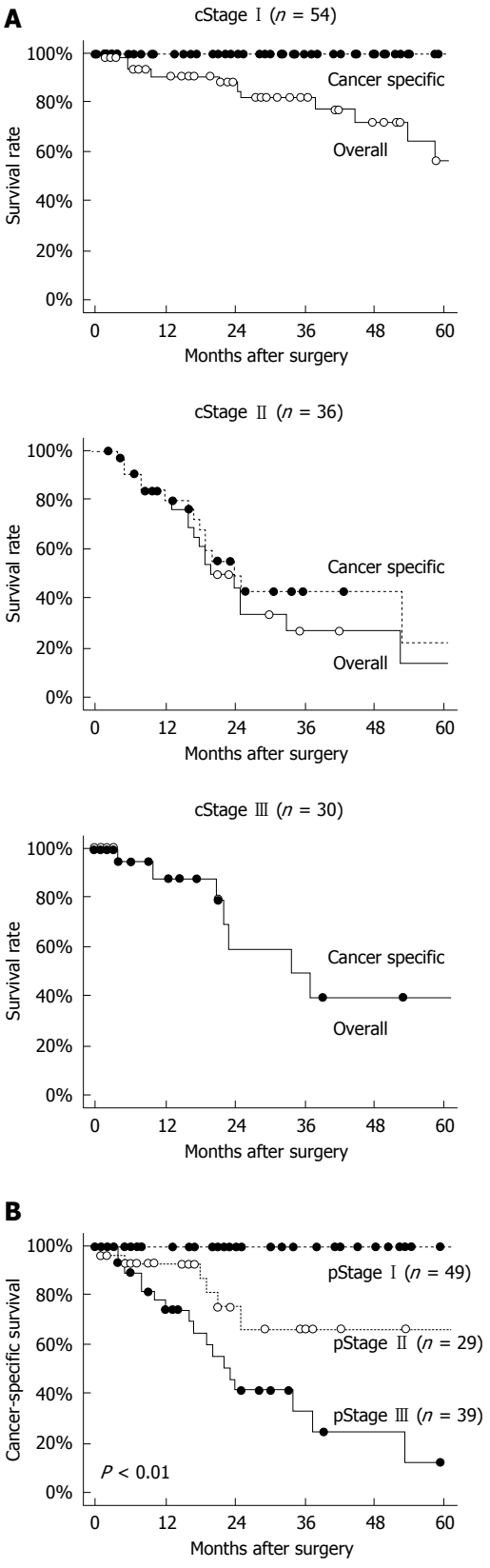



**Figure 1 Survival analysis for patients according to GC stage.** A: Cancer-specific and overall survivals using the Kaplan-Meier method are shown for cStage Ⅰ (*n* = 54), cStage Ⅱ (*n* = 36), and cStage Ⅲ (*n* = 30); B: Cancer-specific survival using the Kaplan-Meier method is shown for each pathological (p)Stage (*P* < 0.01).

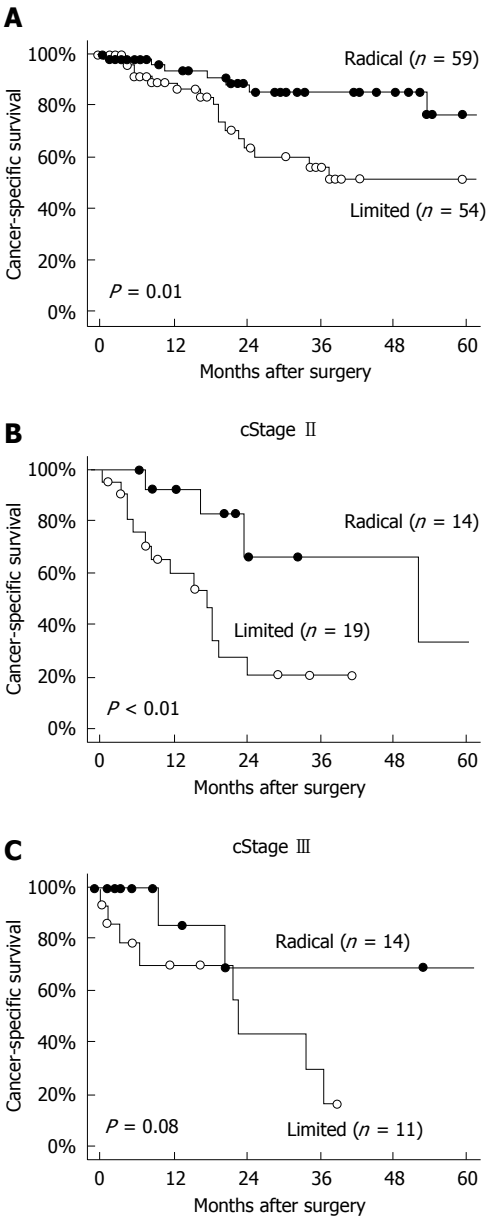



**Figure 2 Survival analysis on cStage Ⅱ and Ⅲ patients who underwent radical or limited lymphadenectomy.** A: Cancer-specific survival using the Kaplan-Meier method is shown for all patients who underwent radical or limited lymphadenectomy; B and C: Cancer-specific survival using the Kaplan-Meier method is shown for patients with cStage Ⅱ (B: *n* = 33) or cStage Ⅲ (C: *n* = 25).

patients with cerebrovascular disease were more likely to have undergone limited lymphadenectomy.

As reported by the Japanese Association of Clinical Cancer Centers, the 5-year relative survival rates of GC patients treated with any surgical therapy between 2004 and 2007 were 96% in cStage Ⅰ, 66.9% in cStage Ⅱ, 48.1% in cStage Ⅲ and 15.7% in cStage Ⅳ[22]. When the results of the present study are generally compared with results of younger GC patients, the cancer-specific survival rate in cStage Ⅰ elderly patients is acceptable, regardless of the surgical extent; however, non-cancer-specific death was frequent in the elderly patient population of the present study and appeared to be affected by surgical therapy and previous histories[3,4,6]. On the other hand, the survival rate of cStage Ⅲ elderly patients was slightly low compared to that which is known for younger GC patients. We attributed this discrepancy to the lack of adjuvant chemotherapy[23,24] and the stage migration of cStage Ⅳ patients due to limited lymphadenectomy[25,26]. The survival rate in cStage Ⅱ elderly patients was similar to that in younger patients[20]. Moreover, cancer-specific survival in cStage Ⅱ patients who underwent radical lymphadenectomy was significantly better than in those who underwent limited lymphadenectomy. This result indicates that radical lymphadenectomy can improve survival in cStage Ⅱ elderly patients[10,12].

There were some limitations to the present study, particularly related to the small number of cases, which must be considered when interpreting the findings. Stage migration should be considered because limited lymphadenectomy was performed more frequently[23,24]. The rate of cancer-specific survival in cStage Ⅰ patients was very high, regardless of lymphadenectomy; however, survival among cStage Ⅱ or Ⅲ patients who underwent limited lymphadenectomy was expected to be improved by stage migration. Therefore, this feature may have affected the results of the present study. Furthermore, overall and cancer-specific survivals were not fully divided by clinical stages, particularly for the cStage Ⅱ or Ⅲ patients. The clinical staging data may have been influenced by a limitation in imaging techniques, the criteria used to reach a diagnosis in each institution, or short follow-up length. In the present study, we were unable to reconfirm details on clinical staging or prognosis in each institution.

In conclusion, although the present study was performed using limited case samples and with some biases, the results obtained showed a trend of elderly GC patients towards surgical therapy. A less-invasive gastrectomy will be permissible, at least for cStage Ⅰ elderly patients, and surgical therapy with radical lymphadenectomy may be effective for cStage Ⅱ elderly patients; however, further studies on non-cancer-specific death or chemotherapy are needed.

## COMMENTS

### Background

The elderly population is increasing worldwide, and therapeutic opportunities for elderly gastric cancer (GC) patients have also expanded. On the other hand, elderly patients may present with existing functional decline in major organs and comorbidities. Although retrospective studies using information of single institutions have evaluated gastrectomy among elderly patient populations, therapeutic strategies for elderly GC patients, particularly those aged 85 or older, are not standardized due to the generally small number of cases.

### Research frontiers

GC patients of age 85 or older have undergone radical gastrectomy with the aim of achieving 5-year survival. However, the decision to perform surgery on an elderly GC patient needs to be made carefully, because the rate of non-

cancer-specific death will be increasing generally and post-operative disorders following gastrectomy may indirectly influence the cause of death. The research hotspot is to examine optimized surgical therapies for elderly GC patients in each clinical disease stage by using a multicenter survey approach.

### Innovations and breakthroughs

There are some limitations in the selection of therapeutic strategies for elderly GC patients due to the small number of cases in a single institution. In the present study, the data for 134 GC patients of age 85 or older who underwent surgery were collected from our 9 related hospitals. The cancer-specific survival rate for cStage Ⅰ elderly patients was acceptable, regardless of the surgical extent; however, the rate of non-cancer-specific deaths due to pneumonia or cardiovascular event was frequent and not negligible. On the other hand, the rate of non-cancer-specific death was not high in cStage Ⅱ or Ⅲ patients, and cancer-specific survival with radical lymphadenectomy was significantly better than that achieved with limited lymphadenectomy in cStage Ⅱ patients.

### Applications

The data in this study suggests that a less-invasive gastrectomy will be permissible for elderly patients, at least for those with cStage Ⅰ GC. Furthermore, this study also provides readers important information regarding surgical therapy with radical lymphadenectomy in elderly GC patients, particularly as related to its effectiveness in cStage Ⅱ elderly patients.

### Terminology

A limited lymphadenectomy for GC is considered for lymphadenectomy less frequently than standard resection, but it has been successfully performed in selected patients with poor general condition or of elderly age. Although the definitive indication of limited lymphadenectomy is not determined, such a limited therapy will be acceptable for some patients, such as the oldest-old patients, after careful considerations of the therapeutic quality or prognosis.

### Peer-review

The authors do a good work, they collected data on GC patients aged 85 or older who underwent surgery in their related hospitals, and examined surgical therapies, clinicopathological features, and survival, which give us some treatment advice for elder gastric cancer patients.

## REFERENCES


1 **Lichtman SM**, Hurria A, Jacobsen PB. Geriatric oncology: an overview. *J Clin Oncol* 2014; **32**: 2521-2522 [PMID: 25071127 DOI: 10.1200/JCO.2014.57.4822]
2 **Ministry of Health, Labour and Welfare**. Abridged life tables for Japan 2014. Available from: URL: http://www.mhlw.go.jp/toukei/saikin/hw/life/life14/index.html
3 **Extermann M**, Overcash J, Lyman GH, Parr J, Balducci L. Comorbidity and functional status are independent in older cancer patients. *J Clin Oncol* 1998; **16**: 1582-1587 [PMID: 9552069 DOI: 10.1200/jco.1998.16.4.1582]
4 **Creditor MC**. Hazards of hospitalization of the elderly. *Ann Intern Med* 1993; **118**: 219-223 [PMID: 8417639 DOI: 10.7326/0003-4819-118-3-199302010-00011]
5 **Smetana GW**, Lawrence VA, Cornell JE; American College of Physicians. Preoperative pulmonary risk stratification for noncardiothoracic surgery: systematic review for the American College of Physicians. *Ann Intern Med* 2006; **144**: 581-595 [PMID: 16618956 DOI: 10.7326/0003-4819-144-8-200604180-00009]
6 **Endo S**, Yoshikawa Y, Hatanaka N, Dousei T, Yamada T, Nishijima J, Kamiike W. Prognostic factors for gastrectomy in elderly patients. *Int Surg* 2014; **99**: 166-173 [PMID: 24670028 DOI: 10.9738/INTSURG-D-13-00016.1]
7 **Japanese Gastric Cancer Association**. Japanese Gastric Cancer Association. Japanese Gastric Cancer Treatment Guidelines 2014 (version 4). Tokyo, Japan, 2014
8 **Gretschel S**, Estevez-Schwarz L, Hünerbein M, Schneider U, Schlag PM. Gastric cancer surgery in elderly patients. *World J Surg* 2006; **30**: 1468-1474 [PMID: 16850149 DOI: 10.1007/s00268-005-0633-5]
9 **Kunisaki C**, Akiyama H, Nomura M, Matsuda G, Otsuka Y, Ono HA, Shimada H. Comparison of surgical outcomes of gastric cancer in elderly and middle-aged patients. *Am J Surg* 2006; **191**: 216-224 [PMID: 16442949 DOI: 10.1016/j.amjsurg.2005.09.001]
10 **Bandoh T**, Isoyama T, Toyoshima H. Total gastrectomy for gastric cancer in the elderly. *Surgery* 1991; **109**: 136-142 [PMID: 1992546]
11 **Endo S**, Dousei T, Yoshikawa Y, Hatanaka N, Kamiike W, Nishijima J. Prognosis of gastric carcinoma patients aged 85 years or older who underwent surgery or who received best supportive care only. *Int J Clin Oncol* 2013; **18**: 1014-1019 [PMID: 23065114 DOI: 10.1007/s10147-012-0482-9]
12 **Kiyokawa T**, Hiki N, Nunobe S, Honda M, Ohashi M, Sano T, Yamaguchi T. Feasibility of Gastrectomy with Standard Lymphadenectomy for Patients Over 85 Years Old with Gastric Cancer. *Ann Surg Oncol* 2015; **22**: 3962-3969 [PMID: 25805234 DOI: 10.1245/s10434-015-4489-0]
13 **Takeshita H**, Ichikawa D, Komatsu S, Kubota T, Okamoto K, Shiozaki A, Fujiwara H, Otsuji E. Surgical outcomes of gastrectomy for elderly patients with gastric cancer. *World J Surg* 2013; **37**: 2891-2898 [PMID: 24081528 DOI: 10.1007/s00268-013-2210-7]
14 **Japanese Gastric Cancer Association**. Japanese classification of gastric carcinoma: 3rd English edition. *Gastric Cancer* 2011; **14**: 101-112 [PMID: 21573743 DOI: 10.1007/s10120-011-0041-5]
15 **Dindo D**, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. *Ann Surg* 2004; **240**: 205-213 [PMID: 15273542 DOI: 10.1097/01.sla.0000133083.54934.ae]
16 **Clavien PA**, Barkun J, de Oliveira ML, Vauthey JN, Dindo D, Schulick RD, de Santibañes E, Pekolj J, Slankamenac K, Bassi C, Graf R, Vonlanthen R, Padbury R, Cameron JL, Makuuchi M. The Clavien-Dindo classification of surgical complications: five-year experience. *Ann Surg* 2009; **250**: 187-196 [PMID: 19638912 DOI: 10.1097/SLA.0b013e3181b13ca2]
17 **Yamada H**, Shinohara T, Takeshita M, Umesaki T, Fujimori Y, Yamagishi K. Postoperative complications in the oldest old gastric cancer patients. *Int J Surg* 2013; **11**: 467-471 [PMID: 23602896 DOI: 10.1016/j.ijsu.2013.04.005]
18 **Isobe T**, Hashimoto K, Kizaki J, Miyagi M, Aoyagi K, Koufuji K, Shirouzu K. Surgical procedures, complications, and prognosis for gastric cancer in the very elderly (& gt; 85): a retrospective study. *Kurume Med J* 2012; **59**: 61-70 [PMID: 23823016 DOI: 10.2739/kurumemedj.59.61]
19 **Ishigami S**, Natsugoe S, Hokita S, Iwashige H, Saihara T, Tokushige M, Aikou T. Strategy of gastric cancer in patients 85 years old and older. *Hepatogastroenterology* 1999; **46**: 2091-2095 [PMID: 10430403]
20 **Endo S**, Yoshikawa Y, Hatanaka N, Tominaga H, Shimizu Y, Hiraoka K, Nishitani A, Irei T, Nakashima S, Park MH, Takahashi H, Wakahara M, Kamiike W. Treatment for gastric carcinoma in the oldest old patients. *Gastric Cancer* 2011; **14**: 139-143 [PMID: 21336856 DOI: 10.1007/s10120-011-0022-8]
21 **Kwon IG**, Cho I, Guner A, Kim HI, Noh SH, Hyung WJ. Minimally invasive surgery as a treatment option for gastric cancer in the elderly: comparison with open surgery for patients 80 years and older. *Surg Endosc* 2015; **29**: 2321-2330 [PMID: 25480603 DOI: 10.1007/s00464-014-3955-2]
22 **Ministry of Health, Labour and Welfare**. The 5-years relative survival rate of gastric cancer patients with any surgical therapies in 2004-2007. Available from: URL: http://www.gunma-cc.jp/sarukihan/seizonritu/seizonritu2007c.html
23 **Sun DS**, Jeon EK, Won HS, Park JC, Shim BY, Park SY, Hong YS, Kim HK, Ko YH. Outcomes in elderly patients treated with a single-agent or combination regimen as first-line chemotherapy for recurrent or metastatic gastric cancer. *Gastric Cancer* 2015; **18**: 644-652 [PMID: 25098925 DOI: 10.1007/s10120-014-0405-8]
24 **Desai AM**, Lichtman SM. Systemic therapy of non-colorectal

gastrointestinal malignancies in the elderly. *Cancer Biol Med* 2015; **12**: 284-291 [PMID: 26779365 DOI: 10.7497/j.issn.2095-3941.2015.0078]

25 **Schwarz RE**, Smith DD. Clinical impact of lymphadenectomy extent in resectable gastric cancer of advanced stage. *Ann Surg Oncol* 2007; **14**: 317-328 [PMID: 17094022 DOI: 10.1245/s10434-006-9218-2]

26 **Bunt AM**, Hermans J, Smit VT, van de Velde CJ, Fleuren GJ, Bruijn JA. Surgical/pathologic-stage migration confounds comparisons of gastric cancer survival rates between Japan and Western countries. *J Clin Oncol* 1995; **13**: 19-25 [PMID: 7799019 DOI: 10.1200/jco.1995.13.1.19]

**P- Reviewer**: Garcia-Olmo D, Huang CM, Lee JI, Nagahara H, Yang MH **S- Editor**: Gong ZM **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/ *World J Gastroenterol* 2017 February 21; 23(7): 1224-1232

DOI: 10.3748/wjg.v23.i7.1224 ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Study

# Post-transplant lymphoproliferative disorder after liver transplantation: Incidence, long-term survival and impact of serum tacrolimus level

Ahad Eshraghian, Mohammad Hadi Imanieh, Seyed Mohsen Dehghani, Saman Nikeghbalian, Alireza Shamsaeefar, Frouzan Barshans, Kourosh Kazemi, Bita Geramizadeh, Seyed Ali Malek-Hosseini

Ahad Eshraghian, Mohammad Hadi Imanieh, Seyed Mohsen Dehghani, Saman Nikeghbalian, Alireza Shamsaeefar, Kourosh Kazemi, Bita Geramizadeh, Seyed Ali Malek-Hosseini, Transplant Research Center, Shiraz University of Medical Sciences, Shiraz 71937-11351, Iran

Ahad Eshraghian, Mohammad Hadi Imanieh, Seyed Mohsen Dehghani, Frouzan Barshans, Gastroenterohepatology Research Center, Namazi Hospital, Shiraz 71937-11351, Iran

Author contributions: Eshraghian A and Imanieh MH contributed to the study concept and design; Eshraghian A, Imanieh MH and Barshans F drafted of the manuscript; Eshraghian A, Imanieh MH, Dehghani SM, Nikeghbalian S, Barshans F, Kazemi K and Malek-Hosseini SA contributed to acquisition, analysis and interpretation of data; Eshraghian A, Imanieh MH, Dehghani SM, Nikeghbalian S, Kazemi K and Malek-Hosseini SA also contributed to the patient care; Eshraghian A, Imanieh MH, Dehghani SM, Nikeghbalian S, Kazemi K, Geramizadeh B and Malek-Hosseini SA critically revised the manuscript for important intellectual content; and Geramizadeh B also contributed to the pathology review, acquisition of data; all the authors contributed to this manuscript.

Conflict-of-interest statement: The authors have no conflict of interest. The study was supported by a grant from Shiraz University of Medical Sciences.

Data sharing statement: No additional data are available.



Manuscript source: Invited manuscript

Correspondence to: Ahad Eshraghian, MD, Gastroenterohepatology Research Center, Namazi Hospital, Fars Province, Shiraz, District 1, Shiraz 71937-11351, Iran. eshraghiana@yahoo.com
Telephone: +98-71-36281442

Received: September 19, 2016
Peer-review started: September 19, 2016
First decision: October 28, 2016
Revised: December 3, 2016
Accepted: January 18, 2017
Article in press: January 18, 2017
Published online: February 21, 2017

## Abstract

***AIM***

To investigate incidence and survival of post-transplant lymphoproliferative disorder (PTLD) patients after liver transplantation.

***METHODS***

A cross-sectional survey was conducted among patients who underwent liver transplantation at Shiraz Transplant Center (Shiraz, Iran) between August 2004 and March 2015. Clinical and laboratory data of patients were collected using a data gathering form.

***RESULTS***

There were 40 cases of PTLD in the pediatric age group and 13 cases in the adult group. The incidence of PTLD was 6.25% in pediatric patients and 1.18% in adult liver transplant recipients. The post-PTLD survival of patients at 6 mo was 75.1% ± 6%, at 1 year was 68.9% ± 6.5% and at 5 years was 39.2% ± 14.2%. Higher serum tacrolimus level was associated with lower post-PTLD survival in pediatric patients

(OR = 1.07, 95%CI: 1.006-1.15, *P* = 0.032). A serum tacrolimus level over 11.1 ng/mL was predictive of post PTLD survival (sensitivity = 90%, specificity = 52%, area under the curve = 0.738, *P* = 0.035).

***CONCLUSION***

Incidence of PTLD in our liver transplant patients is comparable to other centers. Transplant physicians may consider adjustment of tacrolimus dose to maintain its serum level below this cutoff point.

**Key words:** Post-transplant lymphoproliferative disorder; Liver transplantation; Survival; Tacrolimus; Epstein-Barr virus



**Core tip:** Post-transplant lymphoproliferative disorder (PTLD) is one of the complications that may occur after liver transplantation. The present study is a survival analysis of liver transplant patients after PTLD development. The incidence of PTLD was 6.25% in pediatric patients and 1.18% in adult liver transplant recipients. The main new finding is association of serum tacrolimus level with post-PTLD survival. Higher serum tacrolimus level was associated with lower post-PTLD survival in pediatric patients.

Eshraghian A, Imanieh MH, Dehghani SM, Nikeghbalian S, Shamsaeefar A, Barshans F, Kazemi K, Geramizadeh B, Malek-Hosseini SA. Post-transplant lymphoproliferative disorder after liver transplantation: Incidence, long-term survival and impact of serum tacrolimus level. *World J Gastroenterol* 2017; 23(7): 1224-1232 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1224.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1224

## INTRODUCTION

Liver transplantation is an established modality of treatment for end-stage liver diseases of various etiologies. Despite considerable improvement in outcomes of patients, complications frequently occur after transplantation that may have negative impact on survival[1]. Post-transplant lymphoproliferative disorder (PTLD) is one of the complications that may occur after liver transplantation and threatens both graft and patient survival. PTLD is generally believed to be a consequence of relative immunodeficiency state secondary to immunosuppressive regimens in these patients[2]. Immunosuppressive therapy results in depressed T-cell function that predisposes patients to lymphoid proliferation[3]. Epstein-Barr virus (EBV) infection is the other major risk factor for development of PTLD after liver transplantation and the majority of cases (60%-70%) are EBV-positive[4]. While EBV infection has minimal consequences in normal subjects, in liver transplant recipients it is associated with a spectrum of disorders, ranging from reactive monoclonal hyperplasia to aggressive malignant lymphoma[5]. In immunocompetent subjects, the EBV genome remains latent in resting memory B cells after immortalization[6]. However, after transplantation, long-term immunosuppressive therapy results in depressed T-cell function and lack of T-cell inhibition on B-cell proliferation[7]. This may lead to uncontrolled B-cell proliferation and subsequent hyperplasia, and even malignant transformation.

PTLD is more frequently encountered in pediatric patients, and younger age by itself is a known risk factor for PTLD despite controversies[8]. Another proposed risk factor for PTLD development after liver transplantation is hepatitis C virus infection[9]. With a mortality rate ranging from 12% to 60% in different studies, PTLD has imposed considerable negative impact on transplant patients until recently[10-12]. However, outcomes of patients and survival rates have been substantially improved by using new modalities for treatments, such as rituximab (a chimeric anti-CD20 monoclonal antibody) and sirolimus, in addition to reduced-dose immunosuppression[13-15].

This study aimed to investigate incidence, risk factors (including impact of immunosuppressive regimen) and survival of PTLD patients after liver transplantation in Iranian patients.

## MATERIALS AND METHODS

### *Patients*

Shiraz Organ Transplant Center (Shiraz, Iran) is a leading transplant center in Iran, with considerable annual cases of liver transplantation for both adult and pediatric patients. A cross-sectional survey was conducted among the adult and pediatric patients (< 18 years) who underwent liver transplantation at Shiraz Transplant Center between August 2004 and March 2015. Clinical and laboratory data of patients were collected using a data gathering form containing information regarding age, sex, underlying liver disease, type of allograft (deceased donor, living related donor, split liver transplantation), time of liver transplantation and time of PTLD development, survival of patients from date of liver transplantation, survival after PTLD diagnosis, immunosuppressive regimen and dosage, rejection episodes, EBV status before and after transplantation, presenting sign and symptoms, PTLD histology, multi-organ involvement, modality of treatment, response to therapy, and serum level of calcineurin inhibitors (including tacrolimus and cyclosporine). All patients received intravenous methylprednisolone as induction of immunosuppression. Patients received tacrolimus, cyclosporine, mycophenolate mofetil and prednisolone as immunosuppressive therapy during their follow-

**Table 1 Baseline characteristics of patients**

| | Pediatrics | Adults | Overall |
|---|---|---|---|
| Number | 40 | 13 | 53 |
| Mean age in years | 5.05 ± 4.43 | 42 ± 13.39 | 14.11 ± 17.71 |
| Sex, male/female | 23/17 | 10/3 | 33/20 |
| Allograft | | | |
| Living donor | 28 | 0 | 28 |
| Deceased donor | 12 | 13 | 25 |
| Presenting sign and symptoms | | | |
| LAP | 18 | 9 | 27 |
| Fever | 13 | 1 | 14 |
| Abdominal pain | 15 | 3 | 18 |
| Diarrhea | 5 | 0 | 5 |
| Weight loss | 0 | 1 | 1 |
| Cough and dyspnea | 1 | 0 | 1 |
| Bowel obstruction | 1 | 0 | 1 |
| Unilateral weakness | 1 | 0 | 1 |
| Underlying liver disease | | | |
| HBV cirrhosis | 0 | 5 | 5 |
| Cryptogenic cirrhosis | 0 | 2 | 2 |
| PSC | 0 | 2 | 2 |
| HCV cirrhosis | 0 | 1 | 1 |
| AIH | 2 | 1 | 3 |
| Wilson's disease | 1 | 0 | 1 |
| PFIC | 5 | 0 | 5 |
| Crigler-Najjar syndrome | 8 | 0 | 8 |
| Biliary atresia | 12 | 0 | 12 |
| Tyrosinemia | 10 | 0 | 10 |
| Budd-Chiari syndrome | 1 | 1 | 2 |
| Liver metastasis | 0 | 1 | 1 |
| Neonatal hepatitis | 1 | 0 | 1 |
| Immunosuppressive regimen | | | |
| Prednisolone | 39 | 12 | 49 |
| Tacrolimus | 36 | 10 | 46 |
| Mycophenolate mofetil | 15 | 11 | 26 |
| Cyclosporine | 1 | 4 | 5 |
| Sirolimus | 35 | 8 | 43 |

AIH: Autoimmune hepatitis; LAP: Lymphadenopathy; HBV: Hepatitis B virus; HCV: Hepatitis C virus; PFIC: Progressive familial intrahepatic cholestasis; PSC: Primary sclerosing cholangitis.

up. Serum tacrolimus level was measured periodically during follow-up for each patient and the last measured serum tacrolimus levels before diagnosis of PTLD were recorded and analyzed for each patient. Patients were treated with rituximab or chemotherapy based on grade, type and invasiveness of PTLD. Change from tacrolimus to sirolimus was applied for all of the patients diagnosed with PTLD. Thirteen patients with positivity for cytomegalovirus (CMV)-DNA were treated with ganciclovir or valganciclovir.

### PTLD diagnosis

Diagnosis of PTLD was confirmed by tissue biopsies reviewed by expert pathologists. World Health Organization (WHO) classification for tumors of lymphoid tissue was used for PTLD classification[16]. While diagnosis of PTLD was confirmed, patients underwent staging work-up, including CT scans (abdomen, chest and pelvis) and bone marrow aspiration and biopsy to detect possibility of multiple organ involvement. Frozen section or paraffin immunoperoxidase staining or flow cytometry were applied for immunophenotyping of B-cell- and T-cell-associated antigens, as previously described.

Whether PTLD is monoclonal or polyclonal was determined by flow cytometry on fresh cell suspensions checking immunoglobulin light chain restriction, by immunoperoxidase staining on frozen or paraffin-embedded tissues or by southern blot on frozen tissues checking immunoglobulin or T-cell receptor gene rearrangements[17].

Based on these classification, one patient had nodular sclerosis Hodgkin disease, one had plasmacytoma, and others had polymorphic and monomorphic B cell lymphoma.

### Ethics and consent

The study protocol was approved by the institutional review board of Shiraz University of Medical Sciences. The study protocol was carried out in accordance with the Helsinki Declaration as revised in Seoul 2008. Written informed consent was obtained from patients.

### Statistical analysis

Comparisons of continuous variables were performed with the Student's *t*-test, and categorical variables were compared using the chi-square test. Non-parametric Mann-Whitney test was used when appropriate. Data were presented using mean ± standard deviation for numeric variables, and percent and counts for categorical variables. Kaplan-Meier estimates were used for analysis of time to PTLD development and survival after PTLD diagnosis. Kaplan-Meier and Cox regression analyses were used to calculate the influence of probable risk factors on PTLD development and survival. Rejection episodes were considered a time varying statistical variable, and rejections that occurred after PTLD development were excluded. Statistical analysis was performed with SPSS 16.0 (SPSS Inc., Chicago, IL, United States). A $P$ value of $< 0.05$ were considered statistically significant.

## RESULTS

### PTLD characteristics and post-PTLD survival

Overall, 53 patients were diagnosed with PTLD. There were 40 cases of PTLD in the pediatric age group and 13 cases in the adult group. The incidence of PTLD was 6.25% in the pediatric patients and 1.18% in the adult liver transplant recipients. The baseline characteristics of PTLD patients are outlined in Table 1.

The mean overall (adult and pediatric) post-PTLD survival was 66.29 ± 11.86 mo. The post-PTLD survival of patients at 6 mo was 75.1% ± 6%, at 1 year was 68.9% ± 6.5% and at 5 years was 39.2% ± 14.2% (Figure 1A).

The mean post-PTLD survival in adult patients was 82.94 ± 18.58 mo. The post-PTLD survival of adult patients at 6 mo was 83.9% ± 10.4%, at 1 year was 74.6% ± 12.8% and at 5 years was 59.7% ± 16.8% (Figure 1B).

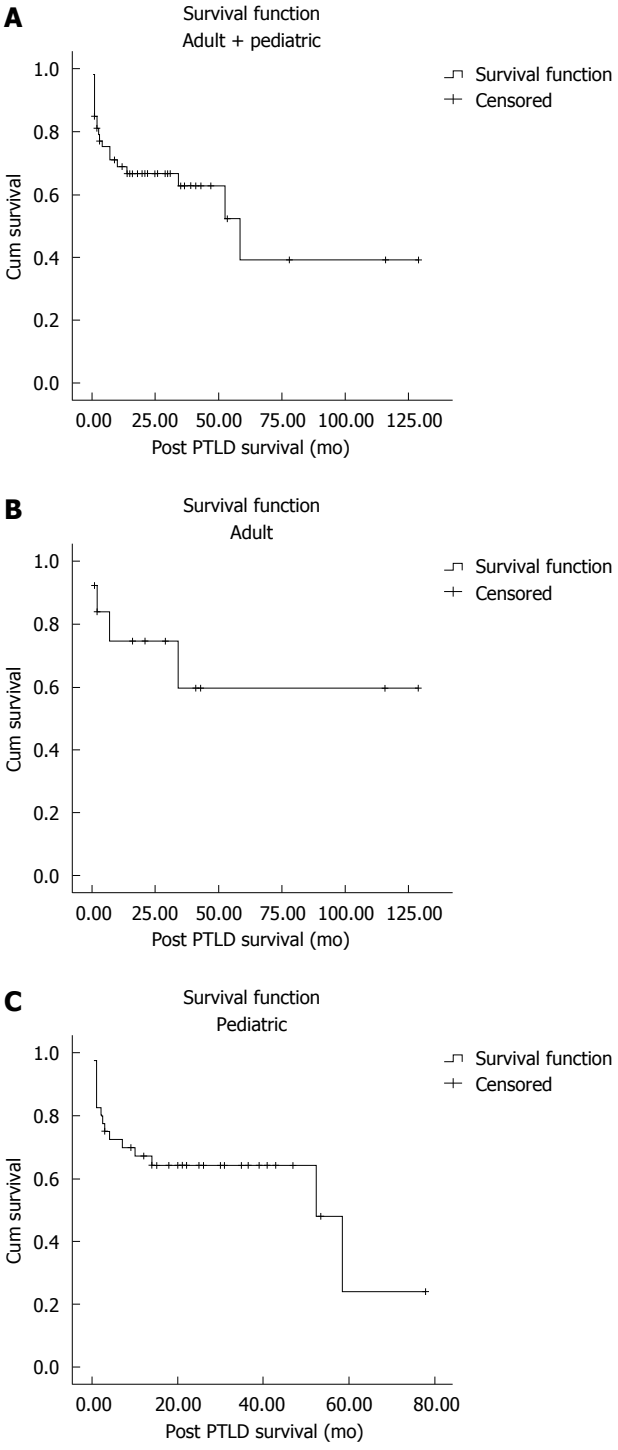



**Figure 1 Post-transplant lymphoproliferative disorder survival.** A: Pediatric and adult patients; B: Adult patients; C: Pediatric patients. PTLD: Post-transplant lymphoproliferative disorder.

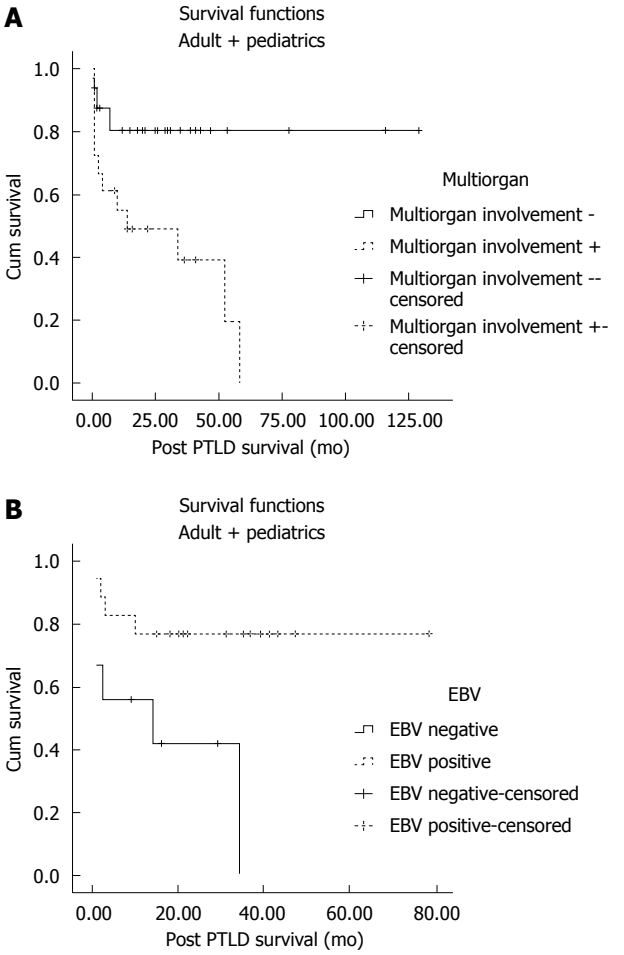



**Figure 2 Post-transplant lymphoproliferative disorder survival of pediatric and adult patients.** A: Impact of multi-organ involvement; B: Impact of EBV status. EBV: Epstein-Barr virus; PTLD: Post-transplant lymphoproliferative disorder.

The mean post-PTLD survival in pediatric patients was 42.61 ± 6.1 mo. The post-PTLD survival of pediatric patients at 6 mo was 72.4% ± 7.1%, at 1 year was 67.1% ± 7.5% and at 5 years was 24.1% ± 18.6% (Figure 1C).

When both pediatric and adult patients were analyzed altogether, multi-organ involvement was significantly associated with lower post-PTLD survival (104.25 ± 9.08 mo *vs* 27.13 ± 6.30 mo, $P$ = 0.002) (Figure 2A). EBV-positive patients with PTLD had significantly higher mean survival compared to EBV-negative PTLD patients (60.58 ± 7.62 mo *vs* 16.72 ± 5.66 mo, $P$ = 0.018) (Figure 2B). Other variables including sex, CMV status, rejection episodes, time to PTLD before or after 1 year, and type of allograft had no significant effect on post-PTLD survival (Table 2).

We also analyzed the influence of different risk factors on pediatric PTLD patients separately. Multi-organ involvement and EBV negativity were significantly associated with lower mean post-PTLD survival in pediatric patients (Table 3). Higher serum tacrolimus level was associated with lower post-PTLD survival in pediatric patients (OR = 1.07, 95%CI: 1.006-1.15, $P$ = 0.032) (Table 4).

### *Impact of multi-organ involvement, and time to PTLD development*

Patients were divided into those who developed PTLD in ⩽ 1 year and those who developed PTLD in ⩾ 1 year. Age, post-PTLD survival, serum tacrolimus level, tacrolimus dose and prednisolone dose were not correlated with time to PTLD development in pediatric

**Table 2 Kaplan-Meier analysis of risk factors and post-transplant lymphoproliferative disorder survival of pediatric and adult patients**

| | Mean survival in mo | *P* value |
|---|---|---|
| Sex | | 0.902 |
| Male | 65.65 ± 13.18 | |
| Female | 36.06 ± 5.30 | |
| Multi-organ involvement | | 0.002 |
| (+) | 27.13 ± 6.30 | |
| (-) | 104.25 ± 9.08 | |
| CMV status | | 0.370 |
| CMV-positive | 51.98 ± 10.50 | |
| CMV-negative | 23.29 ± 5.76 | |
| EBV status | | 0.002 |
| EBV-positive | 60.58 ± 7.62 | |
| EBV-negative | 16.72 ± 5.66 | |
| Rejection episode | | 0.762 |
| (+) | 64.90 ± 13.78 | |
| (-) | 65.86 ± 15.76 | |
| Time to PTLD development in years | | 0.704 |
| ≤ 1 | 62.18 ± 14.03 | |
| ≥ 1 | 75.13 ± 12.49 | |
| Type of allograft | | 0.904 |
| Living donor | 50.56 ± 6.95 | |
| Deceased donor | 60.32 ± 14.33 | |

CMV: Cytomegalovirus; EBV: Epstein-Barr virus; PTLD: Post-transplant lymphoproliferative disorder.

**Table 3 Kaplan-Meier analysis of risk factors and post-transplant lymphoproliferative disorder survival of pediatric patients**

| | Mean survival in mo | *P* value |
|---|---|---|
| Sex | | 0.749 |
| Male | 41.41 ± 7.38 | |
| Female | 35.85 ± 5.76 | |
| Multi-organ involvement | | 0.002 |
| (+) | 25.82 ± 6.90 | |
| (-) | 67.62 ± 5.56 | |
| CMV status | | 0.139 |
| CMV-positive | 58.82 ± 9.56 | |
| CMV-negative | 19.35 ± 6.21 | |
| EBV status | | 0.002 |
| EBV-positive | 60.58 ± 7.62 | |
| EBV-negative | 5.58 ± 2.72 | |
| Rejection episode | | 0.888 |
| (+) | 43.61 ± 8.49 | |
| (-) | 36.02 ± 5.24 | |
| Time to PTLD development in years | | 0.326 |
| ≤ 1 | 39.72 ± 6.86 | |
| ≥ 1 | 36.45 ± 5.30 | |
| Type of allograft | | 0.806 |
| Living donor | 50.56 ± 6.95 | |
| Deceased donor | 37.37 ± 8.11 | |

CMV: Cytomegalovirus; EBV: Epstein-Barr virus; PTLD: Post-transplant lymphoproliferative disorder.

patients ($P > 0.05$) (Table 5). However, multi-organ involvement was more common in patients who developed PTLD within 1 year after liver transplantation ($P = 0.007$) (Table 6). Multi-organ involvement was also more common in pediatric patients who developed PTLD within 1 year after liver transplantation ($P = 0.007$) (Table 6).

**Table 4 Cox regression analysis showing association of different risk factors and post-transplant lymphoproliferative disorder survival of pediatric patients**

| | Mean | OR | 95%CI | *P* value |
|---|---|---|---|---|
| Age in years | 5.05 | 0.94 | 0.82-1.08 | 0.434 |
| Time to PTLD in months | 15.63 | 0.96 | 0.91-1.02 | 0.242 |
| Tacrolimus level | 14.99 | 1.07 | 1.006-1.15 | 0.032 |
| Tacrolimus dose | 3.81 | 1.06 | 0.67-1.66 | 0.797 |
| Prednisolone dose | 10.12 | 0.99 | 0.86-1.13 | 0.897 |

PTLD: Post-transplant lymphoproliferative disorder.

Multi-organ involvement was associated with increased mortality after PTLD development ($P < 0.05$) (Table 7). EBV-positive patients with PTLD had lower mortality when compared to EBV-negative patients ($P < 0.05$) (Table 7).

Multi-organ involvement was not associated with age, serum tacrolimus level, tacrolimus dose, prednisolone dose in univariate analysis (Table 5). EBV-positive patients were less likely to have multi-organ involvement in comparison with EBV- negative patients ($P = 0.008$) (Table 8). Pediatric patients who received liver allograft from deceased donors were more likely to develop PTLD with multi-organ involvement when compared to those receiving liver allograft from living donors ($P = 0.019$) (Table 8).

### *Estimation of a cutoff value for tacrolimus level in pediatric patients*

To estimate a cutoff point value for tacrolimus level in relation to post-PTLD survival in pediatric patients, we used receiver operating characteristic (ROC) curve analysis. A serum tacrolimus of over 11.1 ng/mL was predictive of post-PTLD survival (sensitivity = 90%, specificity = 52%, area under the curve = 0.738, $P = 0.035$).

## DISCUSSION

The present study is one of the largest series of patients with PTLD after liver transplantation. Our study showed that the incidence of PTLD following pediatric liver transplantation was much higher than for adult liver transplantation (6.25% in pediatrics and 1.18% in adults). While previous studies reported PTLD incidence of up to 20% after pediatric liver transplantation[18], recent reported incidence from different studies are lower and range from 10% to 5.5%[19,20]. Since PTLD is mainly considered as a result of interaction of immunosuppression and EBV infection, the decreased incidence in pediatric patients may be secondary to the better monitoring of patients, especially for immunosuppressive regimen and EBV infection. In pediatric patients, our reported incidence is comparable to other studies; however, due to unexplained reasons the incidence of PTLD after

**Table 5 Influence of different continuous variables on time to post-transplant lymphoproliferative disorder development, mortality and multi-organ involvement of post-transplant lymphoproliferative disorder patients**

| | Mean rank PTLD development ⩽ 1 yr | Mean rank PTLD development ⩾ 1 yr | *U* value | *Z* score | *P* value |
|---|---|---|---|---|---|
| Age | 19.85 | 21.85 | 158 | -0.50 | 0.61 |
| Post-PTLD survival | 20.19 | 21.15 | 167 | -0.24 | 0.80 |
| Tacrolimus level | 16.57 | 14.61 | 86 | -0.54 | 0.58 |
| Tacrolimus dose | 19.84 | 18.85 | 154 | -0.27 | 0.78 |
| Prednisolone dose | 20.56 | 18.88 | 154 | -0.44 | 0.65 |
| | Alive patient | Deceased patient | | | |
| Age | 23.19 | 16.47 | 127.5 | -1.78 | 0.74 |
| Tacrolimus level | 13.62 | 21.00 | 55 | -2.11 | 0.03 |
| Tacrolimus dose | 18.96 | 20.43 | 175 | -0.40 | 0.68 |
| Prednisolone dose | 19.65 | 20.57 | 171 | -0.25 | 0.79 |
| Mean time to PTLD | 23.12 | 16.56 | 129 | -1.74 | 0.08 |
| | Multi-organ (+) | Multi-organ (-) | | | |
| Age | 19.50 | 19.50 | 176 | 0.00 | 1.00 |
| Tacrolimus level | 16.50 | 14.74 | 97 | -0.54 | 0.58 |
| Tacrolimus dose | 20.27 | 18.14 | 146 | -0.60 | 0.54 |
| Prednisolone dose | 19.81 | 19.27 | 171 | -0.15 | 0.87 |
| Post-PTLD survival | 15.59 | 22.34 | 113 | -1.85 | 0.06 |
| Mean time to PTLD | 13.62 | 23.77 | 82 | -2.78 | 0.005 |

PTLD: Post-transplant lymphoproliferative disorder.

**Table 6 Influence of different risk factors on time to post-transplant lymphoproliferative disorder development**

| | PTLD development ⩽ 1 yr | PTLD development ⩾ 1 yr | *P* value |
|---|---|---|---|
| All PTLD patients | | | |
| Sex | | | 0.150 |
| Male | 17 | 16 | |
| Female | 14 | 6 | |
| Multi-organ involvement | | | 0.007 |
| (+) | 15 | 3 | |
| (-) | 14 | 18 | |
| CMV status | | | 0.186 |
| CMV-positive | 9 | 4 | |
| CMV-negative | 11 | 1 | |
| EBV status | | | 0.296 |
| EBV-positive | 12 | 5 | |
| EBV-negative | 8 | 1 | |
| Rejection episode | | | 0.399 |
| (+) | 15 | 9 | |
| (-) | 16 | 13 | |
| Mortality | | | 0.324 |
| (+) | 13 | 7 | |
| (-) | 18 | 15 | |
| Type of allograft | | | 0.118 |
| Living donor | 19 | 9 | |
| Deceased donor | 12 | 13 | |
| Pediatric PTLD patients | | | |
| Sex | | | 0.496 |
| Male | 15 | 8 | |
| Female | 12 | 5 | |
| Multi-organ involvement | | | 0.018 |
| (+) | 14 | 2 | |
| (-) | 11 | 11 | |
| CMV status | | | 0.368 |
| CMV-positive | 9 | 3 | |
| CMV-negative | 9 | 1 | |
| EBV status | | | 0.184 |
| EBV-positive | 12 | 5 | |
| EBV-negative | 6 | 0 | |
| Rejection episode | | | 0.587 |
| (+) | 13 | 6 | |
| (-) | 14 | 7 | |
| Mortality | | | 0.120 |
| (+) | 13 | 3 | |
| (-) | 14 | 10 | |
| Type of allograft | | | 0.609 |
| Living donor | 19 | 9 | |
| Deceased donor | 8 | 4 | |

CMV: Cytomegalovirus; EBV: Epstein-Barr virus; PTLD: Post-transplant lymphoproliferative disorder.

adult liver transplantation in our study was lower than previous reports from other centers[21].

Mean post-PTLD survival was higher in the adult patients than in the pediatric patients. This observation is probably due to the long-term survival (> 10 years) of 2 of our adult patients. In a recent study conducted in our center, the 1-year and 5-year overall survival of pediatric liver transplant recipients was found to be 73% and 66% respectively[22]. In this way, the 1-year post-PTLD survival in the pediatric age group is nearly equal to the overall survival of our pediatric patients. However, it should be noted that the 5-year post-PTLD survival in pediatric patients has dramatically declined to 24.1%.

Due to small numbers of adult PTLD patients, the analyses were performed on either pediatric patients or adult plus pediatric patients. We investigated the impact of different variables on post-PTLD survival. As expected, multi-organ involvement was associated with a lower post-PTLD survival and increased mortality. EBV-positive patients had higher mean post-PTLD survival in comparison with EBV-negative subjects. EBV positivity was also associated with lower

**Table 7 Influence of different risk factors on mortality after post-transplant lymphoproliferative disorder**

| | Alive patient | Deceased patient | *P* value |
|---|---|---|---|
| All PTLD patients | | | |
| Sex | | | 0.491 |
| Male | 10 | 13 | |
| Female | 13 | 7 | |
| Multi-organ involvement | | | 0.001 |
| (+) | 6 | 12 | |
| (-) | 26 | 6 | |
| CMV status | | | 0.284 |
| CMV-positive | 9 | 4 | |
| CMV-negative | 6 | 6 | |
| EBV status | | | 0.042 |
| EBV-positive | 13 | 4 | |
| EBV-negative | 3 | 6 | |
| Rejection episode | | | 0.600 |
| (+) | 15 | 9 | |
| (-) | 18 | 11 | |
| Type of allograft | | | 0.485 |
| Living donor | 18 | 10 | |
| Deceased donor | 15 | 10 | |
| Pediatric PTLD patients | | | |
| Sex | | | 0.424 |
| Male | 13 | 10 | |
| Female | 11 | 6 | |
| Multi-organ involvement | | | 0.001 |
| (+) | 5 | 11 | |
| (-) | 19 | 3 | |
| CMV status | | | 0.110 |
| CMV-positive | 9 | 3 | |
| CMV-negative | 4 | 6 | |
| EBV status | | | 0.018 |
| EBV-positive | 13 | 4 | |
| EBV-negative | 1 | 5 | |
| Rejection episode | | | 0.525 |
| (+) | 11 | 8 | |
| (-) | 13 | 8 | |
| Type of allograft | | | 0.309 |
| Living donor | 18 | 10 | |
| Deceased donor | 6 | 6 | |

CMV: Cytomegalovirus; EBV: Epstein-Barr virus; PTLD: Post-transplant lymphoproliferative disorder.

**Table 8 Influence of different risk factors on multi-organ involvement in patients with post-transplant lymphoproliferative disorder**

| | Multi-organ involvement (+) | Multi-organ involvement (-) | *P* value |
|---|---|---|---|
| All PTLD patients | | | |
| Sex | | | 0.421 |
| Male | 12 | 19 | |
| Female | 6 | 13 | |
| CMV status | | | 0.418 |
| CMV-positive | 6 | 7 | |
| CMV-negative | 7 | 5 | |
| EBV status | | | 0.008 |
| EBV-positive | 5 | 11 | |
| EBV-negative | 8 | 1 | |
| Rejection episode | | | 0.448 |
| (+) | 9 | 14 | |
| (-) | 9 | 18 | |
| Type of allograft | | | 0.235 |
| Living donor | 8 | 19 | |
| Deceased donor | 10 | 13 | |
| Pediatric PTL patients | | | |
| Sex | | | 0.206 |
| Male | 11 | 11 | |
| Female | 5 | 11 | |
| CMV status | | | 0.335 |
| CMV-positive | 5 | 7 | |
| CMV-negative | 6 | 4 | |
| EBV status | | | 0.006 |
| EBV-positive | 5 | 11 | |
| EBV-negative | 6 | 0 | |
| Rejection episode | | | 0.520 |
| (+) | 8 | 10 | |
| (-) | 8 | 12 | |
| Type of allograft | | | 0.019 |
| Living donor | 8 | 19 | |
| Deceased donor | 8 | 3 | |

CMV: Cytomegalovirus; EBV: Epstein-Barr virus; PTLD: Post-transplant lymphoproliferative disorder.

mortality, especially among the pediatric age group. These findings may be jeopardized by our other finding that EBV-positive patients had lower probability of multi-organ involvement.

Although up to 30% of PTLD patients are EBV-negative, EBV has been generally considered as responsible for most cases of PTLD. However, the influence of recipient EBV status on outcomes of PTLD patients is conflicting. Some studies have shown that EBV-negative PTLD patients have more malignant appearing disease with an aggressive course and higher mortality rate[23,24]. In univariate analysis, our findings are inconsistent with these mentioned results. However, in regression analysis, EBV status was not associated with post-PTLD survival. Several other studies showed that EBV status had no significant impact on outcomes of PTLD patients, including their survival[25-28]. EBV status was not associated with time of PTLD development in our study. This finding is in contrast with previous reports showing that EBV-negative PTLD occurs later after liver transplantation when compared to EBV-positive PTLD patients[29,30].

Immunosuppressive therapy has been reported to be associated with PTLD development. Treatment of rejection episodes with steroid or OKT3 were risk factors of PTLD development, especially during 1 year after treatment[31,32]. Reducing dose of immunosuppressive medications is another treatment strategy used on PTLD patients in some studies[33,34]. In our study, rejection episode, steroid dose and tacrolimus dose were not associated with PTLD survival, while higher serum tacrolimus level was associated with lower survival. Finally, we showed that a serum tacrolimus cutoff value of over 11.1 ng/mL is associated with post-PTLD survival, having a high sensitivity but a rather low specificity in pediatric patients. Therefore, it might be suggested that transplant physicians consider adjustment of tacrolimus dose to maintain its serum level around this cutoff point.

Although a PTLD series has been published from

our center previously[35], this study is the first that evaluates incidence, survival and associated factors influencing survival of PTLD patients after liver transplantation. This study is also the first that shows the association between serum tacrolimus level and post-PTLD survival, and suggests a serum tacrolimus cutoff point value to adjust tacrolimus dose.

## COMMENTS

### Background

Post-transplant lymphoproliferative disorder (PTLD) is one of the complications after liver transplantation and may threaten both graft and patient survival. This study aimed to investigate incidence and survival of PTLD patients after liver transplantation.

### Research frontiers

Few studies with considerable number of patients have reported survival of PTLD patients after liver transplantation. This study aimed to investigate incidence, risk factors (including impact of immunosuppressive regimen) and survival of PTLD patients after liver transplantation in Iranian patients.

### Innovations and breakthroughs

Multi-organ involvement was associated with a lower post-PTLD survival and increased mortality. Epstein-Barr virus (EBV)-positive patients had higher mean post-PTLD survival in comparison with EBV-negative subjects. EBV status was not associated with time of PTLD development in our study. This finding is in contrast with previous reports showing that EBV-negative PTLD occurs later after liver transplantation when compared to EBV-positive PTLD patients. We showed that a serum tacrolimus cutoff value of 11.1 ng/mL is associated with post-PTLD survival.

### Applications

Adjustment of tacrolimus level to lower than 11.1 ng/mL may help improve post-PTLD survival of patients.

### Peer-review

The reviewer has read with interest the manuscript entitled, "Post-transplant lymphoproliferative disorder after liver transplantation: incidence, long-term survival and impact of serum tacrolimus level". Eshraghian and colleagues performed a retrospective single-center study with a wide recruitment period, including 53 liver transplant patients who developed PTLD (40 pediatric and 13 adult cases). The authors evaluated the risk factors affecting post-PTLD survival of patients. They found that EBV-negative recipients and multi-organ involvement are the two main risk factors of lower post-PTLD survival. They further found within a pediatric recipient cohort that higher serum tacrolimus level was associated with poor survival after PTLD development.

## REFERENCES


1 **Starzl TE**, Fung JJ. Themes of liver transplantation. *Hepatology* 2010; **51**: 1869-1884 [PMID: 20235333 DOI: 10.1002/hep.23595]

2 **Penn I**. Posttransplantation de novo tumors in liver allograft recipients. *Liver Transpl Surg* 1996; **2**: 52-59 [PMID: 9346628]

3 **Vaysberg M**, Lambert SL, Krams SM, Martinez OM. Activation of the JAK/STAT pathway in Epstein Barr virus+-associated posttransplant lymphoproliferative disease: role of interferon-gamma. *Am J Transplant* 2009; **9**: 2292-2302 [PMID: 19656130 DOI: 10.1111/j.1600-6143.2009.02781]

4 **Loren AW**, Porter DL, Stadtmauer EA, Tsai DE. Post-transplant lymphoproliferative disorder: a review. *Bone Marrow Transplant* 2003; **31**: 145-155 [PMID: 12621474 DOI: 10.1038/sj.bmt.1703806]

5 **Cohen JI**. Epstein-Barr virus lymphoproliferative disease associated with acquired immunodeficiency. *Medicine* (Baltimore) 1991; **70**: 137-160 [PMID: 1848644]

6 **Thorley-Lawson DA**, Gross A. Persistence of the Epstein-Barr virus and the origins of associated lymphomas. *N Engl J Med* 2004; **350**: 1328-1337 [PMID: 15044644 DOI: 10.1056/NEJMra032015]

7 **Evens AM**, Roy R, Sterrenberg D, Moll MZ, Chadburn A, Gordon LI. Post-transplantation lymphoproliferative disorders: diagnosis, prognosis, and current approaches to therapy. *Curr Oncol Rep* 2010; **12**: 383-394 [PMID: 20963522 DOI: 10.1007/s11912-010-0132-1]

8 **Duvoux C**, Pageaux GP, Vanlemmens C, Roudot-Thoraval F, Vincens-Rolland AL, Hézode C, Gaulard P, Miguet JP, Larrey D, Dhumeaux D, Cherqui D. Risk factors for lymphoproliferative disorders after liver transplantation in adults: an analysis of 480 patients. *Transplantation* 2002; **74**: 1103-1109 [PMID: 12438954 DOI: 10.1097/01.TP.0000031543.16208.1C]

9 **McLaughlin K**, Wajstaub S, Marotta P, Adams P, Grant DR, Wall WJ, Jevnikar AM, Rizkalla KS. Increased risk for posttransplant lymphoproliferative disease in recipients of liver transplants with hepatitis C. *Liver Transpl* 2000; **6**: 570-574 [PMID: 10980055 DOI: 10.1053/jlts.2000.7578]

10 **Guthery SL**, Heubi JE, Bucuvalas JC, Gross TG, Ryckman FC, Alonso MH, Balistreri WF, Hornung RW. Determination of risk factors for Epstein-Barr virus-associated posttransplant lymphoproliferative disorder in pediatric liver transplant recipients using objective case ascertainment. *Transplantation* 2003; **75**: 987-993 [PMID: 12698085 DOI: 10.1097/01.TP.0000057244.03192.BD]

11 **Newell KA**, Alonso EM, Whitington PF, Bruce DS, Millis JM, Piper JB, Woodle ES, Kelly SM, Koeppen H, Hart J, Rubin CM, Thistlethwaite JR. Posttransplant lymphoproliferative disease in pediatric liver transplantation. Interplay between primary Epstein-Barr virus infection and immunosuppression. *Transplantation* 1996; **62**: 370-375 [PMID: 8779685]

12 **Cacciarelli TV**, Reyes J, Jaffe R, Mazariegos GV, Jain A, Fung JJ, Green M. Primary tacrolimus (FK506) therapy and the long-term risk of post-transplant lymphoproliferative disease in pediatric liver transplant recipients. *Pediatr Transplant* 2001; **5**: 359-364 [PMID: 11560756]

13 **González-Barca E**, Domingo-Domenech E, Capote FJ, Gómez-Codina J, Salar A, Bailen A, Ribera JM, López A, Briones J, Muñoz A, Encuentra M, de Sevilla AF. Prospective phase II trial of extended treatment with rituximab in patients with B-cell post-transplant lymphoproliferative disease. *Haematologica* 2007; **92**: 1489-1494 [PMID: 18024397 DOI: 10.3324/haematol.11360]

14 **Choquet S**, Leblond V, Herbrecht R, Socié G, Stoppa AM, Vandenberghe P, Fischer A, Morschhauser F, Salles G, Feremans W, Vilmer E, Peraldi MN, Lang P, Lebranchu Y, Oksenhendler E, Garnier JL, Lamy T, Jaccard A, Ferrant A, Offner F, Hermine O, Moreau A, Fafi-Kremer S, Morand P, Chatenoud L, Berriot-Varoqueaux N, Bergougnoux L, Milpied N. Efficacy and safety of rituximab in B-cell post-transplantation lymphoproliferative disorders: results of a prospective multicenter phase 2 study. *Blood* 2006; **107**: 3053-3057 [PMID: 16254143 DOI: 10.1182/blood-2005-01-0377]

15 **Majewski M**, Korecka M, Kossev P, Li S, Goldman J, Moore J, Silberstein LE, Nowell PC, Schuler W, Shaw LM, Wasik MA. The immunosuppressive macrolide RAD inhibits growth of human Epstein-Barr virus-transformed B lymphocytes in vitro and in vivo: A potential approach to prevention and treatment of posttransplant lymphoproliferative disorders. *Proc Natl Acad Sci USA* 2000; **97**: 4285-4290 [PMID: 10759564 DOI: 10.1073/pnas.080068597]

16 **Harris N**, Swerdlow S, Frizzera G, Knowles D. Post-transplant lymphoproliferative disorders. In: Jaffe E, Harris N, Stein H, Vardiman J, editors. World Health Organization classification of tumours. Pathology and genetics of tumours of hematopoietic and lymphoid tissues. Lyon: IARC Press, 2001: 264-269

17 **Lust JA**. Molecular genetics and lymphoproliferative disorders. *J Clin Lab Anal* 1996; **10**: 359-367 [PMID: 8951602]

18 **Cox KL**, Lawrence-Miyasaki LS, Garcia-Kennedy R, Lennette ET, Martinez OM, Krams SM, Berquist WE, So SK, Esquivel CO. An increased incidence of Epstein-Barr virus infection and

lymphoproliferative disorder in young children on FK506 after liver transplantation. *Transplantation* 1995; **59**: 524-529 [PMID: 7533344]

19 **Koch DG**, Christiansen L, Lazarchick J, Stuart R, Willner IR, Reuben A. Posttransplantation lymphoproliferative disorder--the great mimic in liver transplantation: appraisal of the clinicopathologic spectrum and the role of Epstein-Barr virus. *Liver Transpl* 2007; **13**: 904-912 [PMID: 17539010 DOI: 10.1002/lt.21152]

20 **Fernández MC**, Bes D, De Dávila M, López S, Cambaceres C, Dip M, Imventarza O. Post-transplant lymphoproliferative disorder after pediatric liver transplantation: characteristics and outcome. *Pediatr Transplant* 2009; **13**: 307-310 [PMID: 18346039 DOI: 10.1111/j.1399-3046.2008.00914.x]

21 **Leblond V**, Choquet S. Lymphoproliferative disorders after liver transplantation. *J Hepatol* 2004; **40**: 728-735 [PMID: 15094218 DOI: 10.1016/j.jhep.2004.03.006]

22 **Haseli N**, Hassanzadeh J, Dehghani SM, Bahador A, Malek Hosseini SA. Long-term survival and its related factors in pediatric liver transplant recipients of shiraz transplant center, shiraz, iran in 2012. *Hepat Mon* 2013; **13**: e10257 [PMID: 24065996 DOI: 10.5812/hepatmon.10257]

23 **Ghobrial IM**, Habermann TM, Macon WR, Ristow KM, Larson TS, Walker RC, Ansell SM, Gores GJ, Stegall MD, McGregor CG. Differences between early and late posttransplant lymphoproliferative disorders in solid organ transplant patients: are they two different diseases? *Transplantation* 2005; **79**: 244-247 [PMID: 15665775]

24 **Leblond V**, Davi F, Charlotte F, Dorent R, Bitker MO, Sutton L, Gandjbakhch I, Binet JL, Raphael M. Posttransplant lymphoproliferative disorders not associated with Epstein-Barr virus: a distinct entity? *J Clin Oncol* 1998; **16**: 2052-2059 [PMID: 9626203]

25 **Evens AM**, David KA, Helenowski I, Nelson B, Kaufman D, Kircher SM, Gimelfarb A, Hattersley E, Mauro LA, Jovanovic B, Chadburn A, Stiff P, Winter JN, Mehta J, Van Besien K, Gregory S, Gordon LI, Shammo JM, Smith SE, Smith SM. Multicenter analysis of 80 solid organ transplantation recipients with post-transplantation lymphoproliferative disease: outcomes and prognostic factors in the modern era. *J Clin Oncol* 2010; **28**: 1038-1046 [PMID: 20085936 DOI: 10.1200/JCO.2009.25.4961]

26 **Kremers WK**, Devarbhavi HC, Wiesner RH, Krom RA, Macon WR, Habermann TM. Post-transplant lymphoproliferative disorders following liver transplantation: incidence, risk factors and survival. *Am J Transplant* 2006; **6**: 1017-1024 [PMID: 16611339 DOI: 10.1111/j.1600-6143.2006]

27 **Maecker B**, Jack T, Zimmermann M, Abdul-Khaliq H, Burdelski M, Fuchs A, Hoyer P, Koepf S, Kraemer U, Laube GF, Müller-Wiefel DE, Netz H, Pohl M, Toenshoff B, Wagner HJ, Wallot M, Welte K, Melter M, Offner G, Klein C. CNS or bone marrow involvement as risk factors for poor survival in post-transplantation lymphoproliferative disorders in children after solid organ transplantation. *J Clin Oncol* 2007; **25**: 4902-4908 [PMID: 17971586 DOI: 10.1200/JCO.2006.10.2392]

28 **Elstrom RL**, Andreadis C, Aqui NA, Ahya VN, Bloom RD, Brozena SC, Olthoff KM, Schuster SJ, Nasta SD, Stadtmauer EA, Tsai DE. Treatment of PTLD with rituximab or chemotherapy. *Am J Transplant* 2006; **6**: 569-576 [PMID: 16468968 DOI: 10.1111/j.1600-6143.2005]

29 **Dotti G**, Fiocchi R, Motta T, Mammana C, Gotti E, Riva S, Cornelli P, Gridelli B, Viero P, Oldani E, Ferrazzi P, Remuzzi G, Barbui T, Rambaldi A. Lymphomas occurring late after solid-organ transplantation: influence of treatment on the clinical outcome. *Transplantation* 2002; **74**: 1095-1102 [PMID: 12438953 DOI: 10.1097/01.TP.0000030637.87507.3C]

30 **Nelson BP**, Nalesnik MA, Bahler DW, Locker J, Fung JJ, Swerdlow SH. Epstein-Barr virus-negative post-transplant lymphoproliferative disorders: a distinct entity? *Am J Surg Pathol* 2000; **24**: 375-385 [PMID: 10716151]

31 **Schubert S**, Renner C, Hammer M, Abdul-Khaliq H, Lehmkuhl HB, Berger F, Hetzer R, Reinke P. Relationship of immunosuppression to Epstein-Barr viral load and lymphoproliferative disease in pediatric heart transplant patients. *J Heart Lung Transplant* 2008; **27**: 100-105 [PMID: 18187094 DOI: 10.1016/j.healun.2007.09.027]

32 **Swinnen LJ**, Costanzo-Nordin MR, Fisher SG, O'Sullivan EJ, Johnson MR, Heroux AL, Dizikes GJ, Pifarre R, Fisher RI. Increased incidence of lymphoproliferative disorder after immunosuppression with the monoclonal antibody OKT3 in cardiac-transplant recipients. *N Engl J Med* 1990; **323**: 1723-1728 [PMID: 2100991 DOI: 10.1056/NEJM199012203232502]

33 **Ganschow R**, Schulz T, Meyer T, Broering DC, Burdelski M. Low-dose immunosuppression reduces the incidence of post-transplant lymphoproliferative disease in pediatric liver graft recipients. *J Pediatr Gastroenterol Nutr* 2004; **38**: 198-203 [PMID: 14734884]

34 **Molmenti EP**, Nagata DE, Roden JS, Squires RH, Molmenti H, Fasola CG, Winick N, Tomlinson G, Lopez MJ, D'Amico L, Dyer HL, Savino AC, Sanchez EQ, Levy MF, Goldstein RM, Andersen JA, Klintmalm GB. Post-transplant lymphoproliferative syndrome in the pediatric liver transplant population. *Am J Transplant* 2001; **1**: 356-359 [PMID: 12099380]

35 **Geramizadeh B**, Malek-Hosseini SA, Bahador A, Salahi H, Nikeghbalian S, Sharifian M, Lankarani KB, Imanieh MH, Dehghani M. Post-transplantation lymphoproliferative disorder after liver transplantation: report of 5 cases among more than 550 liver transplants in Iran. *Arch Iran Med* 2010; **13**: 417-419 [PMID: 20804309]

**P- Reviewer**: Kin T, Salvadori M, Suzuki M **S- Editor**: Qi Y
**L- Editor**: Filipodia **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/

*World J Gastroenterol* 2017 February 21; 23(7): 1233-1240

DOI: 10.3748/wjg.v23.i7.1233

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

ORIGINAL ARTICLE

Retrospective Study

# High levels of serum platelet-derived growth factor-AA and human epidermal growth factor receptor-2 are predictors of colorectal cancer liver metastasis

Hong-Da Pan, Yi-Fan Peng, Gang Xiao, Jin Gu

Hong-Da Pan, Gang Xiao, Department of Gastrointestinal Surgery, Beijing Hospital, National Center of Gerontology, Beijing 100730, China

Yi-Fan Peng, Jin Gu, Department of Colorectal Surgery, Peking University Cancer Hospital and Institute, Beijing Cancer Hospital, Beijing 100142, China

Author contributions: Pan HD and Peng YF performed the majority of experiments, and were also involved in designing the study and writing the manuscript; they contributed equally to this work, and should be regarded as co-first authors; Xiao G and Gu J designed the study and revised the manuscript.

