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

# World Journal of *Gastroenterology*

*World J Gastroenterol* 2015 December 7; 21(45): 12735-12988

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 1377 members, representing a team of worldwide experts in gastroenterology and hepatology. They are from 68 countries, including Algeria (2), Argentina (7), Australia (31), Austria (9), Belgium (11), Brazil (20), Brunei Darussalam (1), Bulgaria (2), Cambodia (1), Canada (26), Chile (4), China (164), Croatia (2), Cuba (1), Czech (6), Denmark (2), Egypt (9), Estonia (2), Finland (6), France (20), Germany (58), Greece (31), Guatemala (1), Hungary (15), Iceland (1), India (33), Indonesia (2), Iran (10), Ireland (9), Israel (18), Italy (194), Japan (150), Jordan (1), Kuwait (1), Lebanon (7), Lithuania (1), Malaysia (1), Mexico (11), Morocco (1), Netherlands (5), New Zealand (4), Nigeria (3), Norway (6), Pakistan (6), Poland (12), Portugal (8), Puerto Rico (1), Qatar (1), Romania (10), Russia (3), Saudi Arabia (2), Singapore (7), Slovenia (2), South Africa (1), South Korea (69), Spain (51), Sri Lanka (1), Sudan (1), Sweden (12), Switzerland (5), Thailand (7), Trinidad and Tobago (1), Tunisia (2), Turkey (55), United Kingdom (49), United States (180), Venezuela (1), and Vietnam (1).

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

**Austria**

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

**Belgium**

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

**Brazil**

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

**Brunei Darussalam**

Vui Heng Chong, *Bandar Seri Begawan*

**Bulgaria**

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

**Cambodia**

Francois Rouet, *Phnom Penh*

**Canada**

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

**Chile**

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

**China**

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

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

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

**Cuba**
Damian Casadesus, *Havana*

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

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

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

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

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

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

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

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

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

**Guatemala**

Carlos Maria Parellada, *Guatemala*

**Hungary**

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

**Iceland**

Tryggvi Bjorn Stefánsson, *Reykjavík*

**India**

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

**Indonesia**

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

**Iran**

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

**Ireland**

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

**Israel**

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

**Italy**

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

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

**Japan**

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

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

**Jordan**
Khaled Ali Jadallah, *Irbid*

**Kuwait**
Islam Khan, *Kuwait*

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

**Lithuania**
Antanas Mickevicius, *Kaunas*

**Malaysia**
Huck Joo Tan, *Petaling Jaya*

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

**Morocco**
Samir Ahboucha, *Khouribga*

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

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

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

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

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

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

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

Filipa F Vale, *Lisbon*

**Puerto Rico**

Caroline B Appleyard, *Ponce*

**Qatar**

Abdulbari Bener, *Doha*

**Romania**

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

**Russia**

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

**Saudi Arabia**

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

**Singapore**

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

**Slovenia**

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

**South Korea**

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

**Spain**

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

**Sri Lanka**

Arjuna Priyadarsin De Silva, *Colombo*

**Sudan**

Ishag Adam, *Khartoum*

**Sweden**

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

**Switzerland**

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

**Thailand**

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

**Trinidad and Tobago**

B Shivananda Nayak, *Mount Hope*

**Tunisia**

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

**Turkey**

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

**United Kingdom**

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

**United States**

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

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

**Venezuela**
Miguel Angel Chiurillo, *Barquisimeto*

**Vietnam**
Van Bang Nguyen, *Hanoi*

World Journal of Gastroenterology

Contents

Weekly Volume 21 Number 45 December 7, 2015

## LETTERS TO THE EDITOR

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
Xiu-Xia Song, Vice Director
*World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
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: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
December 7, 2015



INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm

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

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12735

*World J Gastroenterol* 2015 December 7; 21(45): 12735-12741
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


EDITORIAL

# Interval colorectal carcinoma: An unsolved debate

Mark Benedict, Antonio Galvao Neto, Xuchen Zhang

Mark Benedict, Xuchen Zhang, Department of Pathology, Yale University School of Medicine, New Haven, CT 06520, United States

Antonio Galvao Neto, Department of Pathology, New York University School of Medicine, New York, NY 10016, United States

Xuchen Zhang, Pathology and Laboratory Medicine Service, VA CT Healthcare System, West Haven, CT 06516, United States

Author contributions: Benedict M, Neto AG and Zhang X contributed to the conception and design; Benedict M drafted and revised the manuscript; Zhang X and Neto AG supervised the manuscript.

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



Correspondence to: Xuchen Zhang, MD, PhD, Department of Pathology, Yale University School of Medicine, 310 Cedar Street. PO Box 208023, New Haven, CT 06520, United States. xuchen.zhang@yale.edu
Telephone: +1-203-9325711
Fax: +1-203-9374704

Received: April 30, 2015
Peer-review started: May 8, 2015
First decision: July 10, 2015
Revised: July 16, 2015
Accepted: October 13, 2015
Article in press: October 13, 2015
Published online: December 7, 2015

## Abstract

Colorectal carcinoma (CRC), as the third most common new cancer diagnosis, poses a significant health risk to the population. Interval CRCs are those that appear after a negative screening test or examination. The development of interval CRCs has been shown to be multifactorial: location of exam-academic institution versus community hospital, experience of the endoscopist, quality of the procedure, age of the patient, flat versus polypoid neoplasia, genetics, hereditary gastrointestinal neoplasia, and most significantly missed or incompletely excised lesions. The rate of interval CRCs has decreased in the last decade, which has been ascribed to an increased understanding of interval disease and technological advances in the screening of high risk individuals. In this article, we aim to review the literature with regard to the multifactorial nature of interval CRCs and provide the most recent developments regarding this important gastrointestinal entity.

**Key words:** Colorectal carcinoma; Interval colorectal carcinoma; Post colonoscopy colorectal cancer; Detection; Screening



**Core tip:** Interval colorectal cancers (CRCs) represent a small but important subgroup of colorectal cancers. Interval CRCs are those that appear after a negative screening test or examination. The development of interval CRCs has been shown to be multifactorial. We aim to review the multifactorial nature of interval CRCs and provide the most recent developments regarding this important entity.

Benedict M, Neto AG, Zhang X. Interval colorectal carcinoma: An unsolved debate. *World J Gastroenterol* 2015; 21(45):

12735-12741 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12735.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12735

## INTRODUCTION

Colorectal carcinoma (CRC) is the third most common new cancer diagnosis as well as the third most common cause of death due to cancer[1]. An estimated 96090 new cases of colorectal cancer with 49700 deaths are expected to occur in the United States for 2015[1]. The 5-year survival rate for CRC that is localized at the time of diagnosis is 90% with a decrease to 68% with regional involvement and a precipitous decline to 10% if distant metastases are discovered[2]. Colonoscopy has been an effective measure in the screening and ultimate prevention of CRC with a 30-year decline in new cases and deaths as a result of CRC[3]. There are over 12 million colonoscopic procedures performed in the USA each year with roughly half occurring due to CRC prevention[3]. The current prevention strategy dictates that at 50 years of age a screening colonoscopy be performed and every ten years subsequent to a negative exam[4]. The United States Preventative Services Task Force recommends against the routine screening of any individual after the age of 75[5]. Adenomatous as well as serrated polyps harbor malignant potential and require additional early screening for the development of CRC[5]. Those patients with two or more tubular adenomas that measure less than 10 mm should have a colonoscopic exam every 5 years[5]. A three year repeat colonoscopy is required for patients that have three to 10 adenomas, an adenoma or serrated polyp greater than or equal to 10 mm, an adenoma with villous features or high grade dysplasia, a dysplastic serrated adenoma, or a traditional serrated adenoma[5]. Endoscopic surveillance becomes more tenuous in cases of inflammatory bowel disease (IBD). Inflammatory bowel diseases, specifically ulcerative colitis and Crohn's disease, are damaging processes that result from the constant assault on the bowel by inflammation. Ulcerative colitis characteristically involves the rectum with proximal extension to involve all or just a portion of the colon. However, Crohn's disease is characterized by its patchy involvement of the gastrointestinal tract from the mouth to the anus. There is a bimodal age distribution for inflammatory bowel disease with peak incidences in the age range of 15-30 years and 50-80 years[6]. Inflammatory bowel disease shows its highest prevalence in western countries, with nearly 1.4 million Americans affected[7]. Patients with inflammatory bowel disease are at increased risk for CRC as well as a more rapid progression to CRC[8]. However, the overall incidence of IBD-related CRC has decreased in recent years[9]. Of the conditions which are risk factors for the development of CRC, IBD ranks third behind familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer syndrome (HNPCC/Lynch syndrome)[10]. In the general population, screening colonoscopy seeks to identify dysplastic or premalignant conditions, namely colon polyps, which are typically easily visualized and resected[11]. In stark contrast, dysplasia in IBD is difficult to recognize at colonoscopy, as it is often seen to arise from flat, plaque-like, or occasionally raised polypoid lesions defined as dysplasia-associated lesion or masses (DALM)[11]. In addition to the difficulties posed by flat dysplasia, operator-dependent variability and the quality of examination contribute to the inconsistent effectiveness of colonoscopy, especially in the proximal colon[12]. All of these factors, as well as additional contributing factors that will be subsequently discussed, contribute to what is called interval colorectal carcinoma.