Supported by the Scientific Research Fund of Peking University Cancer Hospital, No. 2013 zizhu-8.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Peking University Cancer Hospital and Institute.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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

Data sharing statement: No additional data are available.



Manuscript source: Unsolicited manuscript

Correspondence to: Jin Gu, MD, FACS, Professor, Department of Colorectal Surgery, Peking University Cancer Hospital and Institute, Beijing Cancer Hospital, No. 52 Fucheng Road, Haidian District, Beijing 100142, China. zlgujin@126.com
Telephone: +86-10-88196086

Received: October 31, 2016
Peer-review started: November 1, 2016
First decision: December 2, 2016
Revised: December 13, 2016
Accepted: January 3, 2017
Article in press: January 3, 2017
Published online: February 21, 2017

## Abstract

### *AIM*

To develop predictive markers in blood for colorectal cancer liver metastasis.

### *METHODS*

Twenty colorectal cancer patients were selected and divided into two groups. Group A consisted of 10 patients whose pathological TNM stage was ⅢC (T3-4N2M0), while another 10 patients with synchronous liver metastasis (TNM stage Ⅳ) were recruited for group B. During the surgical procedure, a 10-mL drainage vein (DV) blood sample was obtained from the DV of the tumor-bearing segment prior to the ligation of the DV. At the same time, a 10-mL peripheral vein (PV) blood sample was collected *via* peripheral venipuncture. The serum levels of 24 molecules that are potentially involved in the mechanism of liver metastasis in both DV blood and PV blood were analyzed by using high-throughput enzyme-linked immunosorbent assay technology.

### *RESULTS*

Univariate analysis revealed that platelet-derived

growth factor AA (PDGFAA) in DV blood (dPDGFAA) ($P$ = 0.001), PDGFAA in PV blood (pPDGFAA) ($P$ = 0.007), and human epidermal growth factor receptor-2 in PV blood (pHER2) ($P$ = 0.001), pMMP7 ($P$ = 0.028), pRANTES ($P$ = 0.013), and pEGF ($P$ = 0.007) were significantly correlated with synchronous liver metastasis. Multivariate analysis identified dPDGFAA (HR = 1.001, $P$ = 0.033) and pHER2 (HR = 1.003, $P$ = 0.019) as independent predictive factors for synchronous liver metastasis. Besides, high peripheral HER2 level may also be a risk factor for metachronous liver metastasis, although the difference did not reach statistical significance ($P$ = 0.06). Significant correlations were found between paired DV and PV blood levels for PDGFAA ($r$ = 0.794, $P$ < 0.001), but not for HER2 ($r$ = 0.189, $P$ = 0.424).

***CONCLUSION***

PDGFAA in tumor drainage and HER2 in PV blood may be useful predictive factors for synchronous liver metastasis of colorectal cancer.

**Key words:** Platelet-derived growth factor AA; Human epidermal growth factor receptor-2; Colorectal cancer; Liver metastasis



**Core tip:** We investigated the serum levels of most commonly studied tumor growth factors that are known to be associated with the mechanism of liver metastasis not only in peripheral vein (PV) blood but also in tumor drainage vein (DV) blood. To our knowledge, this is one of few studies taking DV blood into analysis and comparing the differences in serum molecules between PV blood and DV blood. We found that platelet-derived growth factor AA in tumor drainage and human epidermal growth factor receptor 2 in PV blood may be useful predictive factors for synchronous liver metastasis of colorectal cancer.

Pan HD, Peng YF, Xiao G, Gu J. High levels of serum platelet-derived growth factor-AA and human epidermal growth factor receptor-2 are predictors of colorectal cancer liver metastasis. *World J Gastroenterol* 2017; 23(7): 1233-1240 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1233.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1233

## INTRODUCTION

Liver metastasis occurs in almost 50% of patients with colorectal cancer and it is the leading cause of death from colorectal cancer[1]. Thus, developing useful predictive markers for screening patients at high risk for liver metastasis is of prime importance. Although many studies have reported predictive factors for liver metastasis[2-13], the fundamental pathogenesis remains unclear. The majority of previous studies mainly focused on clinicopathologic characteristics and features in tumor tissue specimens.

Several studies have revealed that circulating factors constitute significant predictors of metastasis in patients with colorectal cancer[2-13]. However, the prognostic value of factors in blood, especially in tumor drainage vein (DV) blood, has received relatively little attention. Theoretically, factors produced by the primary tumor must first go through the metabolism or undergo breakdown in the liver, lungs, and other organs before they can reach the peripheral veins (PV). Therefore, the levels of factors in a tumor DV could provide more accurate information about the primary tumor than assessments performed using PV blood.

This study was undertaken to develop predictive markers in blood for liver metastasis and improve our understanding of its prognosis. We investigated the serum levels of the most commonly studied tumor growth factors that are known to be associated with the mechanism of liver metastasis not only in PV blood but also in tumor DV blood.

## MATERIALS AND METHODS

### *Patients*

This retrospective study was approved by the Institutional Review Board and written informed consent was obtained from each patient before the study. The data of patients who underwent surgery for colorectal cancer in the Department of Colorectal Surgery, Peking University Cancer Hospital, from January 2011 to May 2011 were identified from a surgical database. Patients pathologically diagnosed with colonic or rectal adenocarcinoma, with radiologically confirmed non-metastasis (M0) or synchronous liver metastasis (M1), were included. Patients with extrahepatic metastasis, patients who received prior chemotherapy or radiotherapy, or patients who had a history of cancer within 5 years were excluded from the study.

Twenty patients were selected for the study and they were divided into two groups. Group A consisted of 10 patients whose pathological TNM (tumor-node-metastasis) stage was ⅢC ($T_{3-4}N_2M_0$), while another 10 patients with synchronous liver metastasis (TNM stageⅣ) were recruited for group B. The diagnosis of synchronous liver metastasis was confirmed by intraoperative biopsy, liver magnetic resonance imaging, or positron emission tomography (PET)/computed tomography (CT). Abdominal CT and/or PET scans ruled out extrahepatic metastasis. The patients in group A received curative surgery for the primary colorectal cancer. In group B, nine patients received surgery for the primary tumor only and one patient underwent simultaneous liver resection with curative intent. All of the patients were recommended to receive postoperative chemotherapy.

**Table 1 Patient clinical and pathological characteristics *n* (%)**

| Clinical variable | Group A (*n* = 10) | Group B (*n* = 10) | *P* value |
|---|---|---|---|
| mean age (yr) | 68 (41-79) | 60 (30-73) | 0.150 |
| Male | 6 (60) | 4 (40) | 0.625 |
| pT stage | | | 1.000 |
| pT3 | 6 (60) | 6 (60) | |
| pT4 | 4 (40) | 4 (40) | |
| pN stage | | | 0.168 |
| pN0 | 0 | 1 (10) | |
| pN1 | 0 | 3 (30) | |
| pN2 | 4 (40) | 6 (60) | |
| Location | | | 1.000 |
| Right colon | 4 (40) | 4 (40) | |
| Left colon/rectum | 6 (60) | 6 (60) | |
| Histology differentiation | | | 1.000 |
| Well | 2 (20) | 2 (20) | |
| Moderately | 4 (40) | 4 (40) | |
| Poorly/mucinous | 4 (40) | 4 (40) | |
| Lymphovascular invasion | 6 (60) | 4 (40) | 0.625 |
| Preoperative CEA (median, range) | 1.74 (1.18-11.0) | 9.64 (1.12-512.9) | 0.017 |

Quantitative variables are represented as median (range); qualitative characteristics are represented as *n* (%). CEA: Carcinoembryonic antigen.

### Follow-up

Patients were followed every 3 mo during the first 2 years, and every 6 mo thereafter in the outpatient clinic. At each follow-up, physical examination, detection of serum tumor markers, routine blood tests, and serum chemistry profiling were performed. Proctoscopy, abdominal ultrasonography, abdomen and pelvis CT, and chest radiography were performed every 6-12 mo. The primary endpoints were metastasis and local recurrence.

### Selection of candidate molecules

We searched PubMed using the following keywords: colorectal adenocarcinoma, liver metastasis, and tumor growth factor. Then we reviewed the published relevant articles and selected molecules that could be detected in serum and that could be analyzed by enzyme-linked immunosorbent assay (ELISA) with commercially available antibodies. Twenty-four molecules that are potentially involved in the mechanism of liver metastasis were identified for further investigation, including fibroblast growth factor-b, hepatocyte growth factor, platelet-derived growth factor-BB (PDGFBB), vascular endothelial growth factor (VEGF), matrix metalloproteinase 2, VEGFD, CD30, myeloperoxidase, total plasminogen activator inhibitor 1, regulated on activation normal T cell expressed and secreted (RANTES), Resistin, tissue inhibitor of metalloproteinase (TIMP) 1, TIMP2, vascular cell adhesion molecule 1, epidermal growth factor (EGF), intercellular adhesion molecule 3, platelet-derived growth factor AA (PDGFAA), P-selectin, E-cadherin, osteopontin, MMP9, human epidermal growth factor receptor 2 (HER2), and MMP7.

### Blood sample collection and ELISA

Once the peritoneal cavity was opened, a 10-mL DV blood sample was obtained from the DV of the tumor-bearing segment prior to the ligation of the DV and removal of the tumor. While sampling the DV blood, a 10-mL PV blood sample was collected *via* peripheral venipuncture by a circuit nurse. Blood samples were instantly centrifuged and serum and cells were stored separately at -80 ℃ for later analysis. Measurement of serum levels of all 24 molecules was conducted by ELISA using commercially available kits (LightArray Biotech Co., Ltd) according to the manufacturer's instructions.

### Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Mac, Version 20.0 (Armonk, New York: IBM Corp.). For continuous covariates, means were compared by the Student's *t*-test, while nonparametric data were assessed by the Mann-Whitney *U* test (GraphPad Software Prism 5). Categorical variables were analyzed by the Pearson chi-squared or Fisher's exact test, when appropriate. Variables with a *P* value less than 0.2 during univariate analysis were selected for multivariate analysis using a logistic regression model. Correlations of factor levels in tumor drainage and in PV blood were performed by the Spearman rank correlation analysis. All hypothesis tests were two-sided, with a *P* value less than 0.05 and 0.1 considered statistically significant in univariate and multivariate analysis, respectively.

## RESULTS

### Patient characteristics

The median follow-up period of the patients was 40 mo (range, 38-42 mo), with no patients lost to follow-up. The clinical and pathological characteristics were comparable between the two groups (Table 1). The median age of group A (68 years) was higher than that of group B (60 years), but the difference was not statistically significant ($P$ = 0.150). In both groups A and B, 60% of the patients had pT3 disease, and the remaining 40% had pT4. All patients in group A had pN2, while 60% of patients in group B had pN2, but the difference did not reach statistical significance ($P$ = 0.168). Location and histological differentiation of the tumor were not different between groups A and B. The preoperative CEA level was significantly higher in group B (1.74 ng/mL *vs* 9.64 ng/mL, $P$ = 0.017). No perioperative mortality was reported in either group. Five patients in group A developed metachronous distant metastases, but no local recurrence was observed. One patient in group B who received simultaneous liver resection with curative intent developed

**Table 2 Analyses of serum levels of factors between group A and group B in tumor drainage and peripheral veins**

| | Drainage blood | | | Peripheral blood | | |
|---|---|---|---|---|---|---|
| | Group A (non-metastatic) | Group B (metastatic) | *P* value | Group A (non-metastatic) | Group B (metastatic) | *P* value |
| RANTES (ng/mL) | 52.61 ± 35.37 | 95.17 ± 70.21 | 0.104 | 36.34 ± 23.64 | 66.60 ± 25.55 | 0.013 |
| EGF (pg/mL) | 150.09 ± 70.40 | 270.67 ± 163.70 | 0.053 | 135.16 ± 67.01 | 289.36 ± 147.24 | 0.007 |
| PDGFAA (ng/mL) | 2.30 ± 0.80 | 4.62 ± 1.60 | 0.001 | 2.38 ± 0.85 | 3.99 ± 1.46 | 0.007 |
| HER2 (ng/mL) | 2.54 ± 0.68 | 2.95 ± 1.02 | 0.296 | 2.01 ± 0.55 | 2.91 ± 0.49 | 0.001 |
| MMP7 (ng/mL) | 2.77 ± 1.47 | 4.57 ± 2.70 | 0.080 | 2.20 ± 1.08 | 3.98 ± 2.10 | 0.028 |

Values are presented as mean ± SD. RANTES: Regulated on activation normal T cell expressed and secreted; EGF: Epidermal growth factor; PDGFAA: Platelet-derived growth factor AA; HER2: Human epidermal growth factor receptor 2; MMP7: Matrix metalloproteinase 7.

**Table 3 Correlation between factor levels in peripheral and tumor drainage venous blood**

| Correlation | *r* | *P* value |
|---|---|---|
| dPDGFAA-pPDGFAA | 0.794 | < 0.001 |
| dHER2-pHER2 | 0.189 | 0.424 |
| dRANTES-pRANTES | 0.705 | 0.001 |
| dEGF-pEGF | 0.605 | 0.005 |
| dMMP7-mMMP7 | 0.863 | < 0.001 |
| pEGF-dPDGFAA | 0.678 | 0.001 |
| pEGF-pPDGFAA | 0.887 | < 0.001 |
| pEGF-pHER2 | 0.489 | 0.029 |
| pHER2-pMMP7 | 0.529 | 0.016 |

d means factors in drainage vein blood; p means factors in peripheral vein blood. RANTES: Regulated on activation normal T cell expressed and secreted; EGF: Epidermal growth factor; PDGFAA: Platelet-derived growth factor AA; HER2: Human epidermal growth factor receptor 2; MMP7: Matrix metalloproteinase 7.

a hepatic recurrence.

### *Serum levels of factors related to synchronous liver metastasis*

In the PV, serum levels of RANTES (36.34 ± 23.64 *vs* 66.60 ± 25.55, $P = 0.013$), EGF (135.16 ± 67.01 *vs* 289.36 ± 147.24, $P = 0.007$), PDGFAA (2.38 ± 0.85 *vs* 3.99 ± 1.46, $P = 0.007$), HER2 (2.01 ± 0.55 *vs* 2.91 ± 0.49, $P = 0.001$), and MMP7 (2.20 ± 1.08 *vs* 3.98 ± 2.10, $P = 0.028$) in group B were significantly higher than those in group A. In the DV, only serum level of PDGFAA in group B was significantly higher than that in group A (2.30 ± 0.80 *vs* 4.62 ± 16.00, $P = 0.001$) (Table 2). Other clinicopathological factors were also included in the univariate analysis; however, no significant difference was found (Figure 1).

Variables with a *P*-value less than 0.2 in univariate analysis were selected for multivariate analysis. A logistic regression model was used to identify factors associated with synchronous liver metastasis. The levels of PDGFAA in the DV (HR = 1.001, $P = 0.033$) and HER2 (HR = 1.003, $P = 0.019$) in the PV were identified as independent predictive factors for synchronous liver metastasis.

### *Correlation between factor levels in tumor DV and PV blood*

Factors that showed statistical significance in univariate analysis were selected for correlation analysis using Spearman rank correlation analysis (Table 3). pEGF level was significantly correlated with the level of pPDGFAA ($r = 0.887$, $P < 0.001$). The levels of PDGFAA ($r = 0.794$, $P < 0.001$) and MMP7 ($r = 0.863$, $P < 0.001$) in tumor DV blood samples were highly correlated with those in paired PV blood samples. It was noteworthy that there was a very low correlation between paired tumor DV and PV blood levels for HER2 ($r = 0.189$, $P = 0.424$).

### *Factors associated with metachronous liver metastasis*

In the subgroup analysis of group A, we investigated factors correlated with metachronous liver metastasis by univariate and multivariate analyses; however, no factor was found to be associated with metachronous liver metastasis. The patients who developed metachronous metastasis had higher pHER2 levels than those who did not, but there was no statistical significance (2.33 ng/mL *vs* 1.70 ng/mL, $P = 0.06$).

## DISCUSSION

This study is one of few to analyze serum molecules not only in PV blood but also in DV blood to develop predictive factors for colorectal cancer liver metastasis. Group A and group B consisted of patients with stage ⅢC and stage Ⅳ colorectal cancer, respectively. Except for the TNM stage, other clinicopathological characteristics were comparable between the two groups.

There is a great difference in the 5-year overall survival between stage ⅢC and Ⅳ colorectal cancer (28% *vs* 5.7% for colon cancer[14], and 33.4% *vs* 6.0% for rectal cancer[15]). Second only to stage Ⅳ, stage ⅢC has the worst prognosis in locally advanced colorectal cancer. It is notable that although lymph node metastasis is present in stage ⅢC cases, the disease is still confined to the local region rather than spreads to distant organs. Therefore, we propose the hypothesis that stage Ⅳ tumors may have stronger capabilities of proliferation, colonization, invasion, and angiogenesis than stage ⅢC tumors. There might be some critical factors that trigger the final progression from locally advanced to systemic metastasis. The rationale behind this study is that if the difference

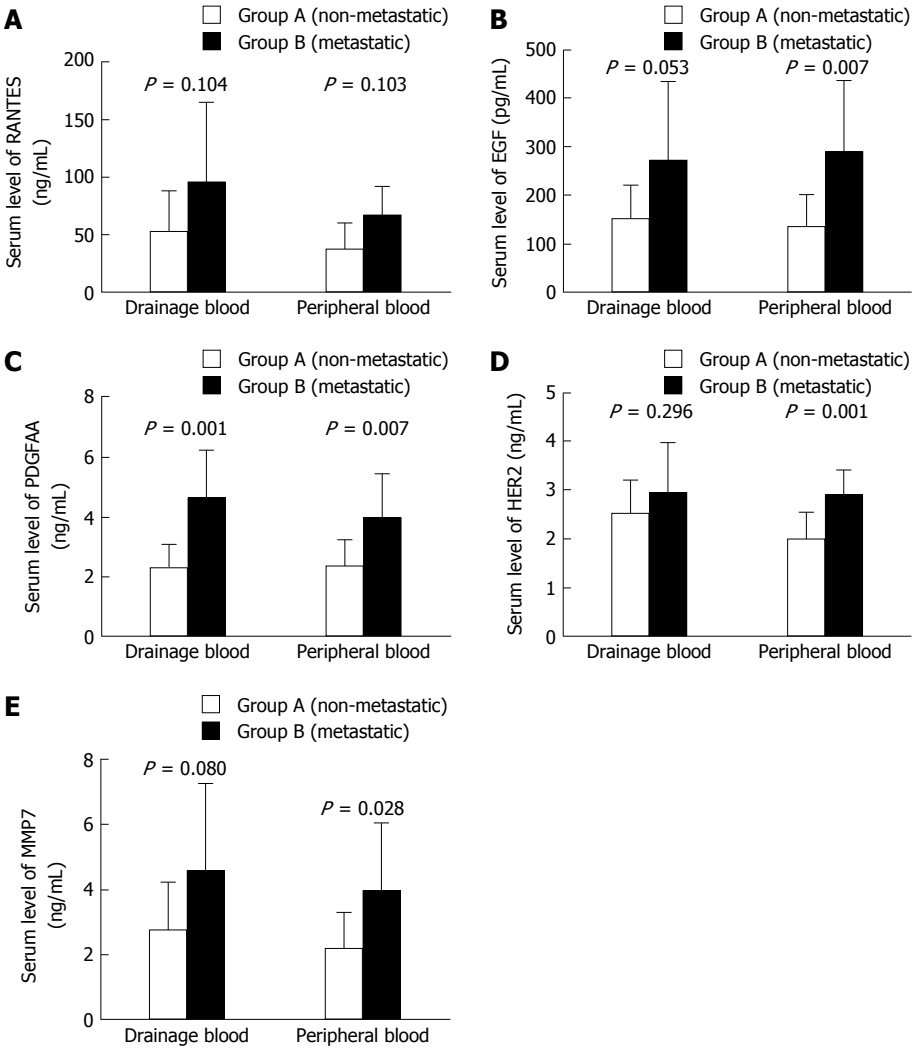



**Figure 1 Serum levels of factors in group A and group B in the tumor drainage and peripheral blood.** A: RANTES; B: EGF; C: PDGFAA; D: HER2; E: MMP7. RANTES: Regulated on activation normal T cell expressed and secreted; EGF: Epidermal growth factor; PDGFAA: Platelet-derived growth factor AA; HER2: Human epidermal growth factor receptor 2; MMP7: Matrix metalloproteinase 7.

between ⅢC and Ⅳ can be identified, we may possibly clarify the underlying mechanism of colorectal cancer liver metastasis.

Many studies have investigated the molecules not only in PV blood, but also in tumor DV blood, and the relationship between recurrence or metastasis of colorectal cancer and those molecules has been explored. Tien *et al*[6] postulated that if the primary colorectal tumor is the exclusive or major source of tumor growth factors, the levels of serum tumor growth factors should be higher in tumor DV blood than in PV blood, and there should be a good correlation for the levels of all tumor growth factors between paired tumor DV and PV blood. After entering the circulation, molecules that are expressed and secreted by the primary tumor may be metabolized and broken down by the liver, lungs, or other organs before they reach the PV.

These authors[6] investigated the relationship between tumor stage and disease recurrence with the level of angiogenic factors in both the DV and PV in patients with colon cancer. The results showed that the level of VEGF in the tumor DV blood was an independent predictor of disease recurrence. A previous study by Min *et al*[5] demonstrated that colon cancer patients with high levels of VEGF and TIMP-1 in the DV had a high risk of metachronous liver metastasis and hepatic recurrence following the resection of synchronous liver metastasis.

The results of the present study have shown that the elevated levels of PDGFAA detected in the DV blood of the primary tumor, and HER2 in the PV blood, were significantly correlated with synchronous liver metastasis. Moreover, high peripheral HER2 level may also be a risk factor for metachronous liver metastasis, although the difference did not reach statistical significance ($P = 0.06$).

Platelet-derived growth factor (PDGF) is one of the numerous growth factors that regulate cell growth and division. In chemical terms, PDGF is a dimeric glycoprotein composed of two A (PDGFAA) or two B (PDGFBB) chains or a combination of the

two (PDGFAB). In particular, it plays a significant role in angiogenesis and its overexpression leads to uncontrolled angiogenesis in the majority of solid organ cancers[16-18]. Several studies have shown that PDGF expression in tumor tissue is correlated with a poor prognosis of colorectal cancer[7,8]. However, there are few reports studying the relationship between serum PDGFAA levels and prognosis in colorectal cancer. Inanç *et al*[10] assessed the serum levels and prognostic role of tumor growth and angiogenic factors in patients with metastatic colorectal cancer treated with chemotherapy. These authors found that PDGFAA was significantly decreased in both patients with a partial response and stable disease.

HER2 (ErbB2) has been shown to play a role in the tumor growth process and it is an established therapeutic target in breast[19-21] and gastric cancers[22-24]. Nevertheless, the role of HER2 in colorectal cancer remains unclear, because conflicting data on the prevalence of HER2 expression have been reported[25-30]. Park *et al*[26] reported that 47.4% of patients with colorectal cancer were determined by immunohistochemistry to overexpress HER2. Patients with tumors with HER2 overexpression had a higher postoperative recurrence rate (39.3% *vs* 14.6%, $P = 0.013$), and its overexpression was associated with poorer 5-year survival rates (55.1% *vs* 78.3%, $P < 0.05$). However, the authors of the EXPERT-C trial reported that only 4.3% of patients had HER2 overexpression, and HER2 does not appear to represent a useful therapeutic target in high-risk rectal cancer[27]. Our previous study[28] showed that HER2 was overexpressed in 15% of rectal cancer patients who received neoadjuvant radiotherapy. Moreover, HER2 overexpression could be a predictive biomarker of distant metastasis in rectal cancer patients after preoperative radiotherapy. A recent study[29] assessed HER2-amplification/overexpression in 1914 stages Ⅱ-Ⅲ and Ⅳ CRC patients. The result showed that HER2 was not associated with overall survival or progression free survival. A higher proportion of HER2-overexpressing cases experienced recurrence, but the difference was not significant. Fusco *et al*[30] considered that HER2 status should be assessed as a putative biomarker of resistance to anti-EGFR therapy in KRAS wild-type patients.

In the correlation analysis, we found significant correlations between paired distal vein and PV blood levels for PDGFAA ($r = 0.794$, $P < 0.001$), but not for HER2 ($r = 0.189$, $P = 0.424$). These findings indicate that the primary tumor was the dominate source of PDGFAA. PDGFAA levels in tumor DV blood provided better prognostic information than those in PV blood. On the other hand, there may be some additional sources producing HER2 outside the primary tumor, perhaps the metastatic tumors in the liver, and therefore the HER2 level detected in the distal vein cannot faithfully reflect the characteristics of the tumor. In addition, a significant correlation was noted between pEGF and pPDGFAA levels, which indicated that EGF and PDGFAA have an interaction effect in the development of colorectal cancer liver metastasis.

### *Limitations*

The limitations of this study are obvious. The study only enrolled a small series of patients and these results require validation. Variations of the mesentery vein are very common, and exposure of the vein needs well-developed surgical skills. Blood from the tumor DV is difficult to obtain, and requires more effort than that from the PV. It is impossible to draw DV blood in non-surgical patients.

In conclusion, the present study analyzed the serum levels of 24 commonly studied tumor growth factors in both tumor drainage and PV blood from patients with colorectal cancer by using high-throughput ELISA technology. We found that PDGFAA in tumor drainage and HER2 in PV blood may be useful predictive factors for synchronous liver metastasis. PDGFAA levels in tumor DV blood provided better prognostic information than those in PV blood. On the contrary, HER2 levels in PV blood reflected tumor characteristics more accurately than those in tumor DV blood.

## COMMENTS

### *Background*

Liver metastasis occurs in almost 50% of patients with colorectal cancer and it is the leading cause of death. Developing useful predictive markers for screening patients at high risk for liver metastasis may optimize our therapy strategy. Previous studies mainly involved clinicopathologic characteristics and factors in tumor tissue specimens, however, the fundamental pathogenesis remains unclear.

### *Research frontiers*

Several studies have revealed that circulating factors constitute significant predictors of metastasis for patients with colorectal cancer. However, the prognostic value of factors in blood, especially in drainage venous (DV) blood, has received relatively little attention.

### *Innovations and breakthroughs*

The authors investigated the serum levels of most commonly studied tumor growth factors that are known to be associated with the mechanism of liver metastasis not only in peripheral venous (PV) blood but also in tumor DV blood. To our knowledge, this is one of few studies taking DV blood into analysis and comparing the differences of serum molecules between PV blood and DV blood.

### *Applications*

The present study provides us with a new angle of predicting liver metastasis of colorectal cancer. Platelet-derived growth factor AA (PDGFAA) in tumor drainage and human epidermal growth factor receptor 2 (HER2) in PV blood may constitute useful predictive factors for synchronous liver metastasis. PDGFAA levels in tumor DV blood provided better prognostic information than those in PV blood, while HER2 levels in PV blood reflected tumor characteristics more accurately than those in tumor DV blood.

### *Terminology*

Platelet-derived growth factor (PDGF) plays a significant role in blood vessel formation, the growth of blood vessels from already-existing blood vessel tissue.

Uncontrolled angiogenesis is a characteristic of cancer. PDGF is a dimeric glycoprotein composed of two A (-AA) or two B (-BB) chains or a combination of the two (-AB). HER2 is a member of the human epidermal growth factor receptor (HER/EGFR/ERBB) family. Amplification or overexpression of this oncogene has been shown to play an important role in the development and progression of certain aggressive types of cancer.

### *Peer-review*

This is an interesting manuscript about the predictors of colorectal cancer liver metastasis. In this study, twenty patients were selected for the study and they were divided into two groups. The serum levels of 24 molecules that are potentially involved in the mechanism of liver metastasis in both DV blood and PV blood were analyzed by using enzyme-linked immunosorbent assay technology.

## REFERENCES


1 **Rees M**, Tekkis PP, Welsh FK, O'Rourke T, John TG. Evaluation of long-term survival after hepatic resection for metastatic colorectal cancer: a multifactorial model of 929 patients. *Ann Surg* 2008; **247**: 125-135 [PMID: 18156932 DOI: 10.1097/SLA.0b013e31815aa2c2]

2 **Yoon SS**, Kim SH, Gonen M, Heffernan NM, Detwiller KY, Jarnagin WR, D'Angelica M, Blumgart LH, Tanabe KK, Dematteo RP. Profile of plasma angiogenic factors before and after hepatectomy for colorectal cancer liver metastases. *Ann Surg Oncol* 2006; **13**: 353-362 [PMID: 16474912 DOI: 10.1245/aso.2006.03.060]

3 **Ivankovics IG**, Fernandes LC, Saad SS, Matos D. Peripheral and mesenteric serum levels of CEA and cytokeratins, staging and histopathological variables in colorectal adenocarcinoma. *World J Gastroenterol* 2008; **14**: 6699-6703 [PMID: 19034974 DOI: 10.3748/wjg.14.6699]

4 **Kanellos I**, Zacharakis E, Kanellos D, Pramateftakis MG, Tsahalis T, Altsitsiadis E, Betsis D. Prognostic significance of CEA levels and detection of CEA mRNA in draining venous blood in patients with colorectal cancer. *J Surg Oncol* 2006; **94**: 3-8 [PMID: 16788936 DOI: 10.1002/jso.20549]

5 **Min BS**, Kim NK, Jeong HC, Chung HC. High levels of serum VEGF and TIMP-1 are correlated with colon cancer liver metastasis and intrahepatic recurrence after liver resection. *Oncol Lett* 2012; **4**: 123-130 [PMID: 22807974 DOI: 10.3892/ol.2012.691]

6 **Tien YW**, Chang KJ, Chiu YF, Huang KW, Lee PH. Comparison of angiogenic factor levels in tumor drainage and peripheral venous blood from colorectal cancer patients. *Ann Surg Oncol* 2006; **13**: 1357-1363 [PMID: 17001510 DOI: 10.1245/s10434-006-9042-8]

7 **Nakamura Y**, Tanaka F, Yoshikawa Y, Mimori K, Inoue H, Yanaga K, Mori M. PDGF-BB is a novel prognostic factor in colorectal cancer. *Ann Surg Oncol* 2008; **15**: 2129-2136 [PMID: 18478301 DOI: 10.1245/s10434-008-9943-9]

8 **Kitadai Y**, Sasaki T, Kuwai T, Nakamura T, Bucana CD, Hamilton SR, Fidler IJ. Expression of activated platelet-derived growth factor receptor in stromal cells of human colon carcinomas is associated with metastatic potential. *Int J Cancer* 2006; **119**: 2567-2574 [PMID: 16988946 DOI: 10.1002/ijc.22229]

9 **Braicu C**, Tudoran O, Balacescu L, Catana C, Neagoe E, Berindan-Neagoe I, Ionescu C. The significance of PDGF expression in serum of colorectal carcinoma patients--correlation with Duke's classification. Can PDGF become a potential biomarker? *Chirurgia* (Bucur) 2013; **108**: 849-854 [PMID: 24331325]

10 **Inanç M**, Er O, Karaca H, Berk V, Ozkan M, Saraymen R, Elmalı F. Prognostic value of tumor growth factor levels during chemotherapy in patients with metastatic colorectal cancer. *Med Oncol* 2012; **29**: 3119-3124 [PMID: 22580817 DOI: 10.1007/s12032-012-0250-8]

11 **Toiyama Y**, Fujikawa H, Kawamura M, Matsushita K, Saigusa S, Tanaka K, Inoue Y, Uchida K, Mohri Y, Kusunoki M. Evaluation of CXCL10 as a novel serum marker for predicting liver metastasis and prognosis in colorectal cancer. *Int J Oncol* 2012; **40**: 560-566 [PMID: 22038159 DOI: 10.3892/ijo.2011.1247]

12 **Beştaş R**, Kaplan MA, Işikdoğan A. The correlation between serum VEGF levels and known prognostic risk factors in colorectal carcinoma. *Hepatogastroenterology* 2014; **61**: 267-271 [PMID: 24901122]

13 **Martins SF**, Garcia EA, Luz MA, Pardal F, Rodrigues M, Filho AL. Clinicopathological correlation and prognostic significance of VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 expression in colorectal cancer. *Cancer Genomics Proteomics* 2013; **10**: 55-67 [PMID: 23603341]

14 **Gunderson LL**, Jessup JM, Sargent DJ, Greene FL, Stewart AK. Revised TN categorization for colon cancer based on national survival outcomes data. *J Clin Oncol* 2010; **28**: 264-271 [PMID: 19949014 DOI: 10.1200/JCO.2009.24.0952]

15 **Gunderson LL**, Jessup JM, Sargent DJ, Greene FL, Stewart A. Revised tumor and node categorization for rectal cancer based on surveillance, epidemiology, and end results and rectal pooled analysis outcomes. *J Clin Oncol* 2010; **28**: 256-263 [PMID: 19949015 DOI: 10.1200/JCO.2009.23.9194]

16 **Yuge R**, Kitadai Y, Shinagawa K, Onoyama M, Tanaka S, Yasui W, Chayama K. mTOR and PDGF pathway blockade inhibits liver metastasis of colorectal cancer by modulating the tumor microenvironment. *Am J Pathol* 2015; **185**: 399-408 [PMID: 25478811 DOI: 10.1016/j.ajpath.2014.10.014]

17 **Liu C**, Li J, Xiang X, Guo L, Tu K, Liu Q, Shah VH, Kang N. PDGF receptor-α promotes TGF-β signaling in hepatic stellate cells via transcriptional and posttranscriptional regulation of TGF-β receptors. *Am J Physiol Gastrointest Liver Physiol* 2014; **307**: G749-G759 [PMID: 25169976 DOI: 10.1152/ajpgi.00138.2014]

18 **Holleran G**, Hall B, O'Regan M, Smith S, McNamara D. Expression of Angiogenic Factors in Patients With Sporadic Small Bowel Angiodysplasia. *J Clin Gastroenterol* 2014; **49**: 831-836 [PMID: 25319741 DOI: 10.1097/mcg.0000000000000260]

19 **Slamon DJ**, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001; **344**: 783-792 [PMID: 11248153 DOI: 10.1056/NEJM200103153441101]

20 **Vogel CL**, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S, Stewart SJ, Press M. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol* 2002; **20**: 719-726 [PMID: 11821453 DOI: 10.1200/JCO.20.3.719]

21 **Romond EH**, Perez EA, Bryant J, Suman VJ, Geyer CE, Davidson NE, Tan-Chiu E, Martino S, Paik S, Kaufman PA, Swain SM, Pisansky TM, Fehrenbacher L, Kutteh LA, Vogel VG, Visscher DW, Yothers G, Jenkins RB, Brown AM, Dakhil SR, Mamounas EP, Lingle WL, Klein PM, Ingle JN, Wolmark N. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med* 2005; **353**: 1673-1684 [PMID: 16236738 DOI: 10.1056/NEJMoa052122]

22 **Gravalos C**, Jimeno A. HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. *Ann Oncol* 2008; **19**: 1523-1529 [PMID: 18441328 DOI: 10.1093/annonc/mdn169]

23 **Hofmann M**, Stoss O, Shi D, Büttner R, van de Vijver M, Kim W, Ochiai A, Rüschoff J, Henkel T. Assessment of a HER2 scoring system for gastric cancer: results from a validation study. *Histopathology* 2008; **52**: 797-805 [PMID: 18422971 DOI: 10.1111/j.1365-2559.2008.03028.x]

24 **Bang YJ**, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschoff J, Kang YK. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* 2010; **376**: 687-697 [PMID: 20728210 DOI: 10.1016/S0140-6736(10)61121-X]

25 **Ingold Heppner B**, Behrens HM, Balschun K, Haag J, Krüger S, Becker T, Röcken C. HER2/neu testing in primary colorectal carcinoma. *Br J Cancer* 2014; **111**: 1977-1984 [PMID: 25211663 DOI: 10.1038/bjc.2014.483]

26 **Park DI**, Kang MS, Oh SJ, Kim HJ, Cho YK, Sohn CI, Jeon WK, Kim BI, Han WK, Kim H, Ryu SH, Sepulveda AR. HER-2/neu overexpression is an independent prognostic factor in colorectal cancer. *Int J Colorectal Dis* 2007; **22**: 491-497 [PMID: 16947041 DOI: 10.1007/s00384-006-0192-8]

27 **Sclafani F**, Roy A, Cunningham D, Wotherspoon A, Peckitt C, Gonzalez de Castro D, Tabernero J, Glimelius B, Cervantes A, Eltahir Z, Oates J, Chau I. HER2 in high-risk rectal cancer patients treated in EXPERT-C, a randomized phase II trial of neoadjuvant capecitabine and oxaliplatin (CAPOX) and chemoradiotherapy (CRT) with or without cetuximab. *Ann Oncol* 2013; **24**: 3123-3128 [PMID: 24146218 DOI: 10.1093/annonc/mdt408]

28 **Yao YF**, Du CZ, Chen N, Chen P, Gu J. Expression of HER-2 in rectal cancers treated with preoperative radiotherapy: a potential biomarker predictive of metastasis. *Dis Colon Rectum* 2014; **57**: 602-607 [PMID: 24819100 DOI: 10.1097/dcr.0000000000000107]

29 **Richman SD**, Southward K, Chambers P, Cross D, Barrett J, Hemmings G, Taylor M, Wood H, Hutchins G, Foster JM, Oumie A, Spink KG, Brown SR, Jones M, Kerr D, Handley K, Gray R, Seymour M, Quirke P. HER2 overexpression and amplification as a potential therapeutic target in colorectal cancer: analysis of 3256 patients enrolled in the QUASAR, FOCUS and PICCOLO colorectal cancer trials. *J Pathol* 2016; **238**: 562-570 [PMID: 26690310 DOI: 10.1002/path.4679]

30 **Fusco N**, Bosari S. HER2 aberrations and heterogeneity in cancers of the digestive system: Implications for pathologists and gastroenterologists. *World J Gastroenterol* 2016; **22**: 7926-7937 [PMID: 27672288 DOI: 10.3748/wjg.v22.i35.7926]

**P- Reviewer**: Jones G, Taylor ME **S- Editor**: Gong ZM
**L- Editor**: Wang TQ **E- Editor**: Zhang FF

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/ *World J Gastroenterol* 2017 February 21; 23(7): 1241-1249

DOI: 10.3748/wjg.v23.i7.1241 ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Observational Study

# Subclinical atherosclerosis is linked to small intestinal bacterial overgrowth *via* vitamin K2-dependent mechanisms

Francesca Romana Ponziani, Maurizio Pompili, Enrico Di Stasio, Maria Assunta Zocco, Antonio Gasbarrini, Roberto Flore

Francesca Romana Ponziani, Maurizio Pompili, Maria Assunta Zocco, Antonio Gasbarrini, Internal Medicine, Gastroenterology and Hepatology, Agostino Gemelli Hospital, 00168 Rome, Italy

Enrico Di Stasio, Biochemistry, Agostino Gemelli Hospital, 00168 Rome, Italy

Roberto Flore, Internal Medicine and Vascular Medicine, Agostino Gemelli Hospital, 00168 Rome, Italy

Author contributions: Ponziani FR collected, analyzed data; Ponziani FR and Flore R drafted the paper; Ponziani FR, Pompili M, Zocco MA and Flore R interpreted data; Di Stasio E analyzed blood samples; Pompili M, Zocco MA, Gasbarrini A and Flore R revised the paper; Gasbarrini A and Flore R supervised the study; all authors approved the final version of the paper.

Institutional review board statement: This study was approved by the Local Institutional Review Board.

Informed consent statement: Patients gave their written consent to participate to this study.

Conflict-of-interest statement: The Authors have no conflicts of interest to declare.

Data sharing statement: the anonymous dataset is available from the corresponding author (francesca.ponziani@yahoo.it) and will be provided on request after obtaining all Authors' agreement.



Manuscript source: Invited manuscript

Correspondence to: Francesca Romana Ponziani, MD, Internal Medicine, Gastroenterology and Hepatology, Agostino Gemelli Hospital, Largo Agostino Gemelli 8, 00168 Rome, Italy. francesca.ponziani@yahoo.it
Telephone: +39-34-71227242
Fax: +39-06-30156265

Received: October 15, 2016
Peer-review started: October 31, 2016
First decision: December 19, 2016
Revised: December 29, 2016
Accepted: January 18, 2017
Article in press: January 18, 2017
Published online: February 21, 2017

## Abstract

***AIM***

To assess the rate of matrix Gla-protein carboxylation in patients with small intestinal bacterial overgrowth (SIBO) and to decipher its association with subclinical atherosclerosis.

***METHODS***

Patients with suspected SIBO who presented with a low risk for cardiovascular disease and showed no evidence of atherosclerotic plaques were included in the study. A glucose breath test was performed in order to confirm the diagnosis of SIBO and vascular assessment was carried out by ultrasound examination. Plasma levels of the inactive form of MGP (dephosphorylated-uncarboxylated matrix Gla-protein) were quantified by ELISA and vitamin K2 intake was estimated using a food frequency questionnaire.

***RESULTS***

Thirty-nine patients were included in the study. SIBO was confirmed in 12/39 (30.8%) patients who also presented with a higher concentration of

dephosphorylated-uncarboxylated matrix Gla-protein (9.5 μg/L *vs* 4.2 μg/L; *P* = 0.004). Arterial stiffness was elevated in the SIBO group (pulse-wave velocity 10.25 m/s *vs* 7.68 m/s; *P* = 0.002) and this phenomenon was observed to correlate linearly with the levels of dephosphorylated-uncarboxylated matrix Gla-protein (β = 0.220, $R^2$ = 0.366, *P* = 0.03). Carotid intima-media thickness and arterial calcifications were not observed to be significantly elevated as compared to controls.

***CONCLUSION***

SIBO is associated with reduced matrix Gla-protein activation as well as arterial stiffening. Both these observations are regarded as important indicators of subclinical atherosclerosis. Hence, screening for SIBO, intestinal decontamination and supplementation with vitamin K2 has the potential to be incorporated into clinical practice as additional preventive measures.

**Key words:** Small intestinal bacterial overgrowth; Vitamin K; Dysbiosis; Atherosclerosis; Cardiovascular disease risk



**Core tip:** The matrix Gla-protein is involved in maintaining vascular health and vitamin K2 is a prerequisite for its activation and function. Intestinal bacteria are the main source of vitamin K2 in humans and small intestinal bacterial overgrowth (SIBO) is associated with altered vitamin K2 metabolism. This study demonstrates that SIBO is associated with increased plasma levels of inactive matrix Gla-protein, which, in turn, correlates directly with early markers of atherosclerotic disease such as increased arterial stiffness. Therefore, SIBO has the potential to serve as an indicator for increased risk of developing an overt cardiovascular disease.

Ponziani FR, Pompili M, Di Stasio E, Zocco MA, Gasbarrini A, Flore R. Subclinical atherosclerosis is linked to small intestinal bacterial overgrowth *via* vitamin K2-dependent mechanisms. *World J Gastroenterol* 2017; 23(7): 1241-1249 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1241.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1241

## INTRODUCTION

The presence of bacteria in the gastrointestinal tract is crucial for maintaining host health. One of the most important functions of gut bacteria is to metabolize carbohydrates, lipids, and proteins derived from food and to synthesize nutrients that are not adequately supplied by the diet.

Vitamin K is a lipophilic vitamin that is present in two main forms: (1) phylloquinone or vitamin K1; and (2) menaquinone (MK) or vitamin K2; the nomenclature is representative of the number of prenyl units contained in these isoforms[1]. Studies conducted on European populations have shown that the bulk of vitamin K intake in Western diets is in the form of phylloquinone (90%) with menaquinones accounting for only 10% (about 7.5% by MK-5 through to MK-13 and 2.5% by MK-4)[2]. This can be partially accounted for the fact that menaquinones are found only in meat, dairy-based foods and fermented soybeans (known as "natto") that are a part of the Japanese diet. Therefore, while MK-4 is obtained by peripheral tissue conversion of menadione, which is produced by intestinal cleavage of phylloquinone[2], the bulk of menaquinones is derived from gut bacteria biosynthesis. In particular, bacterial species, such as *Eggerthella lenta* and *Veillonella,* are involved in the production of MK-7 while *Enterobacteriaceae,* such as *Escherichia coli* and *Shigella,* are responsible for the production of MK8. *Bacteroides* produce MK9-11 and *Prevotella* synthesize MK-5 and MK11-13[2,3].

Vitamin K2 is a cofactor that is involved in the carboxylation of several proteins, such as the matrix Gla-protein (MGP)[4]. The MGP is expressed by chondrocytes, vascular smooth muscle cells, endothelial cells and fibroblasts, and its primary function is to bind calcium crystals present in the vessel wall thereby preventing their nucleation on elastin fibers. Additionally, MGP also prevents osteoblastic differentiation of vascular smooth muscle cells and maintains the composition of the extracellular matrix[5]. Hence it can be concluded that MGP plays a crucial role in maintaining both arterial structure as well as function. Increased levels of the inactive form of MGP (dephosphorylated-uncarboxylated MGP, dp-ucMGP) is widely regarded as one of the best marker for low vitamin K2 status[6] and has been known to be associated with the signs of early vascular disease (intima-media thickening, arterial stiffness, and vascular calcifications)[7-14] and cardiovascular morbidity and mortality[15-17].

Although several recent studies have highlighted that patients with small intestinal bacterial overgrowth (SIBO) have low circulating levels of vitamin K2[18], investigations exploring the clinical relationship between SIBO, MGP activity, and arterial structure and function are still lacking.

The aim of this study was to investigate the rate of MGP carboxylation in patients with SIBO and to decipher its association with the risk of developing subclinical atherosclerosis.

## MATERIALS AND METHODS

During a six month period, all outpatients at the Gastroenterology Division of the Agostino Gemelli Hospital in Rome that presented with clinical signs of SIBO (*e.g.*, bloating, abdominal pain, and diarrhea) were included in the study.

Patient history with an emphasis on the personal

and family history of coronary heart disease, lifestyle, and pharmacotherapy was stringently recorded.

Only subjects that were between 40 and 60 years without any previous cardiovascular events (including myocardial infarction or angina, congestive heart failure, intermittent claudication, previous arterial revascularization, thromboembolic disease and stroke) and with a low cardiovascular disease risk (as per the Framingham risk score up to 8 points) were considered eligible for inclusion in this investigation[19]. The exclusion criteria were (1) current heavy smoking (> 10 cigarettes/d by self-report); (2) morbid obesity (body mass index > 30 kg/m$^2$); (3) dyslipidemia (LDL cholesterol > 160 mg/dL); (4) hypertriglyceridemia (triglycerides > 400 mg/dL); and (5) uncontrolled hypertension (systolic blood pressure > 140 mmHg and/or diastolic blood pressure > 95 mmHg). The patients suffering from diabetes, chronic kidney disease (at any stage), those receiving oral anticoagulant treatment and those afflicted with any carotid, aortic, femoral, and popliteal plaque (according to the Mannheim criteria as detected at the time of ultrasound examination) were also excluded from the protocol[20].

The patients fulfilling the selection criteria underwent a glucose breath test (GBT), filled in a food frequency questionnaire for the assessment of daily vitamin K2 intake, provided a blood sample for the quantification of circulating dp-ucMGP, and underwent ultrasound (US) examination of the non-coronary arterial system.

The study was performed in agreement with the Declaration of Helsinki and subsequent amendments. Written informed consent was obtained from all patients.

### GBT test for SIBO diagnosis

All patients included in the study underwent a GBT to confirm the diagnosis of SIBO[21]. In order to avoid false positive or negative results, each patient was advised to strictly adhere to certain instructions before taking the test. These conditions were (1) laxatives, antibiotics, and prokinetics to be prohibited for at least four weeks before the GBT; (2) consumption of a low-fiber dinner was allowed but the patients were prohibited from eating or drinking eight hours before the test; (3) washing of mouth with antiseptics was done immediately before administration of the test; and (4) smoking, chewing gum, and doing physical exercise were prohibited before and during the test.

The GBT procedure was performed according to the recommendations of the Rome consensus[22]. The procedure was as follows: after the administration of 50 g glucose dissolved in 250 mL of water, hydrogen and methane excretion in the expired air was assessed at 15-min intervals for a total time period of 120 min. These values were then compared to baseline values. SIBO diagnosis was regarded as confirmed if the expired air exhibited an increase of at least 12 parts per million (ppm) above the baseline in hydrogen and/or methane activity.

### Quantification of vitamin K2 daily intake

In order to assess the daily intake of vitamin K2, a food frequency questionnaire obtained from the European Prospective Investigation on Cancer and Nutrition (EPIC) nutrient database project was readapted and used[23].

The questionnaire was structured into three sections. The first section addressed the type and frequency of foods that were consumed by the subjects and included a list of more than 100 items divided into categories (fruit, vegetables, cereals, pasta/bread/rice, soups, meat, fish, eggs, milk/dairy products, fast food products, condiments, sweets, beverages, and vitamins). The frequency of intake of each food item was recorded and ranked (daily, weekly, monthly, yearly, or never).

The second section estimated the serving size and ranked it according to food weight (small, medium, and large portions).

Finally, the last section of the questionnaire investigated cooking habits (*e.g.*, addition of salt to foods, preferred cooking method, *etc.*).

For the purpose of the study, scientific literature was reviewed in order to identify vitamin K2 rich foods that are commonly present in a Western diet. The results of the literature survey were taken into consideration while composing the food questionnaire so as to avoid underestimation of vitamin K2 intake by the study subjects. A list of vitamin K2 content in food is provided as Supplementary Table 1.

Although the food frequency questionnaire only referred to the dietary intake of the previous 12 mo, all subjects participating in the study attested to having followed a stable dietary regimen for the last five years.

### Plasma assay dp-ucMGP

Citrated plasma was separated from whole blood by centrifuging at 1500 × *g* for 10 min. The aliquots of 2 mL each were frozen at -20 ℃ or -80 ℃ within 30 min of blood sampling. For long-term storage exceeding 2 months, all samples were kept at -80 ℃ till use. dp-ucMGP concentration was assessed using a dual-antibody test based on the sandwich ELISA methodology developed by VitaK (inaKtif MGP iSYS kit, Immunodiagnostic Systems Ltd, Boldon, United Kingdom).

### Ultrasound examination

The US and Doppler US (D-US) examination of the non-coronary arterial system was conducted in order to examine for early signs of vascular dysfunction (arterial stiffening) and for the presence of early vascular lesions (intima-media thickening, arterial

calcifications, and subclinical plaques).

Arterial stiffness was assessed by applying an automated radiofrequency-based method (Quality Arterial Stiffness (RF-QAS); Esaote Medical Systems, Genova, Italy) to the D-US examination of the left common carotid artery. Local pulse-wave velocity (PWV) was calculated by combining arterial distension with local distending pressure measure. Assuming a constant difference between mean arterial pressure and diastolic pressure along the arterial tree, the QAS system is able to detect systo-diastolic changes in the arterial diameter following arterial wall movements during the cardiac cycle and to convert local distension variations in modifications of local distending pressure (pulse pressure). PWV was calculated by the Bramwell-Hill equation[24,25], as follows:

$PWV = \sqrt{\Delta P \cdot V / \Delta V \cdot \rho}$

where ΔV and ΔP are changes in volume and pressure, respectively, and ρ is the density of blood. As per the equation, PWV increases when there is an increase in arterial stiffness.

Using the same radiofrequency-based technology outlined above, intima-media thickness (IMT) was measured (Quality Intima-Media Thickness (RF-QIMT); Esaote Medical Systems, Genova, Italy) in a 1 cm long segment of the left common carotid artery, *i.e.*, 1 cm before the bifurcation. To preserve measurement quality, mean IMT values calculated over 6 cardiac cycles were recorded only if the standard error was lower than 20 μm.

The presence of vascular calcifications was investigated by B-mode US in 11 vascular segments (common carotid arteries, common femoral arteries, popliteal arteries, posterior tibial arteries, anterior tibial arteries, and subrenal abdominal aorta) as has been previously described in the literature[26].

### *Statistical analysis*

The Shapiro-Wilk test was performed to verify the normality of data distribution and statistical analysis was carried out using non-parametric tests.

Continuous variables were expressed as median and range (minimum and maximum value) while categorical variables were expressed as frequencies and percentages.

For comparing patients with SIBO with those without, the Mann-Whitney and chi-square tests were applied in order to highlight differences, if any, in baseline characteristics such as sex, age, and vitamin K2 intake. The same tests were also employed to test discrepancies in variables under study (plasma levels of dp-ucMGP, PWV, IMT, vascular calcifications).

Due to the presence of repeat values in the dataset, Kendall's tau-b correlation coefficient was used to investigate the association between dp-ucMGP levels, PWV, IMT, and vascular calcifications. Additionally, data distribution was explored in order to find the best-fit regression model for elucidating the relationship between dp-ucMGP levels and US/D-US parameters. A linear regression was constructed using PWV as dependent variable, and the assumptions were verified by the appropriate diagnostics. Correlations and regression analyses were performed on both the overall population as well as the SIBO group separately.

Statistical analysis was conducted using the R statistics program version 3.1.2[27]. All statistical tests were two-sided and differences were considered significant at *P*-values below 0.05.

## RESULTS

Amongst the 189 patients that were initially recruited into the study, 44 were deemed ineligible as a result of moderate or high Framingham risk score. A further 86 were ruled out as they were found to be afflicted with one or more of the comorbidities listed among the exclusion criteria. Twenty patients refused consent for being a part of this investigation (Figure 1). Eventually, 39 patients were selected to be a part of this study.

Patient characteristics are described in Table 1. The median age of the study group was 53 (41-60) years, and 14 (35.9%) of the participants were male. SIBO was diagnosed in 12/39 (30.8%) patients and the 27 patients without SIBO were regarded as the control group (no-SIBO group). The analysis of the data contained in the food questionnaires revealed that the median vitamin K2 daily intake was approximately 29.5 (8-103.6) μg/d. No differences in median age, sex, and Framingham score could be observed between the SIBO and no-SIBO groups.

Vitamin K2 intake was determined to be 21.2 (8-49.7) μg/d in the SIBO group, which was similar to 31.9 (10.5-103.6) μg/d consumed by the control subjects in the no-SIBO group ($P = 0.111$). The median dp-ucMGP serum level was 4.76 μg/L (1.75–25.7) (Table 2); dp-ucMGP was observed to be significantly increased in patients with SIBO (9.5 μg/L *vs* 4.2 μg/L, $P = 0.02$; Table 2 and Figure 2).

The daily intake of vitamin K2 was not associated with plasma levels of dp-ucMGP ($\tau = -0.08$, $P = 0.441$).

Sixteen of the 39 patients (41%) presented with at least one or more microcalcifications in the explored arterial districts (Supplementary Table 2). It is to be noted however that no arterial plaque was detected. In the overall population, median carotid artery PWV was 8.5 m/s (5.69-14) and median IMT was 661 μm (467-1009). In patients with SIBO, the median PWV was significantly higher than that observed in the no-SIBO group (10.25 m/s *vs* 7.68 m/s; $P = 0.002$; Figure 3) but the median IMT value and the rate of early arterial calcifications did not differ substantially (Table 2). dp-ucMGP levels correlated with arterial stiffness as measured by PWV ($\tau = 0.339$, $P = 0.002$). In particular, regression analysis showed that there was a significant direct linear correlation between dp-ucMGP and PWV ($\beta = 0.219$, $R^2 = 0.293$, $P = 0.0004$; Figure 4).

Additionally, in patients with SIBO, a direct linear

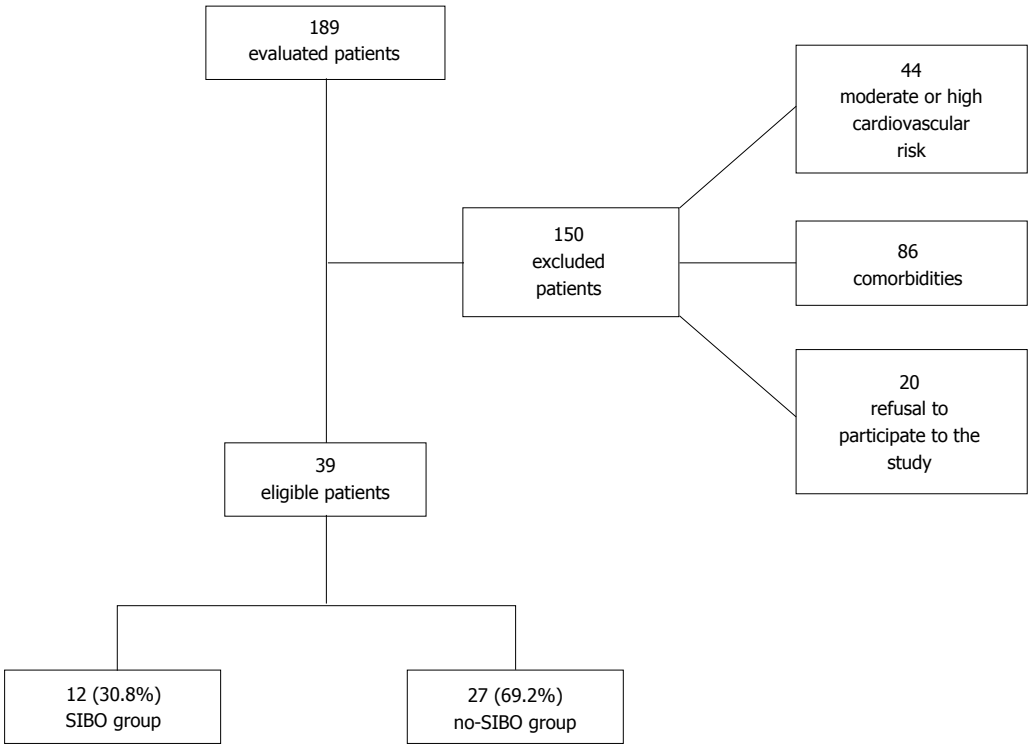



**Figure 1 Study workflow.**

**Table 1 Patients' baseline characteristics**

| | Overall | SIBO ($n$ = 12) | no-SIBO ($n$ = 27) | $P$ value |
|---|---|---|---|---|
| Age | 53 (41-60) | 55 (41-60) | 57 (43-60) | 0.306 |
| Sex (males/females) | 14 (35.9)/25 (64.1) | 2 (16.7)/10 (83.3) | 12 (44.4)/15 (55.6) | 0.095 |
| Vitamin K2 intake (μg/d) | 29.5 (8-103.6)<br>Males: 25.7 (8-103.6)<br>Females 29.6 (10.4-91.8) | 21.2 (8-49.7) | 31.9 (10.5-103.6) | 0.111 |
| Framingham risk score | 5.4 (2-8)<br>Males: 5.8 (2-8)<br>Females: 6.9 (0-8) | 5.5 (2-7) | 5.2 (2-8) | 0.897 |

Data are expressed as median (range) or frequency (%). Comparisons have been performed between SIBO and no-SIBO group. Framingham score was built on age, sex, race, arterial pressure, smoking, diabetes, total blood cholesterol, HDL-cholesterol. SIBO: Small intestinal bacterial overgrowth.

**Table 2 Comparison of circulating levels of dephosphorylated-uncarboxylated matrix Gla-protein and ultrasound parameters (pulse-wave velocity, intima-media thickness, and vascular calcifications) in patients with or without small intestinal bacterial overgrowth**

| | Overall | SIBO ($n$ = 12) | no-SIBO ($n$ = 27) | $P$ value |
|---|---|---|---|---|
| dp-ucMGP (μg/L) | 4.73 (1.75-23.8) | 9.5 (3.6-23.8) | 4.2 (1.75-14.5) | 0.004[a] |
| PWV (m/s) | 8.5 (5.7-14) | 10.25 (5.7-14) | 7.68 (5.6-11) | 0.002[a] |
| IMT (μm) | 661 (467-1009) | 596 (467-1009) | 663 (506-932) | 0.465 |
| Calcifications | 16 (41) | 6 (50) | 10 (37) | 0.609 |

Data are expressed as median (range) or frequency (%), significant comparisons ([a]$P$ < 0.05). SIBO: Small intestinal bacterial overgrowth; dp-ucMGP: Dephosphorylated-uncarboxylated matrix Gla-protein; PWV: Pulse-wave velocity; IMT: Intima-media thickness.

correlation between dp-ucMGP levels and PWV could be verified (β = 0.220, $R^2$ = 0.366, $P$ = 0.03) but no such relation was found between dp-ucMGP and IMT ($P$ = 0.507).

In the no-SIBO group, no significant relationship between dp-ucMGP, arterial stiffness, and IMT could be observed ($P$ = 0.08 and $P$ = 0.415, respectively).

## DISCUSSION

In recent years, the studies on identifying early markers of atherosclerosis have garnered a significant

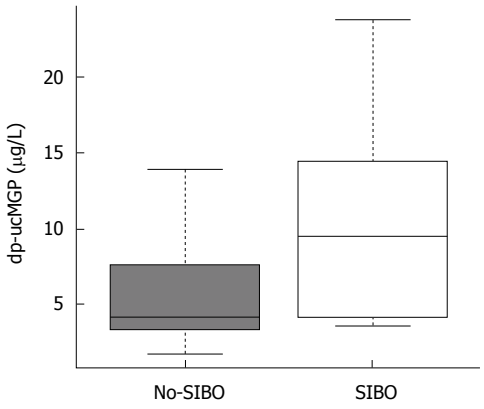



**Figure 2 Plasma concentration of dephosphorylated-uncarboxylated matrix Gla-protein in patients under study.** Patients with small intestinal bacterial overgrowth (SIBO) exhibited significantly higher levels as compared to the patients without SIBO (9.5 μg/L *vs* 4.2 μg/L; *P* = 0.02). Median values are represented by boxplot internal lines and ranges by whiskers. dp-ucMGP: Dephosphorylated-uncarboxylated matrix Gla-protein.

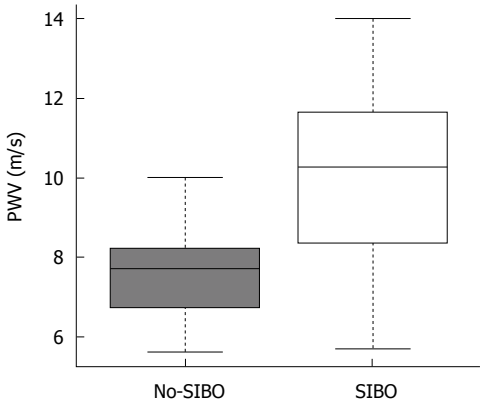



**Figure 3 Pulse-wave velocity values observed in the study population.** Pulse-wave velocity (PWV) was increased in patients with small intestinal bacterial overgrowth (SIBO) compared to the no-SIBO group (10.25 m/s *vs* 7.68 m/s; *P* = 0.002). Median values are represented by boxplot internal lines and ranges by whiskers.

amount of scientific interest. The early identification of vascular dysfunction and lesions has the potential to help individuals that stand to benefit from prevention of disease progression policies[28-30].

For the successful implementation of strategies aimed at prevention of disease formation and progression, it is crucial to recognize pathologies that function as risk factors for the development of atherosclerosis. Several diseases of the gastrointestinal tract, wherein gut bacteria act in a pathogenic capacity, are associated with vascular dysfunction and increase the risk of atherosclerosis in the host[31-33]. Nevertheless, it has been recently demonstrated that metabolic products generated by gut bacteria are implicated in the development of atherosclerotic lesions[34].

MGP is a vitamin K2 dependent protein that helps in preventing calcium accumulation in the arterial wall. Humans need gut bacteria in order to fulfill

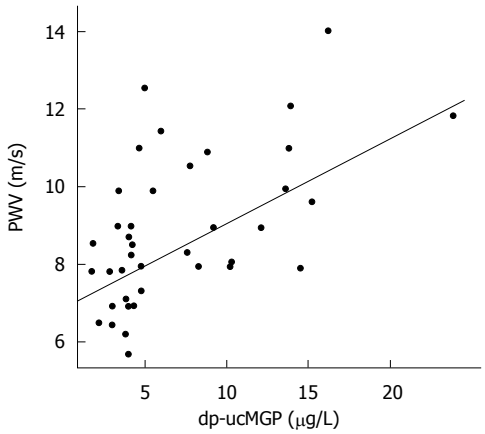



**Figure 4 Linear regression model highlighting the direct correlation between plasma concentration of dephosphorylated-uncarboxylated matrix Gla-protein and pulse-wave velocity (β = 0.219, $R^2$ = 0.293, *P* = 0.0004).** dp-ucMGP: Dephosphorylated-uncarboxylated matrix Gla-protein; PWV: Pulse-wave velocity.

their vitamin K2 requirement as dietary intake is often insufficient. This is especially true in the case of the Western population. SIBO, a condition that is characterized by gut bacteria dysbiosis, is associated with impaired vitamin K metabolism in humans[18,35]. For this reason, patients afflicted with SIBO and/or low vitamin K2 status could hypothetically be at an increased risk for the development of atherosclerotic disease.

To the best of our knowledge, the present study is the first to investigate the consequences of vitamin K2 metabolism derangement on MGP activity in patients with SIBO. When compared to the control group, the SIBO group presented with higher dp-ucMGP serum levels, which is suggestive of either a reduced dietary vitamin K2 intake or an altered vitamin K2 production by intestinal bacteria. It is to be noted that the daily median vitamin K2 intake of patients included in the study was comparable to that of other European populations[36,37] and was similar between patients with SIBO and those without (21.1 μg/d *vs* 31.9 μg/d, *P* = 0.111; Table 2). As plasma vitamin K2 levels are difficult to assess with accuracy[38], we adopted dp-ucMGP levels as surrogate biomarkers for estimating the vitamin K2 nutritional status. Our results strongly indicated that dietary vitamin K2 intake does not correlate with dp-ucMGP serum levels. This observation indirectly confirms the previously reported theory that food is not the primary source of vitamin K2 supplementation in humans and that the gut microbiota is crucial for overcoming dietary insufficiencies under physiologic conditions[2,3].

In all subjects including those in the SIBO group, the serum levels of inactive dp-ucMGP were found to directly correlate with PWV, which is a surrogate parameter of arterial stiffness. However, no significant association was found with either IMT or the presence of calcifications. Atherosclerosis is a progressive

disease characterized by a wide spectrum of vascular changes[39]. While arterial stiffening is an early marker of vascular dysfunction, intima-media thickening and calcifications are the first structural changes that can be detected in atherosclerotic vessels; these changes are usually a manifestation of the adaptive remodeling to flow, wall tension, and lumen diameter alterations[40]. As a result of this, even in the cases that are potentially at low risk for the development of cardiovascular diseases, SIBO appears to cause early vascular dysfunction possibly due to reduced MGP activity. This observation holds true even in the absence of clear signs of structural alterations. The absence of a statistical correlation between dp-ucMGP values and initial signs of vascular remodeling can be explained by the absence of study subjects with medium and high Framingham risk scores. It is reasonable to argue that in the presence of other cardiovascular risk factors, patients with SIBO may be at increased risk for developing structural arterial lesions as compared to patients without SIBO, and that SIBO itself may confer an increased risk of developing an overt cardiovascular disease. Therefore, based on the results obtained in our study, we propose that even asymptomatic patients should be screened for SIBO so as to rule out an additional factor that predisposes patients to cardiovascular diseases. This screening is especially important for patients with known atherosclerotic lesions or previous cardiovascular events as it offers a chance for therapeutic intervention so as to correct a condition that can potentially contribute to disease progression. In patients with SIBO vitamin K2 supplementation and intestinal decontamination, along with additional preventive measures, may be therefore recommended.

The present study suffers from certain limitations. Firstly, information regarding the specific composition of the small intestinal bacteria was not collected due to the invasiveness of the procedure. In addition, at our institution, SIBO is diagnosed by GBT as per the Rome consensus recommendations[18]. We were, therefore, unable to perform metagenomic or metabolomic analyses to assess if the abundance of bacteria specifically involved those involved in vitamin K2 production.

Secondly, instead of computed tomography (CT) scans, US and D-US were used to investigate the presence of vascular calcifications and flow parameters. This was done because US is less harmful, easily reproducible and allows for the quantification of parameters useful for assessing arterial stiffness whereas CT scans require radiation exposure and are also more expensive[41].

Lastly, this study included a relatively small number of patients. A strict adherence to the selection criterion was followed so as to avoid the effect of confounding factors such as treatment with oral vitamin K antagonists, diabetes and kidney disease on MGP carboxylation, or the influence of the previous history of vascular disease and of moderate/high cardiovascular disease risk on the prevalence of vascular calcifications and on the measurement of D-US parameters. The exclusion of patients with comorbidities limited external interactions which allowed us to exclusively evaluate the correlation between SIBO, vitamin K2 metabolism, MGP carboxylation and early arterial dysfunction or vascular lesions without the interference of any confounding factors.