## DEFINITION AND INCIDENCE OF INTERVAL COLORECTAL CARCINOMA

The definition of interval CRC is varied and complex. Therefore, the Expert Working Group on interval CRC of the Colorectal Cancer Screening Committee of the World Endoscopy Organization (WEO) has set out to standardize the nomenclature for its definition[12]. After a literature review, the WEO has defined an interval CRC as "colorectal cancer diagnosed after a colorectal screening examination or test in which no cancer is detected, and before the date of the next recommended exam"[12]. Samadder *et al*[13] conducted a population based-study of Utah residents and observed that 3.4% of all CRCs occurred in 6-36 mo from their index colonoscopy. Singh *et al*[14] looked at 4883 cases of CRCs and concluded that 1 in 13 CRCs may be an early or missed CRC, diagnosed after an index colonoscopy. Whereas, additional data suggests that 1 in 45 of CRCs are of the interval type[15]. Several studies site incidence rates of interval CRC to be as high as 9% of all diagnosed CRCs[15]. Site specific interval CRCs were identified and include (based on nine studies): 4615 proximal interval CRCs out of 53847 total proximal CRCs and 2726 distal interval CRCs out of a total 77922 distal CRCs[10]. This corresponds to 1 in 15 proximal CRCs being interval and 1 in 34 distal CRCs being interval[10]. Proximal interval CRCs are 2.4 times more likely when compared to distal interval CRCs[10]. Sanduleanu *et al*[15] calculated the magnitude of threat posed by interval CRCs to be in the range of 30000 out of one million new cases of CRC diagnosed worldwide each year, based on an average-risk scenario of 1 out of 30 diagnosed CRCs.

## RISK FACTORS

There are several factors that have been implicated in the development of interval CRCs including: technical factors,

biology-related, nonpolypoid colorectal neoplasms, serrated lesions, hereditary cancer syndromes (for example Lynch Syndrome), among others[15].

### Technical factors

Colonoscopy value, defined using both health outcomes and cost, is intimately linked to the physician performing the procedure, university facility versus community practice, site of service, and the engagement of the patient in the colonoscopy sequence[3]. Physician factors have proved most directly related to the risk of an interval CRC. The use of three quality metrics: adenoma detection rate, use of recommended screening and surveillance intervals, and cecal intubation rate are measures which are used to establish this link[3]. A great deal of data exists to support the notion that colonoscopy is less effective in preventing right sided CRCs and that those trained in proper colonoscopic techniques, specifically gastroenterologists, are more effective in the prevention of CRC when compared to other types of physicians[16-22].

Early colorectal neoplasia has been increasingly treated by conventional endoscopic resection, including endoscopic mucosal resection (EMR) and polypectomy[23]. Piecemeal EMR is an accepted treatment option for large adenomatous colorectal neoplasms (> 20 mm) in diameter, however it is more advantageous to resect neoplasia with *en bloc* resection, which results in a more accurate histological assessment[23]. With that being said, endoscopic submucosal dissection (ESD) is an effective, safe, and convenient approach that has gradually been established and has now in more general use[23]. ESD, like polypectomy (discussed subsequently), also falls prey to the problem of incomplete resection and local recurrence[23]. Oka *et al*[23], using data from a multicenter prospective cohort, showed that piecemeal resection is the most important risk factor for a local recurrence after endoscopic resection (ER), irrespective of the method of ER used. They report local recurrence rates of 0%-17.9% for *en bloc* resection and 4.8%-31.4% for piecemeal resection[23]. Hence, the guidelines for CRC screening and surveillance recommend a follow-up colonoscopy at 3-6 mo after piecemeal EMR[23]. Like endoscopic resection, incomplete polypectomy and missed lesions are evolving as a substantial risk factor for the development of interval CRCs. Robertson *et al*[24] showed that 26% of interval CRCs developed in the same anatomical area as where the patient's previous polypectomy occurred. Chen *et al*[25] also showed similar results of interval CRC after incomplete polypectomy. In a study performed by Atkin *et al*[26], 31 of 842 patients with tubulovillous adenomas, specifically of the rectosigmoid, which were most likely incompletely excised, ended up developing interval CRCs. Missed lesions were the most probable cause of interval CRC in 15 out of 28 patients as revealed by le Clercq *et al*[27]. Additionally, using pooled data on 9167 adenoma patients, Robertson *et al*[28] showed that 58 people were diagnosed with CRC within 4 years of colonoscopy and three quarters were likely the result of a missed lesion, incomplete adenoma resection or failed biopsy detection.

Patient related factors also contribute to the risk of interval CRC. Patients with interval CRCs are on average 6 years older than those with non-interval colon cancer and typically have substantial co-morbidities, such as cardiovascular disease or a history of diverticulosis[15]. Patients who are older, frail, and have co-morbidities are more prone to have inadequate bowel preparations, which may explain the increased risk of interval CRCs seen in these individuals[15]. Colonoscopic examination can be difficult in patients with diverticular disease and when coupled with the fact that patients with diverticular disease have a higher risk of harboring adenomas and advanced adenomas in the sigmoid colon, may increase the risk of interval CRC in these persons[29].

### Sessile serrated adenoma/polyps

In order for interval CRCs to occur several conditions need to be fulfilled; namely, a precursor lesion that is rapidly progressive, evades detection, and is difficult to resect[30]. Sessile serrated adenomas (SSA) are the perfect precursor that fulfills these criteria. SSAs without dysplasia are often difficult for endoscopists to detect due to their flat and indistinct nature. SSA prevalence at colonoscopy has always been accepted to be in the realm of 2%, however, recent evidence suggests that these lesions may be more common than previously thought, specifically 4-6 fold higher[31]. Endoscopically, sessile serrated adenomas with dysplasia (SSA-D) are identifiable due to their dysplastic component, which appears to the endoscopist as a typical adenoma; however, when the endoscopist resects the polyp, the dysplastic component is removed leaving the nondysplastic component behind[30]. Endoscopic snare resection of SSAs are often incomplete with studies suggesting that in 31% of cases residual SSAs are left behind, when compared to conventional adenomas, a residual rate of only 7.2% is seen[32]. Large SSAs (1-2 cm) show even greater rates of residual tumor, with one study showing up to 48% of large SSA polypectomies resulting in residual disease[32]. The reason for such concern over an incomplete SSA polypectomy lies in the genetic make-up of these neoplasms. Sessile serrated adenomas commonly have activating mutations of the BRAF proto-oncogene, and develop hypermethylation of the CpG promoter regions of mismatch repair genes (*i.e.,* MLH-1), which leads to microsatellite instability (MSI) and is a well-recognized path to CRC[30]. As discussed earlier, many interval CRCs are proximal in location and are CIMP-H as well as MSI positive, which strongly suggests a role for SSA in the development of interval CRC[30,33]. SSAs have been associated with

proximal MSI CRCs as well as rapid progression times to CRC diagnosis[34]. Sessile serrated adenomas are not only problematic for the endoscopist, they also pose a problem for the pathologist. The frequency of SSA diagnosis varies greatly in the literature and the diagnostic difficulty becomes more apparent due to the fact that the histologic features of microvesicular hyperplastic polyp and SSA overlap[35]. Bettington *et al*[35] showed that in applying strict histologic criteria for the diagnosis of SSA, a 14.7% rate of detection can be achieved with a high rate of reproducibility among pathologists. However, SSAs continue to be underdiagnosed and will lead to inadequate surveillance and will likely contribute to the rate of interval CRCs[35].

### *Interval CRC in inflammatory bowel disease*

Colonoscopy, as described earlier, is the predominant screening and diagnostic test for CRC in the general population. Likewise, colonoscopic examination among those patients with inflammatory bowel disease, specifically Crohn Disease and Ulcerative Colitis, is pivotal in screening this high risk population for CRC. Patients with long-standing inflammatory bowel disease have typically been excluded from studies investigating the rate of early/missed lesions leading to CRC, hence, the rate of early and missed CRCs in this population is still largely unknown[36]. However, in the largest and longest running UC surveillance program in the world (42 year history) has revealed that advanced cancer incidence rate (IR) has consistently decreased over the past four decades, suggesting that the efficacy and use of advanced imaging techniques has led to a greater detection of early neoplasia[37]. Additionally, there has also been a reduction in the incidence rate of high grade dysplasia and low grade dysplasia in the current decade, now 2.1 per 1000 patient-years, down from 4.6 per 1000 patient years[37]. They also found that the risk of interval cancer has rapidly decreased with the steepest decline coming in the last decade, which may be related to the increased use of chromoendoscopy[37]. Chromoendoscopy is the use of image-enhanced techniques, such as the use of dye spraying or optical, to improve the visualization of mucosal structures, and thus improve the recognition of the borders, microvasculature, and surface topography of neoplasia[38]. Patients who underwent chromoendoscopy were found to have a lower risk of developing CRC when compared to those who had never had the procedure[37]. However, other studies do not confirm the benefit of using chromoendoscopy. In a large retrospective study, it was shown that the use of chromoendoscopy with targeted biopsies did not result in an increased neoplasia detection rate when compared to white light endoscopy with random biopsies[39]. With that being said, the majority of the literature seems to suggest a benefit from using chromoendoscopy when compared to standard white light endoscopy[40-43]. Wang *et al*[36] investigated the rate of early/missed CRCs in both IBD patients as well as non-IBD patients. Their findings showed that out of 3589 early/missed lesions, 54 were seen in Crohn's patients, 103 in UC patients, and 3432 in non-IBD patients. Patient's without IBD showed a rate of early/missed CRCs after colonoscopy in the range of 5.8%; however, the rate increased substantially in those patients with IBD to 15.1% for Crohn's and 15.8% for UC[36]. Similar to our discussion of sporadic interval CRC in non-IBD patients, interval CRCs in patients with IBD may be explained again by clinician-dependent factors including: missed lesions, incomplete resection, or deviation from set surveillance protocols[44]. In contrast to non-IBD interval CRC, the presence of active or chronic background inflammation seen in patients with IBD causes diversity in the appearance of dysplastic lesions and thus increases the complexity of the study for the endoscopist[44]. Like SSA, dysplastic lesions in patients with IBD are often flat and easily missed on colonoscopy[44]. The difficulties presented by flat dysplasia in IBD led Maastricht University Medical Center to perform a study where endoscopists were trained on the recognition of nonpolypoid colorectal neoplasms (NP-CRN)[45]. They determined that intensive training with regard to NP-CRNs lead to similar detection rates among their staff gastroenterologists and GI trainees, suggesting that clinical awareness was more important than experience in the detection of flat lesions[45]. Strict adherence to the prescribed colonoscopic surveillance in IBD patients can be tenuous due to a multitude of factors such as: the patient's understanding of cancer risk, disease flares and associated co-morbidity, and disease activity causing a delay in surveillance[44]. Therefore, patient education to include disease course and risk of CRC, adherence to treatment protocols to limit disease flares, and surveillance during quiescent phase may contribute to the reduction of interval CRC in patients with IBD. The biologic factors which underpin the molecular events that underlie the development of CRC in a background of inflammation are still under active investigation. It has been shown that nearly 6% of CRCs arising in those with IBD are small flat invasive lesions with no adjacent adenomatous tissue, which suggests that the progression to CRC may not follow the classic adenoma-carcinoma sequence[44]. Srivastava *et al*[46] looked at the molecular features of 3 unique patients with long standing IBD who developed numerous hyperplastic/serrated colonic polyps. The group revealed that all 3 patients showed retention of MLH-1 and MSH-2 within these polyps, one case showing a loss of MGMT, and no BRAF mutations were present[46]. They proposed that the findings were suggestive of a serrated pathway of carcinogenesis in those with IBD, which is characterized by silencing of MGMT[46].