In conclusion, patients affected by SIBO have higher levels of inactive MGP as well as increased arterial stiffness both of which are early markers for vascular dysfunction. This condition is not influenced by vitamin K2 intake from diet confirming that bacteria are the main source of this vitamin in humans and that vitamin K2 metabolism may be altered as a consequence of small intestinal dysbiosis. Longitudinal studies assessing the role of SIBO as a condition that predisposes patients to the development of atherosclerosis are needed; for this category of patients, vitamin K2 supplementation and the treatment of intestinal dysbiosis may be therapeutic alternatives of significant utility.

## COMMENTS

### Background

Small intestinal bacterial overgrowth (SIBO) is associated with altered vitamin K2 metabolism. Vitamin K2 deficiency leads to a reduced carboxylation of the matrix Gla-protein, which is crucial for maintaining the integrity of the vascular system. Intestinal bacteria are involved in vitamin K2 metabolism.

### Research frontiers

Little is known about vitamin K2 metabolism in patients with intestinal dysbiosis and its association with vascular disease.

### Innovations and breakthroughs

SIBO is associated with both reduced matrix Gla-protein activation as well as arterial stiffening both of which are important signs of subclinical atherosclerosis.

### Applications

Patients should be screened for SIBO so as to rule out an additional factor that predisposes to cardiovascular disease or accelerates its progression. Vitamin K2 supplementation and intestinal decontamination are a valid therapeutic option in case of patients with SIBO.

### Terminology

SIBO is a condition characterized by the presence of more than $10^5$ CFU/mL of bacteria in the small intestine. Matrix Gla-protein binds calcium crystals present in the vessel wall thereby preventing their nucleation on elastin fibers and maintains the composition of the extracellular matrix, preserving optimum arterial structure and function.

### Peer-review

In this manuscript the authors have assessed the role of matrix Gla-protein carboxylation in patients with SIBO and its association with subclinical atherosclerosis.Authors have used non-invasive Glucose Breath test to diagnose SIBO. This is an important study. The idea is novel but the number of patients enrolled is too less to come to this conclusion as mentioned by the authors also.

## REFERENCES


1 **Booth SL**. Vitamin K: food composition and dietary intakes. *Food Nutr Res* 2012; **56** [PMID: 22489217 DOI: 10.3402/fnr.v56i0.5505]
2 **Shearer MJ**, Newman P. Metabolism and cell biology of vitamin K. *Thromb Haemost* 2008; **100**: 530-547 [PMID: 18841274]
3 **Shearer MJ**, Newman P. Recent trends in the metabolism and cell biology of vitamin K with special reference to vitamin K cycling and MK-4 biosynthesis. *J Lipid Res* 2014; **55**: 345-362 [PMID: 24489112 DOI: 10.1194/jlr.R045559]
4 **Gröber U**, Reichrath J, Holick MF, Kisters K. Vitamin K: an old vitamin in a new perspective. *Dermatoendocrinol* 2014; **6**: e968490 [PMID: 26413183 DOI: 10.4161/19381972.2014.968490]
5 **Evrard S**, Delanaye P, Kamel S, Cristol JP, Cavalier E. Vascular calcification: from pathophysiology to biomarkers. *Clin Chim Acta* 2015; **438**: 401-414 [PMID: 25236333]
6 **Schurgers LJ**, Cranenburg EC, Vermeer C. Matrix Gla-protein: the calcification inhibitor in need of vitamin K. *Thromb Haemost* 2008; **100**: 593-603 [PMID: 18841280]
7 **Pivin E**, Ponte B, Pruijm M, Ackermann D, Guessous I, Ehret G, Liu YP, Drummen NE, Knapen MH, Pechere-Bertschi A, Paccaud F, Mohaupt M, Vermeer C, Staessen JA, Vogt B, Martin PY, Burnier M, Bochud M. Inactive Matrix Gla-Protein Is Associated With Arterial Stiffness in an Adult Population-Based Study. *Hypertension* 2015; **66**: 85-92 [PMID: 25987667]
8 **Mayer O**, Seidlerová J, Wohlfahrt P, Filipovský J, Vaněk J, Cífková R, Windrichová J, Topolčan O, Knapen MH, Drummen NE, Vermeer C. Desphospho-uncarboxylated matrix Gla protein is associated with increased aortic stiffness in a general population. *J Hum Hypertens* 2016; **30**: 418-423 [PMID: 26016598 DOI: 10.1038/jhh.2015.55]
9 **Dalmeijer GW**, van der Schouw YT, Vermeer C, Magdeleyns EJ, Schurgers LJ, Beulens JW. Circulating matrix Gla protein is associated with coronary artery calcification and vitamin K status in healthy women. *J Nutr Biochem* 2013; **24**: 624-628 [PMID: 22819559 DOI: 10.1016/j.jnutbio.2012.02.012]
10 **Liabeuf S**, Bourron O, Vemeer C, Theuwissen E, Magdeleyns E, Aubert CE, Brazier M, Mentaverri R, Hartemann A, Massy ZA. Vascular calcification in patients with type 2 diabetes: the involvement of matrix Gla protein. *Cardiovasc Diabetol* 2014; **13**: 85 [PMID: 24762216 DOI: 10.1186/1475-2840-13-85]
11 **Delanaye P**, Krzesinski JM, Warling X, Moonen M, Smelten N, Médart L, Pottel H, Cavalier E. Dephosphorylated-uncarboxylated Matrix Gla protein concentration is predictive of vitamin K status and is correlated with vascular calcification in a cohort of hemodialysis patients. *BMC Nephrol* 2014; **15**: 145 [PMID: 25190488 DOI: 10.1186/1471-2369-15-145]
12 **Schurgers LJ**, Barreto DV, Barreto FC, Liabeuf S, Renard C, Magdeleyns EJ, Vermeer C, Choukroun G, Massy ZA. The circulating inactive form of matrix gla protein is a surrogate marker for vascular calcification in chronic kidney disease: a preliminary report. *Clin J Am Soc Nephrol* 2010; **5**: 568-575 [PMID: 20133489 DOI: 10.2215/CJN.07081009]
13 **Parker BD**, Schurgers LJ, Vermeer C, Schiller NB, Whooley MA, Ix JH. The association of uncarboxylated matrix Gla protein with mitral annular calcification differs by diabetes status: The Heart and Soul study. *Atherosclerosis* 2010; **210**: 320-325 [PMID: 20015492 DOI: 10.1016/j.atherosclerosis.2009.11.023]
14 **Hermans MM**, Vermeer C, Kooman JP, Brandenburg V, Ketteler M, Gladziwa U, Rensma PL, Leunissen KM, Schurgers LJ. Undercarboxylated matrix GLA protein levels are decreased in dialysis patients and related to parameters of calcium-phosphate metabolism and aortic augmentation index. *Blood Purif* 2007; **25**: 395-401 [PMID: 17890861 DOI: 10.1159/000108629]
15 **Mayer O**, Seidlerová J, Vaněk J, Karnosová P, Bruthans J, Filipovský J, Wohlfahrt P, Cífková R, Windrichová J, Knapen MH, Drummen NE, Vermeer C. The abnormal status of uncarboxylated matrix Gla protein species represents an additional mortality risk in heart failure patients with vascular disease. *Int J Cardiol* 2016; **203**: 916-922 [PMID: 26618253 DOI: 10.1016/j.ijcard.2015.10.226]
16 **Mayer O**, Seidlerová J, Bruthans J, Filipovský J, Timoracká K, Vaněk J, Cerná L, Wohlfahrt P, Cífková R, Theuwissen E, Vermeer C. Desphospho-uncarboxylated matrix Gla-protein is associated with mortality risk in patients with chronic stable vascular disease. *Atherosclerosis* 2014; **235**: 162-168 [PMID: 24835435 DOI: 10.1016/j.atherosclerosis.2014.04.027]
17 **Dalmeijer GW**, van der Schouw YT, Magdeleyns EJ, Vermeer C, Verschuren WM, Boer JM, Beulens JW. Circulating desphospho-uncarboxylated matrix γ-carboxyglutamate protein and the risk of coronary heart disease and stroke. *J Thromb Haemost* 2014; **12**: 1028-1034 [PMID: 24826813 DOI: 10.1111/jth.12609]
18 **Giuliano V**, Bassotti G, Mourvaki E, Castellani D, Filippucci E, Sabatino G, Gizzi S, Palmerini F, Galli F, Morelli O, Baldoni M, Morelli A, Iorio A. Small intestinal bacterial overgrowth and warfarin dose requirement variability. *Thromb Res* 2010; **126**: 12-17 [PMID: 20051286 DOI: 10.1016/j.thromres.2009.11.032]
19 **Wilson PW**, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. *Circulation* 1998; **97**: 1837-1847 [PMID: 9603539]
20 **Touboul PJ**, Hennerici MG, Meairs S, Adams H, Amarenco P, Bornstein N, Csiba L, Desvarieux M, Ebrahim S, Hernandez Hernandez R, Jaff M, Kownator S, Naqvi T, Prati P, Rundek T, Sitzer M, Schminke U, Tardif JC, Taylor A, Vicaut E, Woo KS. Mannheim carotid intima-media thickness and plaque consensus (2004-2006-2011). An update on behalf of the advisory board of the 3rd, 4th and 5th watching the risk symposia, at the 13th, 15th and 20th European Stroke Conferences, Mannheim, Germany, 2004, Brussels, Belgium, 2006, and Hamburg, Germany, 2011. *Cerebrovasc Dis* 2012; **34**: 290-296 [PMID: 23128470 DOI: 10.1159/000343145]
21 **Ponziani FR**, Gerardi V, Gasbarrini A. Diagnosis and treatment of small intestinal bacterial overgrowth. *Expert Rev Gastroenterol Hepatol* 2016; **10**: 215-227 [PMID: 26636484 DOI: 10.1586/17474124.2016.1110017]
22 **Gasbarrini A**, Corazza GR, Gasbarrini G, Montalto M, Di Stefano M, Basilisco G, Parodi A, Usai-Satta P, Vernia P, Anania C, Astegiano M, Barbara G, Benini L, Bonazzi P, Capurso G, Certo M, Colecchia A, Cuoco L, Di Sario A, Festi D, Lauritano C, Miceli E, Nardone G, Perri F, Portincasa P, Risicato R, Sorge M, Tursi A. Methodology and indications of H2-breath testing in gastrointestinal diseases: the Rome Consensus Conference. *Aliment Pharmacol Ther* 2009; **29** Suppl 1: 1-49 [PMID: 19344474 DOI: 10.1111/j.1365-2036.2009.03951.x]
23 **World Health Organization**. EPIC study. Available from: URL: http://epic.iarc.fr/about/background.php
24 **Van Bortel LM**, Balkestein EJ, van der Heijden-Spek JJ, Vanmolkot FH, Staessen JA, Kragten JA, Vredeveld JW, Safar ME, Struijker Boudier HA, Hoeks AP. Non-invasive assessment of local arterial pulse pressure: comparison of applanation tonometry and echo-tracking. *J Hypertens* 2001; **19**: 1037-1044 [PMID: 11403351]
25 **Bramwell JC**, Hill AV. The velocity of the pulse wave in man. *Proc R Soc Lond B* 1922; **93**: 298-306
26 **Flore R**, Ponziani FR, Tinelli G, Arena V, Fonnesu C, Nesci A, Santoro L, Tondi P, Santoliquido A. New modalities of ultrasound-based intima-media thickness, arterial stiffness and non-coronary vascular calcifications detection to assess cardiovascular risk. *Eur Rev Med Pharmacol Sci* 2015; **19**: 1430-1441 [PMID: 25967718]
27 **R Core Team**. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available from: URL: http://www.R-project.org/2014
28 **Vlachopoulos C**, Xaplanteris P, Aboyans V, Brodmann M, Cífková R, Cosentino F, De Carlo M, Gallino A, Landmesser U, Laurent S, Lekakis J, Mikhailidis DP, Naka KK, Protogerou AD, Rizzoni D, Schmidt-Trucksäss A, Van Bortel L, Weber T, Yamashina A, Zimlichman R, Boutouyrie P, Cockcroft J, O'Rourke M, Park JB, Schillaci G, Sillesen H, Townsend RR. The role of vascular biomarkers for primary and secondary prevention. A position paper from the European Society of Cardiology Working Group on

peripheral circulation: Endorsed by the Association for Research into Arterial Structure and Physiology (ARTERY) Society. *Atherosclerosis* 2015; **241**: 507-532 [PMID: 26117398 DOI: 10.1016/j.atherosclerosis.2015.05.007]

29 **Koivistoinen T**, Virtanen M, Hutri-Kähönen N, Lehtimäki T, Jula A, Juonala M, Moilanen L, Aatola H, Hyttinen J, Viikari JS, Raitakari OT, Kähönen M. Arterial pulse wave velocity in relation to carotid intima-media thickness, brachial flow-mediated dilation and carotid artery distensibility: the Cardiovascular Risk in Young Finns Study and the Health 2000 Survey. *Atherosclerosis* 2012; **220**: 387-393 [PMID: 21871623 DOI: 10.1016/j.atherosclerosis.2011.08.007]

30 **Boesen ME**, Singh D, Menon BK, Frayne R. A systematic literature review of the effect of carotid atherosclerosis on local vessel stiffness and elasticity. *Atherosclerosis* 2015; **243**: 211-222 [PMID: 26402140 DOI: 10.1016/j.atherosclerosis.2015.09.008]

31 **De Marchi S**, Chiarioni G, Prior M, Arosio E. Young adults with coeliac disease may be at increased risk of early atherosclerosis. *Aliment Pharmacol Ther* 2013; **38**: 162-169 [PMID: 23730933 DOI: 10.1111/apt.12360]

32 **Singh S**, Kullo IJ, Pardi DS, Loftus EV. Epidemiology, risk factors and management of cardiovascular diseases in IBD. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 26-35 [PMID: 25446727 DOI: 10.1038/nrgastro.2014.202]

33 **He C**, Yang Z, Lu NH. Helicobacter pylori-an infectious risk factor for atherosclerosis? *J Atheroscler Thromb* 2014; **21**: 1229-1242 [PMID: 25342566]

34 **Org E**, Mehrabian M, Lusis AJ. Unraveling the environmental and genetic interactions in atherosclerosis: Central role of the gut microbiota. *Atherosclerosis* 2015; **241**: 387-399 [PMID: 26071662 DOI: 10.1016/j.atherosclerosis.2015.05.035]

35 **Scarpellini E**, Gabrielli M, Za T, Lauritano EC, Santoliquido A, Rossi E, Ojetti V, Cammarota G, De Stefano V, Gasbarrini A. The interaction between small intestinal bacterial overgrowth and warfarin treatment. *Am J Gastroenterol* 2009; **104**: 2364-2365 [PMID: 19727103 DOI: 10.1038/ajg.2009.288]

36 **Walther B**, Karl JP, Booth SL, Boyaval P. Menaquinones, bacteria, and the food supply: the relevance of dairy and fermented food products to vitamin K requirements. *Adv Nutr* 2013; **4**: 463-473 [PMID: 23858094 DOI: 10.3945/an.113.003855]

37 **Geleijnse JM**, Vermeer C, Grobbee DE, Schurgers LJ, Knapen MH, van der Meer IM, Hofman A, Witteman JC. Dietary intake of menaquinone is associated with a reduced risk of coronary heart disease: the Rotterdam Study. *J Nutr* 2004; **134**: 3100-3105 [PMID: 15514282]

38 **Schurgers LJ**, Vermeer C. Differential lipoprotein transport pathways of K-vitamins in healthy subjects. *Biochim Biophys Acta* 2002; **1570**: 27-32 [PMID: 11960685]

39 **Sakakura K**, Nakano M, Otsuka F, Ladich E, Kolodgie FD, Virmani R. Pathophysiology of atherosclerosis plaque progression. *Heart Lung Circ* 2013; **22**: 399-411 [PMID: 23541627 DOI: 10.1016/j.hlc.2013.03.001]

40 **Simova I**. Intima-media thickness: Appropriate evaluation and proper measurement, described. 2015 Available from: URL: http://www.escardio.org/Journals/E-Journal-of-Cardiology-Practice/Volume-13/Intima-media-thickness-Appropriate-evaluation-and-proper-measurement-described

41 **Ikonomidis I**, Makavos G, Lekakis J. Arterial stiffness and coronary artery disease. *Curr Opin Cardiol* 2015; **30**: 422-431 [PMID: 26049393 DOI: 10.1097/HCO.0000000000000179]

**P- Reviewer**: Osawa S, Rana SV **S- Editor**: Gong ZM
**L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/

*World J Gastroenterol* 2017 February 21; 23(7): 1250-1261

DOI: 10.3748/wjg.v23.i7.1250

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

ORIGINAL ARTICLE

Observational Study

# B2 adrenergic receptors and morphological changes of the enteric nervous system in colorectal adenocarcinoma

Raluca Niculina Ciurea, Ion Rogoveanu, Daniel Pirici, Georgică-Costinel Târtea, Costin Teodor Streba, Cristina Florescu, Bogdan Cătălin, Ileana Puiu, Elena-Anca Târtea, Cristin Constantin Vere

Raluca Niculina Ciurea, Department of Pathology, University of Medicine and Pharmacy of Craiova, 200349 Craiova, Romania

Ion Rogoveanu, Costin Teodor Streba, Cristin Constantin Vere, Department of Gastroenterology, University of Medicine and Pharmacy of Craiova, 200349 Craiova, Romania

Daniel Pirici, Department of Research Methodology, University of Medicine and Pharmacy of Craiova, 200349 Craiova, Romania

Georgică-Costinel Târtea, Department of Internal Medicine, University of Medicine and Pharmacy of Craiova, 200349 Craiova, Romania

Cristina Florescu, Department of Cardiology, University of Medicine and Pharmacy of Craiova, 200349 Craiova, Romania

Bogdan Cătălin, Elena-Anca Târtea, Department of Physiology, University of Medicine and Pharmacy of Craiova, 200349 Craiova, Romania

Ileana Puiu, Department of Pediatrics, University of Medicine and Pharmacy of Craiova, 200349 Craiova, Romania

Author contributions: Ciurea RN, Rogoveanu I and Pirici D contributed equally to this work; Ciurea RN, Rogoveanu I and Pirici D designed, drafted and performed the research; Streba CT, Florescu C, Cătălin B, Puiu I and Târtea EA participated in writing, editing and reviewing of this manuscript; Târtea GC and Vere CC revised the paper and approved the final version.

Supported by the Romanian National Authority for Scientific Research and Innovation, CNCS - UEFISCDI, project No. PN-II-RU-TE-2014-4-0582, contract No. 160/01.10.2015.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of the University of Medicine and Pharmacy of Craiova, Petru Rareş Street 2, 200349 Craiova, Romania (registration no. 53/19.05.2016).

Informed consent statement: All biological samples from the patients were taken after informed consent.

Conflict-of-interest statement: All the authors declare no conflict of interest.

Data sharing statement: No additional data are available.



Manuscript source: Unsolicited manuscript

Correspondence to: Georgică-Costinel Târtea, MD, PhD, Department of Internal Medicine, University of Medicine and Pharmacy of Craiova, Petru Rareş Street 2, 200349 Craiova, Romania. georgetartea@gmail.com
Telephone: +40-740-303735

Received: November 10, 2016
Peer-review started: November 11, 2016
First decision: December 19, 2016
Revised: December 26, 2016
Accepted: January 11, 2017
Article in press: January 11, 2017
Published online: February 21, 2017

## Abstract

***AIM***

To study the morphology of the enteric nervous system and the expression of beta-2 adrenergic (B2A) receptors in primary colorectal cancer.

***METHODS***

In this study, we included forty-eight patients with primary colorectal cancer and nine patients for control

tissue from the excision of a colonic segment for benign conditions. We determined the clinicopathological features and evaluated the immunohistochemical expression pattern of B2A receptors as well as the morphological changes of the enteric nervous system (ENS). In order to assess statistical differences, we used the student *t*-test for comparing the means of two groups and one-way analysis of variance with Bonferroni's *post hoc* analysis for comparing the means of more than two groups. Correlations were assessed using the Pearson's correlation coefficient.

***RESULTS***

B2A receptors were significantly associated with tumor grading, tumor size, tumor invasion, lymph node metastasis ($P < 0.05$), while there were no statistically significant associations with gender, CRC location and gross appearance ($P > 0.05$). We observed, on one hand, a decrease of the relative area for both Auerbach and Meissner plexuses with the increase of the tumor grading, and on the other hand, an increase of the relative area of other nervous elements not in the Meissner plexus or in the Auerbach plexus with the tumor grading. For G1 tumors we found that epithelial B2A area showed an inverse correlation with the Auerbach plexus areas [$r(14) = -0.531$, $P < 0.05$], while for G2 tumors, epithelial B2A areas showed an indirect variation with both the Auerbach plexus areas [$r(14) = -0.453$, $P < 0.05$] and the Meissner areas [$r(14) = -0.825$, $P < 0.01$]. For G3 tumors, the inverse dependence increased for both Auerbach [$r(14) = -0.587$, $P < 0.05$] and Meissner [$r(14) = -0.934$, $P < 0.05$] plexuses.

***CONCLUSION***

B2A receptors play an important role in colorectal carcinogenesis and can be utilized as prognostic factors. Furthermore, study of the ENS in colorectal cancer may lead to targeted molecular therapies.

**Key words:** Beta-2 adrenergic receptors; Enteric nervous system; Colorectal adenocarcinoma; Spectral unmixing immunohistochemistry; Tumor grading



**Core tip:** In the present study, we provide the first description of beta-2 adrenergic (B2A) receptors found on the nuclear membrane of colon adenocarcinoma cells. B2A receptors have a high level of expression in the neoplastic cells from colorectal adenocarcinoma, and a significant association was found between the expression of B2A receptors and tumor grading. Regarding the enteric nervous system and its associations with colorectal cancer, we observed a decrease of the relative area, both for Auerbach and Meissner plexuses, with the increase of the tumor grading, and an increase of the relative area of other nervous elements with the tumor grading.

Ciurea RN, Rogoveanu I, Pirici D, Târtea GC, Streba CT, Florescu C, Cătălin B, Puiu I, Târtea EA, Vere CC. B2 adrenergic receptors and morphological changes of the enteric nervous system in colorectal adenocarcinoma. *World J Gastroenterol* 2017; 23(7): 1250-1261 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1250.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1250

## INTRODUCTION

Despite the progress of recent years in the field of antineoplastic therapy, colorectal cancer still remains one of the major public health problems. In the United States, at January 1st 2016, out of 15.5 million patients suffering from cancer, 724690 men had cancer of the colon and rectum; this cancer being the third most prevalent in women after breast and uterine corpus cancer[1]. Regarding the mortality, colon and rectum cancer rank as the fourth cause of mortality in the world with 694000 deaths, representing about 8.5% of the total deaths caused by cancer in 2012[2]. Translational studies that identify new cancer prognostic markers, molecular changes that appear at the initiation of carcinogenesis and during the progression phase, as well as treatment targets such as elements of the nervous system, are thus needed[3].

The idea of establishing connections between cancer and psychological influences originates from the Greek doctor Galen, who in the second century before Christ, showed that women with melancholic temperament develop breast cancer more frequently than other temperamental types[4].

The enteric nervous system (ENS) is the most complex part of the autonomic nervous system[5]. It consists of three major ganglionic plexuses (mucous plexus, submucosal plexus also called Meissner, and myenteric plexus also called Auerbach), together with many aganglionar plexuses[6]. The Auerbach plexus is found starting at the esophagus and extending to the terminal part of the digestive tract, between circular and longitudinal muscular layers[7]. The ENS consists of approximately 500 million neurons, more than in the peripheral autonomic nervous system[8]. The ENS is developed from the neural crest, from the sacral and vagal segments of the neural tube[9].

The ENS is connected with the central nervous system *via* sympathetic and parasympathetic pathways, a connection known as the cerebroenteral axis[10]. The background of the connections between the two divisions of the autonomic nervous system is represented by neurotransmitters such as acetylcholine for the parasympathetic nervous system and norepinephrine for the sympathetic nervous system[5]. Although norepinephrine is not found in the enteric neurons, but only in the sympathetic afferent fibers that they receive, Li *et al*[11] showed that norepinephrine transporter, which plays an important

role in mediating the effects of norepinephrine, is expressed by enteric neurons. Some of these neurotransmitters act on receptors which are also expressed by colorectal carcinoma cells[4,11]. B2A receptors are part of the family of catecholamine receptors, and their activation can initiate multiple signaling pathways in colorectal tumorigenesis[12]. Moreover, blocking them may slow down or even stop the progression of the neoplastic process, but in this case, the studies are contradictory[12].

Our aim was to compare the morphological changes of the ENS and to evaluate the expression changes of the B2A receptors in primary colorectal cancer versus normal colic mucosa, and also to investigate significant associations between these parameters and the clinicopathological features.

## MATERIALS AND METHODS

### *Patients and specimens*

In this study, we analyzed specimens from forty-eight consecutive patients who underwent surgery with potentially curative resection for primary colorectal cancer, performed in the 1st Surgery Clinic of the Emergency County Hospital, Craiova, Romania. Patients were diagnosed with colorectal cancer in the 1st Medical Clinic, Gastroenterology Department of the Emergency County Hospital of Craiova between 2015 and 2016. As controls, we used normal colorectal tissue taken from nine patients who were diagnosed with intestinal obstruction or other pathologies that needed surgery in order to perform the excision of a colonic segment. The study was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova (registration no. 53/19.05.2016), according to the Declaration of Helsinki. All patients included in the study provided written acceptance and informed consent. In order to obtain clinical information from the patients, we reviewed their medical records for gender, age, tumor size, sites of the primary tumor (right, transverse, left and sigmoid colon and rectum), T and N stages according to the American Joint Committee on Cancer, histological grading according to World Health Organization criteria including well, moderate, or poor differentiated colorectal adenocarcinoma[13].

### *Tissue processing and immunohistochemistry*

After we reviewed the slides and confirmed the pathologies and tumor gradings, three μm-thick serial sections were cut from each block, deparaffinized in xylene, rehydrated in graded alcohol series, and subjected to enzymatic immunohistochemistry utilizing a rabbit-anti-human anti-beta-2 adrenergic receptor (NBP2-15564, diluted as 1:200, Novus Biologicals, United Kingdom) and a rabbit anti-human anti-S100 (Z0311, diluted as 1:100, Dako, Glostrup, Denmark) primary antibodies. Briefly, the sections were first processed for antigen retrieval by microwaving in citrate buffer pH 6 for 20 min, incubated in 1% hydrogen peroxide in distilled water for 30 min to block the endogenous peroxidase activity, kept for another 30 min in 3% skimmed milk in PBS, then incubated with the primary antibodies at 4°C for 18 h. Next day, the signal was amplified for 30 minutes utilizing a species-specific peroxidase polymer-based system adsorbed for human immunoglobulins (Nikirei-Bioscience, Tokyo, Japan). The signal was finally detected with 3,3'-diaminobenzidine (DAB) (Dako) and the slides were coverslipped in DPX (Sigma-Aldrich, St. Louis, MO, United States) after a hematoxylin and eosin staining. All slides stained for each of the primary antibodies have been processed at the same time for protocol consistency together with control slides stained either with DAB or with hematoxylin and eosin in order to obtain pure spectral signatures of the respective stains (see further below). Negative controls were obtained by omitting the primary antibodies.

### *Image processing and statistics*

In order to evaluate and quantify the immunohistochemical expression of the targets, light microscopy images were acquired on a Nikon Eclipse 90i motorized microscope (Elta90, Bucharest, Romania) equipped with a Nuance FX multispectral camera and the Nuance analysis software (Perkin Elmer, Hopkinton, MA, United States). After building a spectral library from individual slides stained with either hematoxylin or DAB (as described above), we were able to efficiently unmix and characterize the immunohistochemical expression patterns of interest (Figure 1). Furthermore, unmixed DAB signal was quantified as area and integrated optical density (average intensity/density of each area of interest) on 10 random images captured with a 20 × objective, using the Image-Pro Plus AMS 7 image analysis software (Media Cybernetics, Bethesda, MD, United States); the resulting data were averaged for each patient, and finally averaged and compared for each histopathological grade. Stroma was also considered separately in this analysis, by manually defining regions of interest in the captured images prior to their analysis.

The total identifiable submucosal (Meissner's), myenteric (Auerbach's), and intratumoral nervous plexuses, as well as the multiaxonal bundles of nerves (> 20 μm) were imaged on the slides stained for S100 protein, and areas measured by manually defining the respective regions of interest. For referring densitometry data to total histological areas, whole hematoxylin-eosin slides were scanned on a desktop Benq 5560 scanner together with a microscopic stage micrometer at 4.800 dpi against a white background. Image files were loaded in Image-Pro Plus, and after pixel-size calibration for 1 mm against the microscopic stage micrometer, images were segmented and areas measured based on the same RGB profile created to automatically select the tissue on all the slides.

Data obtained after using the Image-Pro Plus AMS software were exported and plotted in Microsoft

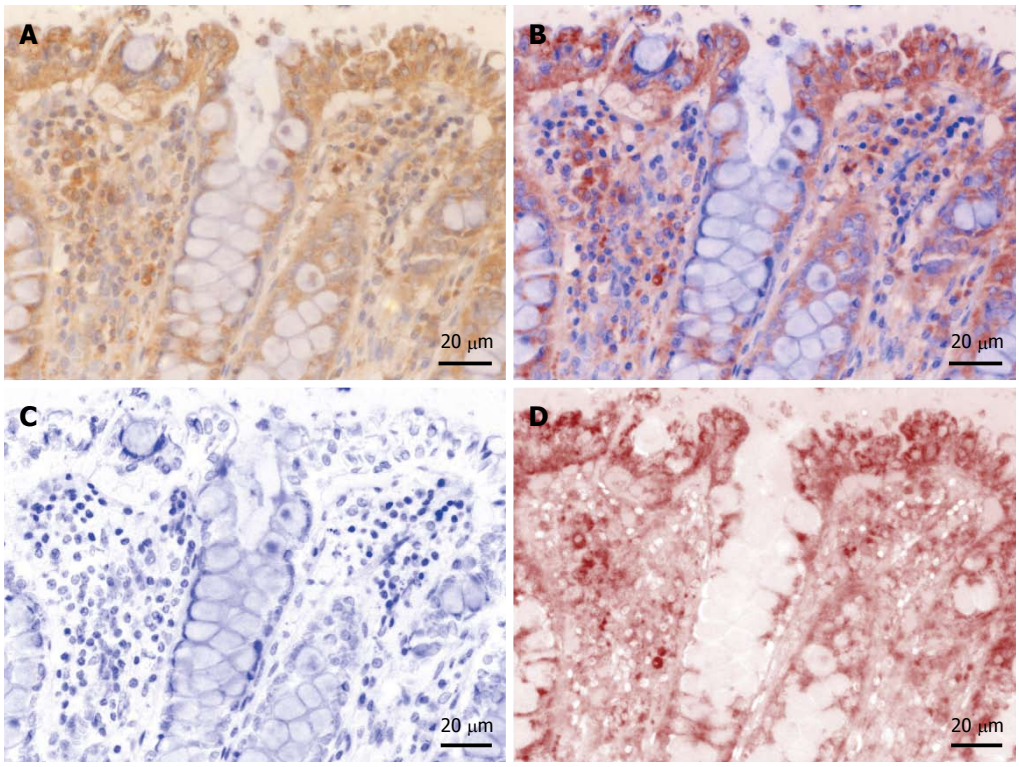



**Figure 1 Example of spectral unmixing for the series of slides.** A: Immunostained for beta-2 adrenergic receptors with DAB and counterstained with hematoxylin; B: Pure DAB and hematoxylin signals are shown either overlapping or (C and D), individually.

Office Excel 2010 (Microsoft Corporation, Redmond, Washington, United States) and were analyzed by using SPSS software (IBM SPSS Statistics, Version 20.0). In order to assess statistical differences we used the student *t*-test for comparing the means of two groups and one-way ANOVA (ANOVA - analysis of variance) with Bonferroni's *post hoc* analysis in order to compare the means of more than two groups. Correlations were assessed using the Pearson's correlation coefficient. Data were reported as mean ± SD or the standard error of the means (SE). In all cases, $P < 0.05$ was used to indicate statistical significance.

## RESULTS

### *Histopathological characterization*

We first sought to characterize the morphological expression of B2A receptor, taking advantage of the multispectral unmixing microscopy (Figure 2). Besides a faint diffuse cytoplasmic reaction on normal colon mucosae, B2A exhibited a granular-like pattern in the cytoplasm of the enterocytes above the nuclei, towards the luminal side of the covering epithelium. In the goblet cells, the signal was localized most frequently below the nucleus. Stromal cells also showed granular staining in their cytoplasmic compartment.

In well differentiated adenocarcinomas, most of the signal was still located towards the luminal side of the tumor cells, although on occasion granules could be clearly identified in the basal pole of the cells.

In moderately differentiated adenocarcinomas, the signal seemed to lose its granular appearance, becoming more diffuse and more intense in the epithelial cells.

In poorly differentiated adenocarcinomas, the intensity of the signal increased in the cytoplasm of the tumor cells, where on a general intense background, one could also identify very intense hotspots surrounding the nuclei with a random-like disposition against them. This time, a clear-cut granular signal could now be observed on occasions in the nuclei or the folds of the nuclear membrane of the tumor cells. A direct counting of the epithelial tumor cells exhibiting this intranuclear/close perinuclear expression pattern of B2A revealed a heterogenous distribution with an average of 12.84% positive- cells [± 12.10% (SD), min = 3%, max = 49%].

On average, there was no signal difference between different tumor stages for the staining of the stromal elements.

B2A receptors were also present in both stromal cells as well as nerves and nervous ganglia. In both submucosal and myenteric plexuses from the normal colon, the marker exhibited a dense-diffuse pattern in the cytoplasm of the ganglion cells, as well as in the satellite cells (Figure 3). Its expression was much fainter in the Schwann cell cytoplasm within the nerve bundles associated with non-tumor tissue. In the tumor tissue, there was a somewhat denser appearance in the cytoplasm of ganglion cells, and interestingly, larger nerve bundles showed a clear-cut increase in signal density compared to non-tumor

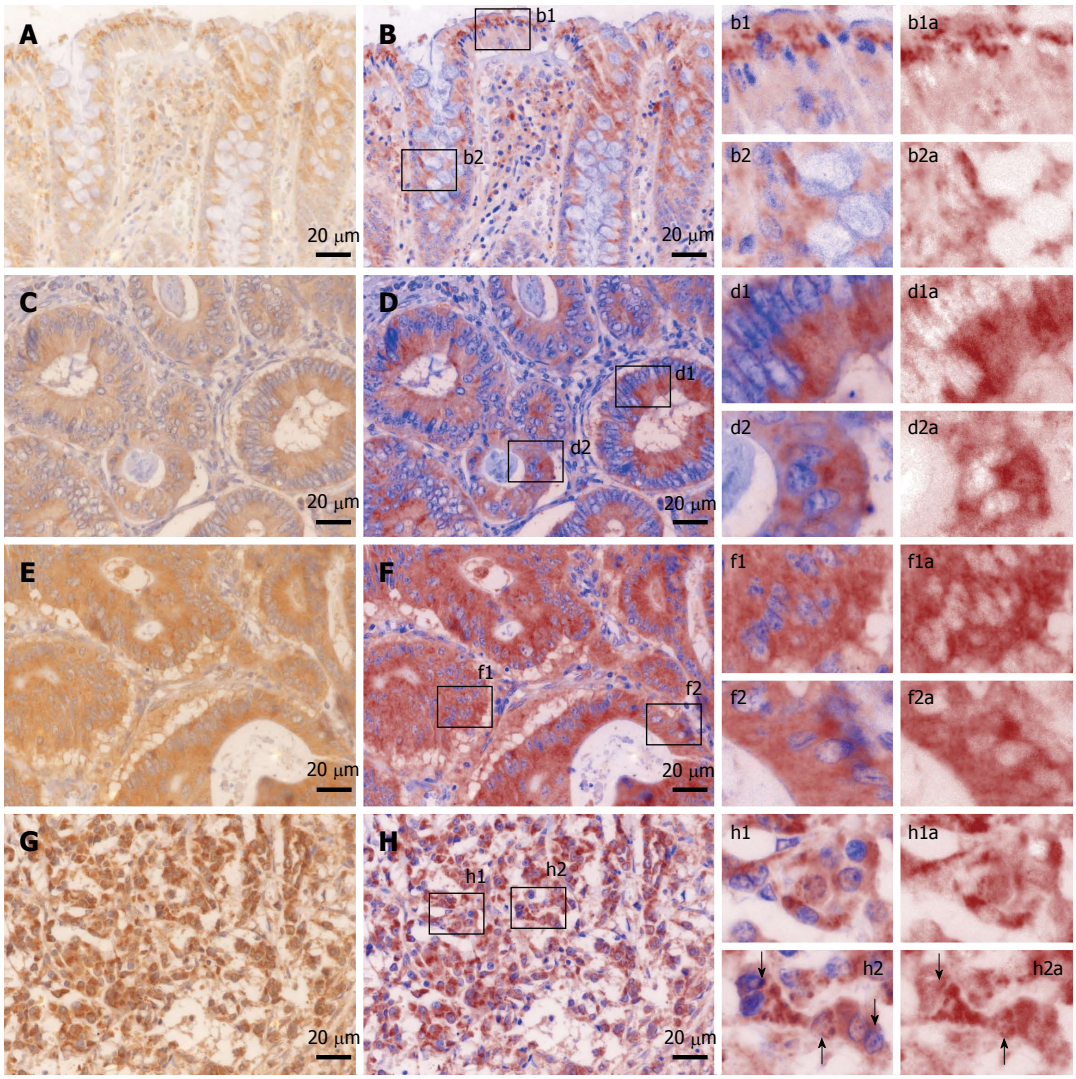



**Figure 2 Analysis of the expression of beta-2 adrenergic receptors in the normal and tumor colon tissue.** RGB (A) and unmixed (B; b1, b1a; b2, b2a) images showing receptor presence mostly as dense granules in the supranuclear sector of the superficial layer of enterocytes (b1; b1a), as well as in the sub-nuclear region of the glandular cells of the crypts (b2; b2a). In well differentiated adenocarcinoma, RGB (C) and unmixed images (D; d1, d1a; d2, d2a) revealed a more dense-diffuse like pattern of expression mostly in the apical sector of the cells (d, d1a), but also in the basal pole of the cells (d2, d2a). In moderately differentiated adenocarcinoma, RGB (E) and unmixed images (F; f1, f1a; f2, f2a) revealed an ever denser and more uniform expression pattern in all the cytoplasm of the tumor cells. In poorly differentiated adenocarcinomas, RGB (G) and unmixed images (H; h1, h1a; h2, h2a) reveal an even denser granular expression pattern, with occasional intra(peri) nuclear localization (arrows in h2 and h2a). Small letters represent enlarged insets from the capital lettered images.

tissue. Smaller nerve bundles (deemed thinner than 20 μm for data stratification) were stained very faintly, as in the control tissue.

### *B2A expression pattern in normal colonic mucosa and in different gradings of colorectal adenocarcinoma*

In this study, nine patients as controls and sixteen for each tumor grading were included. The expression of B2A receptors was analyzed by determining both the area and the integrated optical density (IOD) of the signal, both in the total tissue and in the epithelial and stromal tissue for all the cases included in the study, as described in the Materials and Methods section; this was expressed as the mean ± SD. Regarding the expression of B2A receptors in the total tissue belonging to each patient included in the study, we observed that there was a gradual increase of both the area and IOD (Figure 4A and D) from the normal tissue to G1, G2 and G3 differentiated adenocarcinoma (from 5598.4 ± 3393.9 $\mu m^2$ for area and 862176.0 ± 469798.7 for IOD in normal tissue to 11583.3 ± 5521.3 $\mu m^2$ for area and 1717361.9 ± 886266.2 for IOD in G1, 27891.1 ± 12118.5 $\mu m^2$ for area and 4240529.2 ± 1795221.7 for IOD in G2 and 37218.5 ± 9738.5 $\mu m^2$ for area and 5560460.5 ± 1720879.6 for IOD in G3). In this instance, we observed a statistical significance on one-way analysis of variance with *post hoc* comparisons using the Bonferroni's test between normal tissue and G2, G3 ($P$ = 0.000) and also between G1 and G2, G3 ($P$ = 0.000) both for the area of B2A and for the IOD.

Regarding the expression of B2A receptors only

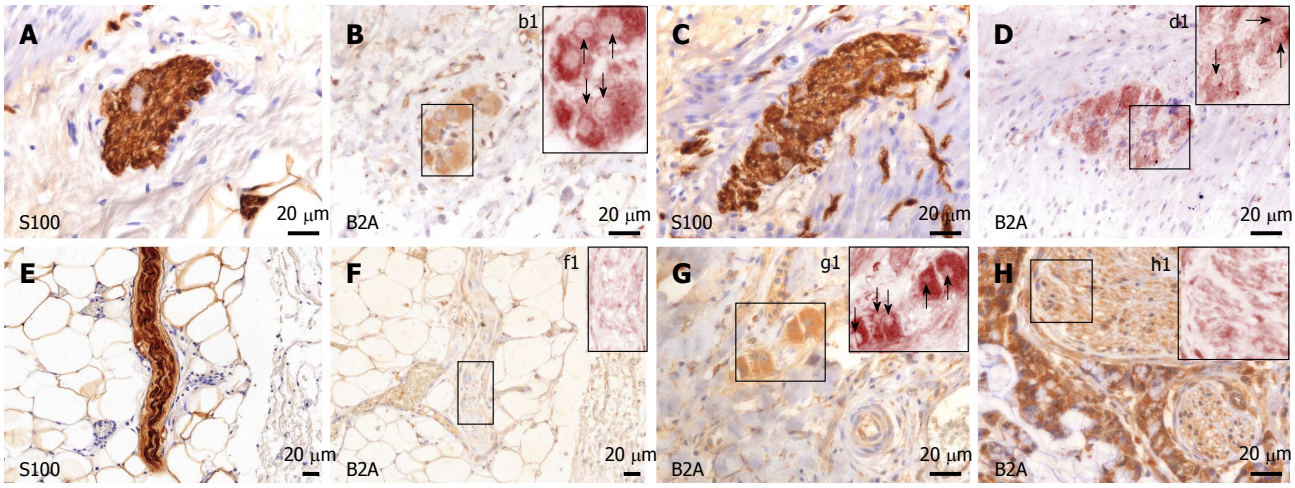



**Figure 3 Expression of beta-2 adrenergic receptors in the normal and tumor peripheral nervous tissue on serial sections immunostained for S100 protein (A, C, E, G) and B2A (B, b1; D; d1; F, f1; H, h1).** Spectral unmixing showed that in the normal submucosal (b1) and myenteric (d1) plexuses, B2A was mostly expressed in the cytoplasm of the ganglion cells, being absent in small nerves dissecting the muscularis proper layer (C and D). Even in larger non-invaded nerves B2A showed a faint expression (E-F, f1 spectral unmixing). However, in invaded ganglia (G, g1) and nerves (H, h1) its expression seems to be expressed in both the cytoplasm of the ganglion cells (arrows in g1 - unmixed), as well as in the larger nerve bundles (h1 - unmixed). Small letters represent enlarged insets from the capital lettered images.

in the epithelium areas, this had a similar growth to its expression in the total tissue (Figure 4B and E); from normal tissue to G1, G2 and G3 differentiated adenocarcinoma (from 3076.5 ± 1895.5 $\mu m^2$ for area and 485440.8 ± 299641.9 for IOD in normal tissue to 7997.6 ± 5344.5 $\mu m^2$ for area and 1255032.6 ± 834235.5 for IOD in G1, 25423.0 ± 11100.6511 $\mu m^2$ for area and 3914461.91 ± 1673240.4 for IOD in G2 and 34670.1 ± 9146.9 $\mu m^2$ for area and 5184455.0 ± 1670704.2 for IOD in G3). Moreover, in this situation, we also observed a statistically significant difference between the normal tissue and G2, G3, similar to the expression of B2A receptors in the total tissue ($P$ = 0.000) and G1, G2 and G3 ($P$ = 0.000), but in addition, we also noticed a statistically significant difference between G2 and G3 ($P$ = 0.047) only for the area, while for IOD there was no significant difference ($P$ = 0.141).

No significant differences between the means of B2A receptor expression and IOD in the stroma of the normal colonic mucosa and different gradings of the colorectal adenocarcinoma could be observed (Figure 4C and F). These data show an increase of the expression of B2A receptors with the tumor grading with regard to the epithelium and not the stroma.

### *Morphological changes of the ENS in colorectal adenocarcinoma*

We analyzed the submucosal (Meissner's), myenteric (Auerbach's) and intratumoral nervous plexuses, as well as those of multiaxonal bundles of nerves (larger than 20 μm) that could not be included either in the Auerbach or in the Meissner plexus, by calculating the percentage average of the area of total nervous tissue separately for both the normal colonic tissue and in different tumor gradings.

The density of the total nervous tissue expressed by percentage area recorded the smallest values in G1 graded tumors (0.129% ± 0.052%), followed by normal colonic tissue (0.184% ± 0.041%), G2 graded tumors (0.355% ± 0.131%) and G3 graded tumors (0.264% ± 0.172%) (Figure 5A). Here, statistical differences between the percentage area from the normal colonic tissue and percentage area from G2 tumors ($P$ = 0.013) were observed.

The density of the Auerbach plexus relative areas revealed a net decrease from the normal tissue (0.136% ± 0.039%) to G1 (0.067% ± 0.043%), G2 (0.094% ± 0.078%) with a minimum for G3 gradings (0.023% ± 0.040%) (Figure 5B). As far as this plexus' density was concerned, significant differences were recorded between the percentage area from the normal colonic tissue and the percentage area from G3 ($P$ = 0.013) tumors.

On the other hand, larger nervous bundles showed about the same pattern for the total analyzed areas (Figure 5C), but with a growth of the percentage nervous area in the normal colonic mucosa (0.013% ± 0.006%), in G1 (0.052% ± 0.033%), G2 (0.248% ± 0.087%) and in G3 (0.241% ± 0.146%) tumors. In this case, significant differences between the percentage nervous area in the normal colonic tissue and the area in G2 ($P$ = 0.001) and G3 tumors ($P$ = 0.002), and also between the percentage nervous area in G1 and the percentage nervous area in G2 ($P$ = 0.004) and G3 tumors ($P$ = 0.008), were recorded.

Furthermore, differences between the relative area of Meissner plexuses revealed the same pattern as for the Auerbach plexuses (Figure 5D), with a maximum in the normal colonic tissue (0.034% ± 0.017%),

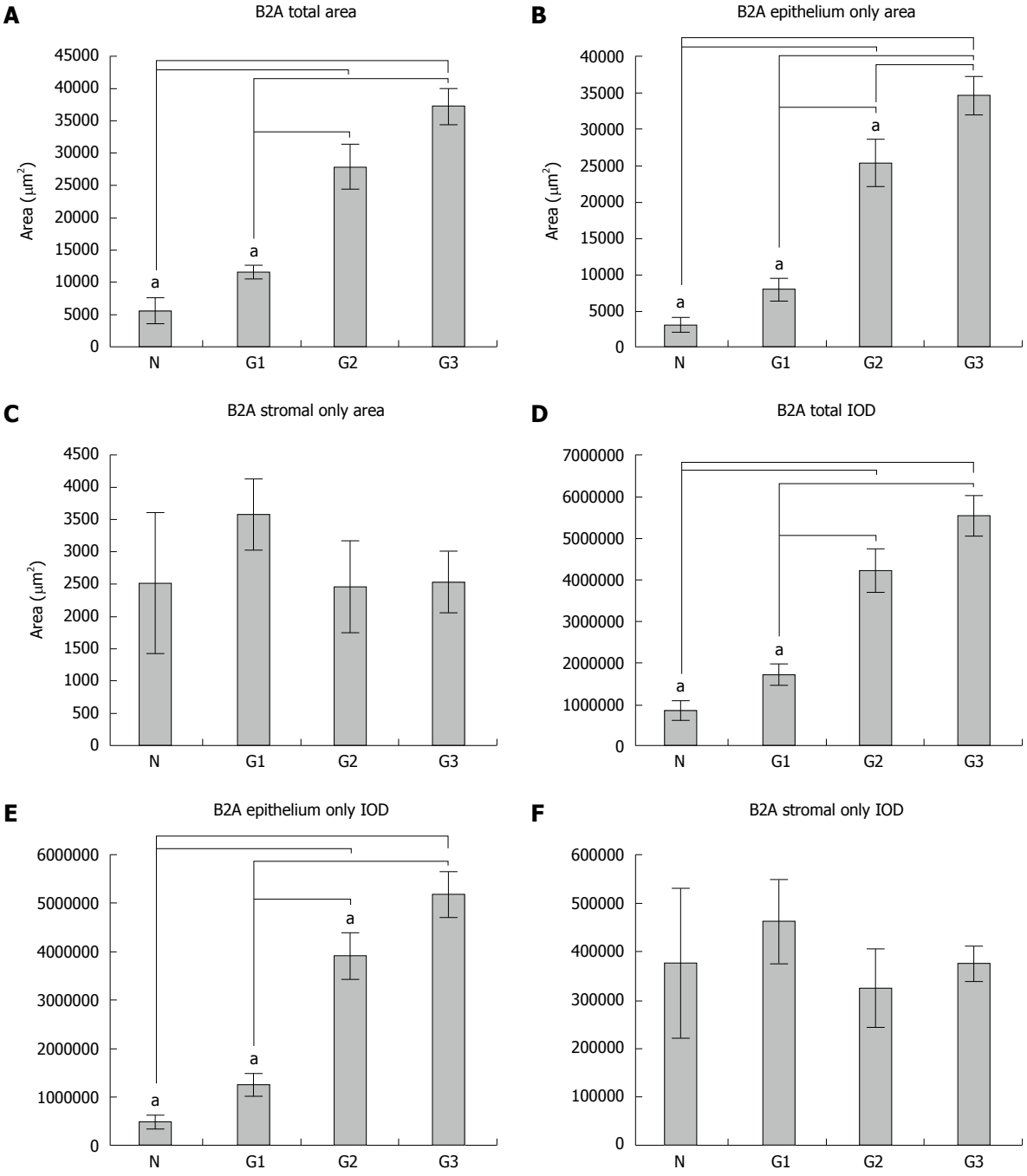



**Figure 4 Analysis of the expression of beta-2 adrenergic receptors.** Total expression area increases gradually from normal tissue to G1, G2 and G3 differentiated adenocarcinoma (A), and this differentiation is driven by the expression in the epithelial areas (B), where assessing stromal signal revealed no differences (C). The same scenario is obtained when evaluating the integrated optical density (IOD) of the signal (D-F). $^{a}P < 0.05$ represents statistical significance on one-way analysis of variance with *post hoc* comparisons using the Bonferroni's test. Error bars represent standard errors of the mean. B2A: Beta-2 adrenergic.

followed by G1 (0.009% ± 0.005%), G2 (0.012% ± 0.013%) and with a minimum in G3 tumors (0.002% ± 0.003%).

These data suggest, on one hand, a decrease of the relative area of both Auerbach and Meissner plexuses with increasing tumor grading, and on the other hand, an increase of the relative area of other nervous elements that could not be included either in the Meissner plexus or in the Auerbach plexus, again with the tumor grading.

### *Relationship between B2A receptor area-IOD and clinicopathological features*

Both B2A receptor expression area and IOD were significantly linked with tumor size, tumor invasion and lymph node metastasis, while there were no statistically significant connections with gender, CRC location and gross appearance. Regarding the involvement of age, there was no significant difference between B2A area in patients under 60 years old and patients over this age, while between B2A IOD

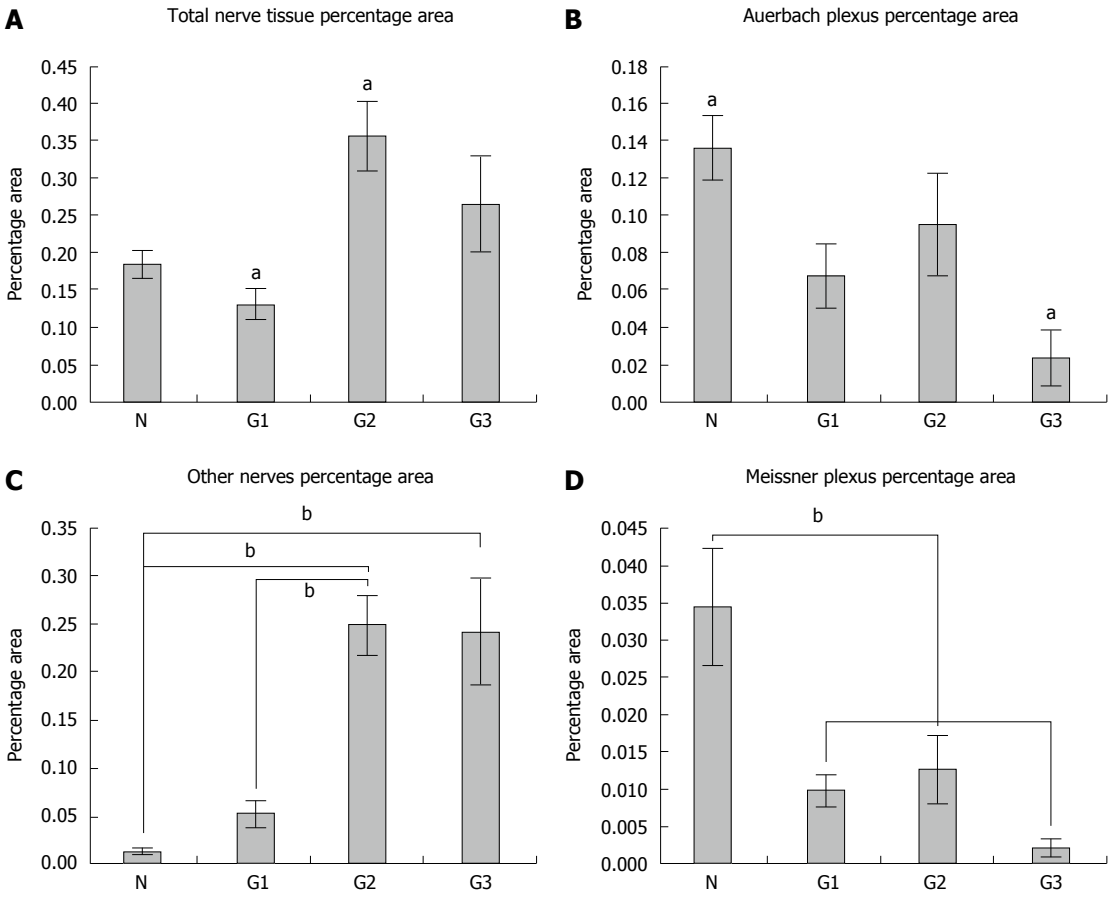



**Figure 5 Comparison of the percentage areas of the total analyzed tissue fragments for the submucosal, myenteric plexuses, as well as for the multi-axonal fiber bundles.** For the above peripheral total nerve elements, G1 graded tumors had the smallest percentage areas, followed by normal colon tissue and G2-G3 differentiated tumors (A); Density of the Auerbach plexuses relative areas revealed a clearcut decrease from normal tissue to G1-G2-G3 gradings, with a minimum for G3 grading (B); Larger nervous bundles showed the same pattern as for the total analyzed areas (C), while differences between the relative areas of Meissner plexuses revealed the same pattern as for the Auerbach plexuses (D), revealing that the total and plexus areas variate in opposite directions when considering increasing gradings. $^{a}P < 0.05$ and $^{b}P < 0.01$ represent statistical significance on one-way analysis of variance with *post hoc* comparisons using the Bonferroni's test. Error bars represent standard errors of the mean.

and patients included in these age groups we found a significant difference ($P = 0.018$). These data are summarized in Table 1.

Moreover, for G1 tumors we found that epithelial B2A area showed an inverse correlation with the Auerbach plexus areas [$r(14) = -0.531$, $P < 0.05$], while for G2 tumors, epithelial B2A areas showed an indirect variation with both the Auerbach plexus areas [$r(14) = -0.453$, $P < 0.05$] and the Meissner areas [$r(14) = -0.825$, $P < 0.01$]. For G3 tumors, the inverse dependence increased for both Auerbach [$r(14) = -0.587$, $P < 0.05$] and Meissner [$r(14) = -0.934$, $P < 0.05$] plexuses. In control tissue, we also found a strong indirect correlation between the B2A signal area and the Auerbach relative areas [$r(7) = -0.897$, $P < 0.01$], but not with the Meissner plexuses.

## DISCUSSION

Both tumor microenvironment and its constituents have an extremely important role in carcinogenesis and in tumor progression. The interaction of neoplastic and of endothelial cells and the extracellular matrix with the cells of the immune system and also with other elements have been carefully analyzed for their contribution as putative molecular targeted (antiangiogenic) therapies[14]. However, the mechanisms that drive the pathogenesis and evolution of neoplasms are far from being understood. In colorectal neoplasm, nervous elements from the tumor microenvironment seem to play an important role. Regarding, for example, perineural invasion, Liebl *et al*[15], in a study that included 673 patients diagnosed with colorectal cancer, concluded that nerve invasion influences in a negative way the survival of the patients with colorectal cancer. However, it is not known if the nervous elements intervene first in the neuro-neoplastic chain, or if this role belongs to neoplastic cells both at the colorectal level and in other localizations of the neoplastic process.

In our study, we approached the neuro-neoplastic interrelationships in colorectal adenocarcinoma, both as a structural characterization (by showing the changes of the ENS) and as an indirect functional

**Table 1 Relationship between beta-2 adrenergic receptor expression and clinicopathological features**

| Clinicopathological features | *n* | B2A only area ($\mu m^2$) mean ± SD | *P* value[1] | B2A only IOD mean ± SD | *P* value[1] |
|---|---|---|---|---|---|
| Gender | | | | | |
| Male | 38 | 25332.4 ± 15753.5 | 0.126 | 2861294.1 ± 1735364.6 | 0.109 |
| Female | 19 | 19450.2 ± 11703.5 | | 3833335.5 ± 2431953.4 | |
| Age group | | | | | |
| < 60 | 15 | 28796.9 ± 13781.3 | 0.085 | 4742202.6 ± 2372372.902 | 0.018[a] |
| ⩾ 60 | 42 | 21584.2 ± 14860.9 | | 3086368.8 ± 2092543.9 | |
| Tumor size | | | | | |
| < 5 cm | 23 | 15778.7 ± 13661.5 | 0.003[a] | 2463149.731 ± 2229187.1 | 0.008[a] |
| ⩾ 5 cm | 25 | 28241.1 ± 13584.4 | | 4188867.1 ± 2074271.4 | |
| CRC location | | | | | |
| Right and transvers colon | 9 | 24817.5 ± 19874.7 | 0.412 | 3818617.6 ± 3131069.5 | 0.375 |
| Left colon, sigmoid and rectum | 39 | 23347.2 ± 14047.3 | | 3492859.3 ± 2140467.2 | |
| Gross appearance | | | | | |
| Exophytic | 20 | 23166.8 ± 14796.3 | 0.429 | 3260358.1 ± 2057001.7 | 0.208 |
| Infiltrative, ulcero-infiltrative | 28 | 24010.8 ± 15114.6 | | 3852705.4 ± 2501409.4 | |
| Tumor invasion | | | | | |
| $T_{1-2}$ | 17 | 11110.3 ± 6784.4 | 0.000[a] | 1650300.5 ± 1004078.8 | 0.000[a] |
| $T_{3-4}$ | 31 | 36025.2 ± 8632.1 | | 5433145.7 ± 1423440.9 | |
| Lymph node metastasis | | | | | |
| $N_{0-1}$ | 21 | 19264.3 ± 12628.1 | 0.010[a] | 2904065.0 ± 1922977.6 | 0.013[a] |
| $N_{\geqslant 2}$ | 27 | 30022.8 ± 15745.7 | | 4498210.2 ± 2467186.4 | |

[1]Student *t*-test. [a]$P < 0.05$ statistically significant. IOD: Integrated optical density; B2A: Beta-2 adrenergic.

characterization (by analyzing the expression of B2A receptors).

Regarding the morphology of the ENS, we found a decrease of the relative area, both for Auerbach and Meissner plexuses, with the increase of the tumor grading and on the other hand, an increase of the relative area of other nervous elements that could not be stratified in the Meissner plexus or in the Auerbach plexus with the tumor grading. A study from 2004 showed that degenerative changes of the intrinsic neurons of the digestive tract cause either direct proportional variation between the size of nervous ganglions and tumor invasion and also an inverse proportional variation with the expression of synaptophysin in the enteric neurons of the colon[16]. However, Godlewski showed that enteric nervous elements were not found in solid colorectal tumors, a finding which in fact supports some symptoms of patients with colorectal carcinoma[17]. Kozlowska *et al*[18] recently discovered that atrophy of the myenteric plexus takes place in the immediate vicinity of the colorectal neoplasm, leading to a decrease both in the size of the nervous ganglia and also in the number of neurons per plexus, in comparison with the nervous plexuses located at a distance from the tumor advancing edge, but this atrophy did not occur in the submucosal plexus. The atrophy of the myenteric plexus is most probably not caused by apoptosis, as both the extrinsic pathway of apoptosis activation *via* caspase 8 (CASP8) and the intrinsic pathway *via* activation of the caspase 3 (CASP3) are not different between the myenteric plexuses situated in the immediate vicinity of the colorectal neoplasm and those which are located at a distance from this process[18]. Many of the symptoms specific to colorectal cancer, such as change in bowel habit, flatulence, bloating and mucus discharge, constipation due to the alteration of the peristalsis, abdominal pain and others[19] can be explained by the structural alterations of the ENS, highlighted both in our study as well as in the studies mentioned above.

Regarding the neuro-neoplastic interrelationships at the colorectal level, we chose to study the expression of B2A receptors, because many intracellular signaling pathways have been discovered *via* this type of receptors up till now; these are involved in the initiation, progression and cancer metastasis. In a previous study of a group of three patients, we have recently reported that the expression of B2A receptors increases with the tumor grading, but a large cohort study is still necessary in order to confirm this observation[20]. Neurotransmitters which act on this type of receptor are mainly adrenaline and noradrenaline. The role of noradrenaline in colorectal cancer development was highlighted by an increase in the locomotor activity of SW 480 colon carcinoma cells and by blocking its effects on this type of cells after using the beta1/2 blocker propranolol[21]. Moreover, the norepinephrine transporter, which plays an important role in mediating the effects of norepinephrine, is expressed by the enteric neurons[11]. Also, adrenaline acts on this type of receptor, being the most important agonist of B2A receptors[22]. Several studies have shown the effects of adrenaline in colorectal adenocarcinoma[23-26]. It is well known that B2A receptors for these neurotransmitters are G protein-coupled receptors[27], and their activation may trigger complex intracellular signaling involved

in the carcinogenesis process, such as the activation of the arachidonic acid cascade and also other pathways *via* the second messenger cyclic adenosine monophosphate (cAMP)[14,28]. Other intracellular signaling pathways are: proto-oncogene tyrosine-protein kinase Src (c-Src), involved in cancer progression and also in promoting other signals; mitogen-activated protein kinase (MAPK); protein kinase B(Akt); extracellular signal-regulated kinases (ERK1/2); cAMP response elements (CREB), which are linked to certain DNA sequences that decrease or increase the transcription of variate genes downstream[12,29,30]. This type of receptor was also studied in other types of cancer including for example pancreas cancer[28], lung cancer[31], breast cancer[32] and prostate cancer[33]. Moreover, our analysis revealed a possible intranuclear/tight perinuclear localization of B2A receptors in poorly differentiated adenocarcinoma, suggesting a connection between its arresting on the downstream of the signaling pathways and more drastic cellular dedifferentiation and loss of function/gain of function signaling misbalances. Although more data are not yet available to clearly show the connection between B2A receptor levels and tumor recurrence, corresponding to the clinical outcomes of these patients, we are following up these patients for future reference. To date, functional B2A receptors have been reportedly found on the nuclear membranes of ventricular myocardiocytes[34], but to our knowledge this is the first description for colon adenocarcinoma cells. Further functional and electron microscopy studies will still be needed to clarify the implications of this observation.

In our study, it was not possible to know for sure if the nervous structures that were deemed as other nerves were actually modified ganglia from the two plexuses or whether they represent sympathetic or parasympathetic afferents or efferents. They could not be catalogued either as ganglia belonging to the Meissner plexus or as ganglia belonging to the Auerbach plexus due to the structural changes caused by the neoplastic process. Neuro-neoplastic functional studies are required in order to find out if these nervous elements found in advanced gradings of colorectal adenocarcinoma secrete adrenaline and noradrenaline, and in this case, the regulation of B2A receptors examined in our study would be paracrine; however, the source of catecholamines could only be represented by the medulla, which seems to be least plausible if we take into account the short half-life of the catecholamines that activate B2A receptors[22].

We showed here that B2A area of expression in the tumor epithelium shows a moderate to strong inverse correlation with the areas of the Auerbach and Meissner plexuses. Although these are components of the stroma, there was no significant correlation between stromal or total B2A area and the number of nervous plexuses. There are too few elements to judge a functional association between these factors, but this is to our knowledge the first report of such a correlation for colon cancer. However, there are studies on breast tumors showing that stress-induced neuroendocrine activation leads to a massive increase of metastasis, and treatment of stressed animals with the β-antagonist propranolol reversed the stress-induced inflammatory infiltrate and inhibited tumor metastasis[32]; all these suggesting that the sympathetic nervous system might act as a regulator for tumor spreading and metastasis.

In conclusion, B2A receptors have a high expression level in the neoplastic cells from colorectal adenocarcinoma, and we found a significant association between the expression of B2A receptors and tumor grading. This shows that B2A receptors may play an important role in antineoplastic therapy or that they can be utilized as a prognostic factor. On the other hand, besides the functional changes caused by B2A receptors, structural changes of the ENS caused by the neoplastic process and the upward trend of the relative area of the nerves in colorectal adenocarcinoma might suggest that studies to develop targeted molecular therapies could be aimed not only at angiogenesis but also at neurogenesis in colorectal cancer; this might represent an important treatment pathway.

## COMMENTS

### Background

Colorectal cancer still remains one of the major health problems worldwide. In this article, the authors wanted to compare the morphological changes of the enteric nervous system (ENS) and to evaluate the changes in expression of the beta-2 adrenergic (B2A) receptors in primary colorectal cancer compared with normal colic mucosa, and also to find significant associations between these parameters and clinicopathological features.

### Research frontiers

The mechanisms that drive the pathogenesis and the evolution of neoplasms are far from being understood. Translational studies that identify new colorectal cancer prognostic markers, molecular changes that appear at the initiation of carcinogenesis and during cancer's progression, as well as treatment targets such as nervous elements, are thus needed.

### Innovations and breakthroughs

The authors provide the first description of B2A receptors found in/on the nuclear membrane of colon adenocarcinoma cells. B2A receptors have a high level of expression in the neoplastic cells from colorectal adenocarcinoma, and a significant association was found between the expression of B2A receptors and tumor grading. Until now, many intracellular signaling pathways have been discovered *via* this type of receptor, which are involved in the initiation, progression and cancer metastasis. On the other hand, in colorectal neoplasm, nervous system elements from the tumor microenvironment seem to play an important role.

### Applications

The present study looks into B2A receptors and morphological changes of the ENS in primary colorectal cancer. B2A receptors may play an important role in antineoplastic therapy, and they can be utilized as a critical prognostic factor. On the other hand, the structural changes of the ENS in colorectal adenocarcinoma may lead to targeted molecular therapies not only regarding

angiogenesis, but also concerning neurogenesis in colorectal cancer.

### Terminology

B2A receptors are G protein-coupled receptors and their activation may trigger complex intracellular signaling involved in the carcinogenesis process. Neurotransmitters which act on this type of receptor are mainly adrenaline and noradrenaline.

### Peer-review

The authors investigated the neuro-neoplastic interrelationship in colon cancer. Authors assessed the expression pattern of B2A receptors and morphological changes of the enteric nervous system in 48 primary colorectal cancers and 9 control non-colon cancer specimens. They concluded that B2A receptors could serve as a prognostic factor in colorectal cancer.

## REFERENCES


1 **Miller KD**, Siegel RL, Lin CC, Mariotto AB, Kramer JL, Rowland JH, Stein KD, Alteri R, Jemal A. Cancer treatment and survivorship statistics, 2016. *CA Cancer J Clin* 2016; **66**: 271-289 [PMID: 27253694 DOI: 10.3322/caac.21349]

2 **Ferlay J**, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015; **136**: E359-E386 [PMID: 25220842 DOI: 10.1002/ijc.29210]

3 **Janakiram NB**, Rao CV. Molecular markers and targets for colorectal cancer prevention. *Acta Pharmacol Sin* 2008; **29**: 1-20 [PMID: 18158862 DOI: 10.1111/j.1745-7254.2008.00742.x]

4 **Fife A**, Beasley PJ, Fertig DL. Psychoneuroimmunology and cancer: historical perspectives and current research. *Adv Neuroimmunol* 1996; **6**: 179-190 [PMID: 8876773 DOI: 10.1016/0960-5428(96)00016-2]

5 **Sayegh AI**, Washington MC. Back to Basics: Regulation of the Gastrointestinal Functions. *J Gastroint Dig Syst* 2012; **2**: 118 [DOI: 10.4172/2161-069X.1000118]

6 **Furness JB**. The enteric nervous system and neurogastroenterology. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 286-294 [PMID: 22392290 DOI: 10.1038/nrgastro.2012.32]

7 **Hansen MB**. The enteric nervous system I: organisation and classification. *Pharmacol Toxicol* 2003; **92**: 105-113 [PMID: 12753424 DOI: 10.1034/j.1600-0773.2003.t01-1-920301.x]

8 **Furness JB**. Integrated Neural and Endocrine Control of Gastrointestinal Function. *Adv Exp Med Biol* 2016; **891**: 159-173 [PMID: 27379644 DOI: 10.1007/978-3-319-27592-5_16]

9 **Lake JI**, Heuckeroth RO. Enteric nervous system development: migration, differentiation, and disease. *Am J Physiol Gastrointest Liver Physiol* 2013; **305**: G1-G24 [PMID: 23639815 DOI: 10.1152/ajpgi.00452.2012]

10 **Mayer EA**, Tillisch K. The brain-gut axis in abdominal pain syndromes. *Annu Rev Med* 2011; **62**: 381-396 [PMID: 21090962 DOI: 10.1146/annurev-med-012309-103958]

11 **Li Z**, Caron MG, Blakely RD, Margolis KG, Gershon MD. Dependence of serotonergic and other nonadrenergic enteric neurons on norepinephrine transporter expression. *J Neurosci* 2010; **30**: 16730-16740 [PMID: 21148012 DOI: 10.1523/JNEUROSCI.2276-10.2010]

12 **Chin CC**, Li JM, Lee KF, Huang YC, Wang KC, Lai HC, Cheng CC, Kuo YH, Shi CS. Selective β2-AR Blockage Suppresses Colorectal Cancer Growth Through Regulation of EGFR-Akt/ERK1/2 Signaling, G1-Phase Arrest, and Apoptosis. *J Cell Physiol* 2016; **231**: 459-472 [PMID: 26189563 DOI: 10.1002/jcp.25092]

13 **Hamilton SR**, Bosman FT, Boffetta P. Carcinoma of the colon and rectum. In: Bosman FT, Carneiro F, Hruban RH, Theise ND. WHO Classification of Tumours of the Digestive System. Lyon: IARC Press, 2010, **8**: 134-146

14 **Jobling P**, Pundavela J, Oliveira SM, Roselli S, Walker MM, Hondermarck H. Nerve-Cancer Cell Cross-talk: A Novel Promoter of Tumor Progression. *Cancer Res* 2015; **75**: 1777-1781 [PMID: 25795709 DOI: 10.1158/0008-5472.CAN-14-3180]

15 **Liebl F**, Demir IE, Rosenberg R, Boldis A, Yildiz E, Kujundzic K, Kehl T, Dischl D, Schuster T, Maak M, Becker K, Langer R, Laschinger M, Friess H, Ceyhan GO. The severity of neural invasion is associated with shortened survival in colon cancer. *Clin Cancer Res* 2013; **19**: 50-61 [PMID: 23147996 DOI: 10.1158/1078-0432.CCR-12-2392]

16 **Sobaniec-Lotowska ME**, Ciołkiewicz M, Pogumirski J, Sulkowski S, Sobczak A. Morphometry of synaptophysin immunoreactive ganglion cells in Auerbach plexus in patients with colorectal cancer. Is this a new prognostic factor? *Folia Neuropathol* 2004; **42**: 167-170 [PMID: 15535035]

17 **Godlewski J**. Morphological changes in the enteric nervous system caused by carcinoma of the human large intestine. *Folia Histochem Cytobiol* 2010; **48**: 157-162 [PMID: 20529833 DOI: 10.2478/v10042-010-0029-8]

18 **Kozlowska A**, Kwiatkowski P, Oponowicz A, Majewski M, Kmiec Z, Godlewski J. Myenteric plexuses atrophy in the vicinity of colorectal cancer tissue is not caused by apoptosis or necrosis. *Folia Histochem Cytobiol* 2016; **54**: 99-107 [PMID: 27439439 DOI: 10.5603/FHC.a2016.0012]

19 **Walter FM**, Emery JD, Mendonca S, Hall N, Morris HC, Mills K, Dobson C, Bankhead C, Johnson M, Abel GA, Rutter MD, Hamilton W, Rubin GP. Symptoms and patient factors associated with longer time to diagnosis for colorectal cancer: results from a prospective cohort study. *Br J Cancer* 2016; **115**: 533-541 [PMID: 27490803 DOI: 10.1038/bjc.2016.221]

20 **Florescu C**, Istrătoaie O, Târtea GC, Pirici D, Streba CT, Cătălin B, Puiu I, Târtea EA, Caragea DC, Ghiluşi MC, Comănescu MV, Rogoveanu I, Vere CC. Neuro-neoplastic interrelationships in colorectal level - immunohistochemical aspect in three cases and review of the literature. *Rom J Morphol Embryol* 2016; **57**: 639-650 [PMID: 27833955]

21 **Masur K**, Niggemann B, Zanker KS, Entschladen F. Norepinephrine-induced migration of SW 480 colon carcinoma cells is inhibited by beta-blockers. *Cancer Res* 2001; **61**: 2866-2869 [PMID: 11306460]

22 **Westfall TC**, Westfall DP. Adrenergic agonists and antagonists. In: Brunton LL, Chabner BA, Knollmann BC . Goodman & Gilman's the pharmacological basis of therapeutics. 12th edition. New York: McGraw-Hill, 2011: 277-334

23 **Yao H**, Duan Z, Wang M, Awonuga AO, Rappolee D, Xie Y. Adrenaline induces chemoresistance in HT-29 colon adenocarcinoma cells. *Cancer Genet Cytogenet* 2009; **190**: 81-87 [PMID: 19380024 DOI: 10.1016/j.cancergencyto.2008.12.009]

24 **Wong HP**, Ho JW, Koo MW, Yu L, Wu WK, Lam EK, Tai EK, Ko JK, Shin VY, Chu KM, Cho CH. Effects of adrenaline in human colon adenocarcinoma HT-29 cells. *Life Sci* 2011; **88**: 1108-1112 [PMID: 21565206 DOI: 10.1016/j.lfs.2011.04.007]

25 **Pu J**, Bai D, Yang X, Lu X, Xu L, Lu J. Adrenaline promotes cell proliferation and increases chemoresistance in colon cancer HT29 cells through induction of miR-155. *Biochem Biophys Res Commun* 2012; **428**: 210-215 [PMID: 23036199 DOI: 10.1016/j.bbrc.2012.09.126]

26 **Coelho M**, Moz M, Correia G, Teixeira A, Medeiros R, Ribeiro L. Antiproliferative effects of β-blockers on human colorectal cancer cells. *Oncol Rep* 2015; **33**: 2513-2520 [PMID: 25812650 DOI: 10.3892/or.2015.3874]

27 **Irina G**, Katrin A, Terence EH. Organizational Complexity of b-adrenergic Receptor Signaling Systems. In: Wang Q, Benos DJ, Balaban R, Simon SA, editors. Advances in Adrenergic Receptor Biology. Elsevier, 2011: 19-49

28 **Zhang D**, Ma QY, Hu HT, Zhang M. β2-adrenergic antagonists suppress pancreatic cancer cell invasion by inhibiting CREB, NFκB and AP-1. *Cancer Biol Ther* 2010; **10**: 19-29 [PMID: 20424515]

29 **Tang J**, Li Z, Lu L, Cho CH. β-Adrenergic system, a backstage manipulator regulating tumour progression and drug target in cancer therapy. *Semin Cancer Biol* 2013; **23**: 533-542 [PMID: 24012659 DOI: 10.1016/j.semcancer.2013.08.009]

30 **Armaiz-Pena GN**, Allen JK, Cruz A, Stone RL, Nick AM, Lin

YG, Han LY, Mangala LS, Villares GJ, Vivas-Mejia P, Rodriguez-Aguayo C, Nagaraja AS, Gharpure KM, Wu Z, English RD, Soman KV, Shahzad MM, Zigler M, Deavers MT, Zien A, Soldatos TG, Jackson DB, Wiktorowicz JE, Torres-Lugo M, Young T, De Geest K, Gallick GE, Bar-Eli M, Lopez-Berestein G, Cole SW, Lopez GE, Lutgendorf SK, Sood AK. Src activation by β-adrenoreceptors is a key switch for tumour metastasis. *Nat Commun* 2013; **4**: 1403 [PMID: 23360994 DOI: 10.1038/ncomms2413]

31 **Al-Wadei HA**, Plummer HK, Ullah MF, Unger B, Brody JR, Schuller HM. Social stress promotes and γ-aminobutyric acid inhibits tumor growth in mouse models of non-small cell lung cancer. *Cancer Prev Res* (Phila) 2012; **5**: 189-196 [PMID: 21955519 DOI: 10.1158/1940-6207.CAPR-11-0177]

32 **Sloan EK**, Priceman SJ, Cox BF, Yu S, Pimentel MA, Tangkanangnukul V, Arevalo JM, Morizono K, Karanikolas BD, Wu L, Sood AK, Cole SW. The sympathetic nervous system induces a metastatic switch in primary breast cancer. *Cancer Res* 2010; **70**: 7042-7052 [PMID: 20823155 DOI: 10.1158/0008-5472.CAN-10-0522]

33 **Ventura S**, Evans BA. Does the autonomic nervous system contribute to the initiation and progression of prostate cancer? *Asian J Androl* 2013; **15**: 715-716 [PMID: 24141535 DOI: 10.1038/aja.2013.114]

34 **Boivin B**, Lavoie C, Vaniotis G, Baragli A, Villeneuve LR, Ethier N, Trieu P, Allen BG, Hébert TE. Functional beta-adrenergic receptor signalling on nuclear membranes in adult rat and mouse ventricular cardiomyocytes. *Cardiovasc Res* 2006; **71**: 69-78 [PMID: 16631628]

**P- Reviewer**: Lakatos PL, Steel JC **S- Editor**: Ma YJ
**L- Editor**: Logan S **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/ *World J Gastroenterol* 2017 February 21; 23(7): 1262-1267

DOI: 10.3748/wjg.v23.i7.1262 ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Observational Study

# Predictors of poor outcomes in patients with wild mushroom-induced acute liver injury

Taerim Kim, Danbi Lee, Jae Ho Lee, Yoon-Seon Lee, Bum Jin Oh, Kyoung Soo Lim, Won Young Kim

Taerim Kim, Jae Ho Lee, Yoon-Seon Lee, Bum Jin Oh, Kyoung Soo Lim, Won Young Kim, Department of Emergency Medicine, University of Ulsan College of Medicine, Asan Medical Center, Seoul 05505, South Korea

Danbi Lee, Department of Gastroenterology, Asan Medical Center, University of Ulsan College of Medicine, Seoul 05505, South Korea

Author contributions: Lee JH, Lee YS, Oh BJ, Lim KS and Kim WY designed the research; Lee D performed the research; Kim T collected data; Kim WY analyzed data; Kim T and Kim WY wrote the paper; all authors have read and approved the final version to be published.

Institutional review board statement: Based on the Declaration of Helsinki, Asan Medical Center Institutional Review Board approved the retrospective use of the clinical, biochemical, and radiographic data for the present study.

Informed consent statement: The requirements for informed consent were waived due to the retrospective design.

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

Data sharing statement: No additional data are available.



Manuscript source: Invited manuscript

Correspondence to: Won Young Kim, MD, PhD, Department of Emergency Medicine, University of Ulsan College of Medicine, Asan Medical Center, 88 Olympic-ro 43-gil, Songpa-gu, Seoul 05505, South Korea. wonpia73@naver.com

Telephone: +82-2-30103350
Fax: +82-2-30103360

Received: November 7, 2016
Peer-review started: November 7, 2016
First decision: December 19, 2016
Revised: January 2, 2017
Accepted: January 11, 2017
Article in press: January 11, 2017
Published online: February 21, 2017

## Abstract

### *AIM*

To identify early predictive markers of poor outcomes in patients with acute liver injury from wild mushroom intoxication.

### *METHODS*

This observational, retrospective record review involved adults aged ⩾ 18 years admitted to emergency department with mushroom intoxication from January 2005 to December 2015. The diagnosis of mushroom intoxication was based on the following: (1) a positive history of recent wild mushroom intake (either raw or cooked); (2) the onset of gastrointestinal symptoms, such as watery diarrhea, vomiting, and/or abdominal pain, after ingestion; and (3) the exclusion of other possible causes of acute liver injury. Acute liver injury was defined by a > 5-fold elevation of liver enzymes or moderate coagulopathy [international normalized ratio (INR) > 2.0]. Clinical and laboratory findings were compared in survivors and non-survivors.

### *RESULTS*

Of 93 patients with mushroom intoxication, 23, 11 men (47.8%) and 12 women (52.2%), of median age 61 years, developed acute liver injury. The overall in-hospital mortality rate was 43.5% (10/23). Among the

laboratory variables, mean serum alkaline phosphatase (73.38 ± 10.89 mg/dL *vs* 180.40 ± 65.39 mg/dL, $P$ < 0.01), total bilirubin (2.312 ± 1.16 mg/dL *vs* 7.16 ± 2.94 mg/dL, $P$ < 0.01) concentrations and indirect/direct bilirubin (2.45 ± 1.39 mg/dL *vs* 0.99 ± 0.45 mg/dL, $P$ < 0.01) ratio as well as prothrombin time (1.88 ± 0.83 mg/dL *vs* 10.43 ± 4.81 mg/dL, $P$ < 0.01), and activated partial thromboplastin time (aPTT; 32.48 ± 7.64 s *vs* 72.58 ± 41.29 s, $P$ = 0.01), were significantly higher in non-survivors than in survivors. Logistic regression analysis showed that total bilirubin concentration (OR = 3.58, 95%CI: 1.25-10.22), indirect/direct bilirubin ratio (OR = 0.14, 95%CI: 0.02-0.94) and aPTT (OR = 1.30, 95%CI: 1.04-1.63) were significantly associated with mortality. All patients with total bilirubin > 5 mg/dL or aPTT > 50 s on day 3 died.

***CONCLUSION***

Monitoring of bilirubin concentrations and aPTT may help in predicting clinical outcomes in patients with acute liver injury from wild mushroom intoxication.

**Key words:** Mushroom; Liver; Outcome; Intoxication; Bilirubin



**Core tip:** Wild mushroom-induced acute liver failure is potentially fatal. Many candidates for liver transplantation progress to multi-organ failure resulting in deterioration while awaiting liver transplantation. Identifying early predictive markers of poor outcomes in patients with acute liver injury resulting from wild mushroom intoxication is critical for improving survival rates. Total bilirubin and activated partial thromboplastin time (aPTT) levels were associated with in-hospital mortality in patients with acute liver injury from wild mushroom intoxication. Monitoring total bilirubin and aPTT as predictors of survival outcomes may determines the need for advanced intervention such as liver transplantation.

Kim T, Lee D, Lee JH, Lee YS, Oh BJ, Lim KS, Kim WY. Predictors of poor outcomes Predictors of poor outcomes in patients with wild mushroom-induced acute liver injury. *World J Gastroenterol* 2017; 23(7): 1262-1267 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1262.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1262

## INTRODUCTION

Although mushroom poisoning is associated with high morbidity and mortality rates[1], little is known about the in-hospital prognosis of these patients during treatment. Wild mushroom-induced acute liver failure (ALF) is potentially fatal, with selected patients requiring liver transplantation[2,3]. Unfortunately, many candidates for transplantation progress to multi-organ failure resulting in deterioration while awaiting liver transplantation[3-5]. Early and precise determination of patients who will not survive without liver transplantation is crucial for improving survival rates[1], indicating the importance of identifying suitable candidates as quickly as possible[5]. Despite several proposed sets of criteria for emergency liver transplantation in patients with ALF, the criteria for emergency liver transplantation in patients with wild mushroom-induced ALF have not been clearly determined[3,6]. This study was therefore designed to identify early predictive markers of poor outcomes in patients with acute liver injury resulting from wild mushroom intoxication.

## MATERIALS AND METHODS

### *Study design and data collection*

This retrospective single-center study was performed at a 2800-bed, university-affiliated, tertiary referral center in Seoul, South Korea. The electronic charts of all adults, aged ⩾ 18 years, with wild mushroom intoxication admitted to the emergency department (ED) from January 2005 to December 2015 were retrospectively reviewed. The diagnosis of mushroom intoxication was based on the following: (1) a positive history of recent intake of wild mushrooms, either raw or cooked; (2) the onset of gastrointestinal symptoms, such as watery diarrhea, vomiting, and/or abdominal pain, after ingestion; and (3) the exclusion of other possible causes of acute liver injury. This study was approved by the ethics committee of our institution, which waived informed consent because of the retrospective design of this study.

Baseline data on patients, including age, gender, medical history, and initial vital signs in the ED, were obtained, as were laboratory data obtained between the time of ED admission (day 1) and hospital day 7. Acute liver injury was defined as a > 5-fold elevation in liver enzymes or moderate coagulopathy (INR > 2.0). The primary outcome was in-hospital mortality, and clinical and laboratory findings were compared in survivors and non-survivors.

### *Statistical analysis*

Continuous variables are presented as median and interquartile range (IQR), and categorical variables as absolute or relative frequencies. Continuous variables were compared in groups of survivors and non-survivors by Mann-Whitney $U$ tests, and categorical variables by Fisher's exact tests. Logistic regression analysis was performed to identify factors associated with in-hospital mortality, with the results of logistic regression analyses summarized as odds ratios (ORs) and 95% confidence intervals (CIs). Because total bilirubin concentrations and activated partial thromboplastin time (aPTT) are numerical data, receiver operating characteristic (ROC) curves were constructed and areas under the curves

**Table 1 Baseline demographic and clinical characteristics of study patients *n* (%)**

| | Total patients (*n* = 23) | Survivors (*n* = 13) | Non-survivors (*n* = 10) | *P* value |
|---|---|---|---|---|
| Age, yr | 61.0 (51.0-66.0) | 61.0 (53.0-69.0) | 52.0 (38.0-62.0) | 0.74 |
| Male | 11 (48) | 6 (46) | 5 (50) | 0.86 |
| Comorbidities | | | | |
| Hypertension | 6 (26) | 5 (38) | 1 (10) | 0.11 |
| Diabetes mellitus | 2 (9) | 2 (17) | 0 (0) | 0.17 |
| Chronic kidney disease | 0 (0) | 0 (0) | 0 (0) | |
| Chronic liver disease | 0 (0) | 0 (0) | 0 (0) | |
| Time from ingestion to hospitalization, d | 3.0 (3.0-3.0) | 3.0 (3.0-3.0) | 3.0 (3.0-3.0) | 0.26 |
| Vital signs, initial | | | | |
| SBP, mmHg | 126.0 (112.0-134.0) | 122.0 (116.0-133.0) | 128.5 (112.0-146.0) | 0.47 |
| DBP, mmHg | 71.0 (66.0-89.0) | 78.0 (67.0-90.0) | 69.5 (66.5-77.5) | 0.59 |
| Heart rate, min | 78.0 (65.0-89.0) | 70.0 (63.0-87.0) | 94.5 (78.5-116.2) | 0.03 |
| Respiratory rate, min | 20.0 (18.0-20.0) | 20.0 (18.0-20.0) | 20.0 (20.0-20.0) | 0.24 |
| Temperature, ℃ | 36.6 (36.1-37.1) | 36.6 (36.2-36.1) | 36.6 (36.1-37.0) | 0.56 |
| $SpO_2$, % | 98.0 (97.0-99.0) | 98.0 (97.0-99.0) | 98.0 (97.0-99.0) | 0.36 |
| Laboratory data | | | | |
| WBC, × $10^3$/mL | 7.5 (5.8-12.2) | 7.0 (6.0-8.3) | 11.3 (6.0-15.6) | 0.11 |
| Hemoglobin, g/dL | 14.1 (13.0-15.4) | 14.2 (13.4-15.4) | 12.8 (10.6-15.0) | 0.12 |
| Platelet, × $10^3$/mL | 172.0 (115.0-223.0) | 197.0 (156.0- 223.0) | 125.5 (87.3-181.3) | 0.06 |
| BUN, mg/dL | 21.0 (9.0-33.0) | 15.0 (10.0-29.0) | 23.0 (10.3-31.5) | 0.66 |
| Cr, mg/dL | 0.90 (0.60-1.30) | 0.70 (0.60-0.90) | 1.70 (1.00-1.80) | 0.23 |
| AST, IU/L | 4568.0 (863.0-6933.0) | 4568.0 (702.0-5471.0) | 5650.0 (2065.3-8155.8) | 0.19 |
| ALT, IU/L | 4750.0 (1561.0-5543.0) | 4436.0 (1561.0-5543.0) | 5002.0 (2235.5-6584.3) | 0.31 |
| γ-GT, IU/L | 38.0 (23.0-71.0) | 26.0 (13.0-53.0) | 55.50 (39.0-85.0) | 0.35 |
| ALP, IU/L | 85.0 (71.0-162.0) | 71.0 (69.0-81.0) | 165.0 (151.0-208.0) | < 0.01 |
| Total bilirubin, mg/dL | 3.50 (1.70-6.40) | 1.80 (1.60-3.00) | 6.80 (6.00-8.80) | < 0.01 |
| Indirect/direct bilirubin ratio | 1.79 (0.5-3.08) | 2.45 (1.06-3.84) | 0.99 (0.54-1.44) | < 0.01 |
| PT-INR, s | 2.90 (1.40-8.80) | 1.60 (1.20-2.60) | 9.70 (7.60-14.30) | < 0.01 |
| aPTT, s | 41.4 (28.9-54.3) | 29.1 (28.9-35.3) | 54.6 (52.6-71.4) | 0.01 |
| Management | | | | |
| Vasopressor | 7 (30) | 0 (0) | 7 (70) | < 0.01 |
| Mechanical ventilation | 6 (26) | 0 (0) | 6 (60) | < 0.05 |
| CRRT | 5 (22) | 0 (0) | 5 (50) | < 0.01 |

Values are expressed as median with interquartile range and *n* (%). SBP: Systolic blood pressure; DBP: Diastolic blood pressure; $SpO_2$: Peripheral capillary oxygen saturation; WBC: White blood cell count; BUN: Blood urea nitrogen; Cr: Creatinine; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γ-GT: Gamma-glutamyl transferase; ALP: Alkaline phosphatase; PT: Prothrombin time; aPTT: Activated partial thromboplastin time; CRRT: Continuous renal replacement therapy.

(AUCs) were evaluated. The cutoff values of total bilirubin and aPTT predicting death were estimated by ROC analyses. A two-sided *P* value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS for Windows, version 21.0 (IBM Corp, Armonk, NY, United States).

## RESULTS

During the study period, 93 patients with wild mushroom intoxication were admitted to the ED, with 23 of these patients developing acute liver injury. These 23 patients consisted of 11 men (47.8%) and 12 women (52.2%), of median age 61 years (IQR, 51-66 years). The median time between mushroom ingestion and hospital admission was 3.0 d. The overall in-hospital mortality rate was 43.5% (10/23).

Table 1 summarizes the demographic characteristics, comorbidities, initial vital signs, and laboratory values of the survivor and non-survivor groups. Mean serum alkaline phosphate (ALP; 73.38 ± 10.89 mg/dL *vs* 180.40 ± 65.39 mg/dL, *P* < 0.01), total bilirubin (2.31 ± 1.16 mg/dL *vs* 7.16 ± 2.94 mg/dL, *P* < 0.01) concentrations and indirect/direct bilirubin (2.45 ± 1.39 *vs* 0.99 ± 0.45, *P* < 0.01) ratio as well as prothrombin time (PT)-INR (1.88 ± 0.83 s *vs* 10.43 ± 4.81 s, *P* < 0.01) and activated partial thromboplastin time (aPTT; 32.48 ± 7.64 s *vs* 72.58 ± 41.29 s, *P* = 0.01), were significantly higher in the non-survivor than in the survivor group. However, other variables, including white blood cell (WBC) and platelet counts and concentrations of hemoglobin (Hb), blood urea nitrogen (BUN), creatinine (Cr), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (γ-GT), did not differ significantly in the survivor and non-survivor groups. Logistic regression analysis showed that elevated total bilirubin concentration (OR = 3.579, 95%CI: 1.253-10.221), indirect/direct bilirubin ratio (OR = 0.14, 95%CI: 0.02-0.94) and prolonged aPTT (OR = 1.301, 95%CI: 1.037-1.634) were significantly associated with patient mortality (Table 2). Figure 1 shows static serum total bilirubin, aPTT, PT INR, and ALP levels in survivors and non-survivors. All patients with total bilirubin > 5 mg/dL

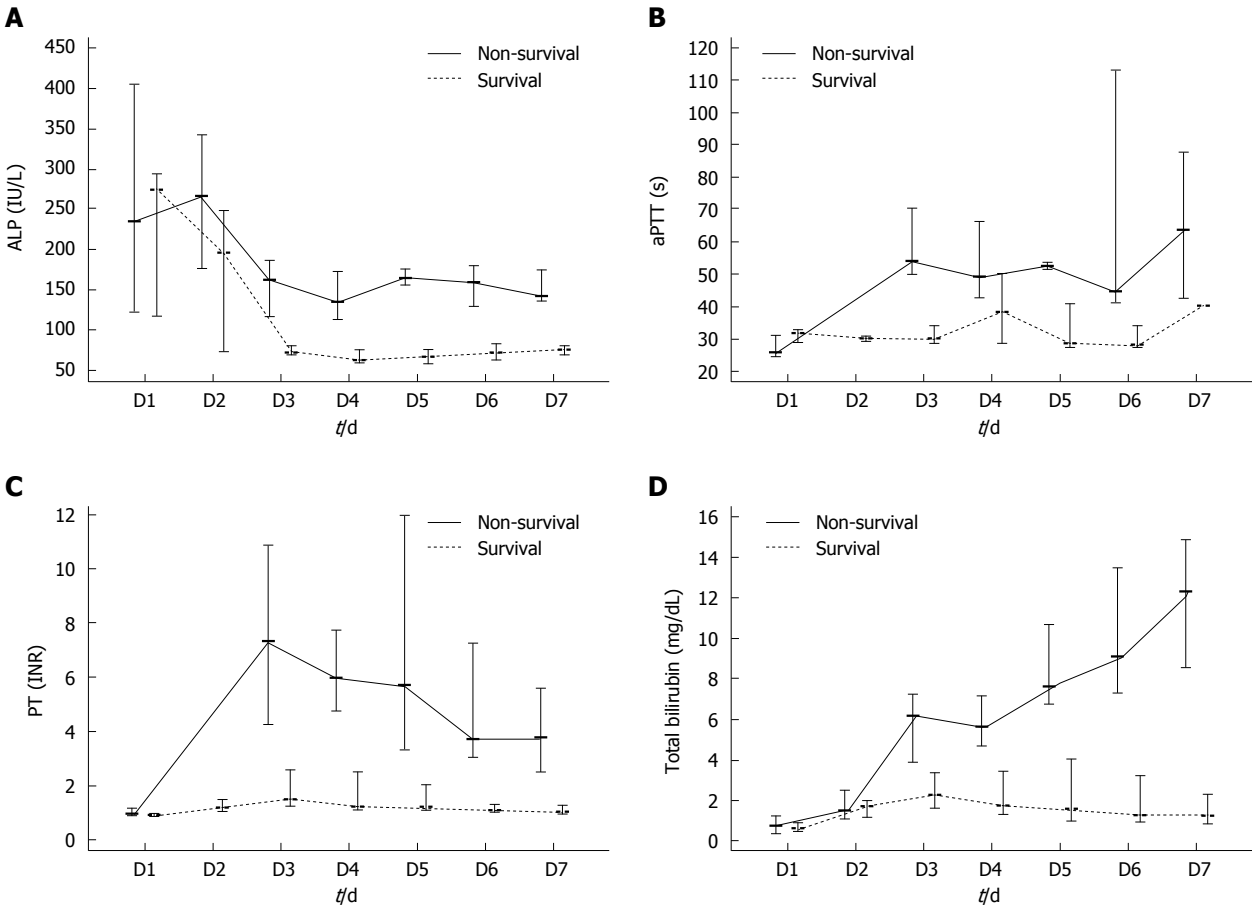



**Figure 1 Static ALP (A), aPTT (B), PT INR (C), and total bilirubin (D) levels in survivors and non-survivors from day 1 to day 7 after wild mushroom ingestion.** ALP: Alkaline phosphatase; aPTT: Activated partial thromboplastin time; PT: Prothrombin time; INR: International normalized ratio.

or aPTT > 50 s on day 3 died in-hospital of ALF.

## DISCUSSION

Poisoning of toxic mushrooms can be divided into seven main categories: amatoxin, gyromitrin, coprine, muscarine, ibotenic acid-muscimol, psilocybin-psilocin and gastrointestinal irritants[7,8]. In South Korea, the vast majority of toxic mushroom ingestions were the amatoxin-containing mushrooms such as Amanita abrupta Peck, Amanita castanopsidis Hongo, Amanita subjunquillea S. Imai, Amanita verna (Bull.) Lam., Amanita virosa (Fr.) Bertill., Conocybe filaris (Fr.) Kühner, Galerina calyptrata P.D. Orton, Galerina helvoliceps (Berk. and M.A. Curtis) Singer[8,9].

This study showed that total bilirubin and aPTT levels were associated with in-hospital mortality in patients with acute liver injury from wild mushroom intoxication. Indeed, all patients with total bilirubin > 5 mg/dL or aPTT > 50 s on day 3 died in-hospital. These results emphasize the critical need for monitoring total bilirubin and aPTT as predictors of survival outcomes and for determining the need for advanced intervention such as liver transplantation.

Although the precise incidence of mushroom poisoning cannot be estimated, the mortality rate in 15 studies involving more than 20 patients from 1980 to 1999 ranges from 4.8% to 34.5%[10]. However, more recent studies report mortality rates of around 10%[11-13]. This is consistent with the results of the present study, which found that 10 (10.8%) of the 93 patients with mushroom poisoning died. These 10 patients constituted 43.5% of the 23 patients with acute liver injury.

**Table 2 Odds ratios for poor outcomes in patients**

| | OR | 95%CI | *P* value |
|---|---|---|---|
| Alkaline phosphatase | 1.21 | (0.91, 1.60) | 0.20 |
| Total bilirubin | 3.58 | (1.25, 10.22) | 0.02 |
| Indirect/direct bilirubin ratio | 0.13 | (0.02, 0.94) | 0.04 |
| Prothrombin time | 789.00 | - | 0.98 |
| Activated partial thromboplastin time | 1.30 | (1.04, 1.63) | 0.02 |

Mushroom-induced acute liver injury is potentially fatal, with the definitive treatment being liver transplantation[2,3]. Laboratory studies showed that urea, AST, ALT, LDH, and total bilirubin concentrations, as well as PT and aPTT, were significantly higher in patients who died than in those who survived[11]. Similarly, patients with ALT or AST concentrations > 2000 IU/mL, or PT > 50 s, were at serious risk of

death[14]. By contrast, we found that AST and ALT levels at admission were not associated with patient mortality. This was not surprising, as AST and ALT levels were shown not to reflect hepatocellular necrosis and hepatotoxicity in patients with ALF[15,16]. Similarly, although serum Cr concentration has been associated with unfavorable outcome[6], we found that serum Cr concentrations did not differ significantly in our groups of survivors and non-survivors. Moreover, in contrast to findings that PT levels were prognostic in patients with liver dysfunction[17], the present study found that PT was not associated with mortality, perhaps because elevated PT may have been masked by the transfusion of coagulation factors into patients hospitalized for mushroom-induced acute liver injury.

Total bilirubin concentration may be a better indicator of the severity of acute liver injury than ALT concentration[18]. High bilirubin concentrations can not only predict short-term mortality, but can also constitute a biochemical marker to improve triage of patients with acute-on-chronic liver failure, especially selecting patients for emerging interventions, such as extracorporeal liver assist devices and possibly improved early phase pharmacological therapies[19].

aPTT measures coagulation via the intrinsic (*i.e.*, contact-activated) pathway[17]. Recent studies show that aPTT reflects failure of coagulation as a multi-organ dysfunction in critically ill patients with ALF. Stravitz *et al*[20] noted monitoring the reaction time by thromboelastography or the aPTT might be more appropriate to assess bleeding risks in patients with ALI and ALF than the INR. Although aPTT is often less valuable than INR for diagnosing liver dysfunction, it may be more suitable for monitoring mushroom-induced liver injury[21].

Deciding whether to transplant livers into patients with wild mushroom-induced ALF continues to be challenging. Although liver transplantation was shown to dramatically increase the survival rate of patients with amatoxin-induced ALF[1], the specific criteria and optimal timing of emergency transplantation remain to be determined[3,6]. Traditionally, transplant decisions are based on King's College[22,23] or Clichy[24] criteria. Other, more specific, criteria have been developed to assess the need for transplantation in patients with amatoxin-induced acute liver injury. Ganzert's criteria suggest that patients with amatoxin poisoning be listed for urgent LT, regardless of the presence of hepatic encephalopathy, if their prothrombin index is < 25% and their serum Cr is > 106 μmol/L on the third day after ingestion[6]. Escudie's criteria suggest that urgent LT be considered in patients showing a reduction in prothrombin index below 10% of normal (INR > 6) 4 or more days after ingestion[25]. Our study showed that both total bilirubin concentration and aPTT level were factors associated with in-hospital mortality in patients with acute liver injury arising from wild mushroom intoxication.

### Limitations

This study had several major limitations. First, we enrolled patients from a single institution who ingested wild mushrooms; therefore, caution should be used when applying our results to other populations, including those in other countries. Second, the retrospective observational design of this study suggests that undetected bias may have been present. Third, because kits that measure amatoxin levels in serum and urine are not commercially available in South Korea, serum alpha-amanitin levels were not analyzed in this study.

This study showed that total bilirubin concentration and aPTT were useful in predicting outcomes in patients with acute liver injury caused by wild mushroom intoxication. Moreover, total bilirubin > 5 mg/dL or aPTT > 50 s on day 3 were prognostic of impending death.

## COMMENTS

### Background

Wild mushroom induced acute liver failure (ALF) is rare but fatal, with selected patients requiring liver transplantation. Many candidates for transplantation progress to multi-organ failure resulting in rapid deterioration while awaiting liver transplantation. However, the specific criteria and optimal timing for emergency liver transplantation in patients with wild mushroom-induced ALF are unclear.

### Research frontiers

Currently, little is known about wild mushroom induced ALF, including its etiology, pathophysiology, clinical outcome, and the in-hospital prognosis in these patients. Few studies have addressed appropriate treatment with wild mushroom induced ALF. Appropriate criteria and precise timing for emergency liver transplantation in patients with wild mushroom-induced ALF are lacking in the medical literature.

### Innovations and breakthroughs

Although prior studies have suggested the association of the prothrombin index and mortality, this study showed that both total bilirubin concentration and activated partial thromboplastin time (aPTT) level were associated with in-hospital mortality in patients with acute liver injury arising from wild mushroom intoxication.

### Applications

This study demonstrates more specific criteria to assess the need for transplantation in patients with amatoxin-induced acute liver injury. Daily monitoring with total bilirubin and aPTT level appears to be of benefit in these patients.

### Terminology

Poisoning of toxic mushrooms in South Korea, the vast majority of toxic mushroom ingestions were the amatoxin-containing mushrooms. Acute liver injury was defined as a > 5-fold elevation in liver enzymes or moderate coagulopathy (INR > 2.0).

### Peer-review

In this study, specific criteria and precise timing for emergency liver transplantation in patients with wild mushroom-induced ALF were assessed. Presence of total bilirubin > 5 mg/dL or aPTT > 50 s on day 3 was prognostic of impending death.

## REFERENCES


1 **Ahishali E**, Boynuegri B, Ozpolat E, Surmeli H, Dolapcioglu C, Dabak R, Bahcebasi ZB, Bayramicli OU. Approach to mushroom intoxication and treatment: can we decrease mortality? *Clin Res Hepatol Gastroenterol* 2012; **36**: 139-145 [PMID: 22189510 DOI: 10.1016/j.clinre.2011.11.004]

2 **Miloh T**, Kerkar N, Parkar S, Emre S, Annunziato R, Mendez C, Arnon R, Suchy F, Rodriguez-Laiz G, Del Rio Martin J, Sturdevant M, Iyer K. Improved outcomes in pediatric liver transplantation for acute liver failure. *Pediatr Transplant* 2010; **14**: 863-869 [PMID: 20609170 DOI: 10.1111/j.1399-3046.2010.01356]

3 **Santi L**, Maggioli C, Mastroroberto M, Tufoni M, Napoli L, Caraceni P. Acute Liver Failure Caused by Amanita phalloides Poisoning. *Int J Hepatol* 2012; **2012**: 487480 [PMID: 22811920 DOI: 10.1155/2012/487480]

4 **Pinson CW**, Daya MR, Benner KG, Norton RL, Deveney KE, Ascher NL, Roberts JP, Lake JR, Kurkchubasche AG, Ragsdale JW. Liver transplantation for severe Amanita phalloides mushroom poisoning. *Am J Surg* 1990; **159**: 493-499 [PMID: 2334013]

5 **Simpson KJ**, Bates CM, Henderson NC, Wigmore SJ, Garden OJ, Lee A, Pollok A, Masterton G, Hayes PC. The utilization of liver transplantation in the management of acute liver failure: comparison between acetaminophen and non-acetaminophen etiologies. *Liver Transpl* 2009; **15**: 600-609 [PMID: 19479803 DOI: 10.1002/lt.21681]

6 **Ganzert M**, Felgenhauer N, Zilker T. Indication of liver transplantation following amatoxin intoxication. *J Hepatol* 2005; **42**: 202-209 [PMID: 15664245 DOI: 10.1016/j.jhep.2004.10.023]

7 **Jo WS**, Hossain MA, Park SC. Toxicological profiles of poisonous, edible, and medicinal mushrooms. *Mycobiology* 2014; **42**: 215-220 [PMID: 25346597 DOI: 10.5941/MYCO.2014.42.3.215]

8 **Sohn CH**. Type and treatment of toxic mushroom poisoning in Korea. *J Korean Med Assoc* 2015; **58**: 818-824

9 **Seok S**, Kim Y, Kim W, Suh J, Jeong M, Lim K, Sohn C, Lee Y. Encyclopedia of poisonous mushrooms. Encyclopedia, 2011

10 **Jander S**, Bischoff J, Woodcock BG. Plasmapheresis in the treatment of Amanita phalloides poisoning: II. A review and recommendations. *Ther Apher* 2000; **4**: 308-312 [PMID: 10975479]

11 **Trabulus S**, Altiparmak MR. Clinical features and outcome of patients with amatoxin-containing mushroom poisoning. *Clin Toxicol* (Phila) 2011; **49**: 303-310 [PMID: 21563906 DOI: 10.3109/15563650.2011.565772]

12 **Bergis D**, Friedrich-Rust M, Zeuzem S, Betz C, Sarrazin C, Bojunga J. Treatment of Amanita phalloides intoxication by fractionated plasma separation and adsorption (Prometheus®). *J Gastrointestin Liver Dis* 2012; **21**: 171-176 [PMID: 22720306]

13 **Roberts DM**, Hall MJ, Falkland MM, Strasser SI, Buckley NA. Amanita phalloides poisoning and treatment with silibinin in the Australian Capital Territory and New South Wales. *Med J Aust* 2013; **198**: 43-47 [PMID: 23330770]

14 **Fantozzi R**, Ledda F, Caramelli L, Moroni F, Blandina P, Masini E, Botti P, Peruzzi S, Zorn M, Mannaioni PF. Clinical findings and follow-up evaluation of an outbreak of mushroom poisoning--survey of Amanita phalloides poisoning. *Klin Wochenschr* 1986; **64**: 38-43 [PMID: 3951164]

15 **Ozer J**, Ratner M, Shaw M, Bailey W, Schomaker S. The current state of serum biomarkers of hepatotoxicity. *Toxicology* 2008; **245**: 194-205 [PMID: 18291570 DOI: 10.1016/j.tox.2007.11.021]

16 **Contreras-Zentella ML**, Hernández-Muñoz R. Is Liver Enzyme Release Really Associated with Cell Necrosis Induced by Oxidant Stress? *Oxid Med Cell Longev* 2016; **2016**: 3529149 [PMID: 26798419 DOI: 10.1155/2016/3529149]

17 **Dufour DR**, Lott JA, Nolte FS, Gretch DR, Koff RS, Seeff LB. Diagnosis and monitoring of hepatic injury. I. Performance characteristics of laboratory tests. *Clin Chem* 2000; **46**: 2027-2049 [PMID: 11106349]

18 **Dufour DR**, Lott JA, Nolte FS, Gretch DR, Koff RS, Seeff LB. Diagnosis and monitoring of hepatic injury. II. Recommendations for use of laboratory tests in screening, diagnosis, and monitoring. *Clin Chem* 2000; **46**: 2050-2068 [PMID: 11106350]

19 **López-Velázquez JA**, Chávez-Tapia NC, Ponciano-Rodríguez G, Sánchez-Valle V, Caldwell SH, Uribe M, Méndez-Sánchez N. Bilirubin alone as a biomarker for short-term mortality in acute-on-chronic liver failure: an important prognostic indicator. *Ann Hepatol* 2013; **13**: 98-104 [PMID: 24378272]

20 **Stravitz RT**, Lisman T, Luketic VA, Sterling RK, Puri P, Fuchs M, Ibrahim A, Lee WM, Sanyal AJ. Minimal effects of acute liver injury/acute liver failure on hemostasis as assessed by thromboelastography. *J Hepatol* 2012; **56**: 129-136 [PMID: 21703173 DOI: 10.1016/j.jhep.2011.04]

21 **Moia M**, Martinelli I, Gridelli B, Langer M, Galmarini D, Mannucci PM. Prognostic value of hemostatic parameters after liver transplantation. *J Hepatol* 1992; **15**: 125-128 [PMID: 1506628]

22 **Bernuau J**. Selection for emergency liver transplantation. *J Hepatol* 1993; **19**: 486-487 [PMID: 8151111]

23 **O'Grady JG**, Alexander GJ, Hayllar KM, Williams R. Early indicators of prognosis in fulminant hepatic failure. *Gastroenterology* 1989; **97**: 439-445 [PMID: 2490426]

24 **Malinchoc M**, Kamath PS, Gordon FD, Peine CJ, Rank J, ter Borg PC. A model to predict poor survival in patients undergoing transjugular intrahepatic portosystemic shunts. *Hepatology* 2000; **31**: 864-871 [PMID: 10733541 DOI: 10.1053/he.2000.5852]

25 **Escudié L**, Francoz C, Vinel JP, Moucari R, Cournot M, Paradis V, Sauvanet A, Belghiti J, Valla D, Bernuau J, Durand F. Amanita phalloides poisoning: reassessment of prognostic factors and indications for emergency liver transplantation. *J Hepatol* 2007; **46**: 466-473 [PMID: 17188393 DOI: 10.1016/j.jhep.2006.10.013]


**P- Reviewer**: Shimizu Y, Villa-Trevino S **S- Editor**: Ma YJ
**L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/

*World J Gastroenterol* 2017 February 21; 23(7): 1268-1277

DOI: 10.3748/wjg.v23.i7.1268

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

ORIGINAL ARTICLE

Observational Study

# Effects of hydrotalcite combined with esomeprazole on gastric ulcer healing quality: A clinical observation study

Rui-Qi Yang, Hua Mao, Li-Yun Huang, Pei-Zhu Su, Min Lu

Rui-Qi Yang, Hua Mao, Li-Yun Huang, Pei-Zhu Su, Min Lu, Department of Digestive Diseases, Zhujiang Hospital of Southern Medical University, Guangzhou 510280, Guangdong Province, China

Author contributions: Yang RQ performed the experiments, analyzed data, interpreted the results of the experiments, prepared figures, and drafted the manuscript; Mao H conceived and designed the research; Huang LY performed the experiments and prepared figures; Su PZ performed the experiments and analyzed data; Lu M performed the experiments and interpreted the results of the experiments; all authors have read and approved the final version to be published.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Zhujiang Hospital of Southern Medical University, Guangzhou, China, approval No. 2014-XHNK-003.

Informed consent statement: All patients gave written informed consent.

Clinical trial registration statement: This study is registered at http://www.chictr.org.cn/usercenter.aspx. The registration identification number is ChiCTR-IPR-16008317.

Conflict-of-interest statement: The authors declared that there is no conflict of interest related to this study.

Data sharing statement: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its supporting information files.



Manuscript source: Unsolicited manuscript

Correspondence to: Dr. Hua Mao, Professor, Department of Digestive Diseases, Zhujiang Hospital of Southern Medical University, No. 253, Middle Gongye Avenue, Guangzhou 510280, Guangdong Province, China. maohua1963@126.com
Telephone: +86-13556196250

Received: May 26, 2016
Peer-review started: May 30, 2016
First decision: July 29, 2016
Revised: August 26, 2016
Accepted: September 14, 2016
Article in press: September 14, 2016
Published online: February 21, 2017

## Abstract

***AIM***

To evaluate the effects of hydrotalcite combined with esomeprazole on gastric ulcer healing quality.

***METHODS***

Forty-eight patients diagnosed with gastric ulcer between June 2014 and February 2016 were randomly allocated to the combination therapy group or monotherapy group. The former received hydrotalcite combined with esomeprazole, and the latter received esomeprazole alone, for 8 wk. Twenty-four healthy volunteers were recruited and acted as the healthy control group. Endoscopic ulcer healing was observed using white light endoscopy and narrow band imaging magnifying endoscopy. The composition of collagen fibers, amount of collagen deposition, expression of factor Ⅷ and TGF-β1, and hydroxyproline content were analyzed by Masson staining, immunohistochemistry, immunofluorescent imaging and ELISA.

***RESULTS***

Following treatment, changes in the gastric microvascular network were statistically different between the combination therapy group and the monotherapy group

($P$ < 0.05). There were significant differences ($P$ < 0.05) in collagen deposition, expression level of Factor Ⅷ and TGF-β1, and hydroxyproline content in the two treatment groups compared with the healthy control group. These parameters in the combination therapy group were significantly higher than in the monotherapy group ($P$ < 0.05). The ratio of collagen Ⅰ to collagen Ⅲ was statistically different among the three groups, and was significantly higher in the combination therapy group than in the monotherapy group ($P$ < 0.05).

***CONCLUSION***

Hydrotalcite combined with esomeprazole is superior to esomeprazole alone in improving gastric ulcer healing quality in terms of improving microvascular morphology, degree of structure maturity and function of regenerated mucosa.

**Key words:** Hydrotalcite; Esomeprazole; Gastric ulcer; Quality of ulcer healing



**Core tip:** Gastric ulcer is mainly caused by an imbalance in defense mechanisms and injurious factors at the gastric mucosa, and has a high recurrence rate. Previous studies comparing the efficacy of hydrotalcite combined with esomeprazole *vs* esomeprazole therapy alone for the treatment of gastric ulcer have shown unclear results. In the present study, we conducted a clinical observation study involving 48 patients diagnosed with gastric ulcer treated with either hydrotalcite combined with esomeprazole or esomeprazole alone. The results showed that hydrotalcite combined with esomeprazole was superior to esomeprazole alone in improving gastric ulcer healing quality.

Yang RQ, Mao H, Huang LY, Su PZ, Lu M. Effects of hydrotalcite combined with esomeprazole on gastric ulcer healing quality: A clinical observation study. *World J Gastroenterol* 2017; 23(7): 1268-1277 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1268.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1268

## INTRODUCTION

Gastric ulcer is mainly caused by an imbalance in defense mechanisms and injurious factors at the gastric mucosa. The use of *Helicobacter pylori* (*H. pylori*) eradication therapy and the wide application of proton pump inhibitors (PPI) have improved the clinical healing rate of gastric ulcer, but its recurrence rate remains high. Tarnawski *et al*[1] proposed the concept of quality of ulcer healing (QOUH) in 1991 for the first time, which considers the fact that tissue regeneration is often incomplete within ulcer scars. QOUH suggests that the evaluation of gastric ulcer healing should focus on whether the structure and function of mucosal and submucosal tissue have been completely regenerated in addition to endoscopic examination and evaluation of ulcer size. It has been shown that ulcer recurrence is closely related to QOUH. Esomeprazole is an $H^+$-$K^+$-ATP enzyme inhibitor, which can effectively inhibit acid, and thereby promotes ulcer repair. However, excessive acid suppression may cause indigestion, gastrointestinal bacterial infection and induce atrophic gastritis or even gastric cancer. Ulcers frequently recur following anti-acid drug treatment alone[2,3]. Therefore, combination therapies for gastric ulcer are favorable. To this end, hydrotalcite[4], a gastric mucosal protective agent, in which the mechanism of action includes the formation of a protective membrane at the ulcer site, provides an $HCO3^-$ reservoir and repairs the mucous bicarbonate barrier. Moreover, it upregulates various protective and repair factors in the gastric tissue such as promoting the formation of prostaglandins, improving mucosal blood flow and metabolism at the ulcer site, thereby enhancing mucosal functional repair. However, previous studies comparing the efficacy of hydrotalcite combined with esomeprazole *vs* esomeprazole therapy alone for the treatment of gastric ulcer did not lead to conclusive results, as these studies merely evaluated the endoscopic morphology of gastric mucosa and not the histologic and ultrastructural changes in the mucosal and submucosal layers. In addition, most studies were conducted using rat models[3,5-7]. Irregular punctate and linear gastric pits are usually abundant in normal gastric mucosa, surrounded by a rich and irregular microvascular network[8]. The morphology of gastric pits and the microvascular network may change corresponding to pathological changes in the gastric mucosa[9,10]. However, these changes cannot be observed under white light endoscopy, but are clearly visible under narrow band imaging (NBI) magnifying endoscopy[11]. Thus, in the present study, we used NBI magnifying endoscopy to observe morphological changes in the gastric pits and microvascular network of regenerated mucosa. The efficacy of hydrotalcite combined with esomeprazole in improving QOUH of adult gastric ulcers was assessed by the maturity of regenerated mucosal tissue. Eradication therapy was also administered to patients with a positive *H. pylori* test.

## MATERIALS AND METHODS

### *Patient selection*

Forty-eight patients diagnosed with gastric ulcer[12] who received treatment in our hospital between June 2014 and December 2015 were randomly divided into the combination therapy group and the monotherapy group. Twenty-four healthy volunteers were recruited into the healthy control group. Exclusion criteria were as follows: (1) patients with bleeding, obstruction, perforation and cancer or other related complications of gastric ulcer; (2) complications such as heart, brain,

**Table 1 Comparison of general parameters, *Helicobacter pylori* positive rate, endoscopic ulcer healing, morphological changes at gastric pits and microvascular networks among the three groups**

| Group | No. | Gender | Age (yr) | *H. pylori* positive rate (BT) | *H. pylori* positive rate (AT) | Total effective rate (AT) | Gastric pits (AT) | Microvascular network changes (AT) |
|---|---|---|---|---|---|---|---|---|
| | | M/F | (mean ± SD)[1] | *n* (%)[2] | *n* (%)[2] | *n* (%)[2] | A + B (%)[2] | Regular (%)[2] |
| Healthy control | 24 | 14/10 | 55.5 ± 12.3 | | | | 23 (95.8%) | 11 (45.8%) |
| Combination therapy | 24 | 13/11[4] | 54.5 ± 11.1[4] | 16 (66.7%)[4] | 2 (8.3%)[4] | 23 (95.8)[4] | 22 (91.7%)[4] | 20 (83.3%)[3] |
| Monotherapy | 24 | 9/15[4] | 53.6 ± 11.2[4] | 14 (58.3%) | 3 (12.5%) | 22 (91.7) | 21 (87.5%) | 13 (54.2%) |

[1]ANOVA test was conducted because these data obey normal distributions; [2]$\chi^2$ test was conducted because these data obey $\chi^2$ distributions; [3]*P* values of post hoc comparison between groups were all lower than 0.05; [4]*P* values between groups were higher than 0.05. BT: Before treatment; AT: After treatment; *H. pylori*: *Helicobacter pylori*.

kidney, lung and other major diseases; (3) recent treatment for gastric ulcer; and (4) contraindications to hydrotalcite or esomeprazole. All subjects were tested for *H. pylori* using the rapid urease test (CLO test; Delta West Pty Ltd., Western Australia). The study was approved by the ethics committee of our hospital (2014-XHNK-003) and registered in the Chinese Clinical Trial Registry (ChiCTR-IPR-16008317). Informed consent was obtained from all patients or their family members.

### Treatment protocol

The combination group received oral hydrotalcite (1.0 g three times a day) combined with oral esomeprazole (20 mg twice a day), and the monotherapy group received oral esomeprazole alone (20 mg twice a day). Treatment duration was eight weeks. *H. pylori*-positive patients also received amoxicillin, clarithromycin and colloidal pectin bismuth for 10-14 d. The healthy control group received no treatment.

### Examinations

Endoscopic ulcer healing was observed first under white light endoscopy, and then gastric pits and microvascular morphology were observed using NBI magnifying endoscopy by the same experienced endoscopist. Following endoscopy, gastric mucosal tissues from patients were biopsied before and after treatment at the same location. Tissues were similarly biopsied from healthy volunteers. Part of the specimen was embedded in paraffin, sectioned and used for Masson, immunofluorescent and immunohistochemical staining. The remainder was preserved at -80 ℃ for subsequent determination of hydroxyproline content by ELISA.

### Statistical analysis

Measurement data following normal distribution were expressed as mean ± SD. These data were compared by analysis of variance (ANOVA) followed by multiple comparisons using the LSD or Dunnett's T3 method based on their determined homogeneity of variance. For data which were not normally distributed, the Kruskal-Wallis test was used followed by multiple comparisons by pairwise methods. Enumeration data were compared using the $\chi^2$ test. *P* values less than 0.05 were considered statistically significant. All data were analyzed using SPSS20.0 (SPSS Inc. United States).

## RESULTS

### Basic clinical characteristics and H. pylori eradication rate

No significant differences in gender and age were observed among the three groups ($P > 0.05$). *H. pylori* positive rates were similar between the combination therapy and monotherapy groups before and after treatment ($P > 0.05$) (Table 1).

### Endoscopic ulcer healing

The standards of Japanese gastroscopy diagnosis and staging criteria were used[13]. A high total effective rate represents high endoscopic ulcer healing quality. The results are shown in Table 1 and Figure 1. The total effective rate was not significantly different between the combination therapy group (95.8%) and the monotherapy group (91.7%) after treatment ($P > 0.05$).

### Morphological changes in gastric pits and mucosal microvascular network

The results are shown in Table 1 and Figure 2. Based on the Sakak standards[14], most gastric pits in the normal gastric mucosa group were classified as type A and B, surrounded by a rich and irregular microvascular network, classified according to the Yao criteria[15]. In the combination therapy and monotherapy groups before treatment, gastric pits in the gastric ulcer mucosa were mostly type C and D, surrounded by a small microvascular network. The types of gastric pits and microvascular networks were not significantly different between the combination therapy and monotherapy groups before treatment ($P > 0.05$). After treatment, the percentage of type A and B gastric pits in the gastric mucosa was not significantly different between the combination therapy and monotherapy groups ($P_{A+B} > 0.05$), but

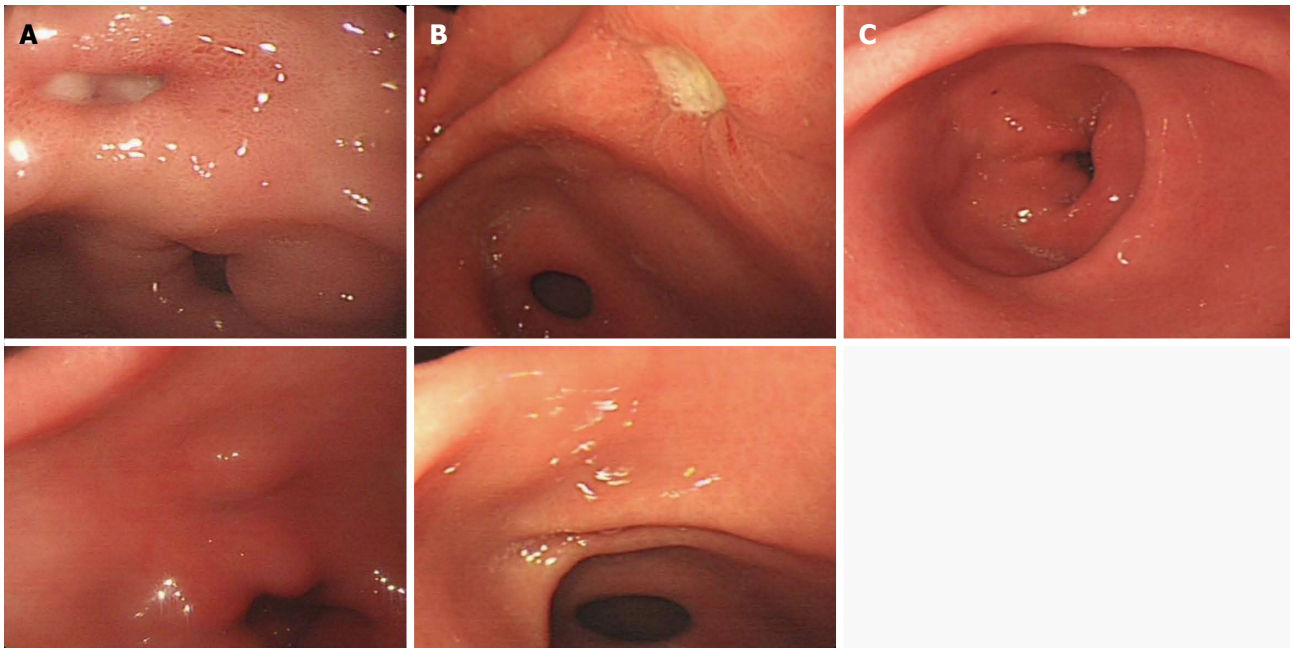



**Figure 1 White light endoscopic assessment of ulcer healing quality.** A: Combination therapy before and after treatment; B: Monotherapy before and after treatment; C: Healthy control group. Both the combination therapy and monotherapy groups had high quality ulcer healing.

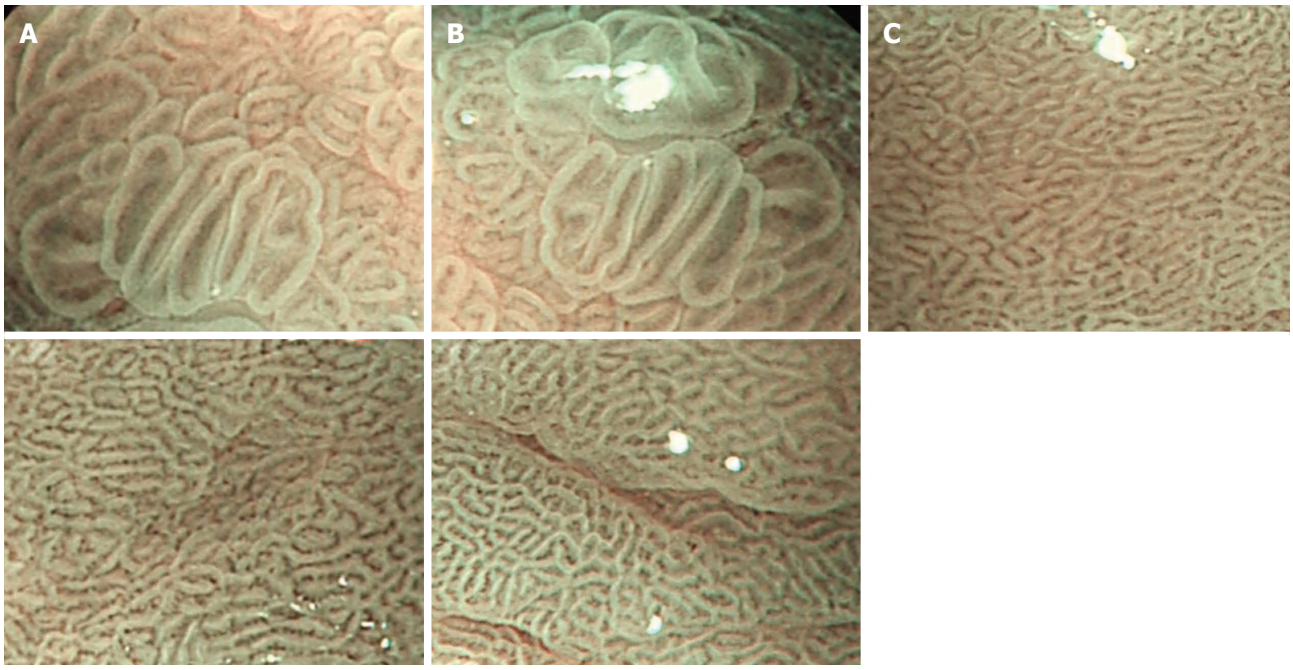



**Figure 2 Gastric pits and microvascular morphology by narrow band imaging magnifying endoscopy.** A: Combination therapy before and after treatment; B: Monotherapy before and after treatment; C: Healthy control group. With regeneration of the gastric mucosal epithelium, the gastric pits and microvascular network were reconstructed. Both the combination therapy and monotherapy groups had improved gastric pit morphology after treatment, which was similar to normal mucosal tissue. However, the percentage of regular microvascular network was much higher.

the percentage of regular microvascular network was significantly different between the two groups ($P < 0.05$), and was significantly higher in the combination therapy group than in the monotherapy group and normal control group.

### *Maturity of regenerated mucosa*

**Collagen deposition area:** Under normal circumstances, collagen fibers are usually distributed in the gastric mucosal and submucosal layers. The area of collagen fiber deposition was evaluated by Masson's trichrome staining, which stains fibrotic tissues blue. Five randomly-chosen fields (× 200 times) of each specimen were imaged. All images were analyzed using Plus6.0 Image-Pro software. Collagen deposition area was measured using integral area and the

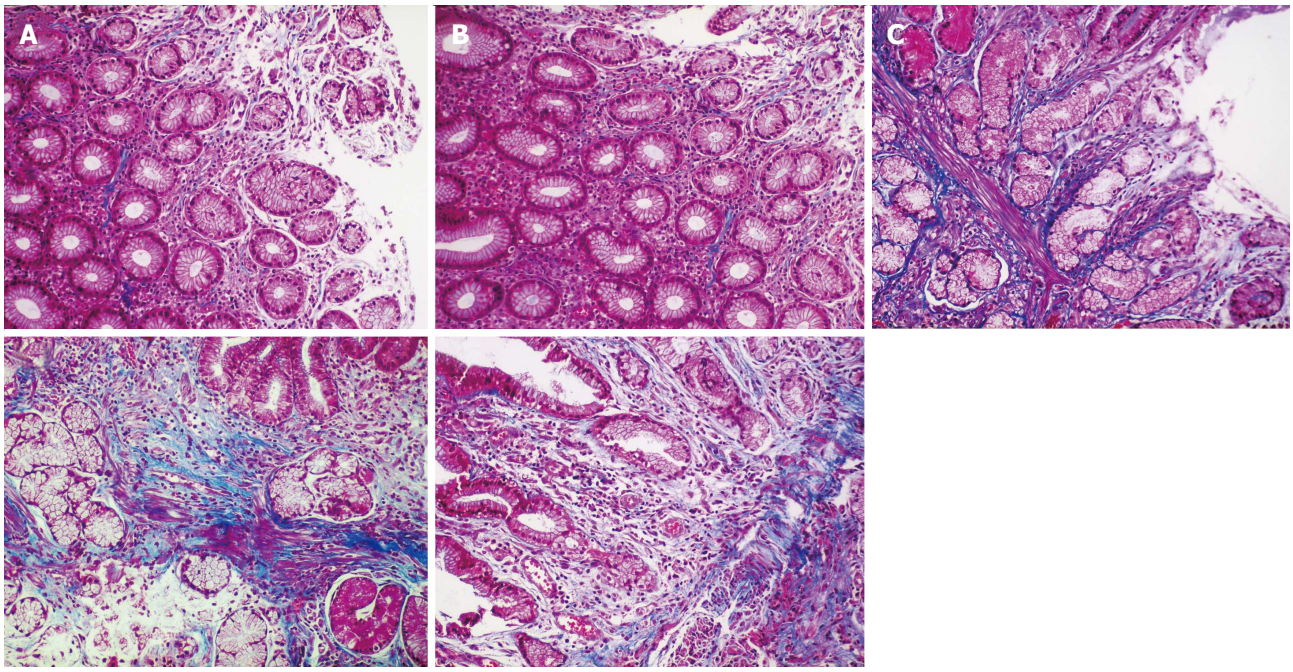



**Figure 3 Gastric mucosal fibrosis was evaluated by Masson's trichrome staining in a 200 × field.** Blue-stained signals represent fibrosis. A: Combination therapy before and after treatment; B: Monotherapy before and after treatment; C: Healthy control group. The area of collagen fiber deposition in the combination therapy group was significantly larger than that in the monotherapy group after treatment, and was similar to that in normal mucosal tissue.

**Table 2 Comparison of collagen fiber area, collagen Ⅰ to collagen Ⅲ ratio, hydroxyproline content, FactorⅧ-positive cells and TGF-β1 expression among the three groups by Masson staining, immunohistochemistry, ELISA assay and immunofluorescent imaging**

| Group | No. | Collagen fiber area | | Collagen Ⅰ to collagen Ⅲ ratio | | Hydroxyproline content (ng/mL) | | FactorⅧ-positive cells | | TGF-β1 expression | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | mean ± SD[1] | *P* value | mean rank[2] | *P* value | mean ± SD[1] | *P* value | mean ± SD[1] | *P* value | mean ± SD[1] | *P* value |
| Healthy control | 24 | 244679.38 ± 210075.67[4] | 0.020[3] | 38.85[4] | 0.031[3] | 1693.72 ± 419.25[4] | 0.013[3] | 23.25 ± 8.24[3] | 0.000[3] | 93984.12 ± 42066.64[4] | 0.016[3] |
| Combination therapy (AT) | 24 | 260909.51 ± 164713.88 | | 39.42 | | 1909.53 ± 828.78 | | 33.54 ± 8.72 | | 101163.85 ± 38130.24 | |
| Monotherapy (AT) | 24 | 135700.75 ± 94175.38[3] | | 25.96[3] | | 1235.32 ± 551.07[3] | | 28.58 ± 6.94[3] | | 72321.44 ± 20030.29[3] | |

[1]ANOVA test was conducted because these data obey normal distributions; [2]Kruskal-Wallis test was conducted because these data did not obey normal distributions; [3]*P* values of post hoc comparison between groups were all lower than 0.05; [4]*P* values between combination group and control group were higher than 0.05. AT: After treatment.

average value was calculated. The area of collagen fiber deposition in the healthy control group was significantly larger than that in the combination therapy and monotherapy groups before treatment ($P < 0.05$). Areas of collagen fiber deposition were not significantly different between the combination therapy and monotherapy groups before treatment ($P > 0.05$, $P = 0.951$), but were significantly different after treatment ($P < 0.05$). Following treatment, the area of collagen fiber deposition in the combination therapy group (260909.51 ± 164713.88) was significantly higher than that in the monotherapy group (135700.75 ± 94175.38) ($P < 0.05$), but was not statistically significant compared with the healthy control group (244679.38 ± 210075.67) ($P > 0.05$) (Table 2, Figure 3).

**Ratio of collagen Ⅰ to collagen Ⅲ:** The ratio of collagen Ⅰ to collagen Ⅲ in the healthy control group was significantly higher than that in the combination therapy and monotherapy groups before treatment ($P < 0.05$). The ratio of collagen Ⅰ to collagen Ⅲ was not significantly different between the combination therapy and monotherapy groups ($P > 0.05$, $P = 0.553$). In contrast, the ratio of collagen Ⅰ to collagen Ⅲ in the combination therapy (mean rank = 39.42) and monotherapy groups (mean rank = 25.96) after treatment were significantly different compared with the healthy control group (mean rank = 38.85) ($P < 0.05$, $P = 0.031$). The ratio in the combination therapy group was significantly higher than that in the monotherapy group ($P < 0.05$), but there was no statistically significant difference between the combination therapy group and the healthy control group ($P > 0.05$, $P = 0.746$), (Table 2, Figure 4).