### *Lynch syndrome and interval CRC*

Lynch syndrome (LS), an autosomal dominant disorder, is characterized by mutations in mismatch

repair (MMR) genes (MLH1, PMS2, MSH2, and MSH6), which causes an increased lifetime risk of developing CRCs as well as other cancers (*i.e.,* endometrial) in the affected host[47]. The recommended surveillance programs for these patients include colonoscopic examination at an interval of 1-2 years starting at the age of 20-25 years[47]. Many factors contribute to the development of an interval CRC in patients with Lynch Syndrome, including compliance to the recommended surveillance protocols. Newton *et al*[48] investigated compliance with large bowel screening in Lynch Syndrome mutation carriers amongst patients in the United Kingdom and found that in only 62% of the cases was the screening colonoscopy performed during the suggest screening interval. They also found a reduced cumulative incidence of CRC, to the age of 70 years, when screening protocols were adhered to; a reduction from 81% in non-screened patients to 25% in screened individuals[48]. Haanstra *et al*[47] showed that in 29 LS patients (all mutational carriers), a total of 31 interval cancers were found within or at 24 mo of previous colonoscopic examination. In 16 of 19 patients with LS, the interval carcinoma was located in a proximal location and when considering all detected interval carcinomas, 65% are found within the right colon[47]. Their study revealed that in all LS patients who developed an interval CRC a MLH1 or MSH2 mutation was identified, and 90% of these CRCs were diagnosed in the 1-2 years after previous colonoscopy[47]. Richter *et al*[49] looked at 42 interval CRCs and showed that 41% of these tumors exhibited DNA microsatellite instability (MSI) and of these 54% exhibited somatic hypermethylation of the MLH1 promoter. They concluded that interval CRCs cannot be distinguished by activation of KRAS, NRAS, BRAF, or PIK3CA oncogenic pathways, however, MSI pathway defects represent a large proportion of interval CRCs with an underlying LS possibly explaining half of these cases[49].

## BIOLOGIC AGGRESSIVENESS AND SURVIVIAL IN INTERVAL-CRCS

As previously discussed, interval CRCs are seen most often in the proximal colon and as demonstrated by Arain *et al*[33], are 2.5 times more likely than non-interval CRCs to be CIMP+ and 2.7 times more likely to show MSI positivity. Other studies as well posit that interval CRC may represent a rapidly growing and aggressive cancer[50-53]. However, other studies have not shown any difference in survival between interval CRC and those with no prior colonoscopic surveillance[13,14]. Erichsen *et al*[50] conducted a population based study among the Danish population from 2000-2009 and found out of 38064 CRC patients, a total of 982 (3%) were interval. When compared to non-interval CRC, interval carcinomas were more often women, were proximal in location, displayed mucinous histology, and had co-morbid conditions (IBD and diverticular disease)[50]. The one year survival rate was similar for those patients with interval CRCs when compared to those who developed CRC after a ten year period from their last colposcopy (68%: interval; 72%: > 10 years before CRC diagnosis, and 71% sporadic)[50]. The five-year survival was close to 40% in all groups[50]. Additionally, interval CRC were less likely to be diagnosed at an advanced stage and interval CRCs were just as likely as detected CRCs to be well-to-moderately differentiated[10]. Interval CRCs, when compared to detected CRCs, were seen to have a 37% lower risk of mortality, which held true for both early-stage and advanced stage cancer[13].

## CONCLUSION

Interval colorectal carcinoma poses a distinct threat, not only to the general population, but also to other population groups such as those with inflammatory bowel disease, hereditary predisposition to gastrointestinal neoplasia, as well as those patients who are more advanced in years with multiple co-morbidities. With a rate that could be as high as nine percent of newly diagnosed CRC being an interval CRC, an estimated 8648 patients will develop a CRC after being screened with colonoscopy in 2015. The etiology of these lesions has been shown to be multifactorial in nature with perhaps the largest risk coming from missed or incompletely excised lesions. There appears to be some disagreement in the literature as to whether interval CRCs are more biologically aggressive due to changes in their molecular make-up. However, there are biological factors that seem to contribute to the development of interval CRC with evidence to suggest that the sessile serrated neoplasia pathway may promote a more rapid development of carcinoma after a screening colonoscopy. Regardless, the overall survival irrespective of tumor biology appears to be similar between interval and detected CRCs. Non-polypoid neoplasia presents a well-defined challenge to the endoscopist as well as the pathologist. Flat lesions are challenging for the endoscopist to discern, but when biopsied, may be miss diagnosed by the pathologist if strict criteria are not adhered to. Improvements in the quality of the endoscopic procedure through the education of the endoscopist is a worthwhile endeavor with a focus on flat lesion recognition. The more widespread use of chromoendoscopy may also be advantageous to many patient groups, most especially those with inflammatory bowel disease. Finally, a greater understanding of the molecular features and biologic behavior of interval CRCs, when coupled with increased endoscopic recognition and complete removal of neoplasia, will likely lead to the greatest improvement and reduction in the rate of diagnosis of carcinoma after a negative colonoscopy.