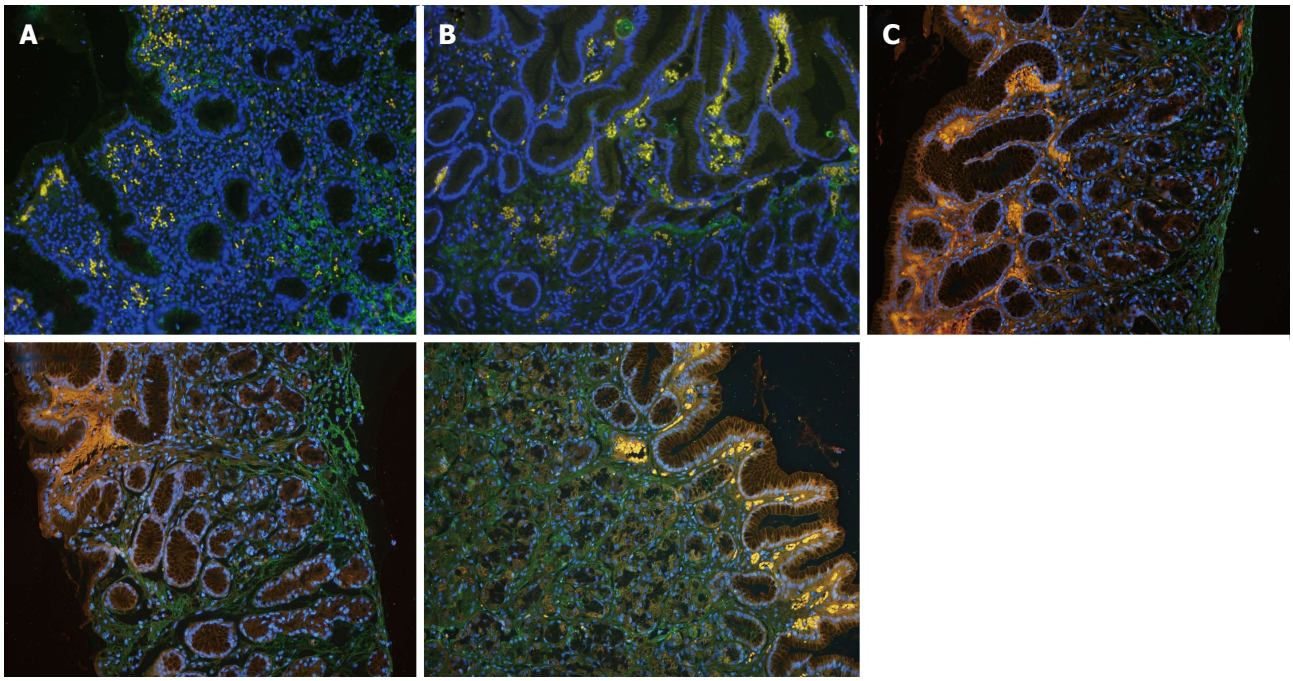



**Figure 4 Expression of collagen Ⅰ and Ⅲ was analyzed using immunofluorescent staining in a 200 × field.** Nuclei were stained blue, collagen Ⅰ stained red, collagen Ⅲ stained green, and the overlap of collagen Ⅰ and collagen Ⅲ stained yellow. A: Results of combination therapy before and after treatment; B: Results of monotherapy before and after treatment; C: Results in the healthy control group. The deposition area of collagen Ⅰ and Ⅲ in the combination therapy group after treatment was similar to that in the normal control group.

**Hydroxyproline content in gastric mucosa:** Hydroxyproline content in the healthy control group was significantly higher than that in the combination therapy group and monotherapy group before treatment ($P < 0.05$). The difference in hydroxyproline content was not statistically significant between the combination therapy and monotherapy groups before treatment ($P > 0.05$, $P = 0.996$), but was significantly different after treatment ($P < 0.05$). Following treatment, hydroxyproline content in the combination therapy group (1909.53 ± 828.78 ng/mL) was significantly higher than that in the monotherapy group (1235.32 ± 551.07 ng/mL) ($P < 0.05$), but was not significantly different compared to the healthy control group (1693.72 ± 419.25 ng/mL), ($P > 0.05$, $P = 0.592$) (Table 2).

**Expression of Factor Ⅷ:** The number of Factor Ⅷ-positive cells in the healthy control group was significantly higher than that in the combination therapy and monotherapy groups before and after treatment ($P < 0.05$). The number of Factor Ⅷ-positive cells was not significantly different between the combination therapy and monotherapy groups before treatment ($P > 0.05$, $P = 0.947$), while that in the combination therapy group (33.54 ± 8.72) was significantly higher than that in the monotherapy group (28.58 ± 6.94) and the healthy control group (23.25 ± 8.24) after treatment ($P < 0.05$), (Table 2, Figure 5).

**Expression of TGF-β1:** The integrated optical density (IOD) of TGF-β1 staining in the healthy control group was significantly higher than that in the combination therapy and monotherapy groups before treatment ($P < 0.05$). The IOD of TGF-β1 staining was not significantly different between the combination therapy and monotherapy groups ($P > 0.05$, $P = 0.992$), but was significantly different after treatment ($P < 0.05$). TGF-β1 expression in the combination therapy group (IOD = 101163.85 ± 38130.24) and the monotherapy group (IOD = 72321.44 ± 20030.29) after treatment was significantly different compared with the healthy control group (IOD = 93984.12 ± 42066.64) ($P < 0.05$). TGF-β1 in the combination therapy group was significantly higher than that in the monotherapy group ($P < 0.05$), but there was no statistically significant difference when compared with the healthy control group ($P < 0.05$), (Table 2, Figure 6).

## DISCUSSION

The gastric ulcer healing process includes the clearance of necrotic debris, growth of granulation tissues, angiogenesis, collagen fiber deposition, scar tissue formation, and epithelial reconstruction with reestablishment of the mucosal microvascular network[16,17]. *H. pylori* eradication therapy and the wide application of PPIs have improved the clinical cure rate of gastric ulcer, but its high recurrence rate remains a clinical challenge. Gastric ulcer mainly

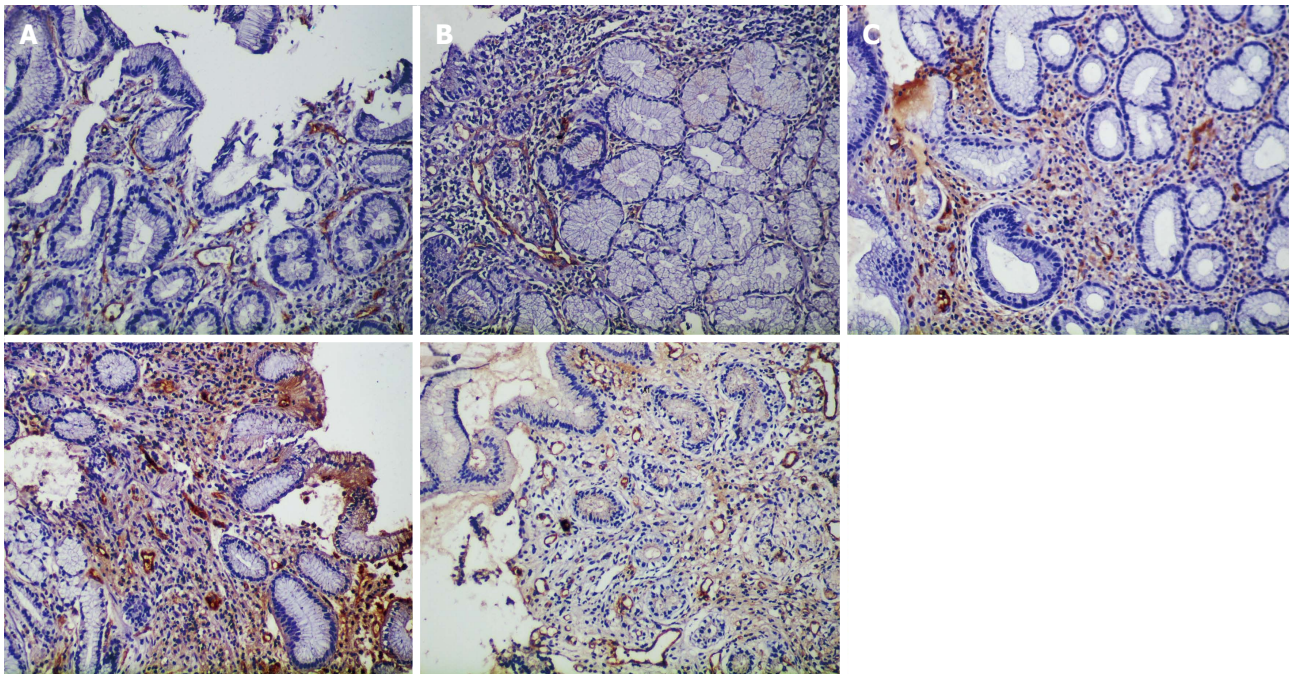



**Figure 5 Expression of FactorⅧ was analyzed using immunohistochemical staining in a 200 × field.** Yellow-stained signals in the cytoplasm and (or) cell membrane represent positive cells. A: Results of combination therapy before and after treatment; B: Results of monotherapy before and after treatment; C: Results in healthy control group. The expression of FactorⅧ in the combination therapy group after treatment was significantly higher than that in the monotherapy and healthy control groups.

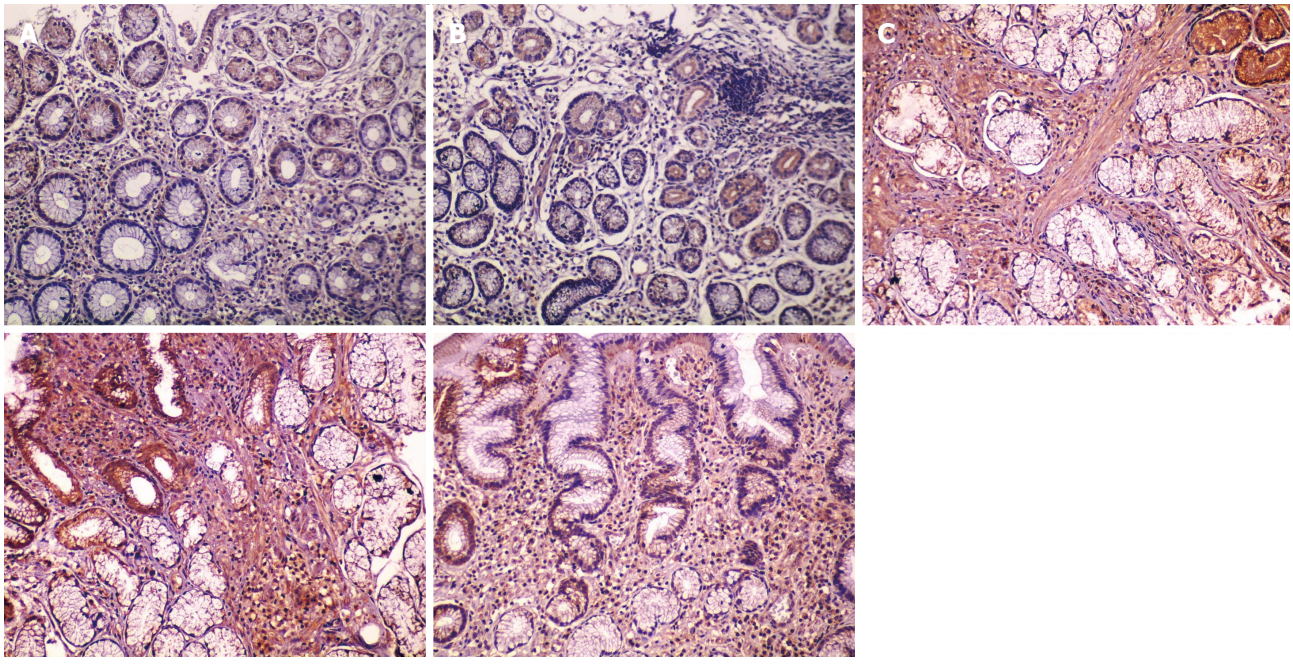



**Figure 6 Expression of TGF-β1 was analyzed using immunohistochemical staining in a 200 × field.** Yellow-stained signals in the cytoplasm and (or) cell membrane represent positive cells. A: Results of combination therapy before and after treatment; B: Results of monotherapy before and after treatment; C: Results in the healthy control group. The expression of TGF-β1 in the combination therapy group was significantly higher than that in the monotherapy group, and similar to that in the healthy control group.

recurs *in situ* or at areas adjacent to the healed ulcer. Tarnawski *et al*[1] showed that the recurrence of gastric ulcer was closely related to QOUH. These authors believe that compromised histological and ultrastructural healing of gastric mucosa impairs the function of mucosal defense, accounting for the pathological basis of ulcer recurrence[5]. In the present study, we observed morphological changes in gastric pits and the microvascular network of regenerated mucosa using NBI magnifying endoscopy, and

evaluated the effects of hydrotalcite combined with esomeprazole on the healing quality of gastric ulcer following *H. pylori* eradication therapy. The results showed that hydrotalcite combined with esomeprazole improved the maturity of regenerated mucosal tissue both structurally and functionally.

This study also showed that the *H. pylori*-positive rate and endoscopic ulcer healing were similar between the combination therapy group and the monotherapy group after treatment. In addition, morphology of the gastric pits under the NBI magnifying endoscope was similar to the normal gastric mucosa in the combination therapy and monotherapy groups. This indicates that both treatments promoted superficial mucosal healing and improved gastric pit morphology.

Previous research[18,19] confirmed that collagen fibers in gastric tissue were mainly composed of collagen Ⅰ and collagen Ⅲ. Type Ⅰ collagen accounts for approximately 80% of collagen fibers and strong expression of collagen Ⅰ is found in newborn granulation tissue. Collagen Ⅰ plays a supportive role in wound healing and excessive deposition can result in scar tissue formation. Collagen Ⅲ accounts for the remaining 20% and strong expression of collagen Ⅲ is found in the active capillary proliferation period, which determines the diameter and elasticity of collagen fibers. In contrast to collagen Ⅰ, deposition of collagen Ⅲ inhibits scar formation and regulates cell proliferation, growth and differentiation. However, over-deposition of collagen fibers in general leads to scar tissue formation and impaired physiological function. Under normal circumstances, the formation and degradation of collagen fibers are in dynamic equilibrium. Research[20] has confirmed that the ratio of collagen Ⅰ to collagen Ⅲ is maintained at 2:1 to 4:1 in normal tissues, which is directly related to the quality of tissue repair. Amino acids in collagen fibers consist mainly of glycine, proline and hydroxyproline[21]. Hydroxyproline is a non-essential amino acid, with just 1%[22,23] distributed in elastic proteins and almost all of the remainder found in collagen. Hydroxyproline accounts for approximately 13.4% of all amino acids in collagen[24]. It is believed that hydroxyproline is unique to collagen fibers, as hydroxyproline content is often used as a quantitative index of collagen deposition in ulcer healing[25].

This study showed that the collagen deposition area, ratio of collagen Ⅰ to collagen Ⅲ and hydroxyproline content in the combination therapy group were significantly higher than those in the monotherapy group after treatment. The levels of these factors in the combination therapy group after treatment were similar to those in healthy control tissues. Therefore, hydrotalcite combined with esomeprazole for the treatment of gastric ulcer promotes deposition of collagen fibers, maintains the ratio of collagen Ⅰ to collagen Ⅲ, and improves the histological structure of regenerated gastric tissues similar to that in normal mucosa tissues, which is consistent with the findings of Griffin *et al*[26]. In conclusion, hydrotalcite combined with esomeprazole was superior to esomeprazole alone in improving ulcer healing quality.

Immunohistochemical staining of factor Ⅷ was performed to measure the number of newly generated microvessels[27,28]. We found that the combination therapy group had higher factor Ⅷ expression and the percentage of regular microvascular network was much higher than that in the monotherapy group after treatment, according to NBI magnifying endoscopy. This may be attributed to the fact that hydrotalcite promotes the expression of various growth factors and stimulates angiogenesis, thus increasing oxygen and nutrient supply to the ulcer site to accelerate ulcer healing[4,29].

Studies have shown that the gastric ulcer healing process is regulated by various growth factors and receptors[3,30]. As a multifunctional growth factor, TGF-β1 plays an important role in tissue remodeling, extracellular matrix formation and angiogenesis, ultimately promoting ulcer repair[31,32]. This study demonstrated that the expression level of TGF-β1 in the combination therapy group was significantly higher than that in the monotherapy group after treatment, which was similar to the level in normal control tissues. This may be due to induced expression of TGF-β1 by hydrotalcite, consistent with the study by Chen *et al*[29].

In conclusion, hydrotalcite combined with esomeprazole is superior to esomeprazole alone in improving the healing quality of gastric ulcer. Specifically, combination therapy improved morphology of the microvascular network, promoted collagen fiber formation, angiogenesis, and the expression of TGF-β1, thereby improving the maturity of regenerated mucosal tissue as well as structure and function of the mucosa. This combination therapy is worthy of wider clinical application. We did not observe the recurrence of gastric ulcer due to our small sample size and short follow-up period. In future, a larger sample size and longer follow-up time will be included to determine whether hydrotalcite combined with esomeprazole can also reduce the recurrence rate of gastric ulcer.

## COMMENTS

### Background

Gastric ulcer is mainly caused by an imbalance in defense mechanisms and injurious factors in the gastric mucosa. The use of *Helicobacter pylori* eradication therapy and the wide application of proton pump inhibitors have improved the clinical healing rate of gastric ulcer, but its high recurrence rate remains a clinical challenge.

### Research frontiers

Gastric ulcers represent a public health problem. However, there are very few studies on human gastric ulcer, and most have focused on endoscopic morphology. A research hot spot is to investigate the effects of hydrotalcite combined with esomeprazole on the healing quality of human gastric ulcer in order to understand the background and trends in the treatment of gastric ulcer.

### Innovations and breakthroughs

Previous studies of quality of ulcer healing (QOUH) concentrated on endoscopic morphology and not on histologic and ultrastructural changes. In addition, most studies were conducted using rat models. In the present study, the authors determined the effects of hydrotalcite combined with esomeprazole on the healing quality of gastric ulcer by observing morphological changes, and the degree of structure maturity and function of regenerated mucosa. The results showed that hydrotalcite combined with esomeprazole was superior to esomeprazole alone in improving the healing quality of gastric ulcer, specifically, the morphology of the microvascular network, collagen fiber formation, angiogenesis, and high expression of TGF-β1.

### Applications

The findings of this study suggest that hydrotalcite combined with esomeprazole was superior to esomeprazole alone in improving the healing quality of gastric ulcer. Furthermore, this study also provides readers with important information regarding the best treatment for gastric ulcer. This combination therapy is worthy of wider clinical application.

### Terminology

Gastric ulcer is a common disease in the department of gastroenterology, and is related to acid secretion. Mucosal injury in gastric ulcer is found in the muscularis mucosa layer, which is a characteristic of chronic ulcer formation. Clinical symptoms are mainly a repeated periodic rhythm of upper abdominal pain, accompanied by belching, bloating discomfort, bleeding, perforation, and even cancer, all of which can seriously harm human health.

### Peer-review

Available studies on QOUH of human are rare. The authors in this study analyzed the healing quality of gastric ulcer, specifically, the morphology of the microvascular network, collagen fiber formation, angiogenesis, and expression of TGF-β1. This study showed that hydrotalcite combined with esomeprazole was superior to esomeprazole alone in improving the QOUH. The results were meaningful and provided important information concerning the background and trends of treatments for gastric ulcer.

## REFERENCES


1 **Tarnawski A**, Stachura J, Krause WJ, Douglass TG, Gergely H. Quality of gastric ulcer healing: a new, emerging concept. *J Clin Gastroenterol* 1991; **13** Suppl 1: S42-S47 [PMID: 1719066]

2 **Fornai M**, Colucci R, Antonioli L, Awwad O, Ugolini C, Tuccori M, Fulceri F, Natale G, Basolo F, Blandizzi C. Effects of esomeprazole on healing of nonsteroidal anti-inflammatory drug (NSAID)-induced gastric ulcers in the presence of a continued NSAID treatment: Characterization of molecular mechanisms. *Pharmacol Res* 2011; **63**: 59-67 [PMID: 20969958 DOI: 10.1016/j.phrs]

3 **Kangwan N**, Park JM, Kim EH, Hahm KB. Quality of healing of gastric ulcers: Natural products beyond acid suppression. *World J Gastrointest Pathophysiol* 2014; **5**: 40-47 [PMID: 24891974 DOI: 10.4291/wjgp.v5.i1.40]

4 **Tarnawski A**, Ahluwalia A, Jones MK. Gastric cytoprotection beyond prostaglandins: cellular and molecular mechanisms of gastroprotective and ulcer healing actions of antacids. *Curr Pharm Des* 2013; **19**: 126-132 [PMID: 22950493 DOI: 10.2174/138161281130601012 6]

5 **Arakawa T**, Watanabe T, Tanigawa T, Tominaga K, Fujiwara Y, Morimoto K. Quality of ulcer healing in gastrointestinal tract: its pathophysiology and clinical relevance. *World J Gastroenterol* 2012; **18**: 4811-4822 [PMID: 23002355 DOI: 10.3748/wjg.v18.i35.4811]

6 **Slomiany BL**, Piotrowski J, Czajkowski A, Yotsumoto F, Slomiany A. Gastric mucosal EGF and PDGF receptor expression with ulcer healing by ebrotidine. *Am J Gastroenterol* 1994; **89**: 894-897 [PMID: 8198101]

7 **Tarnawski AS**, Chai J, Pai R, Chiou SK. Rebamipide activates genes encoding angiogenic growth factors and Cox2 and stimulates angiogenesis: a key to its ulcer healing action? *Dig Dis Sci* 2004; **49**: 202-209 [PMID: 15104358]

8 **Nakagawa S**, Kato M, Shimizu Y, Nakagawa M, Yamamoto J, Luis PA, Kodaira J, Kawarasaki M, Takeda H, Sugiyama T, Asaka M. Relationship between histopathologic gastritis and mucosal microvascularity: observations with magnifying endoscopy. *Gastrointest Endosc* 2003; **58**: 71-75 [PMID: 12838224 DOI: 10.1067/mge.2003.316]

9 **Kawamura M**, Abe S, Oikawa K, Terai S, Saito M, Shibuya D, Kato K, Shimada T, Uedo N, Masuda T. Topographic differences in gastric micromucosal patterns observed by magnifying endoscopy with narrow band imaging. *J Gastroenterol Hepatol* 2011; **26**: 477-483 [PMID: 21155881 DOI: 10.1111/j.1440-1746.2010.06527.x]

10 **Cho JH**, Chang YW, Jang JY, Shim JJ, Lee CK, Dong SH, Kim HJ, Kim BH, Lee TH, Cho JY. Close observation of gastric mucosal pattern by standard endoscopy can predict Helicobacter pylori infection status. *J Gastroenterol Hepatol* 2013; **28**: 279-284 [PMID: 23189930 DOI: 10.1111/jgh.12046]

11 **Tahara T**, Shibata T, Nakamura M, Yoshioka D, Okubo M, Arisawa T, Hirata I. Gastric mucosal pattern by using magnifying narrow-band imaging endoscopy clearly distinguishes histological and serological severity of chronic gastritis. *Gastrointest Endosc* 2009; **70**: 246-253 [PMID: 19386303 DOI: 10.1016/j.gie.2008.11.046]

12 **Bi WP**, Man HB, Man MQ. Efficacy and safety of herbal medicines in treating gastric ulcer: a review. *World J Gastroenterol* 2014; **20**: 17020-17028 [PMID: 25493014 DOI: 10.3748/wjg.v20.i45.17020]

13 **Takemoto T**, Sasaki N, Tada M, Yanai H, Okita K. Evaluation of peptic ulcer healing with a highly magnifying endoscope: potential prognostic and therapeutic implications. *J Clin Gastroenterol* 1991; **13** Suppl 1: S125-S128 [PMID: 1940187]

14 **Sakaki N**, Iida Y, Okazaki Y, Kawamura S, Takemoto T. Magnifying endoscopic observation of the gastric mucosa, particularly in patients with atrophic gastritis. *Endoscopy* 1978; **10**: 269-274 [PMID: 738222 DOI: 10.1055/s-0028-1098307]

15 **Yao K**. The endoscopic diagnosis of early gastric cancer. *Ann Gastroenterol* 2013; **26**: 11-22 [PMID: 24714327]

16 **Tongtawee T**, Kaewpitoon S, Kaewpitoon N, Dechsukhum C, Loyd RA, Matrakool L. Correlation between Gastric Mucosal Morphologic Patterns and Histopathological Severity of Helicobacter pylori Associated Gastritis Using Conventional Narrow Band Imaging Gastroscopy. *Biomed Res Int* 2015; **2015**: 808505 [PMID: 26120585 DOI: 10.1155/2015/808505]

17 **Okubo M**, Tahara T, Shibata T, Nakamura M, Yoshioka D, Maeda Y, Yonemura J, Ishizuka T, Arisawa T, Hirata I. Changes in gastric mucosal patterns seen by magnifying NBI during H. pylori eradication. *J Gastroenterol* 2011; **46**: 175-182 [PMID: 20967557 DOI: 10.1007/s00535-010-0335-0]

18 **Sidgwick GP**, Bayat A. Extracellular matrix molecules implicated in hypertrophic and keloid scarring. *J Eur Acad Dermatol Venereol* 2012; **26**: 141-152 [PMID: 21838832 DOI: 10.1111/j.1468-3083.2011.04200.x]

19 **Wess TJ**. Collagen fibril form and function. *Adv Protein Chem* 2005; **70**: 341-374 [PMID: 15837520 DOI: 10.1016/S0065-3233(05)70010-3]

20 **Bonin EA**, Campos AC, Coelho JC, Matias JE, Malafaia O, Jonasson TH. Effect of pantoprazole administered subcutaneously on the healing of sutured gastric incisions in rats. *Eur Surg Res* 2005; **37**: 250-256 [PMID: 16260877 DOI: 10.1159/000087872]

21 **Di Lullo GA**, Sweeney SM, Korkko J, Ala-Kokko L, San Antonio JD. Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. *J Biol Chem* 2002; **277**: 4223-4231 [PMID: 11704682 DOI: 10.1074/jbc.M110709200]

22 **Heinz A**, Schräder CU, Baud S, Keeley FW, Mithieux SM, Weiss AS, Neubert RH, Schmelzer CE. Molecular-level characterization of elastin-like constructs and human aortic elastin. *Matrix Biol* 2014; **38**: 12-21 [PMID: 25068896 DOI: 10.1016/j.matbio.2014.07.006]

23 **Bochicchio B**, Laurita A, Heinz A, Schmelzer CE, Pepe A. Investigating the role of (2S,4R)-4-hydroxyproline in elastin model peptides. *Biomacromolecules* 2013; **14**: 4278-4288 [PMID: 24127724 DOI: 10.1021/bm4011529]

24 **Gorres KL**, Raines RT. Prolyl 4-hydroxylase. *Crit Rev Biochem Mol Biol* 2010; **45**: 106-124 [PMID: 20199358 DOI: 10.3109/10409231003627991]

25 **Aita H**, Yoneta T, Seto K, Morita H, Hori Y, Takemasa T, Chaki K, Yamada H, Seiki M, Tagashira E. [Studies on the healing promoting action of Z-103 in chronic gastric ulcer models of rats]. *Nihon Yakurigaku Zasshi* 1992; **99**: 345-352 [PMID: 1592317]

26 **Griffin KJ**, Thompson PA, Gottschalk M, Kyllo JH, Rabinovitch A. Combination therapy with sitagliptin and lansoprazole in patients with recent-onset type 1 diabetes (REPAIR-T1D): 12-month results of a multicentre, randomised, placebo-controlled, phase 2 trial. *Lancet Diabetes Endocrinol* 2014; **2**: 710-718 [PMID: 24997559 DOI: 10.1016/S2213-8587(14)70115-9]

27 **Weidner N**. Current pathologic methods for measuring intratumoral microvessel density within breast carcinoma and other solid tumors. *Breast Cancer Res Treat* 1995; **36**: 169-180 [PMID: 8534865]

28 **Saenko EL**, Ananyeva NM, Tuddenham EG, Kemball-Cook G. Factor VIII - novel insights into form and function. *Br J Haematol* 2002; **119**: 323-331 [PMID: 12406064 DOI: 10.1046/j.1365-2141.2002.03793.x]

29 **Chen H**, Li X, Ge Z, Gao Y, Chen X, Cui Y. Rabeprazole combined with hydrotalcite is effective for patients with bile reflux gastritis after cholecystectomy. *Can J Gastroenterol* 2010; **24**: 197-201 [PMID: 20352149]

30 **Tarnawski AS**, Ahluwalia A. Molecular mechanisms of epithelial regeneration and neovascularization during healing of gastric and esophageal ulcers. *Curr Med Chem* 2012; **19**: 16-27 [PMID: 22300072 DOI: 10.2174/092986712803414088]

31 **Penn JW**, Grobbelaar AO, Rolfe KJ. The role of the TGF-β family in wound healing, burns and scarring: a review. *Int J Burns Trauma* 2012; **2**: 18-28 [PMID: 22928164]

32 **Shih SC**, Tseng KW, Lin SC, Kao CR, Chou SY, Wang HY, Chang WH, Chu CH, Wang TE, Chien CL. Expression patterns of transforming growth factor-beta and its receptors in gastric mucosa of patients with refractory gastric ulcer. *World J Gastroenterol* 2005; **11**: 136-141 [PMID: 15609413 DOI: 10.3748/wjg.v11.i1.136]


**P- Reviewer**: Bashashati M, Iida T, Lakatos PL **S- Editor**: Yu J
**L- Editor**: Webster JR **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/

*World J Gastroenterol* 2017 February 21; 23(7): 1278-1288

DOI: 10.3748/wjg.v23.i7.1278

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

SYSTEMATIC REVIEWS

# Success of photodynamic therapy in palliating patients with nonresectable cholangiocarcinoma: A systematic review and meta-analysis

Harsha Moole, Harsha Tathireddy, Sirish Dharmapuri, Vishnu Moole, Raghuveer Boddireddy, Pratyusha Yedama, Sowmya Dharmapuri, Achuta Uppu, Naveen Bondalapati, Abhiram Duvvuri

Harsha Moole, Harsha Tathireddy, Department of Medicine, University of Illinois College of Medicine, Peoria, IL 61637, United States

Sirish Dharmapuri, Department of Medicine, Wilkes-Barre Veterans Affairs Medical Center, Scranton, PA 18711, United States

Vishnu Moole, Raghuveer Boddireddy, Pratyusha Yedama, Sowmya Dharmapuri, Department of Medicine, NTR University of Health Sciences, Andhra Pradesh 520008, India

Achuta Uppu, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York, NY 10461, United States

Naveen Bondalapati, Department of Medicine, Barnes Jewish Christian Medical Group, Christian Hospital, St. Louis, MO 63136, United States

Abhiram Duvvuri, Division of Gastroenterology and Hepatology, Kansas City Veteran Affairs Medical Center, Kansas City, MO 64128, United States

Author contributions: Moole H contributed to conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article, critical revision, and final approval; Tathireddy H, Boddireddy R, Yedama P, Dharmapuri S, Uppu A and Bondalapati N contributed to acquisition of data, analysis and interpretation of data, drafting the article, final approval; Moole V and Dharmapuri S contributed to interpretation of data, revising the article, final approval; Duvvuri A contributed to conception and design of the study, critical revision, final approval.

Conflict-of-interest statement: The authors deny any conflict of interest.

Data sharing statement: No additional data are available.



Manuscript source: Unsolicited manuscript

Correspondence to: Harsha Moole, MD, Clinical Associate, Department of Medicine, University of Illinois College of Medicine, Peoria, IL 61637,
United States. harsha1778@yahoo.co.in
Telephone: +1-309-6557257
Fax: +1-844-8936705

Received: June 21, 2016
Peer-review started: June 21, 2016
First decision: August 22, 2016
Revised: September 9, 2016
Accepted: October 10, 2016
Article in press: October 10, 2016
Published online: February 21, 2017

## Abstract

### *AIM*

To perform a systematic review and meta-analysis on clinical outcomes of photodynamic therapy (PDT) in non-resectable cholangiocarcinoma.

### *METHODS*

Included studies compared outcomes with photodynamic therapy and biliary stenting (PDT group) *vs* biliary stenting only (BS group) in palliation of non-resectable cholangiocarcinoma. Articles were searched in MEDLINE, PubMed, and EMBASE. Pooled proportions were calculated using fixed and random effects model. Heterogeneity among studies was assessed using the $I^2$ statistic.

***RESULTS***

Ten studies ($n$ = 402) that met inclusion criteria were included in this analysis. The $P$ for $\chi^2$ heterogeneity for all the pooled accuracy estimates was > 0.10. Pooled odds ratio for successful biliary drainage (decrease in bilirubin level > 50% within 7days after stenting) in PDT *vs* BS group was 4.39 (95%CI: 2.35-8.19). Survival period in PDT and BS groups were 413.04 d (95%CI: 349.54-476.54) and 183.41 (95%CI: 136.81-230.02) respectively. The change in Karnofsky performance scores after intervention in PDT and BS groups were +6.99 (95%CI: 4.15-9.82) and -3.93 (95%CI: -8.63-0.77) respectively. Odds ratio for post-intervention cholangitis in PDT *vs* BS group was 0.57 (95%CI: 0.35-0.94). In PDT group, 10.51% (95%CI: 6.94-14.72) had photosensitivity reactions that were self-limiting. Subgroup analysis of prospective studies showed similar results, except the incidence of cholangitis was comparable in both groups.

***CONCLUSION***

In palliation of unresectable cholangiocarcinoma, PDT seems to be significantly superior to BS alone. PDT should be used as an adjunct to biliary stenting in these patients.

**Key words:** Photodynamic therapy; Biliary stenting; Unresectable cholangiocarcinoma; Outcome; Systematic review; Meta-analysis



**Core tip:** Role of photodynamic therapy (PDT) in unresectable cholangiocarcinoma has been scarcely described in the past. However most of these studies included patients who also underwent additional palliative measures simultaneously. Hence, overall safety and efficacy of photodynamic therapy is not clear. This is the first systematic review and meta-analysis evaluating exclusively the role of PDT in these patients. PDT with biliary stenting was compared to biliary stenting (BS) alone. PDT seems to be relatively safe and significantly superior to BS alone in this patient population.

Moole H, Tathireddy H, Dharmapuri S, Moole V, Boddireddy R, Yedama P, Dharmapuri S, Uppu A, Bondalapati N, Duvvuri A. Success of photodynamic therapy in palliating patients with nonresectable cholangiocarcinoma: A systematic review and meta-analysis. *World J Gastroenterol* 2017; 23(7): 1278-1288 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1278.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1278

## INTRODUCTION

Cholangiocarcinoma is the primary cancer of bile ducts. It is an aggressive disease with dismal prognosis. It is a rare cancer comprising less than 2% of all cancers but the incidence has been increasing in the past decade[1]. Approximately 60%-70% of these tumors are located within 2 cm from the bifurcation of the common bile duct (hilar cholangiocarcinoma, also called Klatskin tumor), extrahepatic cholangiocarcinoma occur in approximately 20%-30% and intrahepatic in the remaining 5%-10%.

Bismuth-Corelette system is used to classify hilar cholangiocarcinoma into four types (Type Ⅰ-Ⅳ). Bismuth Type Ⅰ is limited to the common hepatic duct below the confluence of the right and left hepatic duct, Type Ⅱ involves tumor infiltration at the confluence without communication between left and right hepatic ducts, Type Ⅲ involves tumor extension into one main hepatic duct and the secondary bile ducts, Type Ⅳ involves bilateral hepatic ducts and the secondary intrahepatic ducts. It is asymptomatic in the early stages and difficult to diagnose.

Complete tumor resection with negative margins (R0) is the only curative option but only 20%-30% of patients are candidates for curative resection[2,3]. Five year survival rates after curative R0 resection is about 30%-50%[2-7]. The remaining 70%-80% present at an advanced stage and are nonresectable due to locally advanced disease (Involvement of vessels or bilateral extension beyond secondary radicals) or presence of distant metastases[3,8,9].

Palliation is the primary management option in these patients. Chemotherapy and radiotherapy have limited role and do not prolong life in advanced cholangiocarcinoma[10]. Palliative biliary decompression by transpapillary or percutaneous insertion of biliary stent alleviates obstructive cholestasis and is currently the standard of care[11-17]. However, stent patency rates are limited by tumor ingrowth or overgrowth[18-22]. Most patients die from complications of obstructive cholestasis such as cholangitis, biliary sepsis or liver failure.

Photodynamic therapy (PDT) is a new local-ablative, tumor-specific treatment that has shown promising results and is now the standard of care for nonresectable cholangiocarcinoma[23-26]. PDT involves administration of a photosensitizing drug with affinity for neoplastic tissue and subsequent selective irradiation with light of a defined wavelength. The resulting interaction between light and photosensitizing agent causes death of tumor cells, and neovascular cells by formation of oxygen free radicals[27-29].

The first case of successful PDT for non resectable cholangiocarcinoma was described by McCaughan *et al*[30] in 1991. Many studies have since then confirmed the significant advantage of using PDT in patients with nonresectable Cholangiocarcinoma. Most of these studies included patients that additionally received other palliative treatments (surgery, radiotherapy, chemotherapy)[23,24]. The aim of this study is to evaluate the success of photodynamic therapy exclusively,

and its impact on survival, morbidity, biliary drainage and quality of life in patients with nonresectable cholangiocarcinoma through a systematic review and meta-analysis of the literature.

## MATERIALS AND METHODS

### Study selection criteria

Studies evaluating the role of PDT as a palliative option in patients with advanced non-resectable cholangiocarcinoma, were included in this meta-analysis. Prospective studies, retrospective studies and randomized controlled trials (RCTs) were included. Subgroup analysis was performed on prospective studies to negate the heterogeneity introduced by retrospective studies. Studies that used PDT as a neo-adjuvant therapy in patients with resectable cholangiocarcinoma were excluded. Studies that used chemotherapy or radiation therapy along with PDT in patients with resectable or unresectable cholangiocarcinoma were excluded. Studies without original data, perspective articles review articles, and expert opinions were excluded from this meta-analysis. Only full text articles, peer reviewed and published in international journals were included in this analysis. If there were duplicate studies, the most complete and latest study was included in this meta-analysis.

### Data collection and extraction

The study design was written in accordance to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement[31]. Articles were systematically searched in Medline, PubMed, Ovid journals, EMABSE, Cumulative Index for Nursing and Allied Health Literature, ACP journal club, DARE, International Pharmaceutical Abstracts, old Medline, Medline nonindexed citations, OVID Healthstar, and Cochrane Central Register of Controlled Trials (CENTRAL). The search was performed for the years 1966 to May 2016. Abstracts were manually searched in the major gastroenterology journals for the past 3 years. Study authors for the abstracts included in this analysis were contacted when the required data for the outcome measures could not be determined from the publications. The MeSH search headings used were "photodynamic therapy", "PDT", "cholangiocarcinoma", "hilar malignancy", "percutaneous trans-hepatic biliary drainage", "malignant biliary obstruction", "biliary drainage", "endoscopic biliary drainage". The reference lists of the included studies were manually searched for any relevant publications. Two authors (HM and VM) independently searched and extracted the data into an abstraction form. Any differences were resolved by mutual agreement. If the disagreement persisted, the final decision was made by a third author (AD) after reviewing the relevant information. The agreement between reviewers for the collected data was quantified using the Cohen's κ[32]. Data was extracted from the selected studies and entered into a standardized data collection form. The following variables were recorded: name and year of study; type of study; median age; male/female distribution; total number of patient included; number of patients that had PDT along with biliary stenting; number of patients that underwent biliary stenting only; PDT agent, PDT route, stent type - metal *vs* plastic, post treatment survival in PDT plus stenting group (in days), post treatment survival in biliary stenting group, over all adverse events (hepatic abscess/cholangitis/perihepatic abscess/drain site infection, photosensitivity) in both groups, cholangitis in both groups, photosensitivity in PDT group, Karnofsky performance scores (pre-treatment, post treatment and change in score after treatment) in both groups, median number of PDT sessions per patient in each study, bilirubin levels scores (pre-treatment, post treatment and change in score after treatment) in both groups.

### Definitions

Successful biliary drainage was defined as a reduction in serum total bilirubin > 50 % at 2 wk and to a value below 3.0 mg/dL at 4 wk follow up. Technical success was defined as successfully placed stent in the appropriate location, confirmed radiographically and/or endoscopically. Stent patency is defined as time interval between biliary stent insertion and the need for an un-anticipated re-interventions.

### Quality of studies

Clinical trials designed with a control and treatment arms can be assessed for quality of the study. A number of criteria have been used to assess this quality of a study (*e.g.*, randomization, selection bias of the arms in the study, concealment of allocation, and blinding of outcome). Jadad score was used to evaluate the quality of randomized studies. Cochrane Collaborations and the Quality of Reporting of Meta-analysis guidelines were followed to assess the quality of studies[33,34]. Quality of retrospective studies were assessed using Newcastle-Ottawa Scale[35].

### Statistical analysis

This meta-analysis was performed by calculating pooled proportions. First the individual study proportion of survival (in days), adverse events, Karnofsky scores *etc*., were transformed into a quantity using Freeman-Tukey variant of the arcsine square root transformed proportion. The pooled proportion is calculated as the back-transform of the weighted mean of the transformed proportions, using inverse arcsine variance weights for the fixed effects model and DerSimonian-Laird weights for the random effects model[36,37]. Random effects model was used if the heterogeneity was significant, and fixed effects model was used if heterogeneity was non-significant. Forest plots were drawn to show the point estimates in each study in

**Table 1 Characteristics of included studies**

| Study | Type | *n* | PDT, *n* | BS, *n* | M/F | Age | Cancer type | PDT agent | PDT route | Stenting route | Stent type | PDT sessions per patient |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ortner *et al*[43], 2003 | RCT | 39 | 20 | 19 | NA | 66 | Non resectable CCA | Photofrin 2 mg/kg | Endoscopic | EBD or PTBD - Double stenting | Plastic | 2 |
| Dumoulin *et al*[44], 2003 | P | 44 | 24 | 20 | 19/25 | 77 | Non resectable CCA | Photofrin 2 mg/kg | Endoscopic | EBD or PTBD | Plastic followed by metal 4 wk later | NA |
| Cheon *et al*[45], 2004 | R | 47 | 27 | 20 | 38/9 | 63 | Non resectable CCA | Photogem | Endoscopic | PTBD in PDT, EBD in other | Plastic | 2 |
| Wiedmann *et al*[46], 2004 | P | 23 | 23 | NA | 15/8 | 68 | Non resectable CCA | Photofrin 2 mg/kg | Endoscopic | EBD or PTBD | Plastic or Metal | 3 |
| Shim *et al*[47], 2005 | P | 24 | 24 | NA | NA | 58 | Non resectable CCA | Photofrin 2 mg/kg | Percutaneous | PTBD | Plastic | 2 |
| Zoepf *et al*[48], 2005 | RCT | 32 | 16 | 16 | 20/12 | 68 | Non resectable CCA | Photosan-3 | Endoscopic or percutaneous | EBD or PTBD | Plastic | 2 |
| Witzigmann *et al*[49], 2006 | P | 124 | 68 | 56 | 59/65 | 69 | Non resectable CCA | Photofrin 2 mg/kg | Endoscopic | EBD or PTBD | Plastic | 2 |
| Prasad *et al*[50], 2007 | R | 25 | 25 | NA | 20/5 | 64 | Non resectable CCA | Photofrin 2 mg/kg | Endoscopic or percutaneous | PTBD | Plastic | 1 |
| Lee *et al*[51], 2012 | R | 33 | 18 | 15 | 24/9 | 66 | Non resectable CCA | Photofrin 2 mg/kg | Endoscopic or percutaneous | EBD or PTBD | Metal | 1 |
| Wagner *et al*[52], 2013 | P | 11 | 11 | NA | 8/3 | 76 | Non resectable CCA | Temoporfin | Endoscopic | EBD or PTBD | Plastic | 1 |

PDT: Photodynamic therapy; BS: Biliary stenting group; EBD: Endoscopic biliary drainage; PTBD: Percutaneous transhepatic biliary drainage; CCA: Cholangiocarcinoma; RCT: Randomized controlled trial; P: Prospective study; R: Retrospective study; NA: Not available.

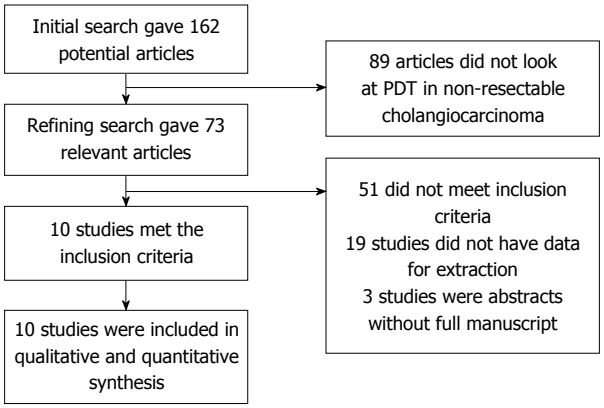



**Figure 1 Study selection.** PDT: Photodynamic therapy.

relation to the summary pooled estimate. The width of the point estimates in the Forest plots indicates the assigned weight to that study. The heterogeneity among studies was tested using $I^2$ statistic and Cochran's $Q$ test based upon inverse variance weights[38]. $I^2$ of 0%-39% was considered as non-significant heterogeneity, 40%-75% as moderate heterogeneity, and 76%-100% as considerable heterogeneity. If $P$ value is > 0.10, it rejects the null hypothesis that the studies are heterogeneous. The effect of publication and selection bias on the summary estimates was tested by both Harbord-Egger bias indicator[39] and Begg bias indicator[40]. Also, funnel plots were constructed to evaluate potential publication bias[41,42]. Microsoft Excel 2013 software was used to perform statistics for this meta-analysis. Subgroup analysis was performed on only prospective studies.

## RESULTS

### *Study selection*

Initial search identified 162 reference articles, in which 73 articles were selected and reviewed. Data was extracted from 10 studies[43-52] ($n$ = 402) which met the inclusion criterion. All the studies are published as full text articles. Figure 1 shows the flow diagram of search results. Among the 10 studies included in this analysis, only two were RCTs[43,48]. Three studies[45,50,51] out of the 10 studies were retrospective studies and the rest were prospective studies[44,46,47,49,52]. Subgroup analysis was performed on all prospective trials.

The total number of patients included in this meta-analysis is 402, with a predominantly male population (65%). Median age of the patients was 68 years. Table 1 shows the baseline characteristics of the studies. The $P$ for $\chi^2$ heterogeneity for all the pooled accuracy estimates was > 0.10. The agreement between reviewers for the collected data gave a Cohen's κ value of 1.0.

Studies evaluating survival of patients followed up with the patients till death. Studies describing the adverse events and quality of life had a median follow up period of three months. All except three studies used Photofrin 2 mg/kg as the PDT agent. Photogem[45], Photosan-3[48], and Temoporfin[52] were the three other PDT agents used. PDT was administered via endoscopic

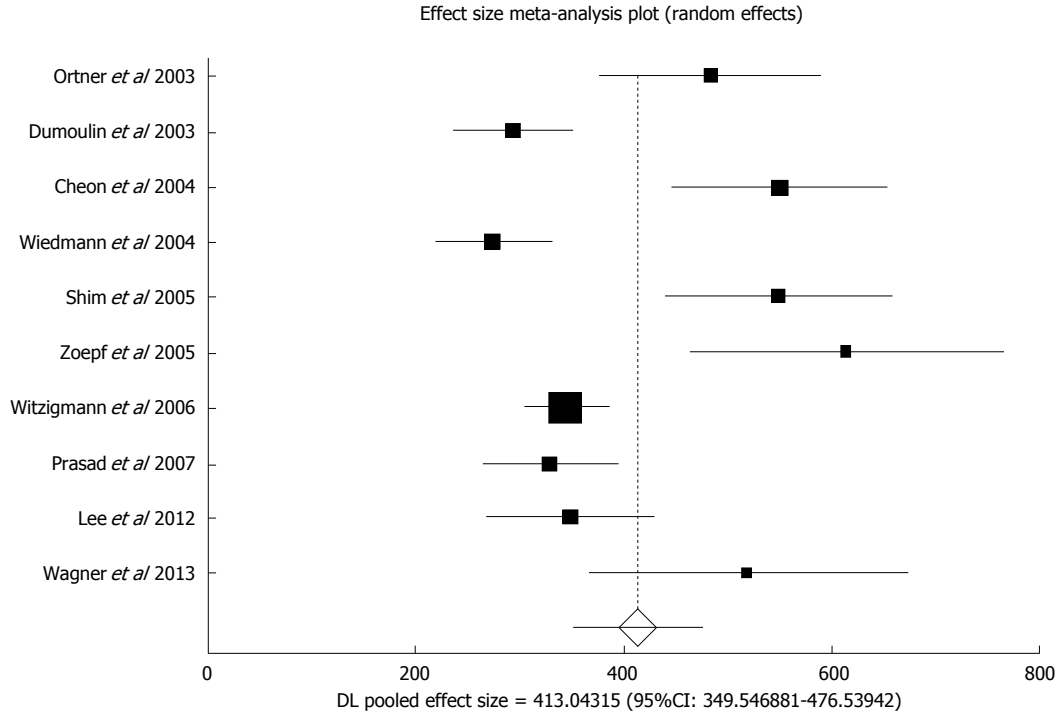



**Figure 2 Forest plot - individual study proportions and the pooled estimate of survival period in photodynamic therapy group.**

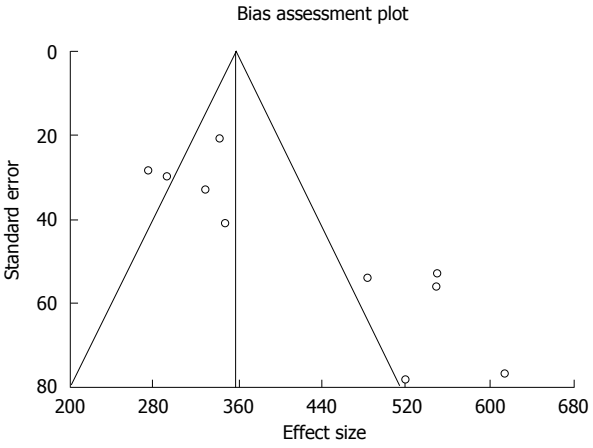



**Figure 3 Funnel plot: Survival in photodynamic therapy group.**

route in six studies[43-46,49,52], percutaneous route in one study[47], and endoscopic or percutaneous route in three studies[48,50,51]. Biliary stenting was performed by endoscopic route (EBD) or percutaneous tranhepatic route (PTBD) in eight studies[43-46,48,49,51,52]. Two studies exclusively used PTBD for biliary drainage[47,50]. Seven studies used plastic biliary stents, one study used only metal stents[43,45,47-50,52], one study used metal and plastic stents[46], and one study used plastic stent followed by metal stent[44]. Median number of PDT sessions per patient was two.

### *Survival benefit and Quality of life with photodynamic therapy*

Data was available in all the ten included studies, to calculate survival period. In the pooled patient population, the survival periods in PDT group (patient received PDT along with biliary stenting) and biliary stenting only group (BS group) were 413.04 d (95%CI: 349.54-476.54) and 183.41 days (95%CI: 136.81 to 230.02) respectively. $I^2$ (inconsistency) = 85.1% (95%CI: 73.5%-90.2%). Egger: bias = 5.09 (95%CI: 2.12-8.07), $P$ = 0.0043. Figures 2 and 3 are forest plot and funnel plot representing the survival in PDT group. Six out of ten studies[43,46-49,52] included data regarding Karnofsky performance scores. The change in Karnofsky performance scores after intervention in PDT and BS groups were +6.99 (95%CI: 4.15-9.82) and -3.93 (95%CI: -8.63-0.77) respectively. $I^2$ (inconsistency) = 97.6% (95%CI: 96.7%-98.1%). Egger: bias = 7.66 (95%CI: -0.22-15.53) $P$ = 0.054. Figure 4 is a forest plot representing the change in Karnofsky scores in PDT group.

### *Biliary drainage outcomes with photodynamic therapy*

Three studies[43,49,51] had data that compared successful biliary drainage in PDT group *vs* BS group. Pooled odds ratio for successful biliary drainage in PDT group *vs* BS group was 4.39 (95%CI: 2.35-8.19). $I^2$ (inconsistency) = 28.8% (95%CI: 0%-79.9%). Horbold-Egger: bias = -1.19 (92.5%CI: -20.32-17.94) $P$ = 0.69. Figure 5 is a forest plot of odds ratio for successful biliary drainage. Figure 6 is an L'Abbe plot for the same variable. Bilirubin levels were assessed in all ten studies that evaluated photodynamic therapy. In the pooled study population, pre-treatment bilirubin levels (mg/dL) in PDT and BS group were 6.36 (95%CI: 5.86-6.87) and 7.83

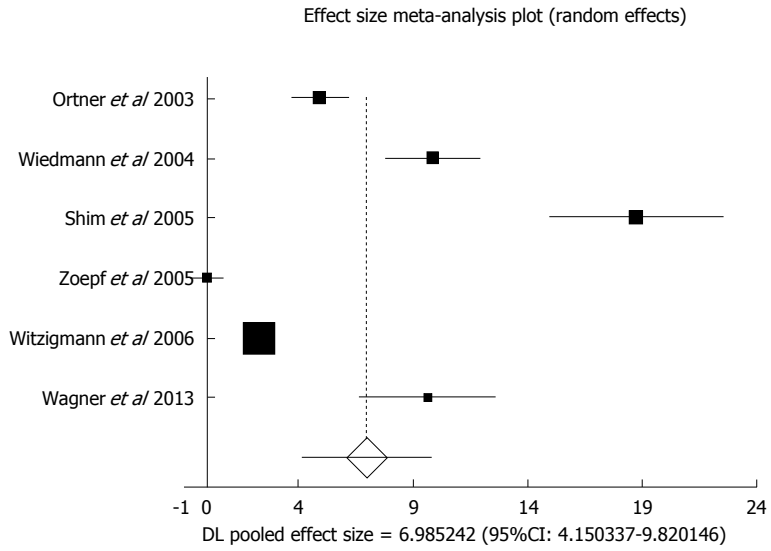



**Figure 4 Forest plot - individual study proportions and the pooled estimate of change in Karnofsky performance scores in photodynamic therapy group.**

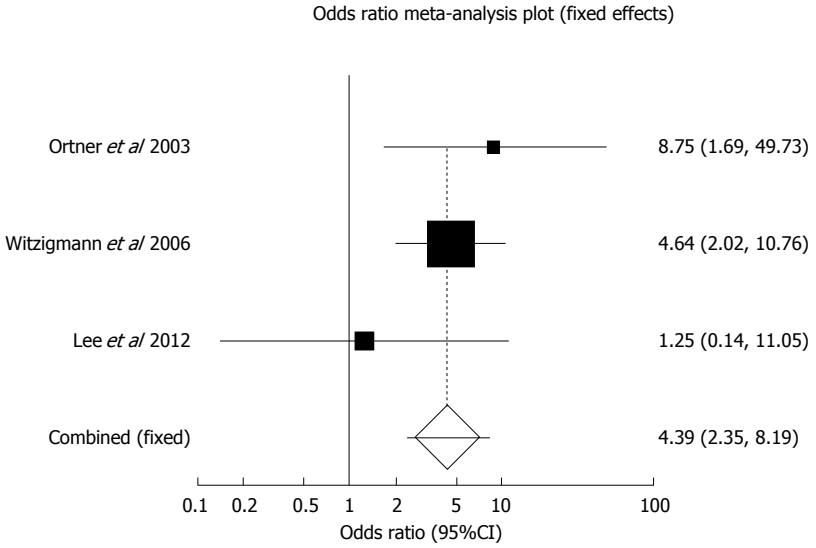



**Figure 5 Forest plot - individual study proportions and the pooled estimate of odds ratio - successful biliary drainage in photodynamic therapy group *vs* biliary stenting group.**

(95%CI: 7.08-8.58) respectively. After the intervention (at median follow up period of 3months), the bilirubin levels decreased by 4.23 (95%CI: 3.86-4.60) and 2.45 (95%CI: 2.08-2.81) in PDT and BS group respectively. $I^2$ (inconsistency) = 97.1% (95%CI: 96.4%-97.7%). Egger: bias = 11.38 (95%CI: 5.28-17.48), $P$ = 0.0026.

### *Adverse events with PDT*

Pooled odds ratio for post-intervention cholangitis episodes in PDT group *vs* BS group was 0.57 (95%CI: 0.35-0.94). $I^2$ (inconsistency) = 48.3% (95%CI: 0%-73.4%). Egger: bias = -0.70 (95%CI: -2.44-1.03), $P$ = 0.38. Figure 7 is a forest plot of odds ratio for cholangitis in PDT group *vs* BS group. Figure 8 is the funnel plot for the same variable. Data regarding photosensitivity secondary to PDT was available in nine studies. One out of ten studies[45] did not have information on photosensitivity reactions. In the pooled proportion of patients in PDT group, 10.51% (95%CI: 6.94-14.72) had photosensitivity reactions that were self-limiting. $I^2$ (inconsistency) = 61.2% (95%CI: 0%-79.5%). Egger: bias = 2.81 (95%CI: 0.38-5.23) $P$ = 0.02. Figure 9 is a forest plot for photosensitivity reactions in PDT group. Due to paucity of data from the individual studies, we were unable to derive at meaningful outcomes regarding overall adverse outcomes and other individual adverse events.

### *Subgroup analysis of prospective studies*

Seven studies[43,44,46-49,52] with 297 patients that met the inclusion criteria were included in this analysis. Median age of the patients was 68years, with 50%

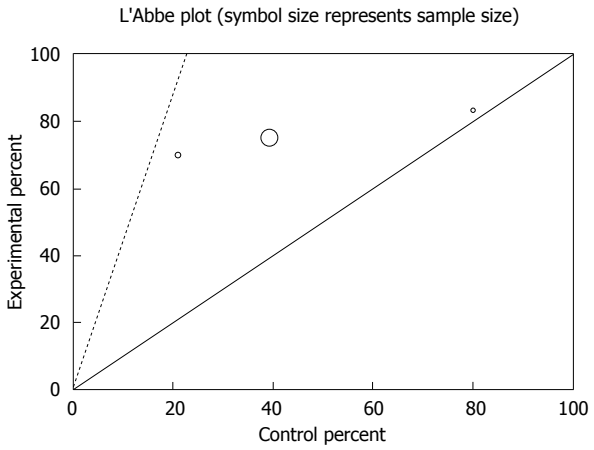



**Figure 6 L'Abbe plot for odds ratio - successful biliary drainage in photodynamic therapy group *vs* biliary stenting group.**

females. The *P* for $\chi^2$ heterogeneity for all the pooled accuracy estimates was > 0.10. Pooled odds ratio for successful biliary drainage in PDT group *vs* BS group was 5.33 (95%CI: 2.71-10.50). In the pooled study population, pre-treatment bilirubin levels (mg/dL) in PDT and BS group were 5.92 (95%CI: 5.35-6.50) and 7.18 (95%CI: 6.38-7.99) respectively. After the intervention (at median follow up period of 3months), the bilirubin levels decreased by 4.35 (95%CI: 3.90-4.81) and 2.08 (95%CI: 1.70-2.45) in PDT and BS group respectively. $I^2$ (inconsistency) = 97.6% (95%CI: 96.8%-98.1%). In the pooled patient population, the survival period in PDT group and BS group were 420.29 (95%CI: 338.69-501.89) and 153.43 (95%CI: 109.09-197.77) respectively. $I^2$ (inconsistency) = 87% (95%CI: 74.2%-91.9%). The change in Karnofsky performance scores after intervention in PDT and BS groups were +7.08 (95%CI: 4.23-9.93) and -2.39 (95%CI: -2.89 to-1.89) respectively. $I^2$ (inconsistency) = 97.6% (95%CI: 96.7%-98.1%). Pooled odds ratio for cholangitis to be 0.78 (95%CI: 0.45-1.35) in PDT vs BS group. $I^2$ (inconsistency) = 45.2% (95%CI: 0%-75.3%). Photosensitivity was present in 11.59% (95%CI: 7.47-16.47) of this PDT subgroup. $I^2$ (inconsistency) = 69% (95%CI: 5.9%-84.1%).

## DISCUSSION

Cholangiocarcinoma is a rare cancer with poor prognosis. About 80% of cholangiocarcinoma present at an advanced stage and are nonresectable. Chemotherapy and radiotherapy, alone do not add any benefit to patient survival and quality of life. Effective palliation by biliary decompression to alleviate symptoms of cholestasis and prevent sepsis is the fundamental goal for most patients with nonresectable cholangiocarcinoma. PDT is a promising and evolving therapy in the management of patients with nonresectable cholangiocarcinoma. Nonresectable cholangiocarcinoma has a median survival time of 3 mo without intervention[53] and 4-10 mo with biliary drainage[3,6,7,19,21,22,53,54]. The current systematic review and meta-analysis shows that PDT combined with biliary stenting improves the success of biliary drainage and improves the survival and quality of life in patients with nonresectable cholangiocarcinoma. For treatment of non resectable cholangiocarcinoma, photosensitizers with the ability to penetrate deep tissue are better compared to those with superficial effect. Chlorine derivatives and hematoporphyrin derivatives usually have a deep tissue penetration.

In the first RCT by Ortner *et al*[43], median survival time after PDT was 493 d compared to 98 d in patients receiving biliary stent alone. Another RCT by Zoepf *et al*[48] showed similar survival benefit in the PDT group 630 days compared to 210 d in the stent only group. Quality of life (Karnofsky index) significantly improved in the study by Ortner *et al*[43] but not in the study by Zoepf *et al*[48] due to the higher performance status of enrolled patients at study entry.

Lee *et al*[51] stated that the duration of metal stent patency was significantly longer after one session of PDT than in the stent-only group. Longer patency of metal stent by PDT translated to better quality of life by decreasing the number of procedures like stent revision or percutaneous drainage. Witzigmann *et al*[49] compared outcomes after palliative PDT and resection therapy. Their study showed that palliative PDT was inferior to complete curative (R0) resection. However, patients with palliative PDT showed similar survival time to that of patients with incomplete resection (R1/R2). Prasad *et al*[50] looked at factors associated with increased survival after PDT and found that presence of visible mass on imaging, low serum albumin and prolonged time period between diagnosis and treatment with PDT to be the predicting factors for early mortality. In non-resectable cholangiocarcinoma patients, the option of liver transplant (with eventual neo-adjuvant therapy) should be considered on a individualized basis, since this option has been studied even in patients with initially non resectable cholangiocarcinoma.

PDT was relatively well tolerated with minimal side effects in most studies. Cholangitis was the most common side effect followed by phototoxicity. All patients who had PDT also had biliary stenting. Hence cholangitis, could be a potential complication of biliary stenting as well. It is difficult to ascertain if cholangitis is a complication of PDT alone.

Strengths of this meta-analysis include the high quality methodology of statistical analysis, high quality methodology used in individual studies. This is an updated meta-analysis to pool the evidence for the utility of PDT plus biliary stenting in palliation of non resectable cholangiocarcinoma. Lu *et al*[55] was the previous meta-analysis on the topic, however several new studies were published after the first meta-

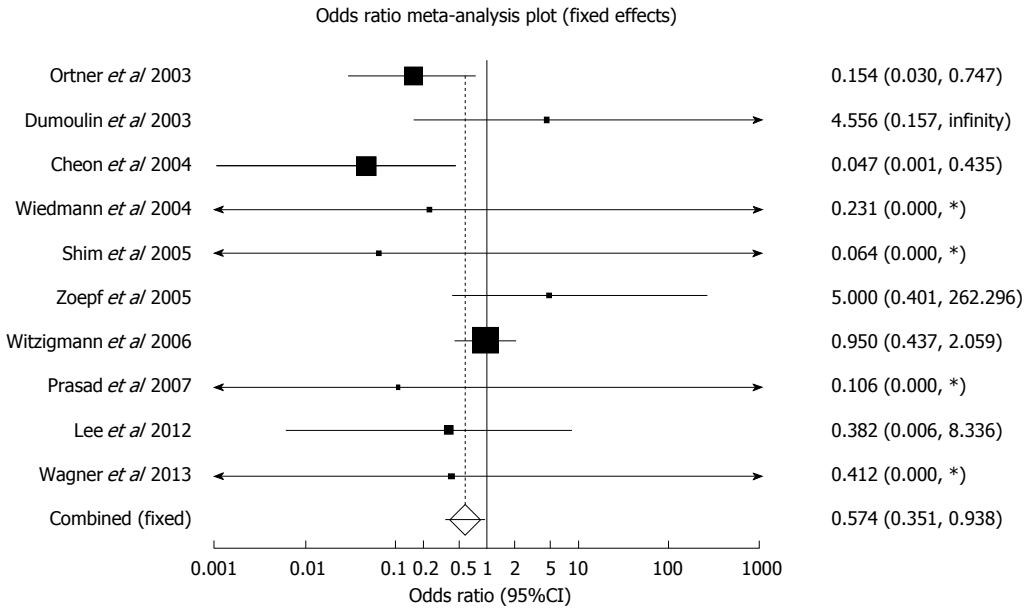



**Figure 7 Forest plot - individual study proportions and the pooled estimate of odds ratio - cholangitis in photodynamic therapy group *vs* biliary stenting group.**

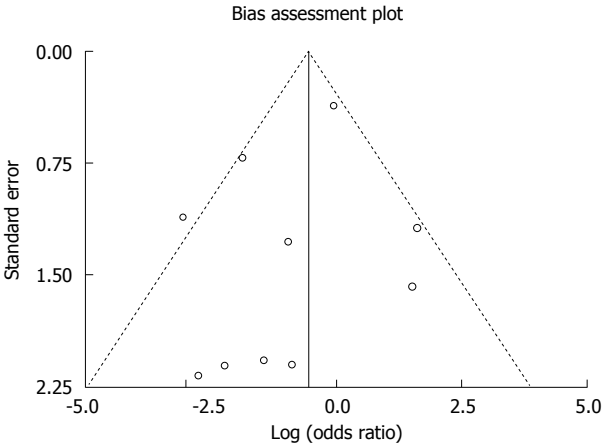



**Figure 8 Funnel plot - odds ratio for cholangitis in photodynamic therapy group *vs* biliary stenting group.**

analysis, that necessitated this updated analysis. Combining chemotherapy with PDT has shown survival benefit compared to PDT alone, in patients with hilar non resectable cholangiocarcinoma[56].

Limitations of this study are: most of the data is synthesized from studies with relatively small sample sizes. Studies differed in the method of PDT (Percutaneous or endoscopic), and the number of sessions of PDT which might have influenced the outcomes. Different types of stents (plastic *vs* metal) were used and route of stenting varied (endoscopic *vs* percutaneous transhepatic approach) among the studies, which could have all affected the outcomes. Retrospective studies were included in this meta-analysis. In order to mitigate this issue, we have performed a sub-group analysis on prospective studies only.

Studies with statistically significant positive results tend to be published and cited. Additionally, smaller studies may show larger treatment effects compared to larger studies. This publication and selection bias may affect the summary estimates. The bias can be estimated using Egger bias indicators and the construction of funnel plots, whose shape can be affected by bias. In the present meta-analysis and systematic review, bias calculations both Egger[39] and Begg *et al*[40] bias indicators showed no statistically significant bias. Furthermore, funnel plots were used to evaluate for publication bias among the studies included in the present analysis.

Granted there is availability of operator expertise and infrastructure availability, we believe that PDT along with biliary stenting is an excellent palliative option for non-resectable cholangiocarcinoma. Based on systematic review of literature, it is evident that in patients with resectable cholangiocarcinoma, surgery would still be the best option. The utility of PDT in this patient population (resectable cholangiocarcinoma) has not shown any additional benefit compared to surgery.

Overall, PDT combined with biliary stenting improves the success of biliary drainage and has a significant benefit in improving the survival period and quality of life. PDT is beneficial, minimally invasive, and well tolerated with a favorable side effect profile. We conclude that PDT with biliary stenting could be offered to all patients with nonresectable cholangiocarcinoma as a palliative option.