## REFERENCES


1 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin* 2015; **65**: 5-29 [PMID: 25559415 DOI: 10.3322/caac.21254]
2 **Richter JM**, Campbell EJ, Chung DC. Interval colorectal cancer after colonoscopy. *Clin Colorectal Cancer* 2015; **14**: 46-51 [PMID: 25510180 DOI: 10.1016/j.clcc.2014.11.001]
3 **Allen JI**. Quality measures for colonoscopy: where should we be in 2015? *Curr Gastroenterol Rep* 2015; **17**: 10 [PMID: 25740247 DOI: 10.1007/s11894-015-0432-6]
4 **Winawer S**, Fletcher R, Rex D, Bond J, Burt R, Ferrucci J, Ganiats T, Levin T, Woolf S, Johnson D, Kirk L, Litin S, Simmang C. Colorectal cancer screening and surveillance: clinical guidelines and rationale-Update based on new evidence. *Gastroenterology* 2003; **124**: 544-560 [PMID: 12557158 DOI: 10.1053/gast.2003.50044]
5 **Short MW**, Layton MC, Teer BN, Domagalski JE. Colorectal cancer screening and surveillance. *Am Fam Physician* 2015; **91**: 93-100 [PMID: 25591210]
6 **Calkins BM**, Lilienfeld AM, Garland CF, Mendeloff AI. Trends in incidence rates of ulcerative colitis and Crohn's disease. *Dig Dis Sci* 1984; **29**: 913-920 [PMID: 6478982]
7 **Loftus EV**. Management of extraintestinal manifestations and other complications of inflammatory bowel disease. *Curr Gastroenterol Rep* 2004; **6**: 506-513 [PMID: 15527681]
8 **Bae SI**, Kim YS. Colon cancer screening and surveillance in inflammatory bowel disease. *Clin Endosc* 2014; **47**: 509-515 [PMID: 25505716 DOI: 10.5946/ce.2014.47.6.509]
9 **Kim ER**, Chang DK. Colorectal cancer in inflammatory bowel disease: the risk, pathogenesis, prevention and diagnosis. *World J Gastroenterol* 2014; **20**: 9872-9881 [PMID: 25110418 DOI: 10.3748/wjg.v20.i29.9872]
10 **Singh S**, Singh PP, Murad MH, Singh H, Samadder NJ. Prevalence, risk factors, and outcomes of interval colorectal cancers: a systematic review and meta-analysis. *Am J Gastroenterol* 2014; **109**: 1375-1389 [PMID: 24957158 DOI: 10.1038/ajg.2014.171]
11 **Mattar MC**, Lough D, Pishvaian MJ, Charabaty A. Current management of inflammatory bowel disease and colorectal cancer. *Gastrointest Cancer Res* 2011; **4**: 53-61 [PMID: 21673876]
12 **Sanduleanu S**, le Clercq CM, Dekker E, Meijer GA, Rabeneck L, Rutter MD, Valori R, Young GP, Schoen RE. Definition and taxonomy of interval colorectal cancers: a proposal for standardising nomenclature. *Gut* 2015; **64**: 1257-1267 [PMID: 25193802 DOI: 10.1136/gutjnl-2014-307992]
13 **Samadder NJ**, Curtin K, Tuohy TM, Pappas L, Boucher K, Provenzale D, Rowe KG, Mineau GP, Smith K, Pimentel R, Kirchhoff AC, Burt RW. Characteristics of missed or interval colorectal cancer and patient survival: a population-based study. *Gastroenterology* 2014; **146**: 950-960 [PMID: 24417818 DOI: 10.1053/j.gastro.2014.01.013]
14 **Singh H**, Nugent Z, Demers AA, Bernstein CN. Rate and predictors of early/missed colorectal cancers after colonoscopy in Manitoba: a population-based study. *Am J Gastroenterol* 2010; **105**: 2588-2596 [PMID: 20877348 DOI: 10.1038/ajg.2010.390]
15 **Sanduleanu S**, Masclee AM, Meijer GA. Interval cancers after colonoscopy-insights and recommendations. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 550-554 [PMID: 22907162 DOI: 10.1038/nrgastro.2012.136]
16 **Baxter NN**, Goldwasser MA, Paszat LF, Saskin R, Urbach DR, Rabeneck L. Association of colonoscopy and death from colorectal cancer. *Ann Intern Med* 2009; **150**: 1-8 [PMID: 19075198]
17 **Brenner H**, Chang-Claude J, Seiler CM, Stürmer T, Hoffmeister M. Does a negative screening colonoscopy ever need to be repeated? *Gut* 2006; **55**: 1145-1150 [PMID: 16469791 DOI: 10.1136/gut.2005.087130]
18 **Lakoff J**, Paszat LF, Saskin R, Rabeneck L. Risk of developing proximal versus distal colorectal cancer after a negative colonoscopy: a population-based study. *Clin Gastroenterol Hepatol* 2008; **6**: 1117-1121; quiz 1064 [PMID: 18691942 DOI: 10.1016/j.cgh.2008.05.016]
19 **Singh H**, Nugent Z, Mahmud SM, Demers AA, Bernstein CN. Predictors of colorectal cancer after negative colonoscopy: a population-based study. *Am J Gastroenterol* 2010; **105**: 663-673; quiz 674 [PMID: 19904239 DOI: 10.1038/ajg.2009.650]
20 **Singh H**, Nugent Z, Demers AA, Kliewer EV, Mahmud SM, Bernstein CN. The reduction in colorectal cancer mortality after colonoscopy varies by site of the cancer. *Gastroenterology* 2010; **139**: 1128-1137 [PMID: 20600026 DOI: 10.1053/j.gastro.2010.06.052]
21 **Brenner H**, Chang-Claude J, Seiler CM, Rickert A, Hoffmeister M. Protection from colorectal cancer after colonoscopy: a population-based, case-control study. *Ann Intern Med* 2011; **154**: 22-30 [PMID: 21200035 DOI: 10.7326/0003-4819-154-1-201101040-00004]
22 **Rabeneck L**, Paszat LF, Saskin R. Endoscopist specialty is associated with incident colorectal cancer after a negative colonoscopy. *Clin Gastroenterol Hepatol* 2010; **8**: 275-279 [PMID: 19879970 DOI: 10.1016/j.cgh.2009.10.022]
23 **Oka S**, Tanaka S, Saito Y, Iishi H, Kudo SE, Ikematsu H, Igarashi M, Saitoh Y, Inoue Y, Kobayashi K, Hisabe T, Tsuruta O, Sano Y, Yamano H, Shimizu S, Yahagi N, Watanabe T, Nakamura H, Fujii T, Ishikawa H, Sugihara K. Local recurrence after endoscopic resection for large colorectal neoplasia: a multicenter prospective study in Japan. *Am J Gastroenterol* 2015; **110**: 697-707 [PMID: 25848926 DOI: 10.1038/ajg.2015.96]
24 **Robertson DJ**, Greenberg ER, Beach M, Sandler RS, Ahnen D, Haile RW, Burke CA, Snover DC, Bresalier RS, McKeown-Eyssen G, Mandel JS, Bond JH, Van Stolk RU, Summers RW, Rothstein R, Church TR, Cole BF, Byers T, Mott L, Baron JA. Colorectal cancer in patients under close colonoscopic surveillance. *Gastroenterology* 2005; **129**: 34-41 [PMID: 16012932]
25 **Chen SC**, Rex DK. Endoscopist can be more powerful than age and male gender in predicting adenoma detection at colonoscopy. *Am J Gastroenterol* 2007; **102**: 856-861 [PMID: 17222317 DOI: 10.1111/j.1572-0241.2006.01054.x]
26 **Atkin WS**, Morson BC, Cuzick J. Long-term risk of colorectal cancer after excision of rectosigmoid adenomas. *N Engl J Med* 1992; **326**: 658-662 [PMID: 1736104 DOI: 10.1056/nejm199203053261002]
27 **le Clercq C**, Rondagh E, Riedl R, Bosman FT, Beets GL, Hameeteman W, Masclee A, Sanduleanu S. Interval Colorectal Cancers Frequently Have Subtle Macroscopic Appearance: A 10 Year-Experience in an Academic Center. *Gastroenterology* 2014; **140**: S112-S113 [DOI: 10.1016/S0016-5085(11)60455-1]
28 **Robertson DJ**, Lieberman DA, Winawer SJ, Ahnen DJ, Baron JA, Schatzkin A, Cross AJ, Zauber AG, Church TR, Lance P, Greenberg ER, Martínez ME. Colorectal cancers soon after colonoscopy: a pooled multicohort analysis. *Gut* 2014; **63**: 949-956 [PMID: 23793224 DOI: 10.1136/gutjnl-2012-303796]
29 **Morini S**, Hassan C, Zullo A, De Francesco V, Festa V, Barberani F, Faleo D, Stroffolini T. Diverticular disease as a risk factor for sigmoid colon adenomas. *Dig Liver Dis* 2002; **34**: 635-639 [PMID: 12405250]
30 **Burgess NG**, Tutticci NJ, Pellise M, Bourke MJ. Sessile serrated adenomas/polyps with cytologic dysplasia: a triple threat for interval cancer. *Gastrointest Endosc* 2014; **80**: 307-310 [PMID: 24890425 DOI: 10.1016/j.gie.2014.03.050]
31 **Tutticci NJ**, Hewett DG, Leggett BA. Prevalence of serrated polyps: implications for significance as colorectal cancer precursors. *Colorectal Cancer* 2013; **2**: 535-547 [DOI: 10.2217/crc.13.70]
32 **Pohl H**, Srivastava A, Bensen SP, Anderson P, Rothstein RI, Gordon SR, Levy LC, Toor A, Mackenzie TA, Rosch T, Robertson DJ. Incomplete polyp resection during colonoscopy-results of the complete adenoma resection (CARE) study. *Gastroenterology* 2013; **144**: 74-80.e1 [PMID: 23022496 DOI: 10.1053/j.gastro.2012.09.043]
33 **Arain MA**, Sawhney M, Sheikh S, Anway R, Thyagarajan B, Bond JH, Shaukat A. CIMP status of interval colon cancers: another piece to the puzzle. *Am J Gastroenterol* 2010; **105**: 1189-1195 [PMID: 20010923]
34 **Goldstein NS**, Bhanot P, Odish E, Hunter S. Hyperplastic-like colon polyps that preceded microsatellite-unstable adenocarcinomas. *Am J Clin Pathol* 2003; **119**: 778-796 [PMID: 12817424 DOI: 10.1309/

drfq-0wfu-f1g1-3ctk]
35 **Bettington M**, Walker N, Rosty C, Brown I, Clouston A, Wockner L, Whitehall V, Leggett B. Critical appraisal of the diagnosis of the sessile serrated adenoma. *Am J Surg Pathol* 2014; **38**: 158-166 [PMID: 24418851 DOI: 10.1097/pas.0000000000000103]
36 **Wang YR**, Cangemi JR, Loftus EV, Picco MF. Rate of early/missed colorectal cancers after colonoscopy in older patients with or without inflammatory bowel disease in the United States. *Am J Gastroenterol* 2013; **108**: 444-449 [PMID: 23295277]
37 **Choi CH**, Rutter MD, Askari A, Lee GH, Warusavitarne J, Moorghen M, Thomas-Gibson S, Saunders BP, Graham TA, Hart AL. Forty-Year Analysis of Colonoscopic Surveillance Program for Neoplasia in Ulcerative Colitis: An Updated Overview. *Am J Gastroenterol* 2015; **110**: 1022-1034 [PMID: 25823771 DOI: 10.1038/ajg.2015.65]
38 **Bartel MJ**, Picco MF, Wallace MB. Chromocolonoscopy. *Gastrointest Endosc Clin N Am* 2015; **25**: 243-260 [PMID: 25839685 DOI: 10.1016/j.giec.2014.11.010]
39 **Mooiweer E**, van der Meulen-de Jong AE, Ponsioen CY, Fidder HH, Siersema PD, Dekker E, Oldenburg B. Chromoendoscopy for Surveillance in Inflammatory Bowel Disease Does Not Increase Neoplasia Detection Compared With Conventional Colonoscopy With Random Biopsies: Results From a Large Retrospective Study. *Am J Gastroenterol* 2015; **110**: 1014-1021 [PMID: 25823770 DOI: 10.1038/ajg.2015.63]
40 **Rutter MD**, Saunders BP, Schofield G, Forbes A, Price AB, Talbot IC. Pancolonic indigo carmine dye spraying for the detection of dysplasia in ulcerative colitis. *Gut* 2004; **53**: 256-260 [PMID: 14724160]
41 **Kiesslich R**, Fritsch J, Holtmann M, Koehler HH, Stolte M, Kanzler S, Nafe B, Jung M, Galle PR, Neurath MF. Methylene blue-aided chromoendoscopy for the detection of intraepithelial neoplasia and colon cancer in ulcerative colitis. *Gastroenterology* 2003; **124**: 880-888 [PMID: 12671882 DOI: 10.1053/gast.2003.50146]
42 **Marion JF**, Waye JD, Present DH, Israel Y, Bodian C, Harpaz N, Chapman M, Itzkowitz S, Steinlauf AF, Abreu MT, Ullman TA, Aisenberg J, Mayer L. Chromoendoscopy-targeted biopsies are superior to standard colonoscopic surveillance for detecting dysplasia in inflammatory bowel disease patients: a prospective endoscopic trial. *Am J Gastroenterol* 2008; **103**: 2342-2349 [PMID: 18844620 DOI: 10.1111/j.1572-0241.2008.01934.x]
43 **Kiesslich R**, Goetz M, Lammersdorf K, Schneider C, Burg J, Stolte M, Vieth M, Nafe B, Galle PR, Neurath MF. Chromoscopy-guided endomicroscopy increases the diagnostic yield of intraepithelial neoplasia in ulcerative colitis. *Gastroenterology* 2007; **132**: 874-882 [PMID: 17383417 DOI: 10.1053/j.gastro.2007.01.048]
44 **Sanduleanu S**, Rutter MD. Interval colorectal cancers in inflammatory bowel disease: the grim statistics and true stories. *Gastrointest Endosc Clin N Am* 2014; **24**: 337-348 [PMID: 24975525 DOI: 10.1016/j.giec.2014.03.001]
45 **Sanduleanu S**, Rondagh EJ, Masclee AA. Development of expertise in the detection and classification of non-polypoid colorectal neoplasia: Experience-based data at an academic GI unit. *Gastrointest Endosc Clin N Am* 2010; **20**: 449-460 [PMID: 20656243 DOI: 10.1016/j.giec.2010.03.006]
46 **Srivastava A**, Redston M, Farraye FA, Yantiss RK, Odze RD. Hyperplastic/serrated polyposis in inflammatory bowel disease: a case series of a previously undescribed entity. *Am J Surg Pathol* 2008; **32**: 296-303 [PMID: 18223333 DOI: 10.1097/PAS.0b013e318150d51b]
47 **Haanstra JF**, Vasen HF, Sanduleanu S, van der Wouden EJ, Koornstra JJ, Kleibeuker JH, de Vos Tot Nederveen Cappel WH. Quality colonoscopy and risk of interval cancer in Lynch syndrome. *Int J Colorectal Dis* 2013; **28**: 1643-1649 [PMID: 23857598 DOI: 10.1007/s00384-013-1745-2]
48 **Newton K**, Green K, Lalloo F, Evans DG, Hill J. Colonoscopy screening compliance and outcomes in patients with Lynch syndrome. *Colorectal Dis* 2015; **17**: 38-46 [PMID: 25213040 DOI: 10.1111/codi.12778]
49 **Richter JM**, Pino MS, Austin TR, Campbell E, Szymonifka J, Russo AL, Hong TS, Borger D, Iafrate AJ, Chung DC. Genetic mechanisms in interval colon cancers. *Dig Dis Sci* 2014; **59**: 2255-2263 [PMID: 24705641 DOI: 10.1007/s10620-014-3134-2]
50 **Erichsen R**, Baron JA, Stoffel EM, Laurberg S, Sandler RS, Sørensen HT. Characteristics and survival of interval and sporadic colorectal cancer patients: a nationwide population-based cohort study. *Am J Gastroenterol* 2013; **108**: 1332-1340 [PMID: 23774154 DOI: 10.1038/ajg.2013.175]
51 **Farrar WD**, Sawhney MS, Nelson DB, Lederle FA, Bond JH. Colorectal cancers found after a complete colonoscopy. *Clin Gastroenterol Hepatol* 2006; **4**: 1259-1264 [PMID: 16996804 DOI: 10.1016/j.cgh.2006.07.012]
52 **Snover DC**. Update on the serrated pathway to colorectal carcinoma. *Hum Pathol* 2011; **42**: 1-10 [PMID: 20869746 DOI: 10.1016/j.humpath.2010.06.002]
53 **Gill MD**, Bramble MG, Hull MA, Mills SJ, Morris E, Bradburn DM, Bury Y, Parker CE, Lee TJ, Rees CJ. Screen-detected colorectal cancers are associated with an improved outcome compared with stage-matched interval cancers. *Br J Cancer* 2014; **111**: 2076-2081 [PMID: 25247322 DOI: 10.1038/bjc.2014.498]

**P- Reviewer**: Gibson J, Jakovljevic Mihajlo B **S- Editor**: Yu J
**L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12742

*World J Gastroenterol* 2015 December 7; 21(45): 12742-12756
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


REVIEW

# *Helicobacter pylori*-induced inflammation and epigenetic changes during gastric carcinogenesis

Manuel A Valenzuela, Jimena Canales, Alejandro H Corvalán, Andrew FG Quest

Manuel A Valenzuela, Jimena Canales, Andrew FG Quest, Laboratorio de Comunicaciones Celulares, Centro de Estudios Moleculares de la Célula, Programa de Biología Celular y Molecular, ICBM, Facultad de Medicina, Universidad de Chile, Santiago 8380453, Chile

Manuel A Valenzuela, Jimena Canales, Alejandro H Corvalán, Andrew FG Quest, Cellular Communication Laboratory, Center for Molecular Studies of the Cell, Advanced Center for Chronic Diseases, School of Medicine, Universidad de Chile, Santiago 8380453, Chile

Alejandro H Corvalán, UC - Centro de Investigación en Oncología, Pontificia Universidad Católica de Chile, Santiago 8330034, Chile

Alejandro H Corvalán, Departamento de Hematología-Oncología, Facultad de Medicine, Pontificia Universidad Católica de Chile, Santiago 8330074, Chile

Author contributions: Valenzuela MA and Quest AFG wrote and edited the review, designed its structure and made conceptual revisions of the different sections; Canales J contributed with a bibliographic revision and discussion in the section "Genes modified by methylation upon *H. pylori* infection", and also designed Figure 1; Corvalán AH contributed with the discussion in the section "Reprimo as a model for epigenetic changes in tumor suppressor genes in gastric cancer" and the clinical findings concerning miRNA biology.

Supported by CONICYT-FONDAP project, No. 15130011 (to Corvalánand AH and Quest AFG); FONDECYT project, No. 1151411 (to Corvalán AH); and a CONICYT PhD fellowship award (to Canales J).

Conflict-of-interest statement: Authors declare no conflict of interests for this article



Correspondence to: Andrew FG Quest, PhD, Cellular Communication Laboratory, Center for Molecular Studies of the Cell, Advanced Center for Chronic Diseases, School of Medicine, Universidad de Chile, Av. Independencia 1027, Santiago 8380453, Chile. aquest@med.uchile.cl
Telephone: +56-2-27382015
Fax: +56-2-27382015

Received: May 27, 2015
Peer-review started: May 29, 2015
First decision: July 14, 2015
Revised: August 8, 2015
Accepted: October 13, 2015
Article in press: October 13, 2015
Published online: December 7, 2015

## Abstract

The sequence of events associated with the development of gastric cancer has been described as "the gastric precancerous cascade". This cascade is a dynamic process that includes lesions, such as atrophic gastritis, intestinal metaplasia and dysplasia. According to this model, *Helicobacter pylori* (*H. pylori*) infection targets the normal gastric mucosa causing non-atrophic gastritis, an initiating lesion that can be cured by clearing *H. pylori* with antibiotics or that may then linger in the case of chronic infection and progress to atrophic gastritis. The presence of virulence factors in the infecting *H. pylori* drives the carcinogenesis process. Independent epidemiological and animal studies have confirmed the sequential progression of these precancerous lesions. Particularly long-term follow-up studies estimated a risk of 0.1% for atrophic gastritis/intestinal metaplasia and 6% in case of dysplasia for the long-term development of gastric cancer. With this in mind, a better understanding of the genetic and epigenetic changes associated with progression of the cascade is critical in determining the risk of gastric cancer associated with *H. pylori* infection. In this review, we will summarize some of the most

relevant mechanisms and focus predominantly but not exclusively on the discussion of gene promoter methylation and miRNAs in this context.

**Key words:** *Helicobacter pylori*; Methylation; Gastric cancer; Epigenetics; MicroRNA



**Core tip:** *Helicobacter pylori* infection increases the risk of developing gastric cancer. Intestinal type gastric cancer is characterized by a histological cascade in which aberrant methylation of CpG islands and deregulation of microRNAs are observed. An exacerbated host response and bacterial virulence factors contribute to these epigenetic changes by enhancing DNA methyl transferase activity *via* nitric oxide production and silencing of tumor suppressor genes and miRNAs. Interestingly, methylated Reprimo DNA is detectable in blood samples and is potentially useful as an early detection marker. Finally, also the role of gamma glutamyl transpeptidase related mechanisms in the loss of the anti-apoptotic protein Survivin and gastric carcinogenesis is discussed.

Valenzuela MA, Canales J, Corvalán AH, Quest AFG. *Helicobacter pylori*-induced inflammation and epigenetic changes during gastric carcinogenesis. *World J Gastroenterol* 2015; 21(45): 12742-12756 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12742.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12742

## PRECANCEROUS CASCADE OF GASTRIC CANCER

*Helicobacter pylori* (*H. pylori*)-is a Gram negative bacteria that colonizes the gastric epithelium of more than 50% of the adult population worldwide and is responsible for 75 % of all gastric cancer cases[1]. Chronic infection with this pathogen is an ethiological agent responsible for gastric pathologies such as chronic gastritis, peptic ulcer, mucosa-associated lymphoid tissue (MALT) lymphoma and increased risk of developing gastric adenocarcinoma[2], the second leading cause of cancer-related deaths worldwide[3]. Histologically, gastric adenocarcinoma is divided into two sub-types following Lauren's classification[4], termed Diffuse and Intestinal-type gastric cancer. For both types, a strong correlation with *H. pylori*-associated inflammation exists[5]. No identifiable precursor lesions are associated with the development of diffuse type gastric cancer; instead, this type of cancer appears to be closely linked to host genetic factors[6]. Alternatively, intestinal type gastric cancer follows a well-defined histological sequence of progression, initiating as chronic gastritis followed by atrophy, intestinal metaplasia, dysplasia and finally terminating in intestinal type gastric cancer[7,8].