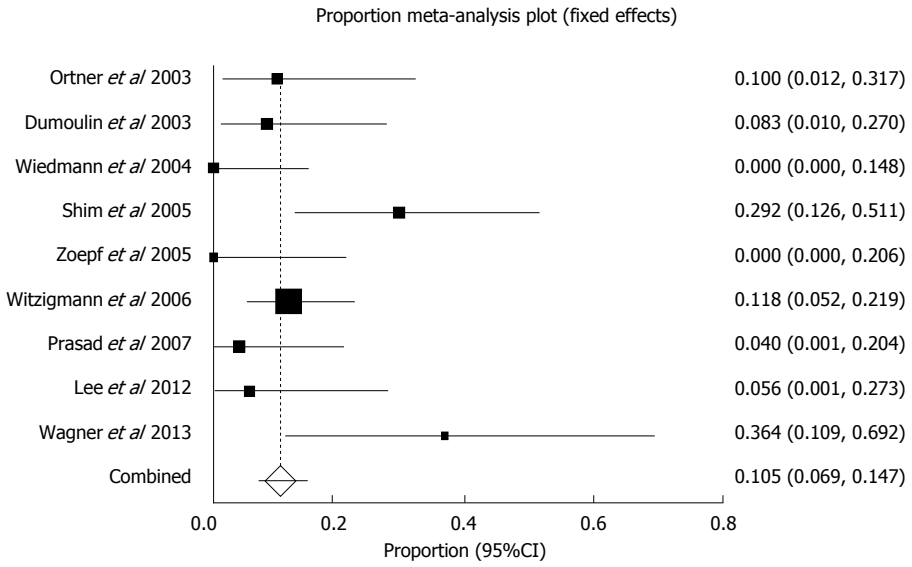



**Figure 9 Forest plot - individual study proportions and the pooled estimate of photosensitivity reactions in photodynamic therapy group.**

## COMMENTS

### Background

Photodynamic therapy (PDT) is a new local-ablative, tumor-specific treatment that has shown promising results and is now the standard of care for nonresectable cholangiocarcinoma.

### Research frontiers

Many studies have confirmed the significant advantage of using PDT in patients with nonresectable Cholangiocarcinoma. Most of these studies included patients that additionally received other palliative treatments (surgery, radiotherapy, chemotherapy).

### Innovations and breakthroughs

PDT combined with biliary stenting improves the success of biliary drainage and has a significant benefit in improving the survival period and quality of life.

### Applications

PDT is beneficial, minimally invasive, and well tolerated with a favorable side effect profile. We conclude that PDT with biliary stenting could be offered to all patients with nonresectable cholangiocarcinoma as a palliative option.

### Peer-review

The manuscript presents a very excellent research in medical treatment of non-resectable cholangiocarcinoma with PDT using meta-analysis approach. The authors have chosen a good set of objective criteria, aggregated enough information and performed well data analysis with high statistic. The language is well written. The study results should be benefits to medicinal field.

## REFERENCES


1 **Shaib Y**, El-Serag HB. The epidemiology of cholangiocarcinoma. *Semin Liver Dis* 2004; **24**: 115-125 [PMID: 15192785 DOI: 10.1055/s-2004-828889]

2 **Rea DJ**, Munoz-Juarez M, Farnell MB, Donohue JH, Que FG, Crownhart B, Larson D, Nagorney DM. Major hepatic resection for hilar cholangiocarcinoma: analysis of 46 patients. *Arch Surg* 2004; **139**: 514-523; discussion 523-525 [PMID: 15136352 DOI: 10.1001/archsurg.139.5.514]

3 **Jarnagin WR**, Fong Y, DeMatteo RP, Gonen M, Burke EC, Bodniewicz BS J, Youssef BA M, Klimstra D, Blumgart LH. Staging, resectability, and outcome in 225 patients with hilar cholangiocarcinoma. *Ann Surg* 2001; **234**: 507-517; discussion 517-519 [PMID: 11573044]

4 **Dinant S**, Gerhards MF, Rauws EA, Busch OR, Gouma DJ, van Gulik TM. Improved outcome of resection of hilar cholangiocarcinoma (Klatskin tumor). *Ann Surg Oncol* 2006; **13**: 872-880 [PMID: 16614876 DOI: 10.1245/ASO.2006.05.053]

5 **Hasegawa S**, Ikai I, Fujii H, Hatano E, Shimahara Y. Surgical resection of hilar cholangiocarcinoma: analysis of survival and postoperative complications. *World J Surg* 2007; **31**: 1256-1263 [PMID: 17453285 DOI: 10.1007/s00268-007-9001-y]

6 **Hemming AW**, Reed AI, Fujita S, Foley DP, Howard RJ. Surgical management of hilar cholangiocarcinoma. *Ann Surg* 2005; **241**: 693-699; discussion 699-702 [PMID: 15849505]

7 **Kawasaki S**, Imamura H, Kobayashi A, Noike T, Miwa S, Miyagawa S. Results of surgical resection for patients with hilar bile duct cancer: application of extended hepatectomy after biliary drainage and hemihepatic portal vein embolization. *Ann Surg* 2003; **238**: 84-92 [PMID: 12832969 DOI: 10.1097/01.SLA.0000074984.83031.02]

8 **Nakeeb A**, Pitt HA, Sohn TA, Coleman J, Abrams RA, Piantadosi S, Hruban RH, Lillemoe KD, Yeo CJ, Cameron JL. Cholangiocarcinoma. A spectrum of intrahepatic, perihilar, and distal tumors. *Ann Surg* 1996; **224**: 463-473; discussion 473-475 [PMID: 8857851]

9 **Nathan H**, Aloia TA, Vauthey JN, Abdalla EK, Zhu AX, Schulick RD, Choti MA, Pawlik TM. A proposed staging system for intrahepatic cholangiocarcinoma. *Ann Surg Oncol* 2009; **16**: 14-22 [PMID: 18987916 DOI: 10.1245/s10434-008-0180-z]

10 **Gao F**, Bai Y, Ma SR, Liu F, Li ZS. Systematic review: photodynamic therapy for unresectable cholangiocarcinoma. *J Hepatobiliary Pancreat Sci* 2010; **17**: 125-131 [PMID: 19455276 DOI: 10.1007/s00534-009-0109-3]

11 **Moole H**, Dhillon S, Volmar F, Cashman M, Bechtold M, Puli S. Mo1378 Is There a Survival and Morbidity Benefit of Covered Over Uncovered Metal Stents in Malignant Biliary Strictures? A Meta-Analysis and Systematic Review. *Gastrointest Endosc* 2015; **5**: AB399 [DOI: 10.1016/j.gie.2015.03.706]

12 **Moole H**, Bechtold M, Puli S. Mo1037 Comparison of Double Layer Stents and Self-Expandable Metal Stents for Malignant Biliary Strictures: A Meta-Analysis and Systematic Review. *Gastrointest Endosc* 2015; **5**: AB347 [DOI: 10.1016/j.gie.2016.03.548]

13 **Moole H**, Cashman M, Volmar F, Dhillon S, Bechtold M, Puli S. Su1601 Are Covered Metal Stents Better than Uncovered Metal Stents for Malignant Biliary Stricture? A Meta-Analysis and Systematic Review. *Gastrointest Endosc* 2015; **5**: AB346 [DOI:

10.1016/j.gie.2015.03.563]
14 **Moole H**, Puli S. Sa1408 Are Metal Stents Better Than Plastic Stents for Malignant Distal Common Bile Duct Stricture? A Meta-Analysis and Systematic Review. *Gastrointest Endosc* 2015; **5**: AB202 [DOI: 10.1016/j.gie.2015.03.179]
15 **Moole H**, Cashman M, Volmar F, Dhillon S, Bechtold M, Puli S. Sa1869 Is There a Morbidity and Survival Benefit for Metal Over Plastic Stents in Managing Malignant Distal Biliary Strictures? A Meta-Analysis and Systematic Review. *Gastroenterology* 2015; **148**: S1030
16 **Moole H**, Puli S. Tu1928 Complications of Metal Vs Plastic Stents in Malignant Distal Biliary Strictures: A Meta-Analysis and Systematic Review. *Gastroenterology* 2015; **148**: S1030 [DOI: 10.1016/S0016-5085(15)33521-6]
17 **Moole H**, Puli S. Su1072 Comparison of Complications for Covered Vs Uncovered Metal Stents in Malignant Biliary Strictures: A Meta-Analysis and Systematic Review. *Gastroenterology* 2015; **148**: S400 [DOI: 10.1016/S0016-5085(15)31344-5]
18 **O'Brien S**, Hatfield AR, Craig PI, Williams SP. A three year follow up of self expanding metal stents in the endoscopic palliation of longterm survivors with malignant biliary obstruction. *Gut* 1995; **36**: 618-621 [PMID: 7537708]
19 **Liu CL**, Lo CM, Lai EC, Fan ST. Endoscopic retrograde cholangiopancreatography and endoscopic endoprosthesis insertion in patients with Klatskin tumors. *Arch Surg* 1998; **133**: 293-296 [PMID: 9517743 DOI: 10.1001/archsurg.133.3.293]
20 **Lai EC**, Lo CM, Liu CL. Endoscopic stenting for malignant biliary obstruction. *World J Surg* 2001; **25**: 1289-1295 [PMID: 11596892 DOI: 10.1007/s00268-001-0112-6]
21 **Ducreux M**, Liguory C, Lefebvre JF, Ink O, Choury A, Fritsch J, Bonnel D, Derhy S, Etienne JP. Management of malignant hilar biliary obstruction by endoscopy. Results and prognostic factors. *Dig Dis Sci* 1992; **37**: 778-783 [PMID: 1373361 DOI: 10.1007/BF01296439]
22 **Polydorou AA**, Cairns SR, Dowsett JF, Hatfield AR, Salmon PR, Cotton PB, Russell RC. Palliation of proximal malignant biliary obstruction by endoscopic endoprosthesis insertion. *Gut* 1991; **32**: 685-689 [PMID: 1711994 DOI: 10.1136/gut.32.6.685]
23 **Ortner MA**. Photodynamic therapy of cholangiocarcinoma cancer. *Gastrointest Endosc Clin N Am* 2000; **10**: 481-486 [PMID: 10899258]
24 **Zoepf T**, Jakobs R, Arnold JC, Apel D, Rosenbaum A, Riemann JF. Photodynamic therapy for palliation of nonresectable bile duct cancer--preliminary results with a new diode laser system. *Am J Gastroenterol* 2001; **96**: 2093-2097 [PMID: 11467637 DOI: 10.1111/j.1572-0241.2001.03968.x]
25 **Rumalla A**, Baron TH, Wang KK, Gores GJ, Stadheim LM, de Groen PC. Endoscopic application of photodynamic therapy for cholangiocarcinoma. *Gastrointest Endosc* 2001; **53**: 500-504 [PMID: 11275896 DOI: 10.1067/mge.2001.113386]
26 **Berr F**, Tannapfel A, Lamesch P, Pahernik S, Wiedmann M, Halm U, Goetz AE, Mössner J, Hauss J. Neoadjuvant photodynamic therapy before curative resection of proximal bile duct carcinoma. *J Hepatol* 2000; **32**: 352-357 [PMID: 10707878 DOI: 10.1016/S0168-8278(00)80083-5]
27 **Nelson JS**, Liaw LH, Orenstein A, Roberts WG, Berns MW. Mechanism of tumor destruction following photodynamic therapy with hematoporphyrin derivative, chlorin, and phthalocyanine. *J Natl Cancer Inst* 1988; **80**: 1599-1605 [PMID: 2973528]
28 **Henderson BW**, Dougherty TJ. How does photodynamic therapy work? *Photochem Photobiol* 1992; **55**: 145-157 [PMID: 1603846 DOI: 10.1111/j.1751-1097.1992.tb04222.x]
29 **Wong Kee Song LM**, Wang KK, Zinsmeister AR. Mono-L-aspartyl chlorin e6 (NPe6) and hematoporphyrin derivative (HpD) in photodynamic therapy administered to a human cholangiocarcinoma model. *Cancer* 1998; **82**: 421-427 [PMID: 9445202 DOI: 10.1002/1097-0142(19980115)82::2<421::AID-CNCR25>3.0.CO;2-O]
30 **McCaughan JS**, Mertens BF, Cho C, Barabash RD, Payton HW. Photodynamic therapy to treat tumors of the extrahepatic biliary ducts. A case report. *Arch Surg* 1991; **126**: 111-113 [PMID: 1824676]
31 **Liberati A**, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, Clarke M, Devereaux PJ, Kleijnen J, Moher D. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. *PLoS Med* 2009; **6**: e1000100 [PMID: 19621070 DOI: 10.1371/journal.pmed.1000100]
32 **Brennan P**, Silman A. Statistical methods for assessing observer variability in clinical measures. *BMJ* 1992; **304**: 1491-1494 [PMID: 1611375]
33 **Jadad AR**, Moore RA, Carroll D, Jenkinson C, Reynolds DJ, Gavaghan DJ, McQuay HJ. Assessing the quality of reports of randomized clinical trials: is blinding necessary? *Control Clin Trials* 1996; **17**: 1-12 [PMID: 8721797 DOI: 10.1016/0197-2456(95)00134-4]
34 **Stroup DF**, Berlin JA, Morton SC, Olkin I, Williamson GD, Rennie D, Moher D, Becker BJ, Sipe TA, Thacker SB. Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. *JAMA* 2000; **283**: 2008-2012 [PMID: 10789670 DOI: 10.1001/jama.283.15.2008]
35 **Wells GA**, Shea B, O'Connell D, Peterson J, Welch V, Losos M, Tugwell P. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomisedstudies in meta-analyses. 2014. (accessed June 20, 2016). Available from: URL: http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp
36 **Stuart A**, Ord JK. Kendall's Advanced Theory of Statistics. 6th ed. London: Edward Arnold, 1994
37 **DerSimonian R**, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177-188 [PMID: 3802833 DOI: 10.1016/0197-2456(86)90046-2]
38 **Deeks JJ**. Systematic reviews of evaluations of diagnostic and screening tests. In: Egger M, Smith GD, Altman DG, editors. Systematic Reviews in Health Care. Meta-analysis in context. London: BMJ Books, 2001
39 **Harbord RM**, Egger M, Sterne JA. A modified test for small-study effects in meta-analyses of controlled trials with binary endpoints. *Stat Med* 2006; **25**: 3443-3457 [PMID: 16345038 DOI: 10.1002/sim.2380]
40 **Begg CB**, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. *Biometrics* 1994; **50**: 1088-1101 [PMID: 7786990 DOI: 10.2307/2533446]
41 **Sterne JA**, Egger M, Smith GD. Systematic reviews in health care: Investigating and dealing with publication and other biases in meta-analysis. *BMJ* 2001; **323**: 101-105 [PMID: 11451790]
42 **Sterne JA**, Egger M. Funnel plots for detecting bias in meta-analysis: guidelines on choice of axis. *J Clin Epidemiol* 2001; **54**: 1046-1055 [PMID: 11576817]
43 **Ortner ME**, Caca K, Berr F, Liebetruth J, Mansmann U, Huster D, Voderholzer W, Schachschal G, Mössner J, Lochs H. Successful photodynamic therapy for nonresectable cholangiocarcinoma: a randomized prospective study. *Gastroenterology* 2003; **125**: 1355-1363 [PMID: 14598251 DOI: 10.1016/j.gastro.2003.07.015]
44 **Dumoulin FL**, Gerhardt T, Fuchs S, Scheurlen C, Neubrand M, Layer G, Sauerbruch T. Phase II study of photodynamic therapy and metal stent as palliative treatment for nonresectable hilar cholangiocarcinoma. *Gastrointest Endosc* 2003; **57**: 860-867 [PMID: 12776033 DOI: 10.1067/mge.2003.250]
45 **Cheon YK**, Cho YD, Baek SH, Cha SW, Moon JH, Kim YS, Lee JS, Lee MS, Shim CS, Kim BS. [Comparison of survival of advanced hilar cholangiocarcinoma after biliary drainage alone versus photodynamic therapy with external drainage]. *Korean J Gastroenterol* 2004; **44**: 280-287 [PMID: 15564808]
46 **Wiedmann M**, Berr F, Schiefke I, Witzigmann H, Kohlhaw K, Mössner J, Caca K. Photodynamic therapy in patients with non-resectable hilar cholangiocarcinoma: 5-year follow-up of a prospective phase II study. *Gastrointest Endosc* 2004; **60**: 68-75 [PMID: 15229428 DOI: 10.1016/S0016-5107(04)01288-X]
47 **Shim CS**, Cheon YK, Cha SW, Bhandari S, Moon JH, Cho

YD, Kim YS, Lee LS, Lee MS, Kim BS. Prospective study of the effectiveness of percutaneous transhepatic photodynamic therapy for advanced bile duct cancer and the role of intraductal ultrasonography in response assessment. *Endoscopy* 2005; **37**: 425-433 [PMID: 15844020 DOI: 10.1055/s-2005-861294]

48 **Zoepf T**, Jakobs R, Arnold JC, Apel D, Riemann JF. Palliation of nonresectable bile duct cancer: improved survival after photodynamic therapy. *Am J Gastroenterol* 2005; **100**: 2426-2430 [PMID: 16279895 DOI: 10.1111/j.1572-0241.2005.00318.x]

49 **Witzigmann H**, Berr F, Ringel U, Caca K, Uhlmann D, Schoppmeyer K, Tannapfel A, Wittekind C, Mossner J, Hauss J, Wiedmann M. Surgical and palliative management and outcome in 184 patients with hilar cholangiocarcinoma: palliative photodynamic therapy plus stenting is comparable to r1/r2 resection. *Ann Surg* 2006; **244**: 230-239 [PMID: 16858185 DOI: 10.1097/01.sla.0000217639.10331.47]

50 **Prasad GA**, Wang KK, Baron TH, Buttar NS, Wongkeesong LM, Roberts LR, LeRoy AJ, Lutzke LS, Borkenhagen LS. Factors associated with increased survival after photodynamic therapy for cholangiocarcinoma. *Clin Gastroenterol Hepatol* 2007; **5**: 743-748 [PMID: 17545000 DOI: 10.1016/j.cgh.2007.02.021]

51 **Lee TY**, Cheon YK, Shim CS, Cho YD. Photodynamic therapy prolongs metal stent patency in patients with unresectable hilar cholangiocarcinoma. *World J Gastroenterol* 2012; **18**: 5589-5594 [PMID: 23112552 DOI: 10.3748/wjg.v18.i39.5589]

52 **Wagner A**, Kiesslich T, Neureiter D, Friesenbichler P, Puespoek A, Denzer UW, Wolkersdörfer GW, Emmanuel K, Lohse AW, Berr F. Photodynamic therapy for hilar bile duct cancer: clinical evidence for improved tumoricidal tissue penetration by temoporfin. *Photochem Photobiol Sci* 2013; **12**: 1065-1073 [PMID: 23558738 DOI: 10.1039/c3pp25425a]

53 **Farley DR**, Weaver AL, Nagorney DM. "Natural history" of unresected cholangiocarcinoma: patient outcome after noncurative intervention. *Mayo Clin Proc* 1995; **70**: 425-429 [PMID: 7537346 DOI: 10.1016/S0025-6196(11)63877-9]

54 **Deviere J**, Baize M, de Toeuf J, Cremer M. Long-term follow-up of patients with hilar malignant stricture treated by endoscopic internal biliary drainage. *Gastrointest Endosc* 1988; **34**: 95-101 [PMID: 2835282]

55 **Lu Y**, Liu L, Wu JC, Bie LK, Gong B. Efficacy and safety of photodynamic therapy for unresectable cholangiocarcinoma: A meta-analysis. *Clin Res Hepatol Gastroenterol* 2015; **39**: 718-724 [PMID: 26070572 DOI: 10.1016/j.clinre.2014.10.015]

56 **Wentrup R**, Winkelmann N, Mitroshkin A, Prager M, Voderholzer W, Schachschal G, Jürgensen C, Büning C. Photodynamic Therapy Plus Chemotherapy Compared with Photodynamic Therapy Alone in Hilar Nonresectable Cholangiocarcinoma. *Gut Liver* 2016; **10**: 470-475 [PMID: 26814610 DOI: 10.5009/gnl15175]

**P- Reviewer**: Garancini M, Leardkamolkarn V, Onori P, Zhang KQ
**S- Editor**: Yu J **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/ *World J Gastroenterol* 2017 February 21; 23(7): 1289-1297

DOI: 10.3748/wjg.v23.i7.1289 ISSN 1007-9327 (print) ISSN 2219-2840 (online)

SYSTEMATIC REVIEWS

# Esophagogastric junction distensibility assessed using the functional lumen imaging probe

Joan W Chen, Joel H Rubenstein

Joan W Chen, Joel H Rubenstein, Department of Gastroenterology and Hepatology, University of Michigan Health Systems, Ann Arbor, MI 48109, United States

Author contributions: Chen JW and Rubenstein JH contributed to the study concept and design, acquisition of data, analysis and interpretation, critical revision, and final approval of the manuscript; Chen JW drafted of the manuscript.

Conflict-of-interest statement: Chen JW and Rubenstein JH have no commercial, personal, political, intellectual, or religious conflict of interest.

Data sharing statement: Technical appendix and dataset available from the corresponding author at chenjoan@med.umich.edu.



Manuscript source: Unsolicited manuscript

Correspondence to: Joan W Chen, MD, MS, Department of Gastroenterology and Hepatology, University of Michigan Health Systems, 3912 Taubman Center, 1500 E. Medical Center Dr., SPC 5362, Ann Arbor, MI 48109, United States. chenjoan@med.umich.edu
Telephone: +1-734-9366400

Received: October 8, 2016
Peer-review started: October 9, 2016
First decision: November 9, 2016
Revised: December 8, 2016
Accepted: December 21, 2016
Article in press: December 21, 2016
Published online: February 21, 2017

## Abstract

***AIM***

To assess reference values in the literature for esophageal distensibility and cross-sectional area in healthy and diseased subjects measured by the functional lumen imaging probe (FLIP).

***METHODS***

Systematic search and review of articles in Medline and Embase pertaining to the use of FLIP in the esophagus was conducted in accordance with the PRISMA guidelines. Cross-sectional area and distensibility at the esophagogastric junction (EGJ) were abstracted for normal subjects, achalasia, and gastroesophageal reflux disease (GERD) patients, stratified by balloon length and volume of inflation.

***RESULTS***

Six achalasia studies ($n$ = 154), 3 GERD ($n$ = 52), and 5 studies including healthy controls ($n$ = 98) were included in the systematic review. Normative data varied widely amongst studies of healthy volunteers. In contrast, studies in achalasia patients uniformly demonstrated low point estimates in distensibility $\leqslant$ 1.6 $mm^2$/mmHg prior to treatment that increased to $\geqslant$ 3.4 $mm^2$/mmHg following treatment at 40mL bag volume. In GERD patients, distensibility fell to the range of untreated achalasia ($\leqslant$ 2.85 $mm^2$/mmHg) following fundoplication.

***CONCLUSION***

FLIP may be a useful tool in assessment of treatment efficacy in achalasia. The drastic drop in EGJ distensibility after fundoplication suggests that FLIP measurements need to be interpreted in the context of esophageal body motility and highlights the importance of pre-operative screening for dysmotility. Future studies using standardized FLIP protocol and balloon

size are needed.

**Key words:** Impedance planimetry; Gastroesophageal reflux disease; Esophageal distensibility; Achalasia



**Core tip:** Functional lumen imaging probe (FLIP) uses impedance planimetry to calculate the distensibility of a hollow organ. In this systematic review, we aimed to assess FLIP reference values for gastroesophageal junction distensibility in healthy and diseased states. We found available normative data to vary widely. In achalasia, patients uniformly demonstrated low distensibility that improved after treatment, highlighting the role of FLIP in assessment of achalasia treatment efficacy. In gastroesophageal reflux disease, distensibility fell to the range of untreated achalasia following fundoplication, emphasizing the importance of pre-operative screening for esophageal body dysmotililty. Future studies using a standardized FLIP protocol and balloon size are needed.

Chen JW, Rubenstein JH. Esophagogastric junction distensibility assessed using the functional lumen imaging probe. *World J Gastroenterol* 2017; 23(7): 1289-1297 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1289.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1289

## INTRODUCTION

The role of the esophagus is to transport ingested material from the mouth into the stomach via complex neuromuscular activities during peristalsis. Esophageal manometry, considered the gold standard tool used to characterize esophageal motor activity, measures pressures in the form of radial squeeze amplitude at defined intervals within the esophageal body and at the lower esophageal sphincter. The technique lacks the ability to measure esophageal wall stiffness and luminal narrowing, which are important properties affecting bolus transit. At the level of the esophagogastric junction (EGJ), where flow is directly related to its ability to open in response to pressure, the biomechanical properties of the esophagus in terms of pressure-geometric data cannot be fully assessed using esophageal manometry.

Over 40 years ago, Harris *et al*[1] introduced the concept of measuring sphincter competence by evaluating resistance to distention rather than focusing on squeeze or tonic contraction. In 2002, Pandolfino *et al*[2] measured EGJ distensibility in GERD patients with hiatal hernia using a combined barostatic/fluoroscopy technique that allowed for measurement of intraluminal diameter at predetermined intraluminal distension pressures. They found that distensibility of the EGJ was significantly increased in GERD patients with hiatal hernia, likely contributing to GERD pathophysiology. In 2004, McMahon *et al*[3] were among the first to use impedance planimetry for measurement of EGJ competence. The investigators used a catheter with multiple electrodes and a bag mounted at the distal end that was placed at the level of the EGJ to generate cross-sectional area (CSA) and pressure data. The data was used to calculate wall tension and EGJ distensibility. Their work demonstrated the feasibility of impedance planimetry in assessing EGJ distensibility. The concept of functional luminal imaging probe (FLIP), involving identifying the narrowest region during distension plotted against bag pressure to provide a graph of compliance, was created.

In the recent years, a new imaging device, EndoFLIP (endolumenal functional lumen imaging probe, Crospon Ltd, Galway, Ireland), has become commercially available. The device consists of a long catheter with several electrodes and a bag mounted distally. Excitation current is generated between adjacent electrodes through a standardized concentration of saline injected into the bag; using impedance planimetry, CSA data are determined at the level of each pair of electrodes along the catheter. In addition, two sensors in the bag measure the intrabag pressure. The CSA-pressure data allows for calculation of distensibility ($mm^2$/mmHg). This device has facilitated assessment of esophageal wall and EGJ distensibility, including studies in healthy volunteers and in patients with GERD, eosinophilic esophagitis, and achalasia. FLIP has also been used intraoperatively for measurements of EGJ distensibility before and after fundoplication for GERD or myotomy for achalasia. However, a major limiting factor for widespread clinical use of FLIP is the lack of a standardized protocol and reference values. A systematic review on this topic has never been performed previously. The aim of our study was to systematically assess reference values in the literature for esophageal distensibility and CSA in healthy and diseased subjects as measured by FLIP.

## MATERIALS AND METHODS

We performed a systematic literature search in PubMed (National Library of Medicine) and EMBASE, including all studies published through December of 2014 that used EndoFLIP impedance planimetry to measure esophageal body or EGJ distensibility or compliance. The search terms used were (Esophagus or GERD or gastroesophageal reflux or achalasia or eosinophilic esophagitis or myotomy or dilation or hiatal hernia or fundoplication) and (FLIP or endoflip or impedance planimetry), and also using the alternative spelling "oesophagus".

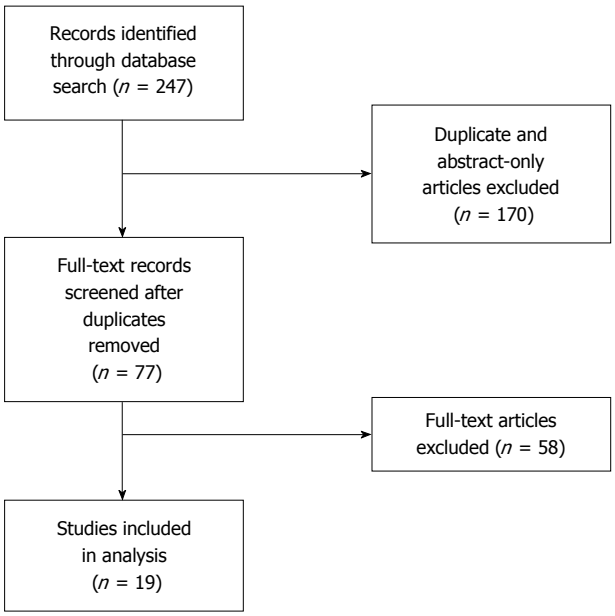



**Figure 1 Flow chart of records identified through literature search and those excluded and included for analysis. Initial literature search identified 247 citations.** After elimination of duplicate citations and abstract-only articles, 77 articles remained. The full-text articles of these 77 citations were reviewed and 19 studies met eligibility requirements for inclusion.

### *Study selection and data extraction*

The studies met inclusion criteria if the following were satisfied: (1) EndoFLIP device was used; (2) EGJ or esophageal body was included in the measurement; and (3) values for distensibility/compliance, diameter, or cross-sectional area were provided. Studies involving healthy controls, GERD (pre- and post-antireflux procedures), hiatal hernia, eosinophilic esophagitis, and achalasia (pre-and post-myotomy, pneumatic dilation, or Botox) patients were included in our review. Excluded studies were those written in a language other than English, FLIP studies performed in the pediatric population or animal studies, or those done on the anorectum, small intestine, or upper esophageal sphincter. Scientific abstracts were excluded due to the lack of detailed EndoFLIP equipment and protocol information.

Study references and citations were collected in EndNote software application (Thomson Reuters, NY, United States) with duplicate publications deleted. Two investigators (Chen JW and Rubenstein JH) reviewed all titles and abstracts independently to assess eligibility. A data collection form was generated in Microsoft Excel (Microsoft, Redmond, WA, United States). For abstracts that appeared eligible on first review, both investigators independently abstracted data from the full articles. After all the data were abstracted, both investigators then compared and confirmed by consensus to account for entry error. Any discrepancies between the reviewers were resolved by joint re-review of the studies.

The database included the primary and secondary aims of the studies, study design, subject types and count, subject age/sex, parameters used to describe distension and diameter, the bag volume at which measurements were taken, location where measurements were taken, length of the FLIP balloon, and any interventions done between measurements taken. Quantitative FLIP data in terms of distension and luminal area/diameter data were recorded in the database.

### *Study quality criteria*

The quality of each included study was assessed based on the Newcastle-Ottawa Scale. This scale measures the quality of studies on a scale of 0-9 *via* the assessment of three main domains: selection of study groups, comparability of groups, and comparability of groups and ascertainment of exposure. Study quality was assessed independently by both investigators and discrepancies were resolved by consensus. Studies with NOS score of 6 or above indicate good quality studies.

### *Statistical analysis*

Included studies were separated into subject type - healthy, GERD, achalasia, and eosinophilic esophagitis (EoE) subjects. Due to the variability of study protocol in terms of balloon length and volume distension at the time of distensibility measurement, which can affect measurement values, we focused our report on the most commonly used balloon sizes and distension volumes in our analysis: EndoFLIP balloon sizes between 7 cm and 10 cm, and volume distension of 30 mL and 40 mL. Given the insufficient number of studies reporting data as mean ± SD, meta-analysis was not possible.

## RESULTS

Our initial literature search identified a combined 247 citations in PubMed and Embase (Figure 1). After elimination of duplicate citations and abstract-only articles, 77 articles remained. The full-text articles of these 77 citations were reviewed and 19 studies met eligibility requirements for inclusion (Table 1). Of the 19 included studies, 8 studies were in patients with achalasia, 5 in GERD patients, 3 in eosinophilic esophagitis patients, and 11 included FLIP measurements in healthy controls. After restricting the data to balloon length (7-10 cm) and 30-40 mL volume distension, remaining were 5 studies including healthy volunteers ($n$ = 98)[4-8], 6 including achalasia patients ($n$ = 154)[8-13], and 3 included GERD patients ($n$ = 52)[6,14,15]. Due to the nature of EndoFLIP as a diagnostic tool currently, studies included were non-randomized observational/cross-sectional studies. The Newcastle-Ottawa Scale (NOS) score for each study is included in Table 1. All included studies had NOS sore between 6 and 8. Four studies including healthy volunteers, two including achalasia patients, and two

**Table 1 Summary of all eligible studies**

| Ref. | Quality Score | *n* (pre-intervention) | *n* (post-intervention) | Bag length (cm) | Bag volume(s) (mL) |
|---|---|---|---|---|---|
| Healthy | | | | | |
| Fukazawa *et al*[4], 2014 | 7 | 9 | N/A | 8 | 20, 40, 50 |
| Lin *et al*[18], 2013 | 6 | 10 | N/A | 16 | 30, 40, 50, 60 |
| Rieder *et al*[8], 2013 | 7 | 4 | N/A | 8 | 30, 40 |
| Rohof *et al*[19], 2012 | 7 | 15 | N/A | 14 | 50 |
| Nathanson *et al*[7], 2012 | 7 | 50 | N/A | 8 | 30 |
| Kwiatek *et al*[5], 2011 | 8 | 15 | N/A | 10 | 20, 30, 40 |
| Kwiatek *et al*[6], 2010 | 6 | 20 | N/A | 7 | 10, 20, 30, 40 |
| Kwiatek *et al*[17], 2010 | 6 | 10 | N/A | 12 | 30, 40, 50, 60 |
| Beaumont *et al*[16], 2009 | 5 | 8 | N/A | 10 | 60 |
| Achalasia | | | | | |
| Teitelbaum *et al*[13], 2014 | 8 | 56 | LHM (20), POEM (36) | 8 | 40 |
| Familiari *et al*[9], 2014 | 7 | 23 | POEM (21) | 8 | 30 |
| Teitelbaum *et al*[12], 2014 | 8 | 31 | LHM (12), POEM (19) | 8 | 30, 40 |
| Teitelbaum *et al*[11], 2013 | 8 | 25 | LHM (11), POEM (14) | 8 | 30, 40, 50 |
| Verlaan *et al*[20], 2013 | 8 | 10 | POEM (8) | 14 | 20, 30, 40, 50 |
| Pandolfino *et al*[10], 2013 | 8 | 23 | treated (31) | 10 | 20, 30, 40 |
| Rieder *et al*[8], 2013 | 7 | 4 | POEM (4) | 8 | 30, 40 |
| Rohof *et al*[19], 2012 | 7 | 30 | Treated (30) | 14 | 50 |
| GERD | | | | | |
| Rinsma *et al*[15], 2014 | 7 | 15 | TIF (15) | 8 | 30 |
| Ilczyszyn *et al*[14], 2014 | 8 | 17 | Nissen (17) | 8 | 30, 40 |
| Kwiatek *et al*[6], 2010 | 6 | 20 | N/A | 7 | 10, 20, 30, 40 |
| Kwiatek *et al*[17], 2010 | 6 | 10 | Fundoplicstoin (10) | 12 | 30, 40, 50, 60 |
| Beaumont *et al*[16], 2009 | 5 | 7 | RFA (7) | 10 | 60 |

including GERD patients were excluded due to use of uncommon balloon length and distension volume[16-20]. FLIP studies including EoE subjects were also excluded as these 3 studies measured FLIP parameters in the esophageal body; in addition, it was unclear if FLIP measurements were taken in the distal, proximal esophagus, or both.

### *Healthy subjects*

Table 2 summarizes results from studies that reported FLIP values in healthy subjects. A total of 98 healthy subjects were included in 5 studies originating from Japan, Australia, and the United States. Healthy volunteers were recruited in each individual study for the purpose of (1) assessing the effects of mosapride on EGJ compliance[4]; (2) comparing EGJ distensibility with achalasia patients undergoing Per-oral Endoscopic Myotomy (POEM)[8]; (3) comparing EGJ distensibility with EoE patients[5]; (4) exploring EGJ distensibility during general anesthesia[7]; and (5) comparing EGJ distensibility with GERD patients[6]. Mean and median values for distensibility and CSA according to endoFLIP bag volume varied amongst studies, and these were included in Table 2. Median distensibility ranged from 0.8 $mm^2$/mmHg to 5.7 $mm^2$/mmHg at 30 and 40 mL balloon volumes. The wide variability in distensibility in healthy subjects is demonstrated in Figure 2A. At 40 mL volume using a 7-10 cm length balloon, the lower limit of distensibility in healthy subjects was 2.4 $mm^2$/mmHg.

### *GERD Subjects*

Five studies included FLIP measurements in GERD patients; three of these included FLIP values measured at 30 mL volume using balloon size 7-10 cm ($n$ = 52). Studies originated from the Netherlands, the United Kingdom, and the United States. Two of the three studies defined GERD patients using clinical symptoms as well as objective studies (endoscopy and/or pH studies)[14,15] and one study defined GERD based on clinical symptoms alone[6]. One study assessed EGJ distensibility before and after Transoral Incisionless Fundoplication (TIF) and one study assessed EGJ properties during Nissen fundoplication. Mean and Median values, standard deviation, and ranges of distensibility and CSA in GERD subjects are summarized in Table 3. Baseline/pretreatment distensibility and CSA did not appear to vary greatly compared to healthy volunteers; however, following either TIF or Nissen fundoplication, there is a reduction in distensibility. Prior to treatment, the point estimate for distensibility ranged from 2.4 to 8 $mm^2$/mmHg at 30-40 mL bag volume. This dropped to 0.97-1.6 $mm^2$/mmHg after fundoplication (Figure 2B).

### *Achalasia subjects*

EndoFLIP balloon size 7-10 cm at 30-40 mL was used in 6 of 8 studies ($n$ = 154) to assess treatment response in achalasia patients[8-13,19,20]. Treatment for achalasia included laparoscopic Heller myotomy (LHM), POEM, and unspecified (including LHM, POEM, and pneumatic dilation). Distensibility and CSA before and after treatment are listed in Table 4. At 30 mL volume distension, the point estimates for distensibility in achalasia pre-treatment (0.8-2.2 $mm^2$/mmHg)

**Table 2 Healthy subjects**

| Healthy | Distensibility[1] (mm²/mmHg) | CSA[1] (mm²) |
|---|---|---|
| 20 mL bag volume | | |
| Kwiatek *et al*[17], 2010 | Median 2 (5%-95% 1-9) | Median 38 (5%-95% 13-94) |
| Fukazawa *et al*[4], 2014 | 2.9 ± 0.6 | 25.2 ± 2.5 |
| Kwiatek *et al*[5], 2011 | Median 0.9 (5%-95% 0.3-1.4) | Median 15 (5%-95% 9-23) |
| 30 mL bag volume | | |
| Kwiatek *et al*[17], 2010 | Median 4 (5%-95% 1-14) | Median 94 (5%-95% 27-225) |
| Reider *et al*[8], 2013 | Median 2.5 (range 2.0-6.3) | Median 64 (range 44-91) |
| Nathanson *et al*[7], 2012 | Median 1.4 | Median 31 |
| Kwiatek *et al*[5], 2011 | Median 0.8 (5%-95% 0.4-2.8) | Median 22 (5%-95% 9-62) |
| Kwiatek *et al*[17], 2010 | N/A | Median 50[2] (5%-95% 50-68) |
| Lin *et al*[18], 2013 | Median 3.2 (5%-95% 1-11.6) | N/A |
| 40 mL bag volume | | |
| Kwiatek *et al*[17], 2010 | N/A | Median 264 (5-95% 99-496) |
| Fukazawa *et al*[4], 2014 | 7.1 ± 0.9 | 163 ± 5.9 |
| Rieder *et al*[8], 2013 | Median 2.7 (range 2.4-8.3) | Median 122 (range 73-171) |
| Kwiatek *et al*[6], 2010 | N/A | Median 50[2] (5%-95% 50-50) |
| Lin *et al*[18], 2013 | Median 5.7 (5%-95% 1.4-15.8) | N/A |
| 50 mL bag volume | | |
| Fukazawa *et al*[4], 2014 | 8.2 ± 0.8 | 259.6 ± 12 |
| Kwiatek *et al*[17], 2010 | N/A | Median 50[2] (5%-95% 50-52) |
| Rohof *et al*[19], 2012 | 6.3 ± 0.7 | N/A |
| Lin *et al*[18], 2013 | Median 5.9 (5-95% 1.6-9.3) | N/A |
| 60 mL bag volume | | |
| Beaumont *et al*[16], 2009 | 3.7 ± 0.9[3] | N/A |
| Kwiatek *et al*[17], 2010 | N/A | Median 93 (5%-95% 50-182) |
| Lin *et al*[18], 2013 | Median 6.3 (5%-95% 2.1-9.5) | N/A |

[1]Values given in mean unless specified otherwise; [2]The minimal detectable CSA was 50 mm²; [3]Values indicate compliance (cm²/cmH₂O).

**Table 3 Gastroesophageal reflux disease subjects**

| GERD | Intervention (*n*) | Intra-Op (Y/N) | Pre-treatment Distensibility[1] (mm²/mmHg) | Post-treatment Distensibility[1] (mm²/mmHg) | Pre-Treatment CSA[1] (mm²) | Post-treatment CSA[1] (mm²) |
|---|---|---|---|---|---|---|
| 20 mL bag volume | | | | | | |
| Kwiatek *et al*[5], 2010 | N/A | N | Median 7 (5%-95% 1-32) | N/A | Median 36 (5%-95% 13-201) | N/A |
| 30 mL bag volume | | | | | | |
| Kwiatek *et al*[6], 2010 | N/A | N | Median 8 (5%-95% 1-46) | N/A | Median 116 (5%-95% 33-344) | N/A |
| Rinsma *et al*[15], 2014 | TIF (15) | Y | 2.4 ± 0.3 | 1.6 ± 0.2 | 40.6 ± 5.4 | 33.6 ± 4.1 |
| Ilczyszyn *et al*[14], 2014 | Nissen (17) | Y | Median 4.23 (IQR 1.60-6.58) | Median 0.972 (IQR 0.715-1.72) | Median 75.1 | |
| Kwiatek *et al*[17], 2010 | Fundoplication (10) | N | N/A | N/A | N/A | Median 51 (5%-95% 50-60) |
| 40 mL bag volume | | | | | | |
| Kwiatek *et al*[17], 2010 | N/A | N | N/A | N/A | Median 180 (5%-95% 86-409) | N/A |
| Ilczyszyn *et al*[14], 2014 | Nissen (17) | Y | Median 3.75 (IQR 2.43-7.21) | Median 1.36 (IQR 0.537-2.85) | Median 124.2 | N/A |
| Kwiatek *et al*[17], 2010 | Fundoplication (10) | N | N/A | N/A | N/A | Median 51 (5%-95% 50-56) |
| 50 mL bag volume | | | | | | |
| Kwiatek *et al*[17], 2010 | Fundoplication (10) | N | N/A | N/A | N/A | Median 61 (5%-95% 52-88) |
| 60 mL bag volume | | | | | | |
| Beaumont *et al*[16], 2009 | Radiofrequency ablation | N | 6.5 ± 0.9[2] | 7.3 ± 1.3[2] | N/A | N/A |
| Kwiatek *et al*[17], 2010 | Fundoplication (10) | N | N/A | N/A | N/A | Median 159 (5%-95% 68-245) |

[1]Values given in mean unless specified otherwise; [2]Values indicate compliance (cm²/cmH₂O). GERD: Gastroesophageal reflux disease.

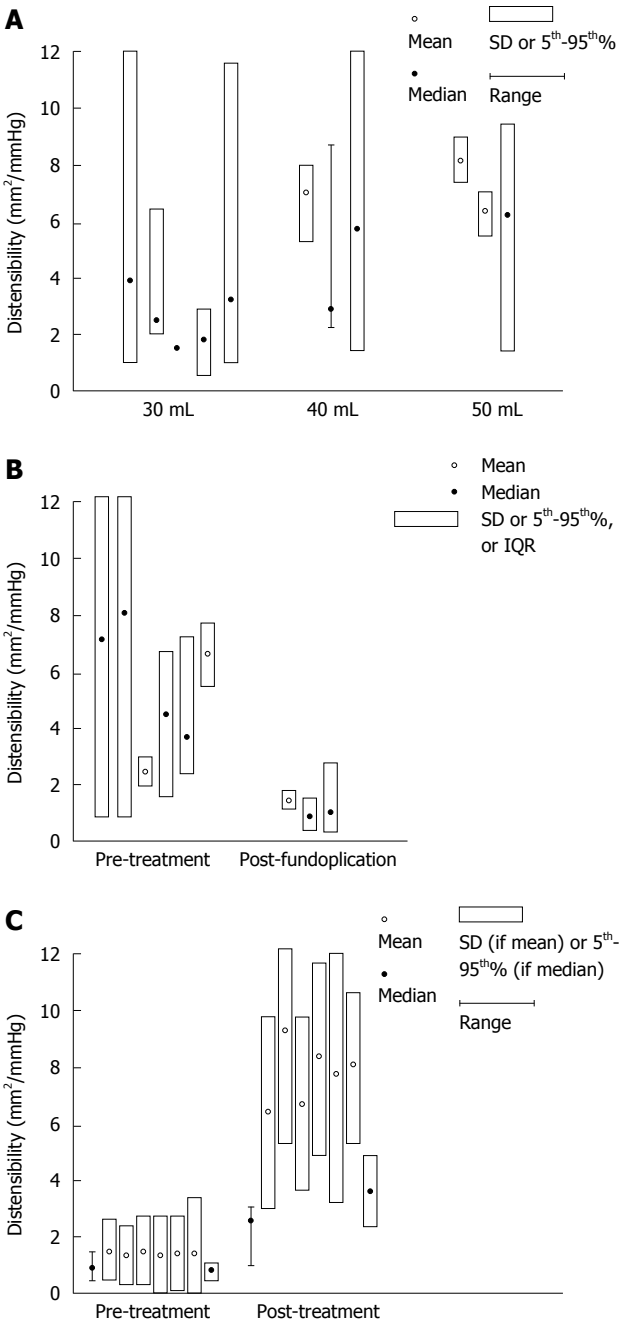



**Figure 2 Healthy subjects.** A: Mean values (open circles) with standard deviations (vertical boxes around means) and median values (closed circles) with 5th to 95th percentile (vertical boxes around medians), and ranges (vertical lines) for distensibility ($mm^2$/mmHg) at FLIP bag volume of 30, 40, and 50 mL in healthy volunteers are shown in this plot; B: Mean values (open circles) with standard deviations (vertical boxes around means) and median values (closed circles) with 5th to 95th percentile (vertical boxes around medians), and ranges (vertical lines) for distensibility ($mm^2$/mmHg) in GERD subjects before and after fundoplication are shown in this plot; C: Mean values (open circles) with standard deviations (vertical boxes around means) and median values (closed circles) with 5th to 95th percentile (vertical boxes around medians), and ranges (vertical lines) for distensibility ($mm^2$/mmHg) in achalasia patients pre- and post-treatment using balloon length 7-10 cm at 40 mL bag volume are shown in this plot. The increase in EGJ distensibility post-achalasia treatment is illustrated here.

overlapped with that in healthy volunteers (0.8-4.0 $mm^2$/mmHg), as did the point estimates for CSA (22 and 32.9 $mm^2$ for achalasia *vs* 22-94 for healthy volunteers). However, at 40 mL volume, there was a clear difference in distensibility between achalasia (point estimates $\leqslant$ 1.6 $mm^2$/mmHg) and healthy volunteers (point estimates 2.7 to 7.1 in 3 studies) and for CSA ($\leqslant$ 41.5 $mm^2$ for achalasia *vs* $\geqslant$ 122.3 $mm^2$ for healthy volunteers). Comparing to pre-treatment, all but one small study ($n$ = 4) demonstrated post-treatment distensibility of 2.2 $mm^2$/mmHg or higher (point estimate of $\geqslant$ 3.4 $mm^2$/mmHg), and CSA of 86 $mm^2$ or higher regardless of the treatment modality. Figure 2C illustrates the rise in EGJ distensibility post-achalasia treatment at 40mL bag volume using balloon length 7-10 cm.

## DISCUSSION

It has become increasing clear that a clinical tool measuring distensibility (essentially resistance to distension), rather than pressure, is useful in assessing the function of EGJ[21]. FLIP offers information on distensibility in the esophagus and can potentially provide information on sphincter dynamics during distension to help distinguish between normal sphincters, patients with reflux disease, and patients with achalasia[22]. This tool has recently become commercially available and has been used to study the biomechanics of esophageal body and sphincter in healthy subjects and in patients with GERD, achalasia, eosinophilic esophagitis, and others. One major factor limiting its widespread use is the lack of reference values for differentiation of normal and diseased conditions. To date, there has not been a systematic review on FLIP measurements in healthy and diseased states. Our study aimed to summarize the currently available FLIP data on healthy subjects and the patient population.

We found that normative data varied widely amongst studies of healthy volunteers. This is potentially due to the variability in balloon sizes and FLIP protocols used, as well as the variable definition of normal subjects. Additionally, Tucker *et al*[23] have demonstrated an inverse association between FLIP measurements (CSA and distensibility) and Body Mass Index (BMI). BMI information was not available and therefore was not controlled in the studies included in this review and may have contributed to the variability of the normative data.

The available data suggests that use of an 8cm balloon (marketed as the 325N catheter) at 40 mL volume is the ideal protocol for assessing EGJ distensibility. In that protocol, healthy patients had distensibility greater than 2.4 $mm^2$/mmHg, and patients with untreated achalasia typically had distensibility less than 3.0 $mm^2$/mmHg with a median of 1.3 $mm^2$/mmHg. Following treatment for achalasia, those patients typically had distensibility greater than 3.0 $mm^2$/mmHg with a median of 7.6 $mm^2$/mmHg. This suggests that FLIP may be a useful tool in

**Table 4 Achalasia subjects**

| Achalasia | Intervention (*n*) | Pre-treatment Distensibility[1] ($mm^2$/mmHg) | Post-treatment Distensibility[1] ($mm^2$/mmHg) | Pre-treatment CSA[1] ($mm^2$) | Post-treatment CSA[1] ($mm^2$) |
|---|---|---|---|---|---|
| 20 mL bag volume | | | | | |
| Pandolfino *et al*[10], 2013 | Untreated (23)<br>Treated[2] (17) | Median 1.1<br>(5%-95% 0.9-1.6) | Median 1.8<br>(5%-95% 1.2-2.2) | N/A | N/A |
| Verlaan *et al*[20], 2013 | 10 | Median 1.4<br>(IQR 1.1-2.4) | POEM: Median 3.0<br>(IQR 1.4-9.4) | N/A | N/A |
| 30 mL bag volume | | | | | |
| Familiari *et al*[9], 2014 | POEM (21) | N/A | N/A | 32.9 ± 23.1 | 102.38 ± 28.2 |
| Teitelbaum *et al*[11], 2014 | LHM (12)<br>POEM (19) | LHM: 2.2 ± 1.7<br>POEM: 1.8 ± 1.4 | LHM: 6.9 ± 3.3<br>POEM: 9.3 ± 4.1 | N/A | N/A |
| Teitelbaum *et al*[12], 2013[3] | LHM (11)<br>POEM (14) | LHM: 1.7 ± 1.5<br>POEM: 1.8 ± 1.1 | LHM: 6.7 ± 4.4<br>POEM: 8.2 ± 3.0 | N/A | N/A |
| Rieder *et al*[8], 2013 | POEM (4) | Median 0.8<br>(range 0.7-1.0) | Median 3.1<br>(range 1.7-3.4) | Median 22<br>(range 20-32) | Median 71.5<br>(range 30-106) |
| Pandolfino *et al*[10], 2013 | Untreated (23)<br>Treated[2] (17) | Median 1.0<br>(5%-95% 0.8-1.2) | Median 2.5<br>(5%-95% 1.3-3.4) | N/A | N/A |
| Verlaan *et al*[20], 2013 | 10 | Median 1.0<br>(IQR 0.8-1.5) | POEM: Median 2.9<br>(IQR 1.3-19.6) | N/A | N/A |
| 40 mL bag volume | | | | | |
| Rieder *et al*[8], 2013 | POEM (4) | Median 1.0<br>(range 0.5-1.4) | Median 2.4<br>(range 1.1-3.0) | Median 41.5<br>(range 20-49) | Median 86<br>(range 41-137) |
| Teitelbaum *et al*[11], 2014 | LHM (12)<br>POEM (19) | LHM: 1.6 ± 1.0<br>POEM: 1.3 ± 1.0 | LHM: 6.3 ± 3.4<br>POEM: 9.2 ± 3.9 | N/A | N/A |
| Teitelbaum *et al*[12], 2014 | LHM (20)<br>POEM (36) | LHM: 1.5 ± 1.2<br>POEM: 1.3 ± 1.4 | LHM 6.6 ± 3.1<br>POEM 8.3 ± 3.4 | N/A | N/A |
| Teitelbaum *et al*[13], 2013[3] | LHM (11)<br>POEM (14) | LHM: 1.4 ± 1.3<br>POEM: 1.4 ± 1.9 | LHM: 7.6 ± 4.4<br>POEM: 7.9 ± 2.7 | LHM 33.5<br>POEM 38.8 | LHM 163.6<br>POEM 163.3 |
| Pandolfino *et al*[10], 2013 | Untreated (23)<br>Treated (17) | Median 0.7<br>(5%-95% 0.5-1.1) | Median 3.4<br>(5%-95% 2.2-4.9) | N/A | N/A |
| Verlaan *et al*[20], 2013 | 10 | Median 1.1<br>(IQR 0.55-2.0) | POEM: Median 4.0<br>(IQR 2.9-12.7) | N/A | N/A |
| 50 mL bag volume | | | | | |
| Teitelbaum *et al*[11], 2013[3] | LHM (11)<br>POEM (14) | LHM: 1.1 ± 0.9<br>POEM: 1.4 ± 2.1 | LHM: 5.7 ± 3.1<br>POEM: 6 ± 1.5 | N/A | N/A |
| Rohof *et al*[19], 2012 | 7 Treated (6 PD, 1 LHM) | 0.7 ± 0.9 | 4.4 ± 0.5 | N/A | N/A |
| Verlaan *et al*[20], 2013 | 10 | Median 1.0<br>(IQR 0.4-2.3) | POEM: Median 6.7<br>(IQR 3.8-16.6) | N/A | N/A |

[1]Values given in mean unless specified otherwise; [2]Treated included patients that underwent pneumatic dilation, Heller myotomy with partial fundoplication, and POEM with good response; [3]Subjects were included in the more recent Teitelbaum study (2014). CSA: Cross-sectional area; LHM: Laparoscopic Heller Myotomy; POEM: Peroral endoscopic myotomy; PD: Pneumatic dilation.

assessment of treatment adequacy intra-operatively or on follow-up of achalasia patients. The role of intra-operative FLIP measurement was highlighted in a study that showed that an extended proximal myotomy was required to normalize distensibility during LHM[12].

CSA and distensibility in GERD patients prior to treatment did not appear to differ from healthy volunteers; however, following anti-reflux procedures, distensibility fell to the range of untreated achalasia ($\leqslant$ 1.6 $mm^2$/mmHg). The drastic drop in EGJ distensibility in GERD patients after antireflux procedures suggests that FLIP EGJ measures should not be interpreted in isolation from data on esophageal body motility, as most of these post-antireflux surgery patients do not exhibit impaired esophageal emptying to the degree of achalasia. This finding also highlights the importance of screening for esophageal motility disorders prior to fundoplication to avoid the heightened risk of pseudoachalasia.

Our systematic review was limited by the overall small number of studies using FLIP to assess distensibility of the esophagus in a heterogeneous population of subjects. There is also a wide variability in the size/length of balloon used and FLIP protocol from study to study. Data comparison was also made difficult due to the variable FLIP parameters reported (*e.g.*, CSA, distensibility, distensibility index, distensibility plateau, *etc.*). Interpretation of distensibility was also made difficult due to the likelihood of non-normally distributed data, as evident by standard deviation larger than mean in several of the studies. Since some studies reporting data as mean ± SD and others reporting median (range), meta-analysis could not be performed.

FLIP provides valuable information regarding esophageal wall compliance and lower esophageal sphincter competency that complement other

diagnostic tools such as esophageal manometry and barium esophagram. From our systematic review of the literature, FLIP may especially have a role in assessment of treatment response in patients with achalasia or GERD patients undergoing intervention. However, future studies in larger number normal subjects and patients using standardized FLIP protocol and balloon size are needed for reliable interpretation of FLIP data. In the meantime, use of an 8 cm balloon at 40 mL volume is the most likely clinically relevant protocol for distinguishing achalasia from normal esophagus, and assessing response to therapy.

## COMMENTS

### Background

The functional lumen imaging probe (FLIP) has been used to assess esophagogastric junction (EGJ) distensibility; however, its routine use in clinical practice is limited by the lack of established reference values.

### Research frontiers

FLIP has been used in research studies to assess the distensibility of esophageal wall and gastroesophageal junction in healthy volunteers and in patients with GERD, eosinophilic esophagitis, and achalasia. However, the clinical role of FLIP is currently still being investigated. Prospective outcome studies using FLIP technology are needed.

### Innovations and breakthroughs

A systematic review of the currently available FLIP data in the literature had not been done prior to this study.

### Applications

This study demonstrated the potential utility of FLIP in assessment of achalasia treatment efficacy. It also highlighted the importance of future studies using a standardized FLIP protocol and balloon size.

### Terminology

Impedance planimetry: an imaging technique involving a conductive fluid-filled bag on a catheter with multiple impedance and pressure sensors. When the catheter-bag device is placed in a hollow tube, impedance measurements between pairs of electrodes are used to estimate the cross-sectional area of the tube. With simultaneous measurement of intrabag pressure, distensibility (smallest cross sectional area/bag pressure) is calculated. EndoFLIP (endolumenal functional lumen imaging probe): A new technology that uses impedance planimetry to measure the cross sectional area and pressure in a hollow organ to determine its distensibility.

### Peer-review

Chen and Rubenstein wrote a systematic review of literature concerning the use of FLIP system to evaluate EGJ in achalasia, GERD patients undergoing surgical procedure and healthy controls. The review is well written and it addresses a novel field with possible evolution in the future. The research strategy is adequate and adherent to the standard of quality.

## REFERENCES


1 **Harris LD**, Pope CE. "Squeeze" vs. resistance: an evaluation of the mechanism of sphincter competence. *J Clin Invest* 1964; **43**: 2272-2278 [PMID: 14234823 DOI: 10.1172/JCI105101]

2 **Pandolfino JE**, Shi G, Curry J, Joehl RJ, Brasseur JG, Kahrilas PJ. Esophagogastric junction distensibility: a factor contributing to sphincter incompetence. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G1052-G1058 [PMID: 12016131 DOI: 10.1152/ajpgi.00279.2001]

3 **McMahon BP**, Frøkjaer JB, Drewes AM, Gregersen H. A new measurement of oesophago-gastric junction competence. *Neurogastroenterol Motil* 2004; **16**: 543-546 [PMID: 15500510 DOI: 10.1111/j.1365-2982.2004.00540.x]

4 **Fukazawa K**, Furuta K, Adachi K, Moritou Y, Saito T, Kusunoki R, Uno G, Shimura S, Aimi M, Ohara S, Ishihara S, Kinoshita Y. Effects of mosapride on esophageal motor activity and esophagogastric junction compliance in healthy volunteers. *J Gastroenterol* 2014; **49**: 1307-1313 [PMID: 24013654 DOI: 10.1007/s00535-013-0876-0]

5 **Kwiatek MA**, Hirano I, Kahrilas PJ, Rothe J, Luger D, Pandolfino JE. Mechanical properties of the esophagus in eosinophilic esophagitis. *Gastroenterology* 2011; **140**: 82-90 [PMID: 20858491 DOI: 10.1053/j.gastro.2010.09.037]

6 **Kwiatek MA**, Pandolfino JE, Hirano I, Kahrilas PJ. Esophagogastric junction distensibility assessed with an endoscopic functional luminal imaging probe (EndoFLIP). *Gastrointest Endosc* 2010; **72**: 272-278 [PMID: 20541755 DOI: 10.1016/j.gie.2010.01.069]

7 **Nathanson LK**, Brunott N, Cavallucci D. Adult esophagogastric junction distensibility during general anesthesia assessed with an endoscopic functional luminal imaging probe (EndoFLIP®). *Surg Endosc* 2012; **26**: 1051-1055 [PMID: 22038169 DOI: 10.1007/s00464-011-1996-3]

8 **Rieder E**, Swanström LL, Perretta S, Lenglinger J, Riegler M, Dunst CM. Intraoperative assessment of esophagogastric junction distensibility during per oral endoscopic myotomy (POEM) for esophageal motility disorders. *Surg Endosc* 2013; **27**: 400-405 [PMID: 22955896 DOI: 10.1007/s00464-012-2484-0]

9 **Familiari P**, Gigante G, Marchese M, Boskoski I, Bove V, Tringali A, Perri V, Onder G, Costamagna G. EndoFLIP system for the intraoperative evaluation of peroral endoscopic myotomy. *United European Gastroenterol J* 2014; **2**: 77-83 [PMID: 24918011 DOI: 10.1177/2050640614521193]

10 **Pandolfino JE**, de Ruigh A, Nicodème F, Xiao Y, Boris L, Kahrilas PJ. Distensibility of the esophagogastric junction assessed with the functional lumen imaging probe (FLIP™) in achalasia patients. *Neurogastroenterol Motil* 2013; **25**: 496-501 [PMID: 23413801 DOI: 10.1111/nmo.12097]

11 **Teitelbaum EN**, Boris L, Arafat FO, Nicodème F, Lin Z, Kahrilas PJ, Pandolfino JE, Soper NJ, Hungness ES. Comparison of esophagogastric junction distensibility changes during POEM and Heller myotomy using intraoperative FLIP. *Surg Endosc* 2013; **27**: 4547-4555 [PMID: 24043641 DOI: 10.1007/s00464-013-3121-2]

12 **Teitelbaum EN**, Soper NJ, Pandolfino JE, Kahrilas PJ, Boris L, Nicodème F, Lin Z, Hungness ES. An extended proximal esophageal myotomy is necessary to normalize EGJ distensibility during Heller myotomy for achalasia, but not POEM. *Surg Endosc* 2014; **28**: 2840-2847 [PMID: 24853854 DOI: 10.1007/s00464-014-3563-1]

13 **Teitelbaum EN**, Soper NJ, Pandolfino JE, Kahrilas PJ, Hirano I, Boris L, Nicodème F, Lin Z, Hungness ES. Esophagogastric junction distensibility measurements during Heller myotomy and POEM for achalasia predict postoperative symptomatic outcomes. *Surg Endosc* 2015; **29**: 522-528 [PMID: 25055891 DOI: 10.1007/s00464-014-3733-1]

14 **Ilczyszyn A**, Botha AJ. Feasibility of esophagogastric junction distensibility measurement during Nissen fundoplication. *Dis Esophagus* 2014; **27**: 637-644 [PMID: 24033477 DOI: 10.1111/dote.12130]

15 **Rinsma NF**, Smeets FG, Bruls DW, Kessing BF, Bouvy ND, Masclee AA, Conchillo JM. Effect of transoral incisionless fundoplication on reflux mechanisms. *Surg Endosc* 2014; **28**: 941-949 [PMID: 24149854 DOI: 10.1007/s00464-013-3250-7]

16 **Beaumont H**, Gondrie JJ, McMahon BP, Pouw RE, Gregersen H, Bergman JJ, Boeckxstaens GE. Stepwise radiofrequency ablation of Barrett's esophagus preserves esophageal inner diameter, compliance, and motility. *Endoscopy* 2009; **41**: 2-8 [PMID: 19160152 DOI: 10.1055/s-0028-1103451]

17 **Kwiatek MA**, Kahrilas K, Soper NJ, Bulsiewicz WJ, McMahon BP, Gregersen H, Pandolfino JE. Esophagogastric junction distensibility after fundoplication assessed with a novel functional

luminal imaging probe. *J Gastrointest Surg* 2010; **14**: 268-276 [PMID: 19911238 DOI: 10.1007/s11605-009-1086-1]
18 **Lin Z**, Nicodème F, Boris L, Lin CY, Kahrilas PJ, Pandolfino JE. Regional variation in distal esophagus distensibility assessed using the functional luminal imaging probe (FLIP). *Neurogastroenterol Motil* 2013; **25**: e765-e771 [PMID: 23965159 DOI: 10.1111/nmo.12205]
19 **Rohof WO**, Hirsch DP, Kessing BF, Boeckxstaens GE. Efficacy of treatment for patients with achalasia depends on the distensibility of the esophagogastric junction. *Gastroenterology* 2012; **143**: 328-335 [PMID: 22562023 DOI: 10.1053/j.gastro.2012.04.048]
20 **Verlaan T**, Rohof WO, Bredenoord AJ, Eberl S, Rösch T, Fockens P. Effect of peroral endoscopic myotomy on esophagogastric junction physiology in patients with achalasia. *Gastrointest Endosc* 2013; **78**: 39-44 [PMID: 23453184 DOI: 10.1016/j.gie.2013.01.006]
21 **Moonen A**, Boeckxstaens G. Measuring mechanical properties of the esophageal wall using impedance planimetry. *Gastrointest Endosc Clin N Am* 2014; **24**: 607-618 [PMID: 25216906 DOI: 10.1016/j.giec.2014.06.001]
22 **McMahon BP**, Rao SS, Gregersen H, Kwiatek MA, Pandolfino JE, Drewes AM, Krarup AL, Lottrup C, Frøkjaer JB. Distensibility testing of the esophagus. *Ann N Y Acad Sci* 2011; **1232**: 331-340 [PMID: 21950823 DOI: 10.1111/j.1749-6632.2011.06069.x]
23 **Tucker E**, Sweis R, Anggiansah A, Wong T, Telakis E, Knowles K, Wright J, Fox M. Measurement of esophago-gastric junction cross-sectional area and distensibility by an endolumenal functional lumen imaging probe for the diagnosis of gastro-esophageal reflux disease. *Neurogastroenterol Motil* 2013; **25**: 904-910 [PMID: 23981175 DOI: 10.1111/nmo.12218]

**P- Reviewer**: Ierardi E, Nicodeme F, Tolone S, Wang BM
**S- Editor**: Qi Y **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/ *World J Gastroenterol* 2017 February 21; 23(7): 1298-1309

DOI: 10.3748/wjg.v23.i7.1298 ISSN 1007-9327 (print) ISSN 2219-2840 (online)

SYSTEMATIC REVIEWS

# Psychological controversies in gastroparesis: A systematic review

Sally Woodhouse, Geoff Hebbard, Simon R Knowles

Sally Woodhouse, Simon R Knowles, Department of Psychological Sciences, Swinburne University of Technology, Hawthorn, Victoria 3122, Australia

Geoff Hebbard, Simon R Knowles, Department of Gastroenterology, the Royal Melbourne Hospital, Victoria 3050, Australia

Geoff Hebbard, Simon R Knowles, Department of Medicine, University of Melbourne, Parkville, Victoria 3010, Australia

Simon R Knowles, Department of Psychiatry, St Vincent's Hospital, Fitzroy, Victoria 3065, Australia

Author contributions: Woodhouse S, Hebbard G and Knowles SR contributed to study concept and design; Woodhouse S performed the literature search; Woodhouse S and Knowles SR reviewed and analyzed the literature; Woodhouse S drafted the paper; Hebbard G and Knowles SR revised the paper.

Conflict-of-interest statement: The authors have no conflicting interests to declare.

Data sharing statement: No additional data are available.



Manuscript source: Invited manuscript

Correspondence to: Simon R Knowles, PhD, Senior Lecturer, Department of Psychological Sciences, Swinburne University of Technology, Hawthorn, Victoria 3122, Australia. sknowles@swin.edu.au
Telephone: +61-3-92148206
Fax: +61-3-98190574

Received: November 30, 2016
Peer-review started: December 2, 2016
First decision: December 28, 2016
Revised: January 18, 2017
Accepted: February 7, 2017
Article in press: February 8, 2017
Published online: February 21, 2017

## Abstract

***AIM***

To systematically review literature addressing three key psychologically-oriented controversies associated with gastroparesis.

***METHODS***

A comprehensive search of PubMed, CINAHL, and PsycINFO databases was performed to identify literature addressing the relationship between gastroparesis and psychological factors. Two researchers independently screened all references. Inclusion criteria were: an adult sample of gastroparesis patients, a quantitative methodology, and at least one of the following: (1) evaluation of the prevalence of psychopathology; (2) an outcome measure of anxiety, depression, or quality of life; and (3) evidence of a psychological intervention. Case studies, review articles, and publications in languages other than English were excluded from the current review.

***RESULTS***

Prevalence of psychopathology was evaluated by three studies ($n$ = 378), which found that combined anxiety/depression was present in 24% of the gastroparesis cohort, severe anxiety in 12.4%, depression in 21.8%-23%, and somatization in 50%. Level of anxiety and depression was included as an outcome measure in six studies ($n$ = 1408), and while limited research made it difficult to determine the level of anxiety and depression in the cohort, a clear positive relationship with gastroparesis symptom severity was evident.

Quality of life was included as an outcome measure in 11 studies (*n* = 2076), with gastroparesis patients reporting lower quality of life than population norms, and a negative relationship between quality of life and symptom severity. One study assessed the use of a psychological intervention for gastroparesis patients (*n* = 120) and found that depression and gastric function were improved in patients who received psychological intervention, however the study had considerable methodological limitations.

***CONCLUSION***

Gastroparesis is associated with significant psychological distress and poor quality of life. Recommendations for future studies and the development of psychological interventions are provided.