One of the proposed mechanisms held responsible for initiation of the intestinal metaplasia/dysplasia/carcinoma sequence, also referred to as the pre-cancerous cascade of gastric cancer, is the loss of the specialized parietal cells, due to alterations in cellular cross-talk and cell differentiation that are crucial for gastric epithelial precursor cell maturation and the coordination of cell migration in the gastric pits[7]. In this local inflammatory environment, bacterial factors altering oncogenic signaling pathways are crucial to propagate the carcinogenic sequence of events. However, bacterial eradication with antibiotics can reverse metaplastic and dysplastic lesions, suggesting that the differentiation/transformation in the gastric mucosa depends on the local environment and cannot be ascribed exclusively to to genetic alterations[7]. Additionally, *H. pylori* alters the expression of tumor suppressor genes by epigenetic deregulation. Consistent with this notion, methylation of such promoter regions is frequently observable *in vivo* during *H. pylori* infection[9,10]. In the following sections, evidence is summarized describing how bacteria-host cell interactions trigger epigenetic changes crucial to understanding the process of *H. pylori*-induced gastric carcinogenesis.

## *H. PYLORI*-INDUCED INFLAMMATION, EPIGENETICS AND GASTRIC CANCER

Persistent inflammation is a prevalent contributing factor to the development of many types of cancer[11-13]. For instance, infection with bacterial and viral agents is known to promote cancer in different epithelia[14]. Among the many potentially relevant intermediates produced during inflammation are molecules, such as cytokines, chemokines, free radicals (ROS and NOS), prostaglandins, growth factors and matrix metalloproteinases (Figure 1). These induce different epigenetic changes, including DNA methylation and histone modifications, which contribute to several steps important in tumorigenesis and progression involving leukocyte recruitment, neo-angiogenesis, proliferation, survival, invasion, and finally metastasis of tumor cells[13,15]. Specifically, *H. pylori*-induced gastric carcinogenesis has been associated with chronic inflammation, characterized by infiltration of neutrophils and macrophages to the gastric epithelium, which favor the accumulation of pro-inflammatory cytokines and reactive oxygen/nitrogen species (ROS/RNS). Accordingly, host factors exacerbating inflammatory responses are also responsible for the final outcome. For instance, polymorphisms present in IL-8, TNF-$\alpha$ and IL-1$\beta$ promoter regions are associated with more severe signs of inflammation and an increased risk of developing gastric cancer[16-19]. *H. pylori*-associated chronic inflammation is linked to

silencing of tumor suppressor genes *via* epigenetic modification[20]. Methylation of CpG islands in the promoter regions of tumor suppressor genes and deregulation of some onco-miRs have emerged as highly relevant players in the context of understanding *H. pylori*-linked gastric carcinogenesis.

Importantly, studies in animal models of *H. pylori*-associated carcinogenesis further support this hypothesis. For instance, a study in the Mongolian gerbil model, demonstrated that *H. pylori* infection promotes DNA methylation as a consequence of chronic inflammation[21]. Interestingly, bacterial eradication did not decrease significantly overall DNA methylation in the gastric epithelium. However, upon suppression of inflammatory responses in gerbils treated with Cyclosporin A, the increased level of methylation was blocked without affecting bacterial colonization[21]. Following a similar rationale, the same authors described that prevention of DNA methylation using 5-aza-2-deoxycytidine decreased the incidence of gastric cancer induced by *H. pylori* infection[22]. However, in this study development of gastric cancer was not completely prevented, suggesting *H. pylori* could promote gastric cancer *via* additional mechanisms beyond aberrant DNA methylation, perhaps by favoring the development of genetic mutations. Likewise, a clinical study using human gastric samples showed that higher levels of DNA methylation in some genes typically hypermethylated in gastric cancer correlated with more severe gastric inflammation and more advanced precancerous lesions[23].

The effect of *H. pylori* induced-inflammation on epigenetic DNA modifications has also been observed *in vitro*. Katayama and colleagues[24] showed that *H. pylori* induces iNOS expression and NO production in macrophages, and the co-culture of macrophages and gastric cells promotes RUNX3 methylation in the gastric cells, without a requirement for direct interaction between gastric cells and the bacteria[24]. Alternatively, Huang *et al*[25] showed that E-cadherin gene methylation occurred in a manner independent of immune cells, because the direct interaction between *H. pylori* and gastric cells increased methylation of this gene. Furthermore, DNA methyl transferase (DNMT) activity was enhanced by increasing NO levels due to iNOS induction in host cells triggered by IL-1β secretion. Moreover, the participation of interleukin-1β in *H. pylori*-induced gastric inflammation and DNA methylation was confirmed using a receptor type 1 knockout (IL-1R1$^{-/-}$) mouse model in which gastritis, NO production and methylation levels were reduced[26]. Despite some discrepancies between these studies, both point towards the importance of the pro-inflammatory molecule NO as a key mediator in the *H. pylori*-induced methylation of genes relevant to the genesis of gastric cancer. In agreement with this conclusion, *H. pylori* infection of iNOS$^{-/-}$ mice lead to a decreased incidence of gastric cancer compared with wild type controls[27].

## DNA DAMAGE AND *H. PYLORI* INFECTION

During gastric cancer progression and similar to other types of cancer, chronic inflammation leads to epigenetic changes characterized by the hypermethylation of driver tumor-suppressor genes, as well as passenger genes, and the hypomethylation of repetitive DNA sequences generating genomic instability[20,28]. Consistent with these observations, dietary supplementation with folic acid (a methyl donor) prevented the decrease in global DNA methylation as well as gastric dysplasia and mucosal inflammation observed during *H. pylori*-associated carcinogenesis in mice[29]. However, for an important proportion of gastric cancers mutations in tumor suppressor genes are also detected, indicating that this type of cancer originates not only as an epigenetic disease. For instance, mutations, particularly in genes like TP53, APC, CTNNB1, CDH1 and KRAS are frequently observed in gastric cancer[30].

Inflammatory processes initiated in response to *H. pylori* infection are accompanied by the formation of reactive oxygen and nitrogen species (ROS/NOS), predominantly by activated neutrophils, macrophages and gastric cells in response to bacterial factors[31,32] (Figure 1). As a consequence, elevated levels of oxidized proteins and fragmented DNA are detected in the gastric mucosa[31]. Nitric oxide produced by inducible oxide synthase (iNOS) increases early in the infected mucosa and, as a consequence, DNA adducts, such as 8-nitroguanine and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG or 8-OHdG) are readily detected in humans and animal models[33,34]. Another mechanism that contributes to mutagenesis of host DNA is the *H. pylori*-induced aberrant NF-κB-dependent expression of activation-induced cytidine deaminase (AID), an enzyme that functions as a DNA- and RNA-editing enzyme in response to *H. pylori* infection[35]. Upregulation of AID favored nucleotide alterations in the TP53 tumor suppressor gene in gastric cells *in vitro*, and similar alterations were also detected in the gastric mucosa of infected patients[35,36]. Studies in animal models indicate that oxidative stress is an important contributing factor to gastric carcinogenesis. For instance, nutritional supplementation with sulphoraphane, a natural compound and activator of the antioxidant transcriptional factor Nrf2, was shown to protect mice against gastric pathologies induced by *H. pylori* infection[37]. Furthermore, similar results were obtained upon supplementation with antioxidants like ascorbic acid in humans[38]. A possible explanation for these observations is that ROS/NOS are responsible for methylation of antioxidant enzyme genes[20]. Also, it is important to consider that ROS accumulation produces directly epigenetic changes. For instance,

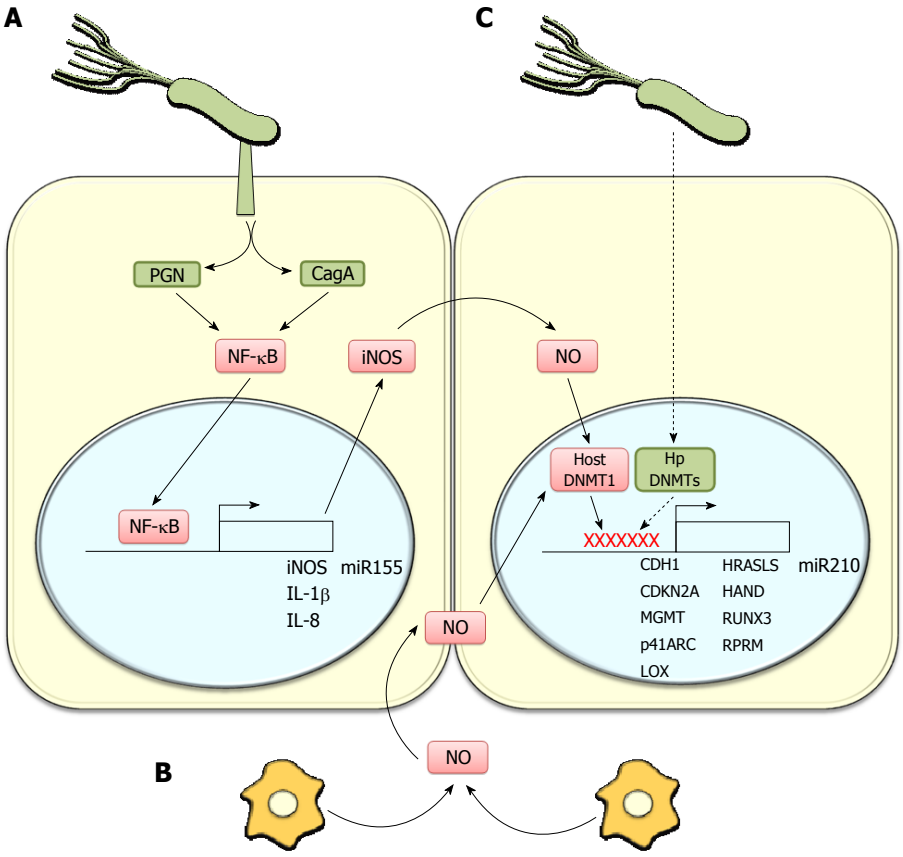



**Figure 1** ***Helicobacter pylori*-linked inflammation promotes methylation of genes associated with tumor suppression.** A: *Helicobacter pylori* (*H. pylori*) induces, *via* injection of the virulence factors CagA and peptidoglycan (PGN), NF-κB activation, promoting a pro-inflammatory response that increases the expression of cytokines (IL-1β, IL-8) and inducible nitric oxide synthase (iNOS). The latter generates nitric oxide (NO) a mediator of inflammation. Furthermore, the *H. pylori*-linked activation of NF-κB promotes miR-155 expression, a microRNA associated with gastric pathologies; B: In addition to the direct effects of *H. pylori* on gastric epithelial cells, the infection activates macrophages, which increase NO production and NO levels in gastric epithelial cells. NO can activate DNA methyltransferase 1 (host DNMT1) to promote DNA methylation in several gene promoter regions; C: Also *H. pylori* DNA Methyltransferases (Hp DNMTs) may directly promote host DNA methylation in the promoter regions of several genes thought to function as tumor suppressors in gastric cancer. Moreover, infection is associated with promoter methylation and decreased expression of miR210, a miRNA which targets mRNAs implicated in stem cell survival, stalling of the DNA repair system, induction of angiogenesis and cellular differentiation, among others.

free radical adducts have been shown to alter DNA cytosine methylation[39] and NO can enhance DNMT activity[25].

## *H. PYLORI* VIRULENCE FACTORS ASSOCIATED WITH INFLAMMATORY RESPONSES AND EPIGENETIC CHANGES IN THE GASTRIC MUCOSA

The experiments in Mongolian gerbils infected with *H. pylori* showing that the presence of the immunosuppressor drug Cyclosporine A reduces the ability of *H. pylori* to induce changes in the methylation status in gastric cells would suggest that *H. pylori* is not directly responsible for triggering aberrant methylation and that these alterations may be attributed to the inflammatory response. However, this point is still controversial because the *H. pylori* genome encodes a large number of cytosine and adenosine-specific type Ⅱ DNA methyl transferases that would be expected to eventually methylate the host DNA because no additional factors are required for enzymatic activity. Relevant examples in this context is m6A or m4C modifications that normally are not considered in current studies[40,41]. Also, epidemiological and basic research data all point towards a number of bacterial factors as being responsible for the observed gastric inflammation, deregulation of cellular signaling pathways and cancer. Some 60% of Western *H. pylori* isolates bear a *cag* pathogenicity island (*cag*PAI)[11], which encodes among other components a type Ⅳ secretion system (T4SS) that is able to transfer macromolecules, peptidoglycans and DNA into the host cells[42] (Figure 1). The presence and functionality of the *cag*PAI correlates very strongly with the development of peptic ulcer and gastric cancer[43]. Interestingly, NF-κB activation is found to be dependent on an intact T4SS secretion system[44]. For instance, one study showed that peptidoglycans injected by the T4SS apparatus were responsible for NF-κB activation *via* the intracellular pathogen recognition receptor NOD1[45].

As mentioned previously, Mongolian gerbils infected

with *H. pylori* undergo histological changes similar to those observed in human gastric carcinogenesis[46]. In a recent study, the inactivation of the peptidoglycan deacetylase (PgdA) enzyme in a particularly carcinogenic *H. pylori* strain (*H. pylori* 7.13, isolated following animal passage)[47], showed that this peptidoglycan modification enhanced the pro-inflammatory activity of this molecule[48]. However, it should be noted that this observation is controversial, because in another study PgdA inactivation contributed to bacterial survival by mitigating the immune response[49]. Together, these studies demonstrate once again the relevance of T4SS functionality in the promotion of inflammation and gastric cancer, although the precise contribution of peptidoglycan-dependent activation of the intracellular receptor NOD1 to aberrant methylation requires further investigation.

On the other hand, the T4SS is also implicated in the injection of another important carcinogenic factor, the immuno-dominant cytotoxin CagA (cytotoxin associated gene A), which is perhaps the best characterized factor in *H. pylori*-associated gastric cancer development[50] (Figure 1) Accordingly, patients infected with CagA+ strains develop more pronounced inflammation and higher levels of IL-8 and IL-1β[51]. CagA (125-145 KDa) is also encoded in the *cag*PAI and is present in 60% of *H. pylori* isolates. After bacterial adhesion, this factor is injected into host cells by the T4SS[52,53]. Once attached to the inner leaflet of the plasma membrane, multimeric CagA is phosphorylated by c-Src and c-Abl kinases on tyrosine residues present in the Glu-Pro-Ile-Tyr-Ala (EPIYA)- motifs of the C-terminal region of the protein[52,53]. Four different EPIYA motifs (A, B, C and D) exist, which determine the genetic variability of CagA due to differences in the number and the sequences of these motifs. In particular, the D motif is exclusively found in East-Asian CagA+ strains, where its presence is considered a major risk for developing gastric cancer[54]. The oncogenic activity of CagA has been demonstrated in a variety of settings where sustained expression of this factor promotes primary gastric cell immortalization[55], intestinal trans-differentiation[8], epithelial-mesenchymal transition[56], loss of cell-cell adhesion[57], extracellular matrix remodeling[58], metastasis[59] and gastrointestinal and hematopoietic neoplasms in mouse models[60]. Signaling pathways may be altered by CagA activity both in phosphorylation-dependent and -independent ways[61]. Phosphorylation-dependent downstream signaling processes include the recruitment of Src homology 2 (SH2)-domain containing molecules[62]. The ensuing events perturb the actin cytoskeleton, and in doing so trigger elongation and scattering of infected host cells *in vitro*, a morphological change referred to as the "hummingbird phenotype"[63]. Phosphorylation-independent signaling induces pro-inflammatory events, mitogenic responses and disruption of cell-cell junctions[62]. Interestingly, the Grb2 adapter can interact with CagA in a phosphorylation-dependent and -independent manner. Furthermore, transgenic expression of CagA mimics the eukaryotic Grb2-associated adaptor protein in Drosophila melanogaster[64]. This interaction promotes Ras activation and in turn stimulates the Raf-MEK-ERK signalling cascade. Particularly, this axis appears to be involved in the CagA phosphorylation-independent persistent activation of transcription factors, such as NF-κB (inflammation)[65-67]. Interestingly, in infected patients, the presence of CagA+ strains is associated with increased levels in the methylation of certain promoters when compared to CagA- infections[68]. Similar observations have also been reported *in vitro*. For instance, ectopic CagA expression can promote silencing of let-7 microRNA by enhancing histone and DNA methylation[69].

The cytotoxin VacA, another potentially relevant secreted bacterial factor, induces vacuole formation in cells treated with purified or recombinant forms of the protein[70,71]. Initially, its activity was associated with apoptosis of gastric cells[72], the inhibition of T and B cell proliferation[73] and induction of autophagy[74] *in vitro*. Despite the many functions attributed to VacA *in vitro*, their relevance *in vivo* is questionable[75]. Of note, vacuolization of cells has been observed in human biopsies and oral administration of partially purified toxin produces mucosal damage in mice[76]. However, infection of gnotobiotic piglets and Mongolian gerbils with isogenic *H. pylori* mutants lacking VacA causes indistinguishable degrees of gastritis in both cases[77]. Despite such results, epidemiology data indicate that the presence of more active variants of the toxin is associated with increased inflammation, peptic ulcers and even cancer[78,79]. Also, a group recently demonstrated that an *H. pylori* strain bearing a more active VacA induced metaplasia in mice[80]. Furthermore, VacA alleles with elevated activity are associated with enhanced secretion of pro-inflammatory cytokines such as TNF-α, MIP-1α, IL-1β, IL-8, IL-10 and IL-13 *in vitro* and expression of the *s1m1* allele correlates with increased inflammation in the gastric mucosa[76,81,82]. In summary, it remains unclear whether VacA effects are relevant during early steps of carcinogenesis in promoting gastric epithelial damage or whether this factor promotes aberrant epigenetic changes during gastric progression and further research is required to resolve this issue.

## THE EPIGENETIC BASIS OF THE GASTRIC PRECANCEROUS CASCADE

The control of gene expression *via* epigenetic mechanisms, including DNA methylation and microRNAs, are important regulatory events that modulate all pathways in the cellular network[83]. Moreover, these epigenetic modifications are considered excellent candidates to explain how environmental factors impact on the genome and cell function and increase the risk of cancer

development[84].

### *DNA methylation in gastric cancer and the precancerous cascade*

DNA methylation, a process whereby cytosine bases are modified with a methyl group in the 5' position when they are followed by a guanine in the DNA sequence, is increasingly considered to be of great importance in gastric carcinogenesis[85]. Currently, a list of gastric cancer genes is emerging that are altered by DNA methylation and considered important in the progression of the cascade[86]. However, only few reports address the role of DNA hypermethylation in the gastric precancerous cascade. Importantly, higher methylation levels are detected in intestinal metaplasia than in atrophic gastritis samples[87,88]. However, no differences were observed in the methylation status between intestinal metaplasia and dysplasia. Further studies have shown methylation in multiple genes in *H. pylori*-induced chronic gastritis in comparison with healthy donors[89,90] suggesting that *H. pylori* infection potently induces aberrant DNA methylation.

As mentioned above, aberrant DNA methylation can be considered as a mechanism to explain how environmental factors alter the susceptibility of individuals to gastric cancer[84]. To support this idea, Chan *et al*[89] and Leung *et al*[91] evaluated changes in DNA methylation of the promoter region of the CDH-1 gene before and after the eradication of *H. pylori*, and in both studies complete regression of DNA methylation after *H. pylori* eradication was observed.