**Key words:** Anxiety; Depression; Gastroparesis; Quality of life; Psychological distress



**Core tip:** Gastroparesis is associated with significant psychological distress and poor quality of life. Literature indicates that quality of life is lower in gastroparesis patients than population norms. Further, gastroparesis symptoms are adversely associated with increased anxiety and depression and impaired quality of life. Rates of psychopathology in gastroparesis cohorts range between 21.8% to 50%. Although a psychological intervention for gastroparesis has found improvements in depression and gastric function, it has not been replicated. Further research into potential mediating factors and the development of psychological interventions for individuals with gastroparesis is warranted.

Woodhouse S, Hebbard G, Knowles SR. Psychological controversies in gastroparesis: A systematic review. *World J Gastroenterol* 2017; 23(7): 1298-1309 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1298.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1298

## INTRODUCTION

Gastroparesis is a gastrointestinal disorder involving delayed gastric emptying in the absence of a mechanical obstruction of the stomach[1]. Patients living with gastroparesis typically experience chronic nausea, vomiting, early satiety, postprandial fullness, and in some cases abdominal pain and fatigue[2-6]. The mean age of diagnosis ranges between 40-45.5 years, with 67%-88% of gastroparesis patients being female[5-12].

In Australia, the prevalence of gastroparesis is unknown, however in 2006 the Australian government provided an estimate that 120000 Australians suffered from severe gastroparesis[13]. The only study to investigate the prevalence of gastroparesis was conducted using medical records in Minnesota (United States) from 1996 to 2006. Jung *et al*[14] found that after adjusting for age and gender (to 2000 US Caucasians), the incidence of definite gastroparesis per 100000 person years was 9.8 in women, and 2.4 in men. In patients over the age of 60 years, the incidence peaked at 10.5 per 100000. It has been estimated that approximately one third of gastroparesis patients will be admitted to hospital for the condition[5], with a disease burden likened to that of Inflammatory Bowel Disease[14]. In terms of financial burden, Wang and colleagues[15] reported that in 1995 the costs of gastroparesis in the United States were 47.7 million dollars (primary diagnosis) and 863.3 million dollars (secondary diagnosis), while in 2004 costs were significantly higher at 208.3 million dollars (primary diagnosis) and 3.3 billion dollars (secondary diagnosis).

Individuals living with chronic gastrointestinal illness must make considerable physical, psychological, and social adjustments in order to manage their often debilitating symptoms[16,17]. Not surprisingly, patients suffering from chronic gastrointestinal conditions frequently report psychological symptoms, such as anxiety, depression, and impaired quality of life (QoL)[17-26]. With limited treatment options available for gastroparesis, the importance of psychological support or intervention has been repeatedly emphasized in the literature[8,27,28]. A systematic review of the gastroparesis literature exploring relationships between psychological distress, psychological processes, and gastroparesis has not yet been conducted.

The current systematic review will explore three key questions in relation to psychological features and processes associated with gastroparesis: (1) what is the prevalence of psychopathology in gastroparesis cohorts and how does it compare to other gastroenterological conditions? (2) what are the levels of anxiety, depression, and QoL in gastroparesis cohorts and do they differ with respect to gastroparesis symptom severity, etiology, degree of gastric retention, and duration of symptoms/disease? And (3) do psychological interventions for gastroparesis patients reduce gastroparesis symptoms, anxiety, depression, and improve QoL?

## MATERIALS AND METHODS

For this review, a comprehensive search of PubMed, CINAHL, and PsycINFO databases was performed. Search criteria used were: ("gastroparesis" OR "gastric delay" OR "gastric emptying" OR "gastric motility" OR "gastric timing") AND ("anxiety" OR "affective state" OR "cognition" OR "control" OR "coping" OR "depression" OR "distress" OR "emotion"

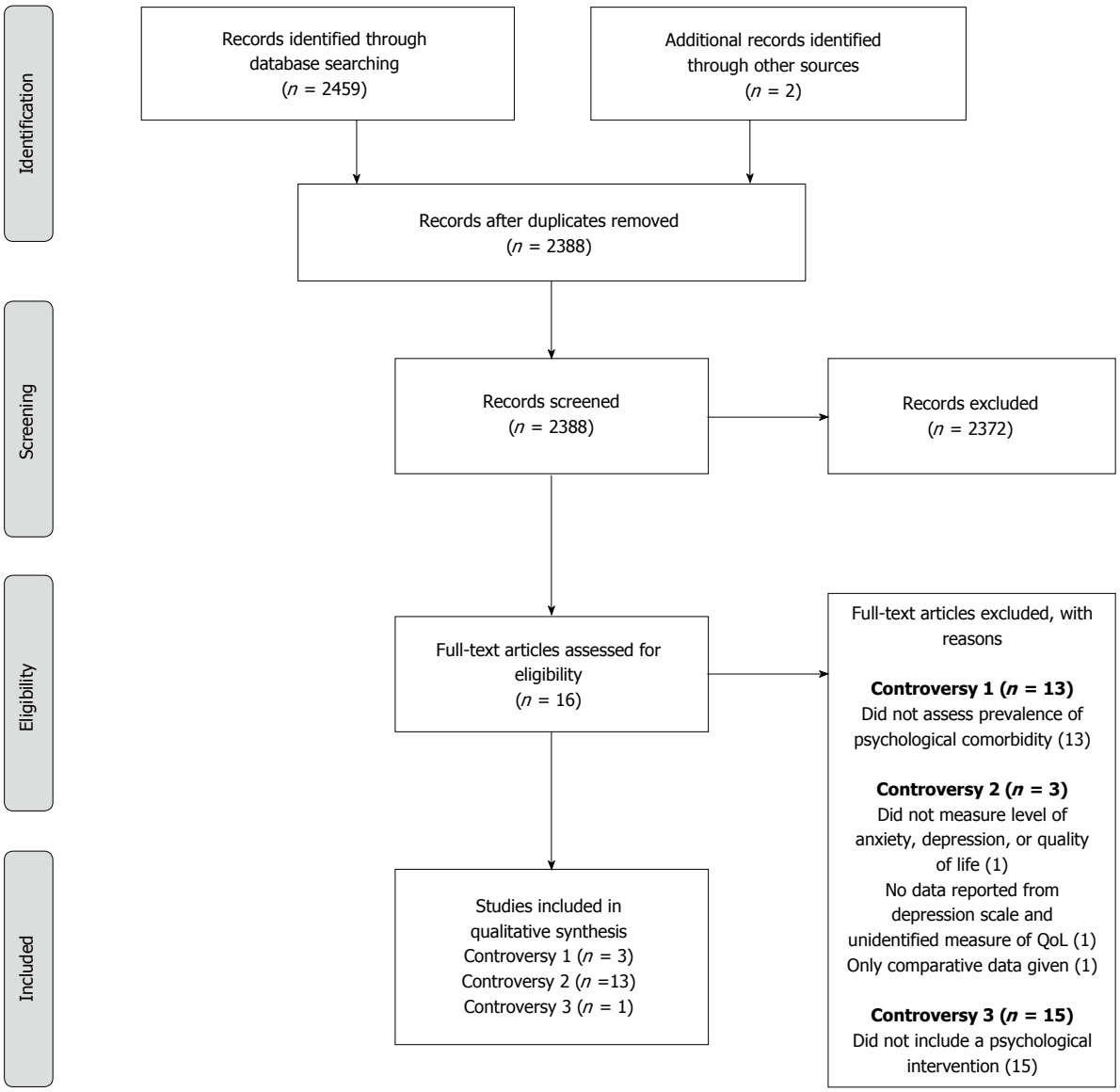



**Figure 1 PRISMA flow diagram, from Moher *et al*[45].** For more information, visit www.prisma-statement.org.

OR "helplessness" OR "illness perception" OR "life events" OR "mastery" OR "mental" OR "mood" OR "neuropsychological" OR "panic" OR "personality" OR "psycholog" OR "psychosocial" OR "quality of life" OR "self-efficacy" OR "stress"). Research papers retrieved through the search were also reviewed for further relevant references.

Inclusion criteria were: an adult sample of gastroparesis patients, a quantitative methodology, and at least one of the following: (1) evaluation of the prevalence of psychopathology; (2) an outcome measure of anxiety, depression, or QoL; and (3) evidence of a psychological intervention. Case studies, review articles, and publications in languages other than English were excluded from the current review.

Two researchers (Woodhouse S, Knowles SR) independently screened all references retrieved through the search and categorized them according to the inclusion and exclusion criteria. The researchers also extracted data from the papers independently, including participant information, methodology, assessment tools, and study outcomes.

## RESULTS

After 73 duplicates were removed, a total of 2388 citations were identified through database searches and review of other relevant references. Of these, 2372 were excluded due to: (1) not meeting the inclusion criteria; or (2) lack of information (Figure 1 for PRISMA diagram). This resulted in a total of 16 research reports which are summarized in Table 1.

Of these reports, three (18.75%) identified the prevalence of psychopathology in a gastroparesis cohort[6,12,14], 13 (81.25%) assessed levels of anxiety, depression, or QoL[2,7,8,10,12,26,29-35], and one (6.25%) involved a psychologically-based intervention for gastroparesis patients[36]. A summary of the studies'

**Table 1 Summary of papers reviewed**

| Ref. | Study characteristics | Participant details | Psychological measures used | Relevant findings | Conclusion |
|---|---|---|---|---|---|
| Soykan *et al*[6] | Cohort study using six-years of hospital records. Demographic and clinical data evaluated at entry to the hospital and most recent follow-up | *n* = 146 (120 females, 26 males). Mean age: 45.0 yr. Etiology: 42 DG, 52 IG, 19 post-surgical, 11 Parkinson's disease, 7 collagen vascular disorders, 6 intestinal pseudo-obstruction, 9 other | CES-D, SCL-90 | 23% of IG patients were thought to be depressed, and 50% displayed significant elevations on gastrointestinal psychosomatic susceptibility | Psychological status may be predictive of response to prokinetic therapy |
| Harrell *et al*[31] | Cross-sectional study with an interview, patients classified into a clinical subgroup based on predominant symptoms | *n* = 100 (87 females, 13 males). Mean age: 48.0 yr. Etiology: unspecified | SF-12 | QoL (subscales and mental/physical component summaries) was significantly diminished in all gastroparesis patients when compared to population norms, but did not differ between groups based on predominant gastroparesis symptoms. QoL negatively correlated with physical symptom scores | Predominant-symptom classification may be useful in the management of gastroparesis |
| Bielefeldt *et al*[8] | Cross-sectional study with a qualitative interview | *n* = 55 (44 females, 11 males). Mean age: 42.4 yr. Etiology: 11 DG, 29 IG, 8 connective tissue disease, 4 post-surgery or trauma, 1 osteogenesis imperfect, 1 mitochondrial myopathy, 1 Marfan syndrome | HADS, SF-12, open-ended interview questions | Patients had moderately elevated scores for anxiety and depression, 74% met screening criteria for anxiety or depression, 29% were above the threshold for clinically relevant affective spectrum disorders, and eighteen patients were receiving chronic anti-depressant medication. Patients demonstrated impaired QoL compared to population norm, with no differences between etiologies. Physical symptoms were inversely related to the physical component score on SF-12. Symptom severity was positively correlated with depression scores, but not anxiety, symptom duration or degree of gastric delay. Qualitative data: patients were asked to describe the impact of gastroparesis on their lives and three main topics were identified: 1) eating out/social functions, 2) fatigue, 3) strain on relationships. Nausea and vomiting were the most troublesome symptoms, and patients also reported a fear of unrelenting disease, as well as frustration/dissatisfaction with healthcare providers | Gastroparesis treatment must focus on improving QoL. The results of this study provide support for the use of psychologically based interventions in gastroparesis |
| Jung *et al*[14] | Cohort study using medical records | Definite gastroparesis = 83 (68 female, 15 males). Mean age at onset: 44.0 yr. Etiology: 21 DG, 41 IG, connective tissue disease 9, hypothyroidism 1, malignancy 2, abdominal surgery 6, provocation drugs 19, end-stage renal disease 4 | None reported. Evidence obtained from medical records. | Of 83 patients with definite gastroparesis, 25 had evidence of comorbid psychiatric illness in their medical records. Twenty patients had "anxiety/depression" and five had "other" | Gastroparesis is difficult to manage and represents a major disease burden |
| Hasler *et al*[7] | Cross-sectional study. Data obtained from the Gastroparesis Registry | *n* = 299 (245 females, 54 males). Mean age: 43.0 yr. Etiology: 100 DG, 199 IG | BDI, STAI | Depression and anxiety scores increased with greater physician-rated, and patient-rated, symptom severity. Nausea and vomiting were greater in patients with more severe depressive symptoms. Bloating and postprandial fullness were greater in patients with more severe depressive symptoms, state and trait anxiety. Higher depression scores were associated with prokinetic or antiemetic drug use, and increased hospitalizations. Higher state anxiety was associated with anxiolytic use, while higher trait anxiety was associated with antidepressant use and increased hospitalizations. Depression and anxiety scores did not differ across etiology or degree of gastric retention. Higher symptom severity score was predictive of higher depression and state anxiety score. Use of anxiolytics was predictive of state anxiety, use of anti-depressants was predictive of greater trait anxiety score, and male gender was predictive of higher state anxiety | The physical and psychological features of gastroparesis both need to be considered in the development of individualized patient treatment plans. Longitudinal studies must be conducted to evaluate the relationship between psychology and gastroparesis, and whether psychological treatment can affect the physical symptoms of gastroparesis |

| | | | | | |
|---|---|---|---|---|---|
| Cherian *et al*[10] | Cross-sectional study | *n* = 68 (58 females, 10 males). Mean age: 42.6 yr. Etiology: 18 DG, 50 IG. 52 Functional Dyspepsia patients also studied | PAGI-QOL | DG patients scored significantly higher than IG patients on the following PAGI-QOL subscales: diet, daily activities, relationships. When pain severity was correlated with QOL subscales, there was a moderate correlation with avoiding physical activity, taking longer to perform daily activities, worry about having stomach problems in public, and depending on others to perform activities | Abdominal pain is an important symptom of gastroparesis and is associated with decreased QoL |
| Hasler *et al*[32] | Cross-sectional study. Data obtained from the Gastroparesis Registry | *n* = 243 (214 females, 29 males). Mean age: 41.0 yr. Etiology: 116 DG, 219 IG | PAGI-QOL, SF-36 | Patients had moderately impaired QoL, with inverse correlation to bloating severity | Bloating is a prevalent symptom in gastroparesis and is associated with impaired physical and mental QoL |
| Parkman *et al*[12] | Cross-sectional study. Data obtained from the Gastroparesis Registry | *n* = 243 (214 females, 29 males). Mean age: 41.0 yr. Etiology: 243 IG | BDI, STAI | 36% of participants demonstrated severe state anxiety, 35% demonstrated severe trait anxiety, and 18% demonstrated severe depression. Overweight IG patients were more likely to have an anxiety disorder. Major depressive disorder was associated with greater symptom severity. Anxiety and depression scores tended to be higher in patients with more severely delayed gastric emptying | Symptoms, gastric retention, current treatment, and psychosocial factors all play a role in the severity of IG |
| Jaffe *et al*[33] | Cross-sectional study | *n* = 59 (52 females, 7 males). Mean age: 43.0 yr. Etiology: 20 DG, 39 IG | PAGI-QOL, SF-36 | Nausea/vomiting subscale of PAGI-SYM correlated with lower scores on the PAGI-QOL. SF-36 scores were significantly decreased in gastroparesis patients compared to population norms | Nausea is a predominant symptom of gastroparesis that is associated with impaired QoL |
| Cherian *et al*[2] | Cross-sectional study | *n* = 156 (126 females, 30 males). Mean age: 41.1 yr. Etiology: 42 DG, 114 IG. 52 FD patients also studied | HADS, PAGI-QOL | Increased fatigue was associated with decreased QoL, increased depression, and decreased anxiety. All but one patient met criteria for depression, and the same was found for anxiety | Fatigue is a significant symptom in gastroparesis and is associated with decreased QoL. Psychiatric interventions may help in fatigue management |
| Hasler *et al*[29] | Cross-sectional study. Data obtained from the Gastroparesis Registry | *n* = 393 (327 females, 66 males). Mean age: 42.9 yr. Etiology: 137 DG, 256, IG | BDI, STAI, PAGI-QOL, SF-36 | Depression and anxiety were higher in those with greater symptom severity. Impaired PAGI- QOL and SF–36 physical component scores related to increased pain and/or discomfort severity | The influence of predominant pain/ discomfort on disease severity is at least as great as predominant nausea/vomiting |
| Friedenberg *et al*[30] | Cross-sectional study | *n* = 255 (212 females, 43 males). Mean age: 42.0 yr. Etiology: 180 IG, 64 DG, 4 post-surgical, 7 other | PAGI-QOL | African American and Hispanic patients had lower scores on clothing and psychological PAGI-QOL subscales than Caucasian patients resulting in lower QoL overall. PAGI-SYM and PAGI-QOL had a negative correlation and 30% of the variation in QoL could be explained by symptom severity | Future population-based studies into the influence of race on symptoms and QoL in gastroparesis are warranted |
| Liu *et al*[36] | Randomized controlled trial with follow-up at 3, 7, 10, and 17 d post intervention | *n* = 120 (70 females, 50 males). Mean age: 60.5 yr. Etiology: 120 post-surgical | CES-D | A group that underwent a mental intervention had faster recovery from post-surgical gastroparesis (*e.g.*, extubation time, eating recovery) compared to a control group. Depression was comparable in groups at baseline, but mental intervention group had lower scores than control at 3, 7, 10, and 17 d post-intervention | Mental intervention is important in post-surgical recovery, and primary nurses should be trained to care for patients physically and psychologically post-surgery |
| Pasricha *et al*[26] | Cross-sectional study. Data obtained from the Gastroparesis Registry | *n* = 262 (215 females, 47 males). Mean age: 44.0 yr. Etiology: 177 IG, 85 DG | PAGI-QOL, BDI, STAI | Mild improvement in QoL from baseline to follow-up at 48 weeks (PAGI-QOL and SF-36 physical and mental component scores), with no significant difference in QoL improvement across etiologies. No significant changes in depression or anxiety levels over the 48-week follow-up period. Moderate to severe depression and the use of anxiolytics at baseline were negative predictors of symptomatic improvement at follow-up, while anti-depressant use was a positive predictor | Less than a third of patients with gastroparesis experience symptomatic improvement over time and QoL remains impaired. Depression is an important predictor of symptomatic improvement |

| | | | | | |
|---|---|---|---|---|---|
| Cutts *et al*[34] | Cross-sectional study | *n* = 235 (186 females, 49 males). Mean age: 47.0 yr. Etiology: 125 IG, 68 DG, 28 post-surgical, 14 unspecified | SF-36 | Reports correlations between SF-36 subscales and gastroparesis symptoms. Negative correlations with Physical Function subscale: bloating severity, bloating frequency, epigastric pain severity. Negative correlations with Bodily Pain subscale: bloating severity, bloating frequency, epigastric pain severity, epigastric pain frequency, epigastric burn frequency. Negative correlations with Social Functioning subscale: epigastric pain frequency, vomiting severity. Negative correlations with Role Emotional subscale: bloating severity, bloating frequency. Negative correlation with mental health subscale: bloating severity. The only positive correlation was between the Role Emotional subscale and epigastric pain severity | Generic and global QoL tools may not accurately reflect the experience of gastroparesis patients |
| Lacy *et al*[35] | Cross-sectional study | *n* = 250 (196 females, 54 males). Mean age: 46.8 yr. Etiology: 126 IG, 37 DG, 34 post-viral, 17 post-surgical, 11 connective tissue disorder, 10 neurologic, 5 post-vaccination, 3 hollow visceral myopathy, 3 vascular, 4 miscellaneous | SF-36 | IG patients had higher physical functioning, mental health, and role-physical scores compared to DG patients. Patients with DG had lower physical component summary scores than patients with IG or other etiologies. Patients with IG had higher mental component summary scores than patients with DG or other etiologies | It is important that gastroparesis interventions aim to lessen pain and improve QoL in patients |

DG: Diabetic gastroparesis; IG: Idiopathic gastroparesis; QoL: Quality of life.

**Table 2 Summary of participant characteristics**

| | *n* |
|---|---|
| Number of studies included in this review | 16 |
| Number of participants identified in the studies | 2967 |
| Disease etiology | |
| Unspecified | 118 |
| Idiopathic | 1850 |
| Diabetic | 761 |
| Post-surgical | 198 |
| Other (*e.g.*, connective tissue disorder, Parkinson's disease) | 151 |
| Gender | |
| Female | 2434 |
| Male | 533 |
| Mean age | 44.6 |

participant characteristics is presented in Table 2.

### *What is the prevalence of psychopathology in gastroparesis cohorts and how does it compare to other gastroenterological conditions?*

Three studies reported on the prevalence of psychopathology in a gastroparesis cohort (*n* = 378). Using the Center for Epidemiologic Studies Depression Scale (CES-D) and the hospital records of 52 idiopathic gastroparesis (IG) patients, Soykan *et al*[6] note that 23% had a history of depression or antidepressant therapy, and 50% displayed clinically significant somatization using the SCL-90. The authors state that somatization was higher in the IG population than in the gastrointestinal population, however the difference was not significant.

In an exploration of the epidemiology of gastroparesis, Jung *et al*[14] identified that 25 out of 83 patients with definite gastroparesis (30%) had evidence of psychiatric comorbidity in their medical records. Twenty of these patients had evidence of anxiety or depression, and five had other psychiatric illness. This study did not compare the prevalence of psychopathology in gastroparesis to other gastroenterological cohorts.

In a larger sample of 243 IG patients, Parkman *et al*[12] identify comorbid major depression in 21.8% of patients, and severe anxiety in 12.4% of patients through face-to-face interviews between patients and study physicians or coordinators. This study did not compare the prevalence of psychopathology in gastroparesis to other gastroenterological cohorts, however it was shown that females were more likely to report comorbid anxiety disorder than males, and patients with severe symptom severity or severe gastric retention were more likely to report major depression than those with milder symptoms. Participants in this study were mainly recruited from tertiary referral centers and therefore may not be representative of the general gastroparesis community.

### *What are the levels of anxiety, depression, and QoL in gastroparesis cohorts and do they differ with respect to gastroparesis symptom severity, etiology, degree of gastric retention, and duration of symptoms/disease?*

**Studies measuring anxiety and/or depression in gastroparesis cohorts:** A total of six studies measured the level of anxiety and/or depression in gastroparesis cohorts (*n* = 1408)[2,7,8,12,26,29]. Of these studies, two used the Hospital Anxiety and Depression Scale (HADS)[2,8]. Bielefeldt *et al*[8] used a cut-off score of > 8 and found that of the 55 participants, 74% met the criteria for either anxiety or depression, and 29% met the criteria for both conditions. No differences

across etiology, gastric retention, or duration of symptoms/disease were reported, however symptom severity did correlate positively with depression score. Cherian *et al*[2] used a cutoff score of > 10 and found that of 156 participants, 99% met the criteria for depression and anxiety. Differences across etiology, symptom severity, gastric retention, and duration of symptoms/disease were not reported in the study.

A further four studies measured depression using the Beck Depression Inventory (BDI)[7,12,26,29]. Parkman *et al*[12] found that the average BDI score was 18.6 and 18% of the 243 IG participants fell into the range of 29-63 to indicate severe depression. Depression levels increased across mild to moderate symptom severity, however no difference was found in depression levels across degree of gastric retention. In a study of 299 gastroparesis patients by Hasler *et al*[7], BDI scores of ⩾ 20 were present in 41.5% of participants. Higher BDI scores were associated with increased gastroparesis severity, nausea and vomiting, bloating, and postprandial fullness. The BDI scores were similar across diabetic gastroparesis (DG) and IG etiology, and degree of gastric retention. Self-reported gastroparesis severity and use of antiemetic/prokinetic medications were predictive of a BDI score ⩾ 20. Another study by Hasler *et al*[29] did not report overall BDI scores, but compared scores across pain severity, etiology, and symptom predominance. Hasler *et al*[29] found that in a study of 393 gastroparesis patients, increased BDI scores were associated with greater pain severity in both DG and IG patients. The most recent study by Pasricha *et al*[26] identified that 41.6% of 262 gastroparesis patients had BDI scores greater than 20, indicating moderate to severe depression. Unlike the aforementioned studies, this study also examined the impact of duration of disease on gastroparesis outcomes, finding no significant change in depression levels after 48 wk of standard medical care for gastroparesis. However, depression level at baseline was a significant predictor of symptomatic improvement at 48 wk.

Finally, four studies used the State-Trait Anxiety Inventory (STAI) to measure anxiety[7,12,26,29]. Parkman *et al*[12] found that the average state anxiety score was 45.2 while trait anxiety was 43.9. Using an STAI score of ⩾ 50 to denote severe anxiety, Parkman *et al*[12] identified that 36% of 243 IG patients reported severe state anxiety, while 35% reported severe trait anxiety. State anxiety levels increased across mild to moderate symptom severity, however no difference was found in state or trait anxiety levels across degree of gastric retention. Hasler *et al*[7] noted that 50.2% of participants reported state anxiety ⩾ 46, and 51.5% reported trait anxiety ⩾ 44. Higher state and trait anxiety was associated with increased gastroparesis severity, bloating, and postprandial fullness. Increased self-reported gastroparesis severity and use of anxiolytic medications were predictive of higher state anxiety, while use of antidepressant medications was predictive of higher trait anxiety. State and trait anxiety were similar across DG and IG etiology, and degree of gastric retention. Hasler *et al*[29] found that increased STAI state and trait scores were associated with greater pain severity in both DG and IG patients. Finally, Pasricha *et al*[26] identified that 32.8% of participants reported state anxiety ⩾ 50 at baseline, and 30.5% reported trait anxiety ⩾ 50 with no significant change in state or trait anxiety levels after 48 wk of standard medical care for gastroparesis. However, use of anxiolytics at baseline was a negative predictor of symptomatic improvement at follow-up.

**Studies measuring QoL in gastroparesis cohorts:** Eleven studies included an outcome measure of QoL ($n$ = 2076)[2,8,10,26,29-35]. The two earliest studies to measure QoL in gastroparesis used the SF-12. Harrell *et al*[31] found that in a sample of 100 gastroparesis patients, SF-12 subscale scores and component summary scores were significantly lower in gastroparesis patients when compared to population norms, with a negative relationship to upper GI symptom severity. Similarly, in a study of 55 gastroparesis patients, Bielefeldt *et al*[8] found that both the physical and mental component scores of the SF-12 were lower than population norms, with no significant difference between DG and IG groups. Symptom severity was negatively correlated with the physical component score. The authors also identified that nausea and bloating severity, combined with the HADS score for depression, best predicted the physical health component score of the SF-12. The influence of gastric retention and duration of symptoms/disease on QoL was not assessed in either study.

Of the five studies that used the SF-36, Jaffe *et al*[33] found that both the mental and physical component scores were impaired in a sample of 59 gastroparesis patients compared to population norms. The study indicated that nausea and vomiting severity was inversely related to QoL, with no significant difference in QoL between DG and IG patients, or across degree of gastric retention. In a larger study of 335 patients, Hasler *et al*[32] noted that physical and mental component scores were negatively correlated to bloating severity, with higher mental component scores predicting greater bloating severity. Another study by Hasler *et al*[29] identified that physical and mental component scores were lower in both DG and IG patients with increased pain/discomfort scores. Additionally, when comparing between pain/discomfort predominant versus nausea/vomiting predominant symptoms, pain predominance was associated with greater impairment in the physical component score.

More recently, Pasricha *et al*[26] identified mild improvement in SF-36 scores (physical and mental components) after 48 wk of standard medical care for gastroparesis. A 2016 study by Cutts *et al*[34] explored the relationships between symptom severity and the SF-36 subscales in a cohort of 235 gastroparesis patients, finding primarily negative correlations

between symptom severity and Physical Functioning, Bodily Pain, Social Functioning, Role Emotional and Mental Health subscales (Table 1 for details). The only positive correlation was between Role Emotional and epigastric pain severity. Finally, the most recent study using the SF-36 was conducted by Lacy *et al*[35] and identified that in 250 gastroparesis patients, those with IG had better physical functioning, mental health, and role-physical than patients with DG. Similarly DG patients had lower physical component summary scores than patients with IG or gastroparesis from other causes, while DG patients and patients with gastroparesis from other causes also had lower mental component summary scores than those with IG.

Seven studies used the Patient Assessment of Upper Gastrointestinal Disorders Quality of Life (PAGI-QOL) to measure QoL. Using this assessment tool, Hasler *et al*[32] reported impaired QoL in individuals with gastroparesis. Cherian *et al*[10] assessed QoL across etiologies and found that, in their sample of 68 patients, IG patients scored significantly lower than DG patients on PAGI-QOL measures of diet, daily activities, and relationships. In addition, significant negative correlations have been identified between the PAGI-QOL and total upper GI symptom severity[30], pain/discomfort severity[10,29], fatigue[2], bloating severity[32], and nausea/vomiting severity[33]. Similar to their findings using the SF-36, Pasricha *et al*[26] identified mild improvement in PAGI-QOL scores after 48 wk of standard medical care for gastroparesis. Despite these improvements in QoL over time, the authors note that QoL remained impaired in relation to the general population.

### *Do psychological interventions involving gastroparesis patients reduce gastroparesis symptoms, anxiety, depression, and improve QoL?*

Only one study[36] involved a psychological intervention for gastroparesis patients. Liu *et al*[36] conducted a randomized controlled trial (RCT) with 120 post-surgical gastroparesis patients. Sixty patients were allocated to a control group that received conventional therapy (gastric tube, fasting, parenteral and enteral nutrition, routine nursing care, health guidance), while another 60 were allocated to a "comprehensive mental intervention" group that received conventional therapy in addition to: supportive mental consultation, bedside symptomatic mental intervention, music and abdominal massage, and mental intervention for patients' families. While the groups had comparable CES-D scores at baseline, the mental intervention group scored significantly lower than the control group on days 3, 7, 10, and 17 after the intervention. The intervention group also had significantly improved gastric function following the intervention compared to the control group. The study did not include measures of anxiety or QoL.

## DISCUSSION

Conclusions are presented according to the key questions of the systematic review. This is followed by a discussion of the strengths and limitations of the literature, and suggestions for future research in the area.

### *Prevalence of psychopathology in gastroparesis*

This review found three studies that investigated the prevalence of psychopathology in gastroparesis patients. The reported prevalence of these psychopathologies were: combined anxiety/depression 24%[14], severe anxiety 12.4%[12], depression 21.8%-23%[6,12], somatization 50%[6], other 5%[14]. Parkman *et al*[12] reported that females were more likely to report comorbid anxiety disorder, and patients with greater symptom severity and gastric delay were more likely to report major depression. Soykan *et al*[6] identified a non-significant difference in the prevalence of somatization in the gastroparesis cohort compared to other gastroenterological cohorts, while Parkman *et al*[12] and Jung *et al*[14] did not make such comparisons.

It must be acknowledged that in addition to using the CES-D, Soykan *et al*[6] assessed whether patients had a medical history of either depression or anti-depressant use, which does not necessarily indicate prevalence of depression. Parkman *et al*[12] only reported on severe anxiety, which is likely to underestimate the prevalence of anxiety in the cohort, and two studies[6,12] only assessed psychopathology in IG patients so findings may not be representative of approximately two-thirds of gastroparesis patients. Finally, all three studies lacked clarity around how patients obtained a psychiatric diagnosis, and two[6,12] limited the psychopathologies that were included in the study. Based on these findings, it can be concluded that while there is psychopathology in gastroparesis patients, there has not been enough research conducted to provide a reliable prevalence rate. Further, no conclusion to date can be made with regard to whether rates of psychopathology are higher or lower in gastroparesis compared to cohorts that are healthy, chronically ill, or have other gastrointestinal conditions.

### *Level of anxiety and/or depression in gastroparesis*

Overall, it is difficult to be definitive regarding the level of anxiety and depression in gastroparesis cohorts given the limited research conducted to date. Based on one study[12], 18% of gastroparesis patients have severe depression, 36% have severe state anxiety, and 35% have severe trait anxiety. Another study reported that 41.6% of patients had moderate to severe levels of depression at baseline, and identified that the percentage of patients scoring equal to or greater than 50 on the STAI at baseline was 32.8% for state anxiety, and 30.5% for trait anxiety[26]. While other studies also measured and reported on anxiety and depression, they did not identify levels of severity. Three studies[7,12,29] indicated that anxiety was positively associated with gastroparesis symptom

severity , and one did not[8], while four[7,8,12,29] indicated that depression increased with gastroparesis symptom severity. Two studies reported on the influence of gender on anxiety and depression levels, with one stating that females displayed less clinically severe depression[12], and the other indicating that male gender was associated with higher state anxiety[7]. One study demonstrated that depression and anxiety levels were similar across DG and IG etiologies[7], and two showed consistency across degree of gastric retention[7,12]. Only one study[26] assessed the influence of duration of symptoms/disease, finding no significant improvement in anxiety or depression levels from baseline to follow-up at 48 wk. However, depression level and use of anxiolytics at baseline were significant predictors of symptomatic improvement at 48 wk.

The six studies that measured anxiety and/or depression in a gastroparesis cohort used a variety of assessment tools and cut-off scores, which makes it difficult to interpret the results as a whole. For example, the two studies employing the HADS each used a different cut-off score and did not give enough information to compare results across the studies. Similarly, of the four studies using the BDI and STAI only two reported the level of anxiety and depression in the sample, while the other two primarily used the scores for correlation analyses. Thus, although studies have been conducted on the severity of anxiety and depression in gastroparesis patients, the lack of consistency and scoring information limits the conclusions that can be made. With this being said, there is evidence to indicate that levels of psychopathology and gastroparesis symptom severity were positively correlated, and that this relationship tends to be consistent across the different forms of gastroparesis.

### *Level of QoL in gastroparesis*

The eleven studies investigating QoL in gastroparesis demonstrated that QoL was lower in gastroparesis patients than population norms[8,31,33], and that there was generally a negative relationship between QoL and gastroparesis symptom severity[2,8,10,29-34], although one study found a weak positive relationship between the Role Emotional subscale of the SF-36 and epigastric pain severity[34]. Two studies found no significant difference in QoL between DG and IG patients[8,33], however one found that IG scored lower than DG on measures of diet, daily activities, and relationships[10], and conversely, another found IG scored higher than DG on both physical and mental components of QoL[35]. Only one study assessed the relationship between degree of gastric retention and QoL, with no significant relationship demonstrated[33]. One study assessed the impact of duration of symptoms/disease, finding a mild improvement in QoL after 48 wk of standard medical care for gastroparesis[26].

Based on these results, it can be concluded that QoL is lower in the gastroparesis cohort than the general population, and greater gastroparesis symptom severity is associated with lower QoL. At this point, there is not enough evidence to make conclusions about the influence of etiology, gastric retention, or duration of symptoms/disease on QoL.

### *Psychological intervention in gastroparesis*

Only one study has reported on a psychological intervention for gastroparesis patients. Liu *et al*[36] found that depression scores and gastric function were significantly improved in patients who received a psychological intervention compared to those who received standard care, however the study had considerable methodological limitations. Firstly, the study was conducted only on post-surgical patients, making the results difficult to generalize to other etiologies. The study also utilized a number of different factors in the intervention condition (*e.g.,* supportive mental consultation, abdominal massage, music) making it impossible to ascertain the impact of any one component of the intervention. Additionally, the study did not utilize long-term follow-up. While the results of this study are promising, there is currently limited evidence for the use of psychological intervention in gastroparesis, and measures of other important psychological factors such as anxiety and QoL have yet to be assessed in this context.

### *Summary of findings and limitations*

Currently the literature indicates that QoL is lower in gastroparesis patients than population norms, and that as gastroparesis symptom severity increases, anxiety and depression also increase while QoL decreases. The studies are few in number, with variability in the assessments used and etiologies studied, making it difficult to form further conclusions. It also appears that five of the 15 studies[7,12,26,29,32] have used overlapping samples as they were all recruited via the Gastroparesis Registry. Consequently, findings may not be reflected across different samples. The evidence for the use of psychological intervention in gastroparesis is minimal and is further weakened by significant methodological limitations in the single relevant study.

Inconsistency in the assessment of gastroparesis must also be considered when interpreting these findings. While the majority of studies used self-report in conjunction with a scintigraphic study where > 60% retention at two hours, and/or > 10% retention at four hours indicated gastroparesis, there was some variation in assessment[6,8,31,34].

### *Future directions*

In order to move forward in understanding this area, future research would benefit from undertaking the following recommendations. When assessing the prevalence of psychopathology in gastroparesis cohorts, studies should consider the broad range of

**Table 3 General recommendations and questions for future research**

| |
|---|
| General recommendations: |
| Identify prevalence of psychological conditions based upon standardized and validated assessment tools (*e.g.*, SCID[37], MINI[38]) |
| Use standardized assessment of gastroparesis (*e.g.*, gastric emptying scintigraphy, PAGI-SYM[39]) |
| Use validated psychological scales to assess, anxiety, depression, stress (*e.g.*, BDI[40], BAI[41], STAI[42], DASS[43]) and QoL measures relevant to individuals with upper gastrointestinal disorders (*e.g.*, PAGI-QoL[44]) |
| Use and provide clear scoring information |
| Report assessment results in a manner that allows comparison across studies (*e.g.*, standardized cut-off scores) |
| Psychological interventions: |
| Randomized control trial design |
| Prior to intervention, power analyses conducted |
| Clear details of intervention content made fully available to allow other researchers to review and undertake accurate replication |
| Gastroparesis-focused interventions |
| Include measures that assess a cost/benefit analysis, engagement of medical services |
| Where possible, patients, assessors, and statistician blinded |
| Independent evaluation of intervention session recordings to ensure protocol/treatment consistency |
| Psychological interventions need to be clearly identified and undertaken by trained and appropriately qualified individuals (*i.e.*, psychologists, psychiatrists) |
| Identify clear inclusion and exclusion criteria |
| Identifying if (and where possible control for) participants have/have not received or are currently receiving psychotherapy (including type, duration *etc.*), using psychotropic medication, are on specialized diets for their gastroparesis |
| Utilize valid measures which can be accurately compared to other intervention studies |
| Evaluate participant engagement in therapy (*e.g.*, % attendance to sessions, completion of homework) |
| Evaluate differences between completers versus non-completers |
| Include long-term post-therapy efficacy review time points (*i.e.*, 1 and 2 yr post-intervention) |
| Future research questions: |
| What is the prevalence of psychopathology in gastroparesis compared to other gastroenterological cohorts? |
| What psychological processes act as moderating/mediating factors between gastroparesis symptom activity and outcome variables such as QoL, anxiety, and depression (*e.g.*, personality, coping style, self-efficacy)? |
| How may gender impact upon the presentation and course of gastroparesis and associated psychological distress? |
| How may historical and current stressors and/or traumas impact upon the presentation and course of gastroparesis? |
| To what extent does duration of symptoms/disease influence the relationship between gastroparesis and psychological distress? |

QoL: Quality of life.

psychopathologies, which should be diagnosed by an appropriately qualified individual. To gain greater insight into the relationship between psychological factors and gastroparesis, studies should use standardized assessment tools and cut off scores, and provide clear scoring information. Studies are also invited to look beyond basic correlation analyses, and explore possible mediating factors. Information regarding mediating factors would be especially useful in designing individualized psychological interventions for gastroparesis patients. To promote consistency and future comparison, recommendations for studies are summarized in Table 3, along with suggestions for the development of psychological interventions and future research questions.

In conclusion, increased levels of psychopathology are evident in patients suffering from gastroparesis, with associations between the severity of psychological factors and the severity of gastroparesis symptoms. Although only one study has utilized a psychologically-based intervention for gastroparesis patients to date, the intervention was associated with improvement in both gastroparesis symptoms and levels of depression. The results of this systematic review indicate the importance of further research into the relationship between psychological factors and gastroparesis, especially given that current medical treatments for gastroparesis are limited. In particular, further exploration of the prevalence of psychopathology in gastroparesis compared to other conditions is warranted, as well as an assessment of the factors that may mediate an individual's ability to adapt to, and manage, gastroparesis.

## COMMENTS

### Background

Gastroparesis is a gastrointestinal disorder involving delayed gastric emptying in the absence of a mechanical obstruction of the stomach. Typical symptoms include: chronic nausea, vomiting, early satiety, postprandial fullness, and in some cases abdominal pain and fatigue. Patients suffering from chronic gastrointestinal conditions frequently report psychological symptoms, such as anxiety, depression, and impaired quality of life (QoL).

### Research frontiers

With limited treatment options available for gastroparesis, the importance of psychological support or intervention for gastroparesis patients has been repeatedly emphasized in the literature. This is the first systematic review of the literature to explore the relationship between psychological factors and gastroparesis.

### Innovations and breakthroughs

This systematic review reveals that QoL is lower in gastroparesis patients than population norms, and that as gastroparesis symptom severity increases, anxiety and depression also increase while QoL decreases. Recommendations for the development of future research questions and psychological interventions are provided to encourage progress in this important research area.

### Applications

The results of this systematic review indicate that further exploration of the prevalence of psychopathology in gastroparesis is warranted, as well as an assessment of the factors that may mediate an individual's ability to adapt to, and manage, gastroparesis. Better understanding of these factors will assist in the development of targeted psychological support programs for the gastroparesis cohort.

### Peer-review

This paper conducted for systematic review of psychological aspects of gastroparesis. Authors concluded that "gastroparesis is associated with significant psychological distress and poor quality of life. Recommendations for future studies and the development of psychological interventions are provided".

## REFERENCES


1 **Tack J**. Gastric motility disorders. In: Weinstein WM, Hawkey CJ, Bosch J, editors. Clinical Gastroenterolology and Hepatology. St Louis, MO: Elsevier, 2005: 261-266

2 **Cherian D**, Paladugu S, Pathikonda M, Parkman HP. Fatigue: a prevalent symptom in gastroparesis. *Dig Dis Sci* 2012; **57**: 2088-2095 [PMID: 22669206 DOI: 10.1007/s10620-012-2241-1]

3 **Tang DM**, Friedenberg FK. Gastroparesis: approach, diagnostic evaluation, and management. *Dis Mon* 2011; **57**: 74-101 [PMID: 21329779 DOI: 10.1016/j.disamonth.2010.12.007]

4 **Cherian D**, Parkman HP. Nausea and vomiting in diabetic and idiopathic gastroparesis. *Neurogastroenterol Motil* 2012; **24**: 217-222, e103 [PMID: 22118574 DOI: 10.1111/j.1365-2982.2011.01828.x]

5 **Dudekula A**, O'Connell M, Bielefeldt K. Hospitalizations and testing in gastroparesis. *J Gastroenterol Hepatol* 2011; **26**: 1275-1282 [PMID: 21443672 DOI: 10.1111/j.1440-1746.2011.06735.x]

6 **Soykan I**, Sivri B, Sarosiek I, Kiernan B, McCallum RW. Demography, clinical characteristics, psychological and abuse profiles, treatment, and long-term follow-up of patients with gastroparesis. *Dig Dis Sci* 1998; **43**: 2398-2404 [PMID: 9824125]

7 **Hasler WL**, Parkman HP, Wilson LA, Pasricha PJ, Koch KL, Abell TL, Snape WJ, Farrugia G, Lee L, Tonascia J, Unalp-Arida A, Hamilton F. Psychological dysfunction is associated with symptom severity but not disease etiology or degree of gastric retention in patients with gastroparesis. *Am J Gastroenterol* 2010; **105**: 2357-2367 [PMID: 20588262 DOI: 10.1038/ajg.2010.253]

8 **Bielefeldt K**, Raza N, Zickmund SL. Different faces of gastroparesis. *World J Gastroenterol* 2009; **15**: 6052-6060 [PMID: 20027677 DOI: 10.3748/wjg.15.6052]

9 **Borges CM**, Secaf M, Troncon LE. Clinical features and severity of gastric emptying delay in Brazilian patients with gastroparesis. *Arq Gastroenterol* 2013; **50**: 270-276 [PMID: 24474228 DOI: 10.1590/S0004-28032013000400006]

10 **Cherian D**, Sachdeva P, Fisher RS, Parkman HP. Abdominal pain is a frequent symptom of gastroparesis. *Clin Gastroenterol Hepatol* 2010; **8**: 676-681 [PMID: 20472097 DOI: 10.1016/j.cgh.2010.04.027]

11 **Karamanolis G**, Caenepeel P, Arts J, Tack J. Determinants of symptom pattern in idiopathic severely delayed gastric emptying: gastric emptying rate or proximal stomach dysfunction? *Gut* 2007; **56**: 29-36 [PMID: 16840507 DOI: 10.1136/gut.2005.089508]

12 **Parkman HP**, Yates K, Hasler WL, Nguyen L, Pasricha PJ, Snape WJ, Farrugia G, Koch KL, Abell TL, McCallum RW, Lee L, Unalp-Arida A, Tonascia J, Hamilton F. Clinical features of idiopathic gastroparesis vary with sex, body mass, symptom onset, delay in gastric emptying, and gastroparesis severity. *Gastroenterology* 2011; **140**: 101-115 [PMID: 20965184 DOI: 10.1053/j.gastro.2010.10.015]

13 **Department of Health and Ageing**. Enterra Therapy Gastric Electrical Stimulation (GES) System for the treatment of the symptoms of medically refractory gastroparesis. Canberra, ACT: HealthPACT, 2006. Available from: URL: http://www.horizonscanning.gov.au/internet/horizon/publishing.nsf/Content/211ABF81A69CA39DCA2575AD0080F3DC/$File/HS Enterra Therapy.pdf

14 **Jung HK**, Choung RS, Locke GR, Schleck CD, Zinsmeister AR, Szarka LA, Mullan B, Talley NJ. The incidence, prevalence, and outcomes of patients with gastroparesis in Olmsted County, Minnesota, from 1996 to 2006. *Gastroenterology* 2009; **136**: 1225-1233 [PMID: 19249393 DOI: 10.1053/j.gastro.2008.12.047]

15 **Wang YR**, Fisher RS, Parkman HP. Gastroparesis-related hospitalizations in the United States: trends, characteristics, and outcomes, 1995-2004. *Am J Gastroenterol* 2008; **103**: 313-322 [PMID: 18047541 DOI: 10.1111/j.1572-0241.2007.01658.x]

16 **Moss-Morris R**. Adjusting to chronic illness: time for a unified theory. *Br J Health Psychol* 2013; **18**: 681-686 [PMID: 24118260 DOI: 10.1111/bjhp.12072]

17 **Creed F**, Levy RL, Bradley LA, Francisconi C, Drossman DA, Naliboff BD, Olden KW. Psychosocial aspects of Functional Gastrointestinal Disorders. In: Drossman DA, Corazziari E, Delvaux M, Spiller R, Talley NJ, Thompson WG, Whitehead WE, editors. Rome III: The Functional Gastrointestinal Disorders. 3 ed. Lawrence, KS: Allen Press, Inc, 2006: 295-368

18 **Aro P**, Talley NJ, Agréus L, Johansson SE, Bolling-Sternevald E, Storskrubb T, Ronkainen J. Functional dyspepsia impairs quality of life in the adult population. *Aliment Pharmacol Ther* 2011; **33**: 1215-1224 [PMID: 21443537 DOI: 10.1111/j.1365-2036.2011.04640.x]

19 **Mikocka-Walus AA**, Turnbull DA, Moulding NT, Wilson IG, Andrews JM, Holtmann GJ. Controversies surrounding the comorbidity of depression and anxiety in inflammatory bowel disease patients: a literature review. *Inflamm Bowel Dis* 2007; **13**: 225-234 [PMID: 17206706 DOI: 10.1002/ibd.20062]

20 **Van Oudenhove L**, Vandenberghe J, Vos R, Fischler B, Demyttenaere K, Tack J. Abuse history, depression, and somatization are associated with gastric sensitivity and gastric emptying in functional dyspepsia. *Psychosom Med* 2011; **73**: 648-655 [PMID: 21949416 DOI: 10.1097/PSY.0b013e31822f32bf]

21 **Haug TT**, Wilhelmsen I, Berstad A, Ursin H. Life events and stress in patients with functional dyspepsia compared with patients with duodenal ulcer and healthy controls. *Scand J Gastroenterol* 1995; **30**: 524-530 [PMID: 7569758 DOI: 10.3109/00365529509089784]

22 **Frank L**, Kleinman L, Rentz A, Ciesla G, Kim JJ, Zacker C. Health-related quality of life associated with irritable bowel syndrome: comparison with other chronic diseases. *Clin Ther* 2002; **24**: 675-689; discussion 674 [PMID: 12017411 DOI: 10.1016/S0149-2918(02)85143-8]

23 **Gralnek IM**, Hays RD, Kilbourne A, Naliboff B, Mayer EA. The impact of irritable bowel syndrome on health-related quality of life. *Gastroenterology* 2000; **119**: 654-660 [PMID: 10982758 DOI: 10.1053/gast.2000.16484]

24 **Haag S**, Senf W, Häuser W, Tagay S, Grandt D, Heuft G, Gerken G, Talley NJ, Holtmann G. Impairment of health-related quality of life in functional dyspepsia and chronic liver disease: the influence of depression and anxiety. *Aliment Pharmacol Ther* 2008; **27**: 561-571 [PMID: 18208571 DOI: 10.1111/j.1365-2036.2008.03619.x]

25 **Glise H**, Wiklund I. Health-related quality of life and gastrointestinal disease. *J Gastroenterol Hepatol* 2002; **17** Suppl: S72-S84 [PMID: 12000595 DOI: 10.1046/j.1440-1746.17.s1.6.x]

26 **Pasricha PJ**, Yates KP, Nguyen L, Clarke J, Abell TL, Farrugia G, Hasler WL, Koch KL, Snape WJ, McCallum RW, Sarosiek I, Tonascia J, Miriel LA, Lee L, Hamilton F, Parkman HP. Outcomes and Factors Associated With Reduced Symptoms in Patients With Gastroparesis. *Gastroenterology* 2015; **149**: 1762-1774.e4 [PMID: 26299414 DOI: 10.1053/j.gastro.2015.08.008]

27 **Rashed H**, Cutts T, Abell T, Cowings P, Toscano W, El-Gammal A, Adl D. Predictors of response to a behavioral treatment in patients with chronic gastric motility disorders. *Dig Dis Sci* 2002; **47**: 1020-1026 [PMID: 12018897]

28 **Abell TL**, Bernstein RK, Cutts T, Farrugia G, Forster J, Hasler WL, McCallum RW, Olden KW, Parkman HP, Parrish CR, Pasricha PJ, Prather CM, Soffer EE, Twillman R, Vinik AI. Treatment of gastroparesis: a multidisciplinary clinical review.

*Neurogastroenterol Motil* 2006; **18**: 263-283 [PMID: 16553582 DOI: 10.1111/j.1365-2982.2006.00760.x]

29 **Hasler WL**, Wilson LA, Parkman HP, Koch KL, Abell TL, Nguyen L, Pasricha PJ, Snape WJ, McCallum RW, Sarosiek I, Farrugia G, Calles J, Lee L, Tonascia J, Unalp-Arida A, Hamilton F. Factors related to abdominal pain in gastroparesis: contrast to patients with predominant nausea and vomiting. *Neurogastroenterol Motil* 2013; **25**: 427-438, e300-1 [PMID: 23414452 DOI: 10.1111/nmo.12091]

30 **Friedenberg FK**, Kowalczyk M, Parkman HP. The influence of race on symptom severity and quality of life in gastroparesis. *J Clin Gastroenterol* 2013; **47**: 757-761 [PMID: 23426455 DOI: 10.1097/MCG.0b013e3182819aae]

31 **Harrell SP**, Studts JL, Dryden GW, Eversmann J, Cai L, Wo JM. A novel classification scheme for gastroparesis based on predominant-symptom presentation. *J Clin Gastroenterol* 2008; **42**: 455-459 [PMID: 18344894 DOI: 10.1097/MCG.0b013e31815ed084]

32 **Hasler WL**, Wilson LA, Parkman HP, Nguyen L, Abell TL, Koch KL, Pasricha PJ, Snape WJ, Farrugia G, Lee L, Tonascia J, Unalp-Arida A, Hamilton F. Bloating in gastroparesis: severity, impact, and associated factors. *Am J Gastroenterol* 2011; **106**: 1492-1502 [PMID: 21483459 DOI: 10.1038/ajg.2011.81]

33 **Jaffe JK**, Paladugu S, Gaughan JP, Parkman HP. Characteristics of nausea and its effects on quality of life in diabetic and idiopathic gastroparesis. *J Clin Gastroenterol* 2011; **45**: 317-321 [PMID: 20733513 DOI: 10.1097/MCG.0b013e3181eeb5e9]

34 **Cutts T**, Holmes S, Kedar A, Beatty K, K Mohammad M, Abell T. Twenty-five years of advocacy for patients with gastroparesis: support group therapy and patient reported outcome tool development. *BMC Gastroenterol* 2016; **16**: 107 [PMID: 27576470 DOI: 10.1186/s12876-016-0523-3]

35 **Lacy BE**, Crowell MD, Mathis C, Bauer D, Heinberg LJ. Gastroparesis: Quality of Life and Health Care Utilization. *J Clin Gastroenterol* 2016; Epub ahead of print [PMID: 27775961 DOI: 10.1097/mcg.0000000000000728]

36 **Liu Y**, Song X, Zhang Y, Zhou L, Ni R. The effects of comprehensive mental intervention on the recovery time of patients with postsurgical gastroparesis syndrome. *J Clin Nurs* 2014; **23**: 3138-3147 [PMID: 24702786 DOI: 10.1111/jocn.12554]

37 **First MB**, Spitzer RL, Miriam G, Williams JBW. Structured Clinical Interview for DSM-IV-TR Axis I Disorders, Research Version, Non-patient Edition. New York: Biometrics Research, New York State Psychiatric Institute, 2002

38 **Sheehan DV**, Lecrubier Y, Sheehan KH, Amorim P, Janavs J, Weiller E, Hergueta T, Baker R, Dunbar GC. The Mini-International Neuropsychiatric Interview (M.I.N.I.): the development and validation of a structured diagnostic psychiatric interview for DSM-IV and ICD-10. *J Clin Psychiatry* 1998; **59** Suppl 20: 22-33; quiz 34-57 [PMID: 9881538]

39 **Rentz AM**, Kahrilas P, Stanghellini V, Tack J, Talley NJ, de la Loge C, Trudeau E, Dubois D, Revicki DA. Development and psychometric evaluation of the patient assessment of upper gastrointestinal symptom severity index (PAGI-SYM) in patients with upper gastrointestinal disorders. *Qual Life Res* 2004; **13**: 1737-1749 [PMID: 15651544 DOI: 10.1007/s11136-004-9567-x]

40 **Beck AT**, Steer RA, Ball R, Ranieri W. Comparison of Beck Depression Inventories -IA and -II in psychiatric outpatients. *J Pers Assess* 1996; **67**: 588-597 [PMID: 8991972 DOI: 10.1207/s15327752jpa6703_13]

41 **Beck AT**, Epstein N, Brown G, Steer RA. An inventory for measuring clinical anxiety: psychometric properties. *J Consult Clin Psychol* 1988; **56**: 893-897 [PMID: 3204199 DOI: 10.1037/0022-006X.56.6.893]

42 **Spielberger CD**, Gorsuch RL, Lushene R, Vagg PR, Jacobs GA. Manual for the State-Trait Anxiety Inventory. Palo Alto, CA: Consulting Psychologists Press, 1983

43 **Lovibond PF**, Lovibond SH. The structure of negative emotional states: comparison of the Depression Anxiety Stress Scales (DASS) with the Beck Depression and Anxiety Inventories. *Behav Res Ther* 1995; **33**: 335-343 [PMID: 7726811 DOI: 10.1016/0005-7967(94)00075-U]

44 **de la Loge C**, Trudeau E, Marquis P, Kahrilas P, Stanghellini V, Talley NJ, Tack J, Revicki DA, Rentz AM, Dubois D. Cross-cultural development and validation of a patient self-administered questionnaire to assess quality of life in upper gastrointestinal disorders: the PAGI-QOL. *Qual Life Res* 2004; **13**: 1751-1762 [PMID: 15651545 DOI: 10.1007/s11136-004-8751-3]

45 **Moher D**, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *BMJ* 2009; **339**: b2535 [PMID: 19622551 DOI: 10.1371/journal.pmed.1000097]

**P- Reviewer**: Choi YS, Isik A, Okubo Y **S- Editor**: Qi Y
**L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/ *World J Gastroenterol* 2017 February 21; 23(7): 1310-1318

DOI: 10.3748/wjg.v23.i7.1310 ISSN 1007-9327 (print) ISSN 2219-2840 (online)

META-ANALYSIS

# Prognostic value of circulating tumor cells in esophageal cancer

Hai-Tao Xu, Jing Miao, Jian-Wei Liu, Lian-Guo Zhang, Qing-Guang Zhang

Hai-Tao Xu, Jian-Wei Liu, Lian-Guo Zhang, Qing-Guang Zhang, Department of Thoracic Surgery, Affiliated Hospital of Binzhou Medical University, Binzhou 256603, Shandong Province, China

Jing Miao, Department of Neonatal Intensive Care Unit, Binzhou People's Hospital, Binzhou 256603, Shandong Province, China

Author contributions: Xu HT and Miao J contributed equally to this work as co-first authors; Xu HT designed the research, analyzed the data and wrote the manuscript; Miao J designed the research, analyzed the data and wrote the manuscript; Liu JW performed the research and contributed analytical tools; Zhang LG performed the research; Zhang QG conceived and designed the research, and revised the manuscript as corresponding author.

Conflict-of-interest statement: The authors declare that this is no conflict of interest related to this study.

Data sharing statement: No additional data are available.



Manuscript source: Unsolicited manuscript

Correspondence to: Qing-Guang Zhang, MD, Department of Thoracic Surgery, Affiliated Hospital of Binzhou Medical University, No. 661, Huanghe Erlu, Binzhou 256603, Shandong Province, China. drzhangqingg@126.com
Telephone: +86-543-3258731
Fax: +86-543-3257792

Received: December 19, 2016
Peer-review started: December 21, 2016
First decision: January 10, 2017
Revised: January 20, 2017
Accepted: February 7, 2017

Article in press: February 8, 2017
Published online: February 21, 2017

## Abstract

***AIM***

To perform a meta-analysis of the related studies to assess whether circulating tumor cells (CTCs) can be used as a prognostic marker of esophageal cancer.

***METHODS***

PubMed, Embase, Cochrane Library and references in relevant studies were searched to assess the prognostic relevance of CTCs in patients with esophageal cancer. The primary outcome assessed was overall survival (OS). The meta-analysis was performed using the random effects model, with hazard ratio (HR), risk ratio (RR) and 95% confidence intervals (95%CIs) as effect measures.

***RESULTS***

Nine eligible studies were included involving a total of 911 esophageal cancer patients. Overall analyses revealed that CTCs-positivity predicted disease progression (HR = 2.77, 95%CI: 1.75-4.40, $P < 0.0001$) and reduced OS (HR = 2.67, 95%CI: 1.99-3.58, $P < 0.00001$). Further subgroup analyses demonstrated that CTCs-positive patients also had poor OS in different subsets. Moreover, CTCs-positivity was also significantly associated with TNM stage (RR = 1.48, 95%CI: 1.07-2.06, $P = 0.02$) and T stage (RR = 1.44, 95%CI: 1.13-1.84, $P = 0.003$) in esophageal cancer.

***CONCLUSION***

Detection of CTCs at baseline indicates poor prognosis in patients with esophageal cancer. However, this finding relies on data from observational studies and is potentially subject to selection bias. Prospective trials are warranted.

**Key words:** Circulating tumor cells; Esophageal cancer; Prognosis; Meta-analysis



**Core tip:** The clinical validity of circulating tumor cells (CTCs) is still controversial and inconclusive in patients with esophageal cancer. Our meta-analysis provides strong evidence that detection of CTCs in peripheral blood at baseline is an independent prognosticator of poor survival outcomes in esophageal cancer patients.

Xu HT, Miao J, Liu JW, Zhang LG, Zhang QG. Prognostic value of circulating tumor cells in esophageal cancer. *World J Gastroenterol* 2017; 23(7): 1310-1318 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1310.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1310

## INTRODUCTION

Esophageal cancer (EC) is the eighth most common malignant tumor worldwide[1] and is the sixth leading cause of cancer death[2]. However, the early diagnosis of EC is difficult due to the lack of specific symptoms in the early stages. In most cases, the disease is already at an advanced stage at presentation. EC ranks fourth among cancer-related deaths in China[3]. Surgical resection is the main treatment for EC, with a postoperative 5-year survival rate of only 34%-36%[2]. New treatment strategies including neoadjuvant radiochemotherapy[4,5], preoperative neoadjuvant chemotherapy[6,7], and three-field lymph node dissection[8] are helpful for improving the 5-year survival rate of EC patients. However, the outcomes are still unsatisfactory, and many patients die of local recurrence and distant metastasis[9]. Therefore, biomarkers which can be used to identify the recurrence or metastasis of EC are needed to facilitate timely diagnosis and treatment strategies and thus improve the prognosis of EC patients.

In the early stage of EC metastasis or recurrence, the clinical manifestations are occult and cannot be effectively predicted by routine laboratory tests. In recent years, circulating tumor cells (CTCs) have been recognized as the cause of tumor metastasis or recurrence[10,11]. CTCs are the cells that are shed from a primary or metastatic tumor into the peripheral circulation. Most CTCs will be cleared by the human immune system, whereas a small number of surviving CTCs can reach other parts of the body via the bloodstream, resulting in tumor metastasis[12]. CTCs can remain non-proliferative for a long period of time and can resist the anti-tumor effect of chemotherapy drugs[13,14]. At present, many studies on the correlations between CTC positivity and the prognoses of breast cancer[15], colorectal cancer[16], gastric cancer[17], and lung cancer[18] have shown that CTCs-positivity can indicate poor prognosis.

Although many studies have demonstrated the relationship between CTCs and the prognosis and clinicopathological features of EC, their findings had certain limitations due to differences in CTC detection methods and EC treatment strategies. In addition, the role of CTC detection before surgical or non-surgical treatment in EC patients remains unclear. Therefore, in this meta-analysis, we summarized and analyzed the prognostic value of CTCs in EC patients before and after treatment in a quantitative and comprehensive manner.

## MATERIALS AND METHODS

### *Literature search and selection criteria*

A literature search of related studies was conducted using the PubMed, Embase, and Cochrane Library databases. The following search terms were used: (1) "circulating tumor cells" or "CTCs"; (2) "esophageal cancer" using MeSH or free words; and (3) a combination of (1) and (2). The last search was conducted on November 17, 2016.

Two authors, Xu HT and Miao J, independently retrieved the titles and abstracts of the primary studies identified in the electronic search. In addition, references of potentially relevant studies were examined. Duplicate studies were excluded.

The inclusion criteria were as follows: (1) population: patients with esophageal cancer; (2) intervention: CTCs-positivity; (3) comparison: CTCs-negativity; (4) outcome: the primary outcome assessed was overall survival (OS), and clinicopathological characteristics and other prognostic outcomes were assessed as secondary outcomes; (5) design: randomized controlled trials (RCTs) or observational studies; (6) samples used in these studies should be collected from peripheral blood (PB) and at baseline; and (7) sufficient data to calculate hazard ratio (HR) or risk ratio (RR) with 95% confidence intervals (95%CIs) as comparable effect estimates.

Exclusion criteria included the following: (1) review articles, letters, comments and case reports; and (2) studies where it was impossible to retrieve or calculate data of interest.

### *Data extraction and quality assessment*

Data extraction was performed by Xu HT and Miao J independently. The following information was extracted from each study: (1) first author, year of publication, country and study type; (2) number and characteristics of patients in both the CTCs positivity and negativity groups; and (3) outcome data including follow-up period, OS and other prognostic outcomes such as disease-free survival (DFS) or progression-free survival (PFS) or relapse-free survival (RFS).

All relevant texts, tables and figures were reviewed for data extraction, and entered into an Excel file.

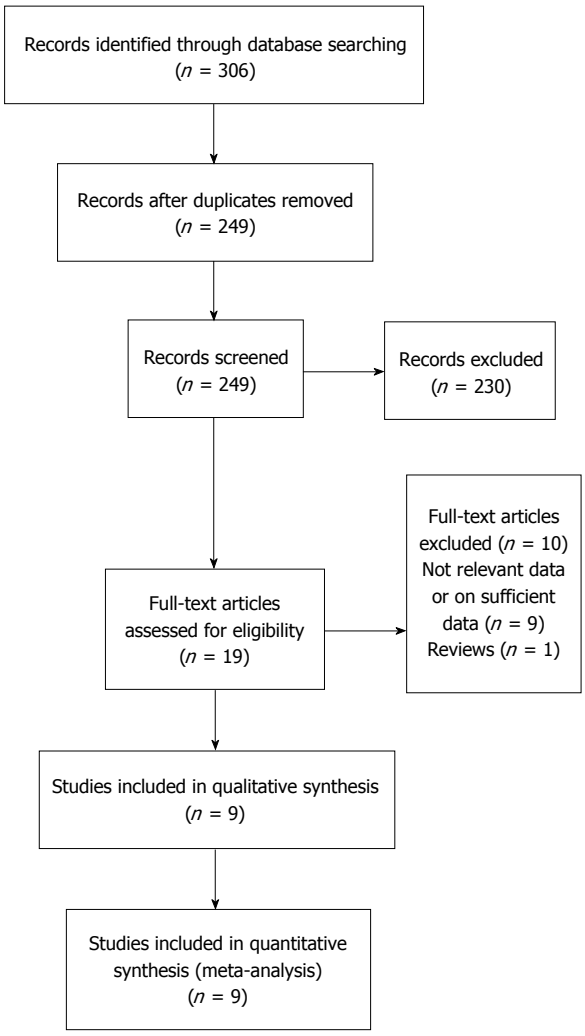



**Figure 1 Selection process for studies included in the meta-analysis.**

Furthermore, we included only the most recent or complete studies to avoid duplication of information. Discrepancies between the two reviewers were resolved by discussion and consensus.

The Cochrane risk of bias tool was adopted to assess the risk of bias in RCTs[19]. Observational studies were evaluated using the Newcastle-Ottawa Scale[20].

### Statistical analysis

Analyses were performed using Cochrane RevMan 5.3. For time-to-event data, HR and 95%CIs were obtained directly or indirectly from studies according to the method described by Tierney[21]. RR was used to compare dichotomous variables. Heterogeneity was tested using the $I^2$ statistic. Studies with an $I^2$ statistic of 0%, 25%, 50% and 75% represented no, low, moderate and high heterogeneity[22,23]. The random effect models were used for the analysis, as this model can obtain more conservative results and better fit the multi-center clinical studies due to the existence of heterogeneity[24]. The Generic Inverse Variance method was used to calculate pooled HRs and 95%CIs. The Mantel-Haenszel method was used to calculate pooled RRs and 95%CIs.

Moreover, a sensitivity analysis was conducted by deleting each study individually to evaluate the quality and consistency of the results. Visual inspection of the funnel plot was carried out to assess publication bias.

In addition, subgroup analyses of the studies were conducted according to region (Asia *vs* non-Asia), curative method (surgery *vs* non-surgery), and method used to detect CTCs (CellSearch *vs* RT-PCR *vs* other methods). The subgroup analyses were performed only for OS.

## RESULTS

### Study selection

A total of 306 studies were identified from the initial database search. Fifty-seven studies were excluded due to duplicates, and 230 studies were excluded for various reasons based on the titles and abstracts (reviews, case reports, or clearly irrelevant to the analysis). Ten studies were excluded due to reviews or the lack of an outcome of interest. In total, 9 studies were included in the meta-analysis[25-33]. The selection process is shown in Figure 1.

### Study characteristics

The main characteristics of the included studies are shown in Table 1. The studies were conducted in three countries (China, Japan and Germany) and published between 2009 and 2016. Of the included studies, none were RCTs, 5 were prospective cohort studies[25,27-29,31], and 4 were cohort studies[25,30,32,33]. The sample size ranged from 38 to 244 (CTCs-positive group, $n$ = 336; CTCs-negative group, $n$ = 575). All studies assessed CTCs at baseline. Of the 9 studies, 7 studies contained data on clinicopathological characteristics[26-31,33], 8 had HRs for OS[25-29,31-33], 2 had HRs for PFS[25,30], 2 had HRs for DFS[26,31], and 2 had HRs for RFS[27,33].

### Quality assessment

Assessment of risk of bias in the studies is shown in Table 2. Based on the Newcastle-Ottawa Scale to assess the risk of bias in cohort studies, 7 studies were rated as having a total score of > 5[25-29,31,32], and 2 as having a score of ⩽ 5, indicating a high risk of bias[30,33].

### Correlation between CTCs and OS

The HRs for OS were available in 8 studies[25-29,31-33], including 779 EC patients. The pooled results showed that CTCs-positive EC patients had significantly poorer OS than CTCs-negative patients (HR = 2.67, 95%CI: 1.99-3.58, $P$ < 0.00001), and heterogeneity was statistically nonsignificant ($I^2$ = 14%, $P$ = 0.32) (Figure 2).

We performed subgroup analyses to further assess whether CTCs status had prognostic value in different subsets (Table 3). We first evaluated the effects of CTCs status on OS regarding region and found that

**Table 1 Characteristics of the included studies**

| Ref. | Country | Study design | Number (M/F) | Age, yr (range) | Population | Detection method | Sample time | Rate% (n/N)[1] | Cut-off criteria | Curative method | Follow-up | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Li *et al*[26], 2016 | China | Cohort | 140 (117/23) | 62.8 ± 8.5 (36-78) | ESCC | Fluorescent IHC | Baseline | 44 (62/140) | > 2/5 mL | Surgery | 3 yr | OS, DFS |
| Su *et al*[25], 2016 | China | Prospective cohort | 57 (55/2) | 54 (36-78) | EC | Flow cytometry | Baseline | 50 (29/57) | ⩾ 21/mL | CCRT | 3 yr | OS, PFS |
| Reeh *et al*[27], 2015 | Germany | Prospective cohort | 100 (77/23) | 66 (32-85) | EC | CellSearch | Baseline | 18 (18/100) | ⩾ 1/7.5 mL | Surgery | 37.5 mo (median) | OS, RFS |
| Matsushita *et al*[28], 2015 | Japan | Prospective cohort | 90 (78/12) | 65 (46-98) | ESCC | CellSearch | Baseline and after treatment | 27 (25/90) | ⩾ 1/7.5 mL | Chemotherapy or CRT | 10.3 mo (median), range 0.3-36.4 mo | OS |
| Tanaka *et al*[29], 2015 | Japan | Prospective cohort | 38 (30/8) | 63 (43-87) | EC | CellSearch | Baseline and after treatment | 50 (19/38) | ⩾ 2/7.5 mL | Chemotherapy or CRT | 19 mo (median) | OS |
| Yin *et al*[30], 2012 | China | Cohort | 72 (54/18) | 63 (46-83) | ESCC | RT-PCR | Baseline and after treatment | 69 (50/72) | Expression of any one of CEA, CK19, survivin | Radiotherapy | 2 yr | PFS |
| Tanaka *et al*[31], 2010 | Japan | Prospective cohort | 244 (212/32) | 64 (NR) | ESCC | RT-PCR | Baseline and after treatment | 13 (34/244) | Expression of any one of CEA, SCCA | Surgery | 24.3 mo (median) | OS, DFS |
| Hoffmann *et al*[32], 2010 | Germany | Cohort | 62 (53/9) | 61 (NR) | EC | RT-PCR | Baseline | 77 (48/62) | Expression of survivin | Surgery | 3 yr (median) | OS |
| Gao *et al*[33], 2009[2] | China | Cohort | 108 (85/23) | 58.9 (36-82) | ESCC | RT-PCR | Baseline | 47 (51/108) | Expression of survivin | Surgery | 19.5 mo (median), range, 1-33 mo | OS, RFS |

[1]Positive CTCs at baseline; [2]Only 48 patients were available for follow-up. M/F: Male/Female; ESCC: Esophageal squamous cell carcinoma; EC: Esophageal cancer; IHC: Immunohistochemistry; CCRT: Concurrent chemoradiotherapy; CRT: Chemoradiotherapy; OS: Overall survival; DFS: Disease-free survival; PFS: Progression-free survival; RFS: Relapse-free survival; CEA: Carcinoembryonic antigen; CK19: Cytokeratin 19; SCCA: Squamous cell carcinoma antigen.

for both Asians and non-Asians, detection of CTCs predicted a poor prognosis (Asian: HR = 2.46, 95%CI: 1.77-3.40, $I^2$ = 14%, $P$ < 0.00001; non-Asian: HR = 3.74, 95%CI: 1.98-7.05, $I^2$ = 0%, $P$ < 0.0001). We then determined the effects of CTCs status on OS with regard to curative method and discovered that for both surgery and non-surgery, detection of CTCs at baseline indicated an increased risk of poor prognosis (surgery: HR = 2.81, 95%CI: 1.72-4.58, $I^2$ = 50%, $P$ < 0.0001; non-surgery: HR = 2.70, 95%CI: 1.70-4.30, $I^2$ = 0%, $P$ < 0.0001). We also assessed the effects of CTCs status on OS with regard to detection method and found that CTCs detection by CellSearch or RT-PCR or other methods indicated a worse prognosis (CellSearch: HR = 2.91, 95%CI: 1.78-4.74, $I^2$ = 0%, $P$ < 0.0001; RT-PCR: HR = 3.44, 95%CI: 1.42-8.34, $I^2$ = 70%, $P$ = 0.006; other methods: HR = 2.22, 95%CI: 1.38-3.58, $I^2$ = 0%, $P$ = 0.001). In addition, the stratified results showed that compared to CTCs-negative patients, CTCs-positive patients had a higher risk for poor OS in these subgroups.

The funnel plot did not show obvious asymmetry (Figure 3). Therefore, no significant publication bias was observed.

### *Correlation between CTCs and disease progression (DFS, RFS and PFS)*

The HRs for disease progression (DFS, RFS and PFS) were available in 6 studies[25-27,30,31,33], involving 661 EC patients. The overall analysis revealed that compared with CTCs-negative EC patients, the CTCs-positive patients had a higher risk of disease progression (HR = 2.77, 95%CI: 1.75-4.40, $I^2$ = 55%, $P$ < 0.0001) (Figure 4). Sensitivity analyses confirmed the stability of our results, and indicated that our results were not obviously affected or dominated by a single study.

### *Correlation between CTCs and clinicopathological characteristics*

Six studies reported the relationship between CTCs status and TNM stage[26,27,29-31,33]. Pooled analysis showed that CTCs-positivity in stage Ⅲ and Ⅳ was greater than that in Ⅰ and Ⅱ (RR = 1.48, 95%CI: 1.07-2.06, $I^2$ = 47%, $P$ = 0.02) as shown in Figure 5A. Studies assessed by pooled analysis showed a significant association

**Table 2 Assessment of the risk of bias in each cohort study using the Newcastle-Ottawa scale**

| Study | Selection | | | | Comparability | Outcome | | | Total score |
|---|---|---|---|---|---|---|---|---|---|
| | Exposed cohort | Non-exposed cohort | Ascertainment of exposure | Outcome of interest | | Assessment of outcome | Length of follow-up | Adequacy of follow-up | |
| Li *et al*[26], 2016 | NS | S | S | S | NS | S | S | S | 6 |
| Su *et al*[25], 2016 | S | S | S | S | NS | S | S | S | 7 |
| Reeh *et al*[27], 2015 | S | S | S | S | NS | S | S | S | 7 |
| Matsushita *et al*[28], 2015 | NS | S | S | S | NS | S | S | S | 6 |
| Tanaka *et al*[29], 2015 | S | S | S | S | NS | S | NS | S | 6 |
| Yin *et al*[30], 2012 | NS | S | S | S | NS | S | NS | S | 5 |
| Tanaka *et al*[31], 2010 | NS | S | S | S | NS | S | S | S | 6 |
| Hoffmann *et al*[32], 2010 | S | S | S | S | NS | S | S | S | 7 |
| Gao *et al*[33], 2009 | NS | S | S | S | NS | S | NS | NS | 4 |

A higher overall score corresponds to a lower risk of bias; a score of ⩽ 5 (out of 9) indicates a high risk of bias. S: The study is satisfied the item; NS: The study is not satisfied the item.

**Table 3 Subgroup analyses of the effects of circulating tumor cells on overall survival in esophageal cancer patients**

| | No. of studies | No. of patients | HR (95%CI) | *P* value | Heterogeneity | |
|---|---|---|---|---|---|---|
| | | | | | $I^2$ | *P* value |
| Region | | | | | | |
| Asia | 6 | 637 | 2.46 (1.77-3.40) | < 0.00001 | 14% | 0.33 |
| Non-Asia | 2 | 162 | 3.74 (1.98-7.05) | < 0.0001 | 0% | 0.36 |
| Curative method | | | | | | |
| Surgery | 5 | 594 | 2.81 (1.72-4.58) | < 0.0001 | 50% | 0.09 |
| Non-surgery | 3 | 185 | 2.70 (1.70-4.30) | < 0.0001 | 0% | 0.95 |
| Detection method | | | | | | |
| CellSearch | 3 | 228 | 2.91 (1.78-4.74) | < 0.0001 | 0% | 0.93 |
| RT-PCR | 3 | 354 | 3.44 (1.42-8.34) | 0.006 | 70% | 0.04 |
| Other methods | 2 | 197 | 2.22 (1.38-3.58) | 0.001 | 0% | 0.44 |

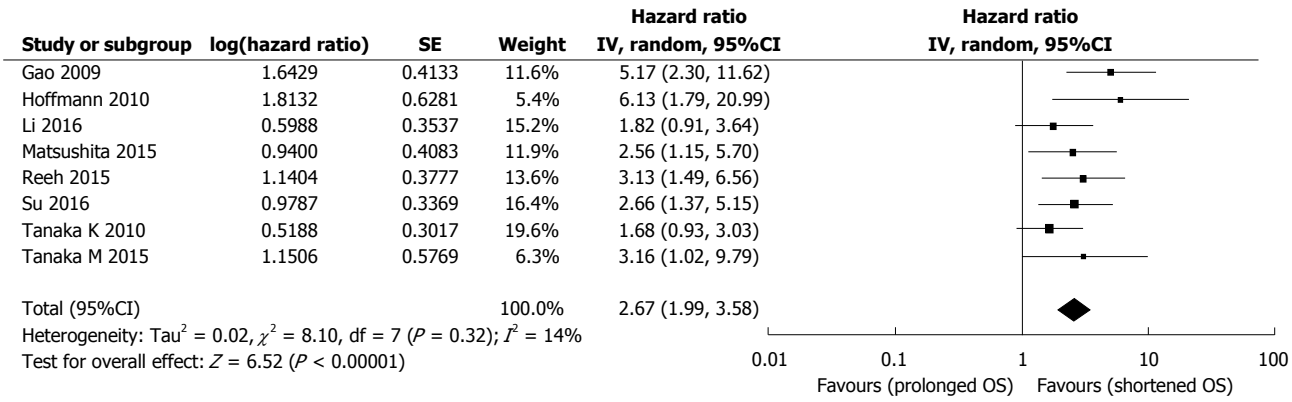



**Figure 2 Forest plots of the hazard ratios for overall survival.** OS: Overall survival; IV: Inverse variance; df: Degrees of freedom.

between CTCs-positivity and T stage (RR = 1.44, 95%CI: 1.13-1.84, $I^2$ = 0%, *P* = 0.003) (Figure 5B)[26-29,31,33], but a non-significant association between CTCs-positivity and histological differentiation (RR = 1.01, 95%CI: 0.79-1.30, $I^2$ = 0%, *P* = 0.93) (Figure 5C)[26,27,30,31]. Six studies assessed the relationship between CTCs-positivity and N stage (RR = 1.47, 95%CI: 1.09-1.98, $I^2$ = 36%, *P* = 0.01) [26-28,30,31,33]. However, when the Yin 2012 study was removed, no statistical significance in the five remaining studies was observed (RR = 1.51, 95%CI: 0.98-2.32, $I^2$ = 48%, *P* = 0.06).

## DISCUSSION

Although radical surgery and neoadjuvant radiochemotherapy have been widely used in EC patients, metastasis or recurrence of EC still poses a significant challenge for doctors and patients. Therefore, biomarkers which can be used to identify the recurrence or metastasis of EC are needed to facilitate the timely diagnosis and treatment strategies for EC patients. CTCs, released by primary tumors, are regarded as a key stage of tumorigenesis[34]; their further development leads to metastatic lesions, which

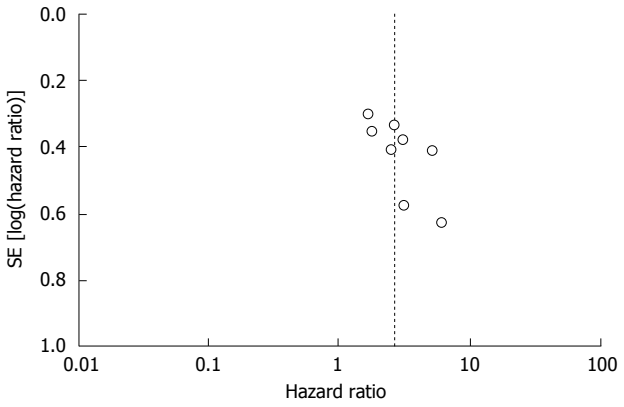



**Figure 3 Funnel plot of the studies on overall survival.**

can be explained by the "seed and soil" theory[35]. It is generally believed that lymph node metastasis occurs prior to blood-borne metastasis; however, the detection of CTCs in early tumors indicates that blood-borne metastasis can occur even in the early stage of tumorigenesis, *i.e.,* before the occurrence of lymph node metastasis[36]. In one study[31], peripheral venous blood CTCs were detected before and after the surgical treatment of EC; it was found that the preoperative positive expression of CTCs was not correlated with prognosis, whereas the postoperative positive expression of CTCs was significantly correlated with prognosis. However, in another study[27], positive CTCs in peripheral venous blood before treatment were thought to be correlated with prognosis. Thus, the value of CTC detection before surgical or non-surgical treatment in EC patients requires further investigation.

In this meta-analysis, we conducted a comprehensive literature search and demonstrated that detection of peripheral venous blood CTCs in EC patients can predict disease progression and poor prognosis. Compared with a previous study[37], in the present study, we included most recent literature and restricted the CTCs detection time to "before treatment". It is well known that EC patients who have received surgical treatment or non-surgical treatment had different prognoses. Our subgroup analysis of EC treatment further showed that detection of CTCs before treatment is valuable for predicting the prognosis of EC patients, and the expression of CTCs was not significantly correlated with treatment mode. In addition, considering the differences in treatment mode and observation time, we combined DFS, RFS, and PFS as "disease progression" and carried out a subgroup analysis accordingly.

Regional differences and differences in treatment modes and CTCs examination methods can also result in clinical heterogeneity; in this regard, we also carried out a subgroup analysis. As shown in our study, there was no significant heterogeneity when the relationship between CTCs status and the OS of EC patients was analyzed, and subgroup analysis also showed stable results. However, heterogeneity was found in the relationship between CTC status and disease progression. The sensitivity analysis showed that the exclusion of any of the articles did not affect the outcome, and the heterogeneity was generated from the difference in the clinical observation time points among different studies. Also, different studies came to different conclusions regarding the relationship between CTC status and clinicopathological factors. Li *et al*[26] and Matsushita *et al*[28] concluded that positive CTC expression was correlated with TNM stage, but not with T stage, N stage, and the degree of differentiation; Reeh *et al*[27] and Tanaka *et al*[31] found that the CTC status was not correlated with TNM stage, T stage, and lymph node metastasis; and other studies[33,38] found that CTC positivity was correlated with N stage. In the present study, we found that CTC status was associated with TNM stage and T stage, but not with lymph node metastasis or degree of differentiation. Because different TNM staging methods were used among studies, and the clinical stages and pathological stages also differed, heterogeneity was inevitable in the present study. The results were unstable during the pooled analysis of the relationship between N stage and CTC status. However, when one article by Yin *et al*[30] 2012 was removed, the research outcome changed, which may be explained by the fact that the N stage in five other articles[26-28,31,33] was the pathological stage, whereas Yin *et al*[30] used clinical N stage.

Our meta-analysis had some limitations. First, potential biases such as gender, age, and race could not be avoided or controlled during the pooled analysis,which contains females are less susceptive to this type of cancer,white man in certain countries are more susceptible and in Asia(specifically in China) the squamous esophageal cancer is detected more than esophageal adenocarcinoma *etc*. Second, although no publication bias was found during analysis of the relationship between CTC positivity and the survival rate of EC patients, all the included articles were published in the English literature, which may have led to the omission of some non-English literature with negative results. In addition, most studies were conducted in Asian countries and areas and hence were less representative. Third, some of the included literature did not explicitly provide HR and 95%CI values, which had to be extracted from the relevant data and curves in the literature. Fourth, the differences in CTC detection methods, EC therapeutic approaches, and EC staging methods could also have affected the judgment of prognosis. Despite these limitations, we still demonstrated the relationships between CTCs and the prognosis and clinicopathological factors of EC.

In conclusion, CTC detection may be a valuable tool for improving the prognosis of EC patients, and it may be possible to carry out individualized therapy based on the results of CTC detection in the future. For postoperative EC patients, CTCs monitoring can provide individualized clinical information during the

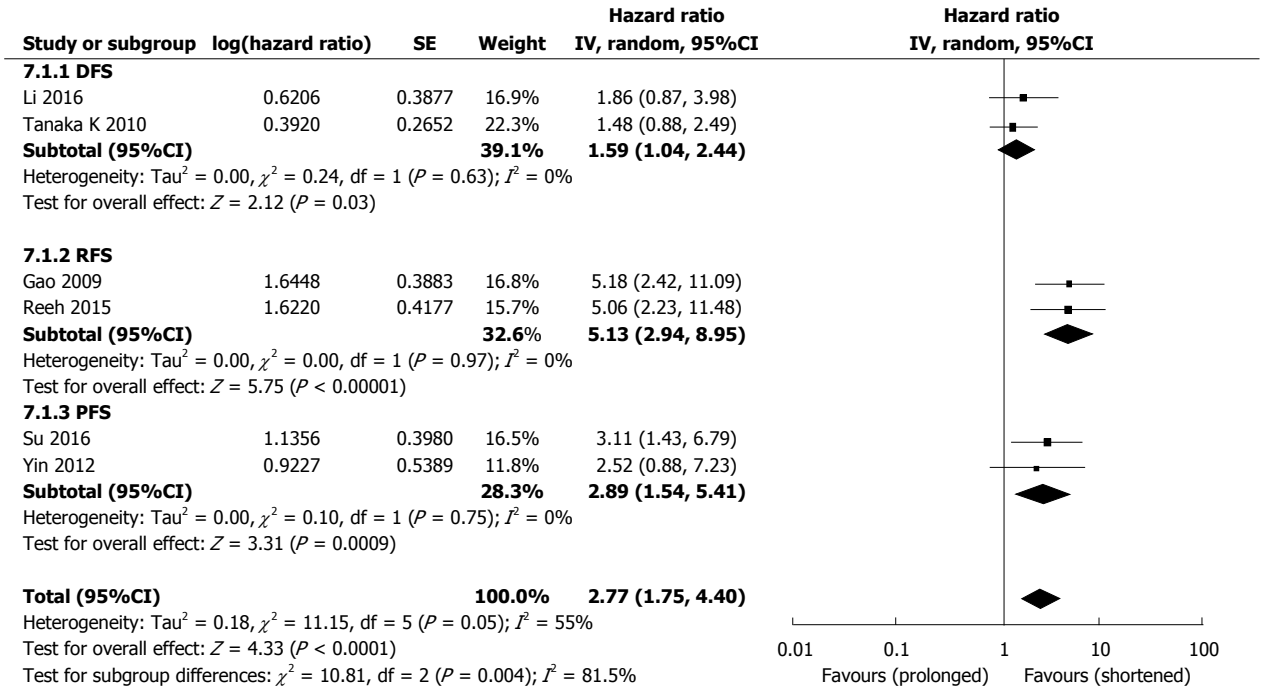



**Figure 4 Forest plots of the hazard ratios for disease progression.** HRs: Hazard Ratios; DFS: Disease-Free Survival; PFS: Progression-Free Survival; RFS: Relapse-Free Survival; IV: Inverse variance; df: degrees of freedom.

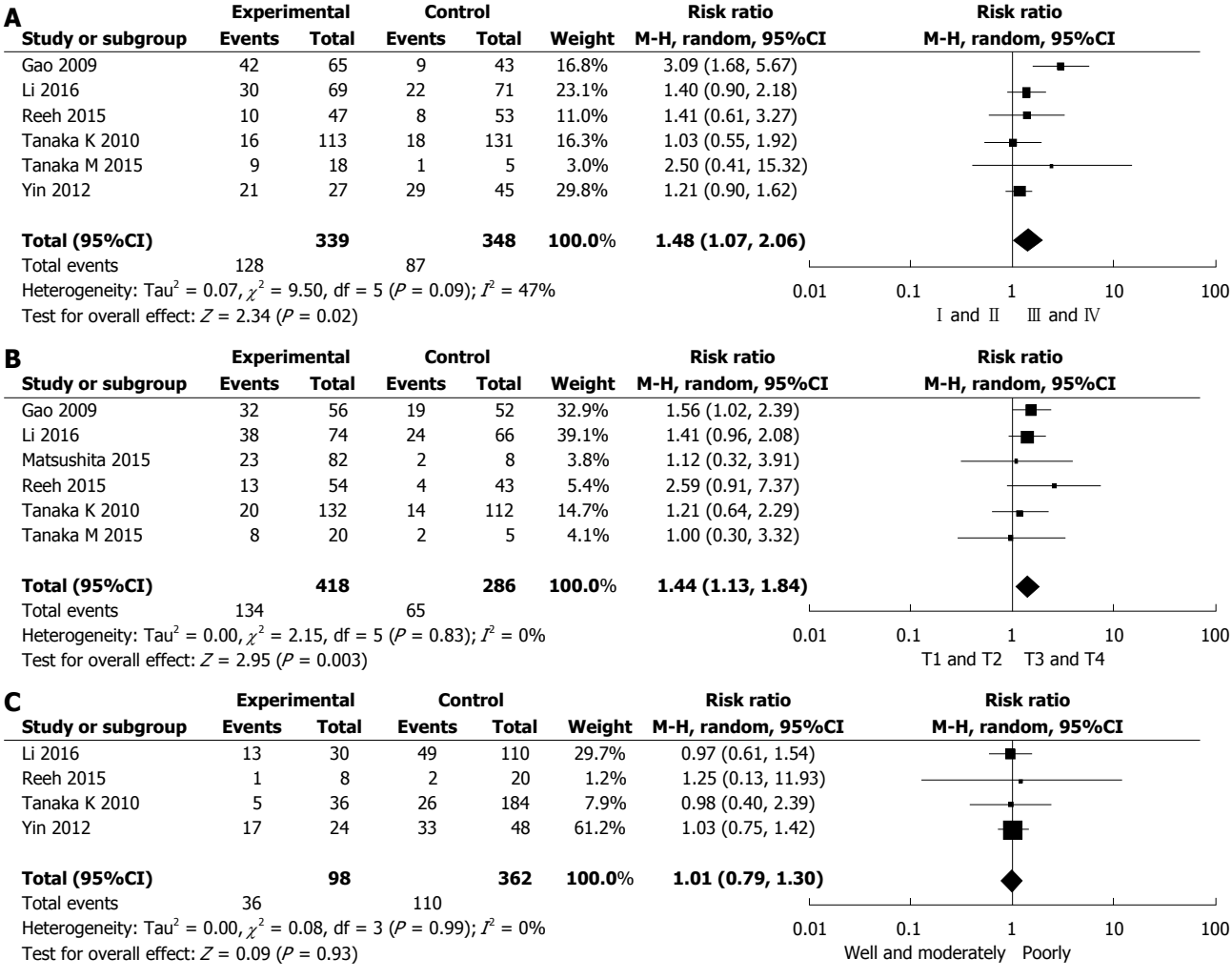



**Figure 5 Forest plots of the RRs for clinicopathological characteristics.** A: TNM stage; B: T stage; C: Histological differentiation. RRs: Risk Rataios; M-H: Mantel-Haenszel; df: Degrees of freedom.

follow-up. For EC patients requiring radiochemotherapy, CTCs monitoring may help identify the risk of tumor progression and enable some patients to benefit from second-line therapy. However, some problems still need to be addressed before CTC detection is applied in clinical settings. First, a variety of methods have been developed for detecting CTCs[39]; therefore, multi-center studies are required to standardize the CTC detection techniques and define CTCs reference values. Second, the CTC detection results may be negative in peripheral blood samples in some EC patients with dominant metastasis[40,41], which may be because the detection markers for metastatic lesions are not expressed or because the expression levels of these markers are below the detection thresholds. Therefore, efforts should be made to optimize the detection platforms for CTCs to improve the sensitivity of CTC detection. Finally, the value of CTC detection in predicting prognosis and the risk of EC recurrence/metastasis still need to be validated in large multi-center clinical trials.

## COMMENTS

### Background

Circulating tumor cells are cells released from the primary tumor into peripheral blood and are considered to be the main cause of tumor metastasis. The prognostic role of circulating tumor cells in esophageal cancer has been widely investigated.

### Research frontiers

The clinical validity of circulating tumor cells is still controversial and inconclusive in patients with esophageal cancer. The aim of this meta-analysis was to include available studies to assess whether circulating tumor cells can be used as a prognostic marker in esophageal cancer.

### Innovations and breakthroughs

This meta-analysis provides strong evidence to indicate that detection of circulating tumor cells in peripheral blood at baseline is an independent prognosticator of poor survival outcomes in esophageal cancer patients. More convincing results will be obtained by increasing the sample size.

### Applications

Circulating tumor cells detected in peripheral blood can predict aggressive disease progression and poor overall survival in patients with esophageal cancer.

### Peer-review

It is a well written article. Statistical analysis is comprehensive and well presented. The paper will attract attention of clinical scientists and surgeons in the field of esophageal cancer worldwide.

## REFERENCES


1 **Torre LA**, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87-108 [PMID: 25651787 DOI: 10.3322/caac.21262]

2 **Pennathur A**, Gibson MK, Jobe BA, Luketich JD. Oesophageal carcinoma. *Lancet* 2013; **381**: 400-412 [PMID: 23374478 DOI: 10.1016/S0140-6736(12)60643-6]

3 **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]

4 **van Hagen P**, Hulshof MC, van Lanschot JJ, Steyerberg EW, van Berge Henegouwen MI, Wijnhoven BP, Richel DJ, Nieuwenhuijzen GA, Hospers GA, Bonenkamp JJ, Cuesta MA, Blaisse RJ, Busch OR, ten Kate FJ, Creemers GJ, Punt CJ, Plukker JT, Verheul HM, Spillenaar Bilgen EJ, van Dekken H, van der Sangen MJ, Rozema T, Biermann K, Beukema JC, Piet AH, van Rij CM, Reinders JG, Tilanus HW, van der Gaast A. Preoperative chemoradiotherapy for esophageal or junctional cancer. *N Engl J Med* 2012; **366**: 2074-2084 [PMID: 22646630 DOI: 10.1056/NEJMoa1112088]

5 **Shapiro J**, van Lanschot JJ, Hulshof MC, van Hagen P, van Berge Henegouwen MI, Wijnhoven BP, van Laarhoven HW, Nieuwenhuijzen GA, Hospers GA, Bonenkamp JJ, Cuesta MA, Blaisse RJ, Busch OR, Ten Kate FJ, Creemers GJ, Punt CJ, Plukker JT, Verheul HM, Bilgen EJ, van Dekken H, van der Sangen MJ, Rozema T, Biermann K, Beukema JC, Piet AH, van Rij CM, Reinders JG, Tilanus HW, Steyerberg EW, van der Gaast A. Neoadjuvant chemoradiotherapy plus surgery versus surgery alone for oesophageal or junctional cancer (CROSS): long-term results of a randomised controlled trial. *Lancet Oncol* 2015; **16**: 1090-1098 [PMID: 26254683 DOI: 10.1016/S1470-2045(15)00040-6]

6 **Cunningham D**, Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, Scarffe JH, Lofts FJ, Falk SJ, Iveson TJ, Smith DB, Langley RE, Verma M, Weeden S, Chua YJ. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. *N Engl J Med* 2006; **355**: 11-20 [PMID: 16822992 DOI: 10.1056/NEJMoa055531]

7 **van der Sluis PC**, Ubink I, van der Horst S, Boonstra JJ, Voest EE, Ruurda JP, Borel Rinkes IH, Wiezer MJ, Schipper ME, Siersema PD, Los M, Lolkema MP, van Hillegersberg R. Safety, efficacy, and long-term follow-up evaluation of perioperative epirubicin, Cisplatin, and capecitabine chemotherapy in esophageal resection for adenocarcinoma. *Ann Surg Oncol* 2015; **22**: 1555-1563 [PMID: 25564156 DOI: 10.1245/s10434-014-4120-9]

8 **Ma GW**, Situ DR, Ma QL, Long H, Zhang LJ, Lin P, Rong TH. Three-field vs two-field lymph node dissection for esophageal cancer: a meta-analysis. *World J Gastroenterol* 2014; **20**: 18022-18030 [PMID: 25548502 DOI: 10.3748/wjg.v20.i47.18022]

9 **Zhu ZJ**, Hu Y, Zhao YF, Chen XZ, Chen LQ, Chen YT. Early recurrence and death after esophagectomy in patients with esophageal squamous cell carcinoma. *Ann Thorac Surg* 2011; **91**: 1502-1508 [PMID: 21354552 DOI: 10.1016/j.athoracsur.2011.01.007]

10 **Izbicki JR**, Hosch SB, Pichlmeier U, Rehders A, Busch C, Niendorf A, Passlick B, Broelsch CE, Pantel K. Prognostic value of immunohistochemically identifiable tumor cells in lymph nodes of patients with completely resected esophageal cancer. *N Engl J Med* 1997; **337**: 1188-1194 [PMID: 9337377 DOI: 10.1056/NEJM199710233371702]

11 **Sclafani F**, Smyth E, Cunningham D, Chau I, Turner A, Watkins D. A pilot study assessing the incidence and clinical significance of circulating tumor cells in esophagogastric cancers. *Clin Colorectal Cancer* 2014; **13**: 94-99 [PMID: 24332356 DOI: 10.1016/j.clcc.2013.11.003]

12 **Peeters DJ**, Brouwer A, Van den Eynden GG, Rutten A, Onstenk W, Sieuwerts AM, Van Laere SJ, Huget P, Pauwels P, Peeters M, Vermeulen PB, Dirix LY. Circulating tumour cells and lung microvascular tumour cell retention in patients with metastatic breast and cervical cancer. *Cancer Lett* 2015; **356**: 872-879 [PMID: 25449778 DOI: 10.1016/j.canlet.2014.10.039]

13 **Dean M**, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer* 2005; **5**: 275-284 [PMID: 15803154 DOI: 10.1038/nrc1590]

14 **Abdallah EA**, Fanelli MF, Buim ME, Machado Netto MC, Gasparini Junior JL, Souza E Silva V, Dettino AL, Mingues NB, Romero JV, Ocea LM, Rocha BM, Alves VS, Araújo DV, Chinen LT. Thymidylate synthase expression in circulating tumor cells: a new tool to predict 5-fluorouracil resistance in metastatic colorectal cancer patients. *Int J Cancer* 2015; **137**: 1397-1405 [PMID: 25721610 DOI: 10.1002/ijc.29495]

15 **Lu YJ**, Wang P, Wang X, Peng J, Zhu YW, Shen N. The significant prognostic value of circulating tumor cells in triple-negative breast

cancer: a meta-analysis. *Oncotarget* 2016; **7**: 37361-37369 [PMID: 27008698 DOI: 10.18632/oncotarget.8156]

16 **Rahbari NN**, Aigner M, Thorlund K, Mollberg N, Motschall E, Jensen K, Diener MK, Büchler MW, Koch M, Weitz J. Meta-analysis shows that detection of circulating tumor cells indicates poor prognosis in patients with colorectal cancer. *Gastroenterology* 2010; **138**: 1714-1726 [PMID: 20100481 DOI: 10.1053/j.gastro.2010.01.008]

17 **Huang X**, Gao P, Sun J, Chen X, Song Y, Zhao J, Xu H, Wang Z. Clinicopathological and prognostic significance of circulating tumor cells in patients with gastric cancer: a meta-analysis. *Int J Cancer* 2015; **136**: 21-33 [PMID: 24803400 DOI: 10.1002/ijc.28954]

18 **Wang J**, Wang K, Xu J, Huang J, Zhang T. Prognostic significance of circulating tumor cells in non-small-cell lung cancer patients: a meta-analysis. *PLoS One* 2013; **8**: e78070 [PMID: 24223761 DOI: 10.1371/journal.pone.0078070]

19 **Higgins JP**, Altman DG, Gøtzsche PC, Jüni P, Moher D, Oxman AD, Savovic J, Schulz KF, Weeks L, Sterne JA. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. *BMJ* 2011; **343**: d5928 [PMID: 22008217 DOI: 10.1136/bmj.d5928]

20 **Wells GA**, Shea B, O'Connell D, Peterson J, Wekch V, Losos M, Tugwell P. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analysis. Ottawa Hospital Research Institute website. Available from: URL: http: //www.ohri.ca/programs/clinical_epidemiology/oxford.asp.

21 **Tierney JF**, Stewart LA, Ghersi D, Burdett S, Sydes MR. Practical methods for incorporating summary time-to-event data into meta-analysis. *Trials* 2007; **8**: 16 [PMID: 17555582 DOI: 10.1186/1745-6215-8-16]

22 **Higgins JP**, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. *BMJ* 2003; **327**: 557-560 [PMID: 12958120 DOI: 10.1136/bmj.327.7414.557]

23 **Gu WJ**, Wang F, Tang L, Liu JC. Single-dose etomidate does not increase mortality in patients with sepsis: a systematic review and meta-analysis of randomized controlled trials and observational studies. *Chest* 2015; **147**: 335-346 [PMID: 25255427 DOI: 10.1378/chest.14-1012]

24 **Schmidt FL**, Oh IS, Hayes TL. Fixed- versus random-effects models in meta-analysis: model properties and an empirical comparison of differences in results. *Br J Math Stat Psychol* 2009; **62**: 97-128 [PMID: 18001516 DOI: 10.1348/000711007X255327]

25 **Su PJ**, Wu MH, Wang HM, Lee CL, Huang WK, Wu CE, Chang HK, Chao YK, Tseng CK, Chiu TK, Lin NM, Ye SR, Lee JY, Hsieh CH. Circulating Tumour Cells as an Independent Prognostic Factor in Patients with Advanced Oesophageal Squamous Cell Carcinoma Undergoing Chemoradiotherapy. *Sci Rep* 2016; **6**: 31423 [PMID: 27530152 DOI: 10.1038/srep31423]

26 **Li SP**, Guan QL, Zhao D, Pei GJ, Su HX, Du LN, He JX, Liu ZC. Detection of Circulating Tumor Cells by Fluorescent Immunohistochemistry in Patients with Esophageal Squamous Cell Carcinoma: Potential Clinical Applications. *Med Sci Monit* 2016; **22**: 1654-1662 [PMID: 27184872 DOI: 10.12659/MSM.898335]

27 **Reeh M**, Effenberger KE, Koenig AM, Riethdorf S, Eichstädt D, Vettorazzi E, Uzunoglu FG, Vashist YK, Izbicki JR, Pantel K, Bockhorn M. Circulating Tumor Cells as a Biomarker for Preoperative Prognostic Staging in Patients With Esophageal Cancer. *Ann Surg* 2015; **261**: 1124-1130 [PMID: 25607767 DOI: 10.1097/SLA.0000000000001130]

28 **Matsushita D**, Uenosono Y, Arigami T, Yanagita S, Nishizono Y, Hagihara T, Hirata M, Haraguchi N, Arima H, Kijima Y, Kurahara H, Maemura K, Okumura H, Ishigami S, Natsugoe S. Clinical Significance of Circulating Tumor Cells in Peripheral Blood of Patients with Esophageal Squamous Cell Carcinoma. *Ann Surg Oncol* 2015; **22**: 3674-3680 [PMID: 25652049 DOI: 10.1245/s10434-015-4392-8]

29 **Tanaka M**, Takeuchi H, Osaki Y, Hiraiwa K, Nakamura R, Oyama T, Takahashi T, Wada N, Kawakubo H, Saikawa Y, Omori T, Kitagawa Y. Prognostic significance of circulating tumor cells in patients with advanced esophageal cancer. *Esophagus* 2015; **12**: 352-359 [DOI: 10.1007/s10388-014-0482-0]

30 **Yin XD**, Yuan X, Xue JJ, Wang R, Zhang ZR, Tong JD. Clinical significance of carcinoembryonic antigen-, cytokeratin 19-, or survivin-positive circulating tumor cells in the peripheral blood of esophageal squamous cell carcinoma patients treated with radiotherapy. *Dis Esophagus* 2012; **25**: 750-756 [PMID: 22394149 DOI: 10.1111/j.1442-2050.2012.01326.x]

31 **Tanaka K**, Yano M, Motoori M, Kishi K, Miyashiro I, Shingai T, Gotoh K, Noura S, Takahashi H, Ohue M, Yamada T, Ohigashi H, Yamamoto T, Yamasaki T, Doki Y, Ishikawa O. CEA-antigen and SCC-antigen mRNA expression in peripheral blood predict hematogenous recurrence after resection in patients with esophageal cancer. *Ann Surg Oncol* 2010; **17**: 2779-2786 [PMID: 20411433 DOI: 10.1245/s10434-010-1075-3]

32 **Hoffmann AC**, Vallböhmer D, Grimminger P, Metzger R, Prenzel KL, Hoelscher AH, Brabender J. Preoperative survivin mRNA detection in peripheral blood is an independent predictor of outcome in esophageal carcinoma. *Pharmacogenomics* 2010; **11**: 341-347 [PMID: 20235790 DOI: 10.2217/pgs.09.164]

33 **Cao M**, Yie SM, Wu SM, Chen S, Lou B, He X, Ye SR, Xie K, Rao L, Gao E, Ye NY. Detection of survivin-expressing circulating cancer cells in the peripheral blood of patients with esophageal squamous cell carcinoma and its clinical significance. *Clin Exp Metastasis* 2009; **26**: 751-758 [PMID: 19521785 DOI: 10.1007/s10585-009-9274-7]

34 **Chaffer CL**, Weinberg RA. A perspective on cancer cell metastasis. *Science* 2011; **331**: 1559-1564 [PMID: 21436443 DOI: 10.1126/science.1203543]

35 **Fidler IJ**, Kripke ML. The challenge of targeting metastasis. *Cancer Metastasis Rev* 2015; **34**: 635-641 [PMID: 26328524 DOI: 10.1007/s10555-015-9586-9]

36 **Klein CA**. Parallel progression of primary tumours and metastases. *Nat Rev Cancer* 2009; **9**: 302-312 [PMID: 19308069 DOI: 10.1038/nrc2627]

37 **Qiao GL**, Qi WX, Jiang WH, Chen Y, Ma LJ. Prognostic significance of circulating tumor cells in esophageal carcinoma: a meta-analysis. *Onco Targets Ther* 2016; **9**: 1889-1897 [PMID: 27099520 DOI: 10.2147/OTT.S100005]

38 **Kaganoi J**, Shimada Y, Kano M, Okumura T, Watanabe G, Imamura M. Detection of circulating oesophageal squamous cancer cells in peripheral blood and its impact on prognosis. *Br J Surg* 2004; **91**: 1055-1060 [PMID: 15286970 DOI: 10.1002/bjs.4593]

39 **Bobek V**, Matkowski R, Gürlich R, Grabowski K, Szelachowska J, Lischke R, Schützner J, Harustiak T, Pazdro A, Rzechonek A, Kolostova K. Cultivation of circulating tumor cells in esophageal cancer. *Folia Histochem Cytobiol* 2014; **52**: 171-177 [PMID: 25308732 DOI: 10.5603/FHC.2014.0020]

40 **Yamaguchi T**, Okumura T, Hirano K, Watanabe T, Nagata T, Shimada Y, Tsukada K. Detection of circulating tumor cells by p75NTR expression in patients with esophageal cancer. *World J Surg Oncol* 2016; **14**: 40 [PMID: 26897248 DOI: 10.1186/s12957-016-0793-9]

41 **Li H**, Song P, Zou B, Liu M, Cui K, Zhou P, Li S, Zhang B. Circulating Tumor Cell Analyses in Patients With Esophageal Squamous Cell Carcinoma Using Epithelial Marker-Dependent and -Independent Approaches. *Medicine* (Baltimore) 2015; **94**: e1565 [PMID: 26402816 DOI: 10.1097/MD.0000000000001565]

**P- Reviewer**: Nabissi M, Sukocheva OA, Vinh-Hung V
**S- Editor**: Qi Y **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/

*World J Gastroenterol* 2017 February 21; 23(7): 1319-1324

DOI: 10.3748/wjg.v23.i7.1319

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

CASE REPORT

# Clinical and immunologic effects of faecal microbiota transplantation in a patient with collagenous colitis

Sezin Günaltay, Lech Rademacher, Elisabeth Hultgren Hörnquist, Johan Bohr

Sezin Günaltay, Elisabeth Hultgren Hörnquist, Johan Bohr, Faculty of Medicine and Health, School of Medical Sciences, Örebro University, SE-70185 Örebro, Sweden

Lech Rademacher, Johan Bohr, Division of Gastroenterology, Department of Medicine, Örebro University Hospital, SE-70185 Örebro, Sweden

Lech Rademacher, Department of Medicine, Avesta Hospital, SE-774 82 Avesta, Sweden

Author contributions: Bohr J performed the first two fecal microbiota transplantations and collected colonic mucosal biopsies; Rademacher L performed the third; Günaltay S carried out experiments and data analyses; Günaltay S, Rademacher L and Bohr J drafted the manuscript; Günaltay S, Rademacher L, Hultgren Hörnquist E and Bohr J created the study design, coordination, and data analyses; Günaltay S, Rademacher L, Hultgren Hörnquist E and Bohr J finalized the manuscript; all authors read and approved the final manuscript.

Supported by Örebro University Hospital Research Foundation (Nyckelfonden).

Institutional review board statement: This case report was exempt from the Institutional Review Board standards at Örebro University in Örebro, Sweden.

Informed consent statement: The patient was informed of the study protocol before treatment and colonoscopy, and gave her written consent to donate tissue samples for research purposes and undergo treatment with FMT.

Conflict-of-interest statement: The authors declare that there is no conflict of interest regarding the publication of this case report.



Manuscript source: Unsolicited manuscript

Correspondence to: Johan Bohr, MD, PhD, Division of Gastroenterology, Department of Medicine, Örebro University Hospital, SE-70185 Örebro, Sweden. johan.bohr@regionorebrolan.se
Telephone: +46-19-6021000
Fax: +46-19-6021774

Received: September 27, 2016
Peer-review started: September 28, 2016
First decision: October 20, 2016
Revised: November 20, 2016
Accepted: January 2, 2017
Article in press: January 3, 2017
Published online: February 21, 2017

## Abstract

One to six percent of patients with microscopic colitis are refractory to medical treatment. The effect of faecal microbiota transplantation (FMT) in active collagenous colitis (CC) has, to the best of our knowledge, never been reported before. Here, we report the effect of repeated FMT in a patient with CC. The patient presented with severe symptoms including profuse diarrhea and profound weight loss. Although she responded to budesonide in the beginning, she became gradually refractory to medical treatment, and was therefore treated with FMT. The patient remained in remission for 11 mo after the third faecal transplantation. The immunomodulatory effect of the therapy was evaluated using flow cytometry, which showed alterations in the profile of intraepithelial and lamina propria lymphocyte subsets after the second transplantation. Our observations indicate that FMT can have an effect in CC, which support the hypothesis that luminal factors, influencing the intestinal microbiota, are involved in the pathogenesis of CC.

**Key words:** Faecal microbiota transplantation; Microbiota;

Collagenous colitis; Flow cytometry; Lymphocytes



**Core tip:** Collagenous colitis (CC) is characterized by chronic watery diarrhea and inflammation in the colonic mucosa. The treatment is based on budesonide or immunomodulatory treatment in moderate to severe cases. However, some patients do not respond to the treatment. The aim of this article is to report the effect of repeated faecal microbiota transplantation in a CC patient who remained in remission 11 mo after the repeated transplantations, which also caused alterations in the lymphocyte subsets.

Günaltay S, Rademacher L, Hultgren Hörnquist E, Bohr J. Clinical and immunologic effects of faecal microbiota transplantation in a patient with collagenous colitis. *World J Gastroenterol* 2017; 23(7): 1319-1324 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1319.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1319

## INTRODUCTION

Microscopic colitis (MC) is a chronic inflammatory disease of the colon, characterized clinically by chronic watery diarrhea, abdominal pain and weight loss[1]. Based on histopathological features in the colon mucosa, MC comprises primarily two entities; collagenous colitis (CC) and lymphocytic colitis (LC). Both CC and LC are characterized by inflammation in the lamina propria of the colon mucosa. CC, however, is distinguished from LC by a colonic subepithelial collagen band of 10 μm or more in thickness, whereas LC has a more pronounced lymphocyte infiltration in the colonic epithelium[2]. The pathogenesis of MC is not known, but it is thought to be multifactorial, involving a mucosal immune response to luminal factors in genetically predisposed individuals. Defects in the epithelial barrier function may lead to transmucosal passage of antigens and bacteria, and consequently leading to inflammation as seen in MC[3-5]. A role for the colonic microbiota or an invasive microorganism in the disease pathogenesis is supported by the observation that some CC patients clinically improve after treatment with antibiotics of different categories for concurrent infections[6].

The treatment of MC was earlier based on retrospective studies: loperamide, cholestyramine, sulphasalazine and 5-aminosalicylic acid were the standard alternatives[6]. During the last decades, it has become evident that budesonide is a very efficient drug and the drug of choice for MC with a moderate to severe course, alternatively azathioprine in steroid refractory cases[7,8]. However, one to six percent of MC patients are refractory to conventional medical treatment[9], and surgery with an ileostomy has hitherto been the only option in severe medically refractory cases[10].

Faecal microbiota transplantation (FMT) is a method to modify microbiota dysbiosis, and studies have shown that it can have effect in patients with ulcerative colitis, resulting in maintained clinical as well as endoscopic and histopathologic remission[11-14]. For recurrent *Clostridium difficile* infection, FMT has for some decades, been established as an effective treatment[15-18]. Despite microbiota showing dysbiosis in CC patients[19,20], FMT as a possible treatment for CC has not been reported before. We here present a case with medically refractory CC, who responded to FMT.

## CASE REPORT

### *Patient and donor characteristics, procedures and clinical results of FMT*

The patient was a 72 year old female who suffered from frequent watery diarrhea and was diagnosed with CC six months after debut of symptoms in 2008. The diagnosis was based on clinical findings and histopathological evaluation which showed an increased number of lymphocytes in the lamina propria, in the epithelium and in a few crypts. There was a thickened collagenous band subepithelially. The findings were diffusely distributed in the whole colon[2,7]. She had normal biochemical and hematologic parameters, whereas faecal calprotectin levels were increased from 160 mg/kg in 2009 to 500 mg/kg when the CC deteriorated in 2013. Stool cultures were tested for *Clostridium difficile* at diagnosis, before the first and before the second FMT, and were also negative for *Salmonella species, Shigella dysenteriae, Campylobacter jejuni,* and *Yersinia enterocolitica*. Based on histopathologic examination of duodenal biopsies, celiac disease was excluded. Initial treatment with loperamide had only little effect, and cholestyramine, had no effect. The patient responded well to budesonide, and was in remission for 2 years. Gradually, however, the effect of budesonide declined during 2013, and her symptoms worsened with profuse watery diarrhea, severe fecal incontinence, fatigue, and a weight loss of totally 15 kg, though, without biochemical signs of malabsorption. She tried mesalazine without effect, and azathioprine, but did not tolerate it. She had doxycycline in November 2013, 100 mg per day for 10 d as treatment for olecranon bursitis, which resulted in decreased numbers of daily stools, but the effect only lasted one week after the cessation of the antibiotic. She had one additional doxycycline cure in 2014, also with good but temporary antidiarrheal effect. The details for treatments are summarized in Table 1. The patient then learned about the possible effect of FMT in ulcerative colitis, and was interested in trying this treatment. As FMT has not been documented in CC, she was offered this

**Table 1 Summary of the treatment and results in a collagenous colitis patient**

| Treatment | Result of the treatment |
|---|---|
| Loperamide 2-4 mg per day | Little effect |
| Cholestyramine, 4 g, three times per day | No effect |
| Mesalazine 800 mg twice per day for 4 mo | No effect |
| Budesonide capsules 3 mg 1-3 times per day | Good but declining effect after 2 yr of treatment |
| Azathioprine | Side effects (liver toxicity) |
| Doxycycline, 100 mg per day for 10 d, given twice | Good but temporary effect |
| First FMT, November 2014 | General improvement for 2 wk |
| 200 mL filtrate infused as an enema in the rectum for five consecutive mornings | Not fulfilling the criteria for remission[21] |
| Second FMT, March 2015 | General improvement for 4 wk |
| 300 mL filtrate instilled in cecum | Not fulfilling the criteria for remission |
| Third FMT, May 2015 | Remission with normalized stools and normal BMI achieved for 11 mo |
| 300 mL filtrate instilled in cecum | |

Histopathologic findings before the first transplant and after the second transplant showed typical and similar features of CC. CC: Collagenous colitis; BMI: Body mass index; FMT: Faecal microbiota transplantation.

treatment after thorough information about possible side effects. The patient was informed of the study protocol before treatment and colonoscopy, and gave her written consent to donate tissue samples for research purposes and undergo treatment with FMT.

The patient's husband was selected as the donor of faecal microbiota. His feces were screened and found negative for *Salmonella species, Shigella dysenteriae, Campylobacter jejuni, Yersinia enterocolitica* and *Clostridium difficile* by culture. Furthermore, tests for haematology and liver enzymes were negative, as well as blood tests for hepatitis A, B and C and varicella zoster virus. The presence of serum antibodies against Epstein-Barr virus and cytomegalovirus indicated earlier, but no ongoing, infections.

The faecal sample from the donor was obtained 2-3 h before transplantation. Two tablespoons of feces were diluted and mixed in 500 mL 0.9% NaCl. The homogenized solution was filtered twice through a pre-sterilized metal sieve. At the first instillation procedure, 200 mL of the filtrate was infused over 1 h as an enema into the rectum of the patient, for five consecutive mornings according to our standard procedure for *C. difficile* treatment. Since the first FMT did not improve the clinical status of the patient, at the second and third instillation procedures, 300 mL filtrate was infused *via* a colonoscope into the cecum over 10 min (Table 1)[1].

Colonic mucosal biopsies were collected before the first FMT in November 2014 and 1 mo after the second FMT in March 2015. Colonic biopsy specimens for immunological studies were taken from the hepatic flexure, and stored in PBS on ice for a maximum of 20 h until lymphocyte isolation and analysis were done. Routine biopsy specimens were obtained from the proximal, transverse, and distal colon for histopathologic confirmation of the diagnosis. The third FMT, also with cecal instillation, was performed in May 2015.

The patient felt generally better for 2 wk after the first FMT in November 2014, without any change in the number of daily stools. After the second FMT in March 2015, the patient felt an improvement with loose rather than watery stools for one month. The histopathology before the first transplant and after the second transplant showed typical and unaltered features of CC. After the third FMT in May 2015, remission, as defined by Hjortswang *et al*[21], was achieved for 11 mo, with 2 normal stools daily, and a weight gain from 48 till 55 kg. After 11 mo, the patient gradually relapsed, but has been in remission with a medication of budesonide, which did not have any effect before the FMTs. The patient have had no adverse effects from any of the FMTs. The course is summarized in Table 1.

### *Analysis of immunomodulatory effect of FMT using flow cytometry*

The isolation of intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) were performed as described in our previous study[22]. 200000 cells/mL were stained with fluorochrome-conjugated antibodies, and corresponding fluorochrome-conjugated isotype controls were used to eliminate non-specific staining[22]. Surface labeled cells were fixed and permeabilized using the Nuclear Factor Fixation and Permeabilization Buffer Set according to the manufacturer's instructions (Biolegend, San Diego, CA, United States) and thereafter stained with anti-Ki67-PE[22], anti-FoxP3-PE (clone 259D/C7; BD Biosciences) or isotype control (IgG1κ-PE, BD Biosciences). A minimum of 100,000 events was collected on a Coulter Epics Altra flow cytometer (Beckman Coulter, Danvers, MA, United States) and analyzed using Kaluza software v1.3 (Beckman Coulter) based on gated $CD3^+$ lymphocytes. IEL samples were only analyzed for CD3, CD4, and CD8 markers as the number of events obtained were less than 1000 for the rest of the markers[22].

The immunomodulatory effect of FMT was assessed according to immunophenotypes of colonic mucosal T cells before the first and after the second FMTs (Table 2). Although no major changes were observed in the proportions of $CD3^+CD4^+$ and $CD3^+CD8^+$ LPLs after the second FMT, the proportion of $CD4^+$ and $CD8^+$ activated/memory $CD45RO^+$ LPLs were decreased, whereas the proportion of $CD4^+$ and $CD8^+$ naïve $CD45RA^+$ T cells was increased and unchanged, respectively. The proportion of proliferating $CD4^+$ and $CD8^+$ $Ki67^+$ LPLs were increased after the second FMT. The proportion of $CD4^+$ $FoxP3^+$ lamina propria regulatory T cells (Treg) was increased 3.5 times after the second FMT, whereas the proportion of $CD8^+$ $Foxp3^+$ T cells was decreased. The proportion

**Table 2 Percentages of lamina propria and intraepithelial T cell subsets expressing different markers detected by flow cytometry before and after faecal microbiota transplantation**

| Cell phenotype | Before first FMT | After second FMT |
|---|---|---|
| Percentages of lamina propria T cell subsets | | |
| $CD3^+CD4^+$ | 53.0 | 51.1 |
| $CD3^+CD4^+CD45RO^+$ | 37.2 | 29.2 |
| $CD3^+CD4^+CD45RA^+$ | 4.0 | 8.4 |
| $CD3^+CD4^+Ki67^+$ | 1.2 | 2.9 |
| $CD3^+CD4^+Foxp3^+$ | 2.9 | 10.2 |
| $CD3^+CD8^+$ | 40.7 | 38.9 |
| $CD3^+CD8^+CD45RO^+$ | 18.8 | 7.4 |
| $CD3^+CD8^+CD45RA^+$ | 19.5 | 18.1 |
| $CD3^+CD8^+$ $Ki67^+$ | 1.2 | 2.9 |
| $CD3^+CD8^+$ $Foxp3^+$ | 2.9 | 1.4 |
| Percentages of intraepithelial T cell subsets | | |
| $CD3^+CD4^+$ | 13.3 | 22.1 |
| $CD3^+CD8^+$ | 77.9 | 62.4 |

FMT: Faecal microbiota transplantation.

of $CD3^+CD8^+$ IELs was decreased after the second FMT, whereas the proportion of $CD3^+CD4^+$ IELs was increased.

## DISCUSSION

In an earlier study, we showed that diversion of the faecal stream in CC patients led to clinical and histopathological remission indicating a possible role of luminal factors in initiating the inflammation in CC[10]. Our observation showing that FMT can result in clinical improvement of CC, supports the hypothesis that luminal factors, including or influencing the intestinal microbiota, are involved in the pathogenesis of CC. Efficacy of FMT has been shown in ulcerative colitis[11-14] and in recurrent infections with *Clostridium difficile* in the colon[15-17]. Although the pathophysiological mechanisms of FMT are not known in detail, it is shown that FMT can restore a dysbiosis in the gut[23]. The colonic microbiota in CC are reported to be disturbed[19,20], and thus a potential target for modification by FMT. To the best of our knowledge, this is the first work demonstrating beneficial effects of FMT in the clinical management of CC.

The recorded increased proportion of $CD3^+CD8^+$ IELs compared to controls in the patient before FMT is in accordance with our previous studies in CC patients[22,24]. This cell type was decreased after the second FMT indicating a reduction in the excessive cytotoxic activity against microbes[4,25,26]. The increased proportions of $CD3^+CD4^+$ IELs after two FMTs indicate improved tissue repair[27,28], which in turn may contribute to remission. The decreased proportions of $CD4^+$ and $CD8^+$ activated/memory $CD45RO^+$ cells after the second FMT suggest reduced immune responses due to the altered microbiota[29]. The increase in regulatory $Foxp3^+CD4^+$ cells, similarly to our and others' previous studies[24,30], is likely important to ameliorate the ongoing inflammation, whereas the role of $Foxp3^+CD8^+$ T cells in CC pathology remains elusive. Increased proportions of proliferating $Ki67^+$ $CD4^+$ LPLs after the second FMT may be due to the increased proportions of $Foxp3^+$ regulatory T cells.

According to the T lymphocyte subset profile, the immune response in the mucosa was likely still activated, which may partly explain why the patient relapsed and required a third FMT to reach a long-term clinical remission. We did not collect new biopsies after the last FMT due to the risk of cleansing the colon, as the biopsies were collected from the right flexure.

In conclusion, this case study may represent a novelty in the clinical management of MC. We used FMT with a good clinical effect, and it suggests a new indication for the microbiota-related therapeutic concept. Although this is only a case-report, we believe that FMT in MC should be further studied to explore the potential of this approach.

## COMMENTS

### *Case characteristics*

A 72-year-old female who suffered from frequent watery diarrhea and was diagnosed with collagenous colitis (CC) six months after debut of symptoms in 2008.

### *Laboratory diagnosis*

She had normal biochemical and hematologic parameters, whereas faecal calprotectin levels were increased from 160 mg/kg in 2009 to 500 mg/kg when the CC deteriorated in 2013. All stool cultures were negative for *Salmonella species, Shigella dysenteriae, Campylobacter jejuni, Yersinia enterocolitica* and *Clostridium difficile*.

### *Imaging diagnosis*

The colonoscopy was normal.

### *Pathological diagnosis*

Histopathological evaluation showed an increased number of lymphocytes in the lamina propria, in the epithelium and in a few crypts. There was a thickened collagenous band subepithelially. The findings were diffusely distributed in the whole colon.

### *Treatment*

The patient responded only temporarily to medical treatment. Accordingly, she had repeated fecal microbiota transplantations.

### *Related reports*

Fecal microbiota transplantation (FMT) was reported for the first time in 1958. Since then, FMT has been performed for various indications, such as *Clostridium difficile*-infection (CDI), inflammatory bowel disease, irritable bowel syndrome, and metabolic syndrome. FMT for CDI has been established as an effective therapy when compared to treatment with antibiotics. Despite dysbiosis in CC patients, FMT as a possible treatment for CC has not been reported before.

### *Term explanation*

FMT is a process of transplantation of fecal bacteria from a healthy individual into a recipient to modify microbiota dysbiosis.

### *Experiences and lessons*

FMT is apparently a potential treatment for refractory severe CC.

### *Peer-review*

The manuscript from Günaltay *et al*. presents a case of a patient with collagenous colitis receiving faecal microbiota transplantation. The case is interesting and describes a novel application of a procedure already established in other conditions.

## REFERENCES


1 **Nyhlin N**, Wickbom A, Montgomery SM, Tysk C, Bohr J. Long-term prognosis of clinical symptoms and health-related quality of life in microscopic colitis: a case-control study. *Aliment Pharmacol Ther* 2014; **39**: 963-972 [PMID: 24612051 DOI: 10.1111/apt.12685]

2 **Langner C**, Aust D, Ensari A, Villanacci V, Becheanu G, Miehlke S, Geboes K, Münch A. Histology of microscopic colitis-review with a practical approach for pathologists. *Histopathology* 2015; **66**: 613-626 [PMID: 25381724 DOI: 10.1111/his.12592]

3 **Barmeyer C**, Erko I, Fromm A, Bojarski C, Allers K, Moos V, Zeitz M, Fromm M, Schulzke JD. Ion transport and barrier function are disturbed in microscopic colitis. *Ann N Y Acad Sci* 2012; **1258**: 143-148 [PMID: 22731727 DOI: 10.1111/j.1749-6632.2012.06631.x]

4 **Münch A**, Söderholm JD, Ost A, Ström M. Increased transmucosal uptake of E. coli K12 in collagenous colitis persists after budesonide treatment. *Am J Gastroenterol* 2009; **104**: 679-685 [PMID: 19209166 DOI: 10.1038/ajg.2008.95]

5 **Taha Y**, Carlson M, Thorn M, Loof L, Raab Y. Evidence of local eosinophil activation and altered mucosal permeability in collagenous colitis. *Dig Dis Sci* 2001; **46**: 888-897 [PMID: 11330429]

6 **Bohr J**, Tysk C, Eriksson S, Abrahamsson H, Järnerot G. Collagenous colitis: a retrospective study of clinical presentation and treatment in 163 patients. *Gut* 1996; **39**: 846-851 [PMID: 9038667]

7 **Bohr J**, Wickbom A, Hegedus A, Nyhlin N, Hultgren Hörnquist E, Tysk C. Diagnosis and management of microscopic colitis: current perspectives. *Clin Exp Gastroenterol* 2014; **7**: 273-284 [PMID: 25170275 DOI: 10.2147/CEG.S63905]

8 **Pardi DS**, Loftus EV, Tremaine WJ, Sandborn WJ. Treatment of refractory microscopic colitis with azathioprine and 6-mercaptopurine. *Gastroenterology* 2001; **120**: 1483-1484 [PMID: 11313319]

9 **Esteve M**, Mahadevan U, Sainz E, Rodriguez E, Salas A, Fernández-Bañares F. Efficacy of anti-TNF therapies in refractory severe microscopic colitis. *J Crohns Colitis* 2011; **5**: 612-618 [PMID: 22115383 DOI: 10.1016/j.crohns.2011.05.001]

10 **Järnerot G**, Tysk C, Bohr J, Eriksson S. Collagenous colitis and fecal stream diversion. *Gastroenterology* 1995; **109**: 449-455 [PMID: 7615194]

11 **Colman RJ**, Rubin DT. Fecal microbiota transplantation as therapy for inflammatory bowel disease: a systematic review and meta-analysis. *J Crohns Colitis* 2014; **8**: 1569-1581 [PMID: 25223604 DOI: 10.1016/j.crohns.2014.08.006]

12 **Moayyedi P**, Surette MG, Kim PT, Libertucci J, Wolfe M, Onischi C, Armstrong D, Marshall JK, Kassam Z, Reinisch W, Lee CH. Fecal Microbiota Transplantation Induces Remission in Patients With Active Ulcerative Colitis in a Randomized Controlled Trial. *Gastroenterology* 2015; **149**: 102-109.e6 [PMID: 25857665 DOI: 10.1053/j.gastro.2015.04.001]

13 **Rossen NG**, MacDonald JK, de Vries EM, D'Haens GR, de Vos WM, Zoetendal EG, Ponsioen CY. Fecal microbiota transplantation as novel therapy in gastroenterology: A systematic review. *World J Gastroenterol* 2015; **21**: 5359-5371 [PMID: 25954111 DOI: 10.3748/wjg.v21.i17.5359]

14 **Vermeire S**, Joossens M, Verbeke K, Wang J, Machiels K, Sabino J, Ferrante M, Van Assche G, Rutgeerts P, Raes J. Donor Species Richness Determines Faecal Microbiota Transplantation Success in Inflammatory Bowel Disease. *J Crohns Colitis* 2016; **10**: 387-394 [PMID: 26519463 DOI: 10.1093/ecco-jcc/jjv203]

15 **Agrawal M**, Aroniadis OC, Brandt LJ, Kelly C, Freeman S, Surawicz C, Broussard E, Stollman N, Giovanelli A, Smith B, Yen E, Trivedi A, Hubble L, Kao D, Borody T, Finlayson S, Ray A, Smith R. The Long-term Efficacy and Safety of Fecal Microbiota Transplant for Recurrent, Severe, and Complicated Clostridium difficile Infection in 146 Elderly Individuals. *J Clin Gastroenterol* 2016; **50**: 403-407 [PMID: 26352106 DOI: 10.1097/MCG.0000000000000410]

16 **Girotra M**, Garg S, Anand R, Song Y, Dutta SK. Fecal Microbiota Transplantation for Recurrent Clostridium difficile Infection in the Elderly: Long-Term Outcomes and Microbiota Changes. *Dig Dis Sci* 2016; **61**: 3007-3015 [PMID: 27447476 DOI: 10.1007/s10620-016-4229-8]

17 **Kelly CR**, Khoruts A, Staley C, Sadowsky MJ, Abd M, Alani M, Bakow B, Curran P, McKenney J, Tisch A, Reinert SE, Machan JT, Brandt LJ. Effect of Fecal Microbiota Transplantation on Recurrence in Multiply Recurrent Clostridium difficile Infection: A Randomized Trial. *Ann Intern Med* 2016; **165**: 609-616 [PMID: 27547925 DOI: 10.7326/m16-0271]

18 **Khoruts A**, Rank KM, Newman KM, Viskocil K, Vaughn BP, Hamilton MJ, Sadowsky MJ. Inflammatory Bowel Disease Affects the Outcome of Fecal Microbiota Transplantation for Recurrent Clostridium difficile Infection. *Clin Gastroenterol Hepatol* 2016; **14**: 1433-1438 [PMID: 26905904 DOI: 10.1016/j.cgh.2016.02.018]

19 **Carstens A**, Dicksved J, Nelson R, Andreasson A, Bohr J, Tysk C, Agreus L, Engstrand L, Halfvarson J. Intestinal Dysbiosis in Collagenous Colitis. *Gastroenterology* 2015; 148: S-715

20 **Fischer H**, Holst E, Karlsson F, Benoni C, Toth E, Olesen M, Lindén M, Sjöberg K. Altered microbiota in microscopic colitis. *Gut* 2015; **64**: 1185-1186 [PMID: 25841239 DOI: 10.1136/gutjnl-2014-308956]

21 **Hjortswang H**, Tysk C, Bohr J, Benoni C, Kilander A, Larsson L, Vigren L, Ström M. Defining clinical criteria for clinical remission and disease activity in collagenous colitis. *Inflamm Bowel Dis* 2009; **15**: 1875-1881 [PMID: 19504614 DOI: 10.1002/ibd.20977]

22 **Kumawat AK**, Strid H, Elgbratt K, Tysk C, Bohr J, Hultgren Hörnquist E. Microscopic colitis patients have increased proportions of Ki67(+) proliferating and CD45RO(+) active/memory CD8(+) and CD4(+)8(+) mucosal T cells. *J Crohns Colitis* 2013; **7**: 694-705 [PMID: 22995775 DOI: 10.1016/j.crohns.2012.08.014]

23 **Duranti S**, Gaiani F, Mancabelli L, Milani C, Grandi A, Bolchi A, Santoni A, Lugli GA, Ferrario C, Mangifesta M, Viappiani A, Bertoni S, Vivo V, Serafini F, Barbaro MR, Fugazza A, Barbara G, Gioiosa L, Palanza P, Cantoni AM, de'Angelis GL, Barocelli E, de'Angelis N, van Sinderen D, Ventura M, Turroni F. Elucidating the gut microbiome of ulcerative colitis: bifidobacteria as novel microbial biomarkers. *FEMS Microbiol Ecol* 2016; **92**: pii: fiw191 [PMID: 27604252 DOI: 10.1093/femsec/fiw191]

24 **Göranzon C**, Kumawat AK, Hultgren-Hörnqvist E, Tysk C, Eriksson S, Bohr J, Nyhlin N. Immunohistochemical characterization of lymphocytes in microscopic colitis. *J Crohns Colitis* 2013; **7**: e434-e442 [PMID: 23523417 DOI: 10.1016/j.crohns.2013.02.007]

25 **Bohr J**, Nordfelth R, Järnerot G, Tysk C. Yersinia species in collagenous colitis: a serologic study. *Scand J Gastroenterol* 2002; **37**: 711-714 [PMID: 12126251]

26 **Erim T**, Alazmi WM, O'Loughlin CJ, Barkin JS. Collagenous colitis associated with Clostridium difficile: a cause effect? *Dig Dis Sci* 2003; **48**: 1374-1375 [PMID: 12870798]

27 **Epple HJ**, Allers K, Tröger H, Kühl A, Erben U, Fromm M, Zeitz M, Loddenkemper C, Schulzke JD, Schneider T. Acute HIV infection induces mucosal infiltration with CD4+ and CD8+ T cells, epithelial apoptosis, and a mucosal barrier defect. *Gastroenterology* 2010; **139**: 1289-1300 [PMID: 20600014 DOI: 10.1053/j.gastro.2010.06.065]

28 **Poussier P**, Ning T, Banerjee D, Julius M. A unique subset of self-specific intraintestinal T cells maintains gut integrity. *J Exp Med* 2002; **195**: 1491-1497 [PMID: 12045247]

29 **Karlsson M**, Linton L, Lampinen M, Karlén P, Glise H, Befrits R, Janczewska I, Carlson M, Winqvist O, Eberhardson M. Naïve T

cells correlate with mucosal healing in patients with inflammatory bowel disease. *Scand J Gastroenterol* 2014; **49**: 66-74 [PMID: 24188321 DOI: 10.3109/00365521.2013.853829]
30 **Carrasco A**, Esteve M, Salas A, Pedrosa E, Rosinach M, Aceituno M, Zabana Y, Fernández-Bañares F. Immunological Differences between Lymphocytic and Collagenous Colitis. *J Crohns Colitis* 2016; **10**: 1055-1066 [PMID: 26928959 DOI: 10.1093/ecco-jcc/jjw058]

**P- Reviewer**: Abignano G, Freeman HJ, Hourigan SK, Lakatos PL
**S- Editor**: Gong ZM **L- Editor**: A **E- Editor**: Wang CH

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