### *Genes modified by methylation upon H. pylori infection*

**Genes linked to intercellular junctions:** As indicated above, extensive evidence is available linking gastric cancer to the silencing of the CDH1 gene by aberrant methylation. E-cadherin is a well-established tumor suppressor and represents an important component of adherent junctions[92]. A number of studies have pointed to the existence of a connection between the *H. pylori* infection status and CDH1 methylation in pre-cancer lesions. For instance, *H. pylori* infection and aberrant DNA methylation of CpG islands in the CDH1 promoter correlated in dyspeptic patients[89]. Perri *et al*[93] confirmed this observation, demonstrating that the CDH-1 promoter is also hypermethylated in gastritis patients infected with *H. pylori*, and that this was reduced following bacterial eradication. Interestingly, an *in vitro* study showed that *H. pylori* infection augmented E-cadherin promoter methylation *via* activation of the IL-1β receptor, iNOS induction and subsequently increased DNMT activity as a consequence of NO production[25] (Figure 1).

Vezatin (VEZT), another Adherent junction protein, is also silenced by gene promoter hypermethylation and is proposed to represent a novel tumor suppressor in gastric cancer[94]. As for CDH-1, the VEZT promoter is found to be hypermethylated in biopsies from *H. pylori*-positive chronic gastritis patients when compared with the non-infected control group. Additionally, an *in vitro* study demonstrated that *H. pylori* infection of the non-transformed gastric epithelial GES-1 cell line was associated with VEZT promoter methylation and a reduction in Vezatin mRNA levels was also observed[94]. Aberrant methylation of the VEZT promoter is also observed in other types of cancer, and re-expression of the protein reduces malignancy of these cells[94], indicating that Vezatin might be an important target during *H. pylori*-associated aberrant methylation and gastric carcinogenesis.

Connexin 32 (Cx32) and Connexin 43 (Cx43) are structural components of gap junctions between epithelial cells[95]. Wang and colleagues demonstrated that expression of these connexins was decreased in gastric pre-neoplastic lesions associated with *H. pylori* infection when compared to non-infected individuals and that these changes were closely associated with methylation in the respective promoter regions. Furthermore, hypermethylation of Cx32 and Cx43 promoters increased during the progression from pre-neoplasic to neoplastic lesions in infected patients[96].

**Genes linked to cell cycle regulation:** CDKN2A, another important tumor suppressor gene that is normally methylated in gastric cancer, encodes the p16 (INK4A) protein, which is involved in cell cycle arrest in the G1 phase[92]. Maekita *et al*[90] showed that methylation of this promoter was significantly elevated in the mucosa of *H. pylori*-positive compared with *H. pylori*-negative healthy volunteers[90]. Additionally, another study demonstrated that methylation levels of CDKN2A in the gastric mucosa did not differ from those observed in intestinal metaplasia patients, indicating that CDKN2A promoter methylation is an early event during *H. pylori*-associated gastric carcinogenesis. Furthermore, eradication of *H. pylori* infection reverted the aberrant methylation status of this gene[93].

**Genes linked to DNA repair:** The gene hMHL-1 (the human homolog of *E. coli* MutL), encoding a DNA repair protein in humans, is also hypermethylated in the promoter region following *H. pylori* gastric infection. However, this event appears to occur in later stages of cancer progression, as revealed in a study of intestinal metaplasia lesions[93]. In a similar study, higher levels of CpG methylation were observed for the promoter of a gene encoding another DNA repair protein, O6-methylguanine DNA methyltransferase (MGMT), in the gastric mucosa of gastritis patients compared to control patients. Following *H. pylori* eradication, methylation was reduced from 70% to 48% of the cases, coincident with significantly reduced CpG methylation and increased MGMT expression. Furthermore, in this same study, the authors demonstrated that MGMT methylation was remarkably higher in those patients infected with CagA bearing strains[68]. A recent study showed that hMHL-1 and MGMT promoters are hypermethylated in biopsies from *H.*

*pylori*-associated chronic gastritis adult patients, but not in biopsy samples from infected children, and that hypermethylation was associated with *H. pylori* infection in the case of the MGMT promoter[97].

**Genes linked to inflammation**: TFF-2 gene encodes the Trefoil factor 2 protein, involved in wound healing and modulating the inflammatory response in the stomach[98,99]. Chronic *H. pylori* infection was shown to enhance promoter methylation of TFF-2 gene in the gastric mucosa. Furthermore, methylation of the TFF2 promoter is an early event that increases throughout gastric tumor progression[100]. On the other hand, the pro-inflammatory enzyme cycloxygenase-2 (COX-2) has been described to be hypermethylated in gastritis associated with *H. pylori* infection and bacterial eradication reverts completely the methylation[93]. COX enzymes are important to maintain tissue homeostasis and wound healing in the gastric mucosa. Therefore, methylation-mediated silencing of these genes might tend to favor the appearance of pre-neoplasic lesions.

**Genes encoding transcriptional factors**: RUNX3 is a transcription factor that is thought to function as a tumor suppressor by regulating the expression of several cancer-related genes, including p53, p27 and caspase-3, among others[101,102]. The RUNX3 promoter is hypermethylated in pre-neoplasic gastric lesions, increasing progressively from chronic atrophic gastritis to gastric cancer[103]. Additionally, *H. pylori* infection contributed to inactivation of the RUNX3 gene in gastric epithelial cells by augmenting promoter hypermethylation[103]. Moreover, an *in vitro* study confirmed that *H. pylori* promotes RUNX3 promoter methylation *via* NO produced during co-incubation with macrophages[24].

FOXD3 is a member of the forkhead box (Fox) transcription factor family, whose promoter is hypermethylated in gastric mucosa infected with *H. pylori*. The evidence provided indicates that silencing of FOXD3 could be important during gastric carcinogenesis given that ectopic expression of FOXD3 in cancer cell lines reduces cell proliferation and their invasive capacity[104].

The upstream stimulatory factors, USF1 and USF2, are pleiotropic transcriptional factors that regulate the expression of genes linked to immune responses, cell cycle control and cell proliferation[105]. Bussière and colleagues demonstrated *in vitro* that *H. pylori* decreases the expression of USF1 and USF2 genes by hypermethylation of their promoters regions. These observations were confirmed in gastric tissue samples from infected mice, where they observed decreased expression of USF1 and USF2 and hypermethylation of USF1 and USF2 promoters in *H. pylori*-associated gastritis[106]. These factors control cell growth and have been shown to block cMyc/Ras-mediated transformation of primary rat cells[105].

For the transcription factors GATA-4 and GATA-5, both tumor suppressors, a higher methylation status was detected in gastric samples from *H. pylori* infected patients than in those from non-infected patients. Changes in methylation were particularly notable for the GATA-4 promoter. In addition, among gastric tissues with or without chronic gastritis, those with GATA-4 methylation were from *H. pylori* infected patients and no GATA-4 methylation was observed in biopsies from individuals without *H. pylori* infection[107].

**Other tumor suppressor genes:** The LOX and HRASLS genes encode a lysyl oxidase and HRAS-like suppressor proteins, respectively. The promoters of these genes are highly methylated in *H. pylori*-infected patients as compared to those without infection. In the same study, higher levels of methylation were observed for the CpG isIands present in the promoter regions of the genes THBD, HAND1 and FLN in *H. pylori*-positive than in *H. pylori*-negative individuals[90]. On the other hand, Perri *et al*[93] determined that APC is hypermethylated in gastritis associated with *H. pylori* infection and that bacterial eradication was sufficient to reduce methylation of the promoter. p41ARC is a protein that regulates Arp2/3 complex formation and is necessary for cell migration[108]. For p41ARC, a higher degree of methylation was observed in an exonic CpG island in *H. pylori*-positive individuals than in non-infected individuals[90]. The tumor suppressor gene WWOX encodes the WW-domain containing oxidoreductase, a protein frequently down-regulated in several cancers. An *in vitro* study demonstrated that in gastric cancer cell lines infected with *H. pylori*, methylation of WWOX is elevated and, furthermore, in this model methylation was associated with *H. pylori*-enhanced expression of DNMT1 and DNMT3[109].

## MICRORNAS IN GASTRIC CANCER, THE PRECANCEROUS CASCADE AND *H. PYLORI* INFECTION

### *MicroRNA biology*

MiRNAs are 20-24 nucleotides single-strand RNAs that alter the stability and translation of target mRNAs and, in doing so, modulate gene expression at the post-transcriptional level[83,110]. A considerable number of reports in the literature suggest that miRNAs control several processes during carcinogenesis that favor tumor development and progression[83]. Reduced translation and stability of messenger RNAs (mRNAs) can be triggered by miRNA binding to the 3' untranslated region (3'UTR) of messenger RNAs (mRNAs). Most miRNAs are found in introns and intergenic regions and possess their own promoter and regulatory elements[111]. miRNAs have many physiological functions and control a variety of biological processes, including cellular differentiation, development, proliferation, metabolism, apoptosis and immune responses[112]. Not surprisingly, deregulation of miRNA levels is observed in

many pathological conditions beyond cancer, including neurodegenerative and cardiovascular diseases[113,114]. Notably, miRNA genes are frequently found in regions of chromosomal instability (amplification, translocation or deletion) or near chromosomal breakpoints[115]. In tumorigenesis, the target mRNAs affected by miRNAs include those involved in inflammation, cell cycle regulation, stress responses (autophagy), differentiation (epithelial-mesenchymal transition, EMT), apoptosis and invasion[116-118]. Several of the relevant target genes include oncogenes or tumor suppressors that are up-regulated or downregulated, respectively, in tumor progression and metastasis[112]. In addition to their function in physiological and pathological processes, miRNAs play an important role during microbial infection caused by viruses, bacteria, parasites and fungi[119].

### *MiRNA signatures in gastric cancer*

Tong *et al*[120] reported on frequent aberrant expression of miRNAs in gastric cancer. An association between miRNA expression and alterations in cell proliferation (miR-139, by targeting the CXCR4 gene; and miR-1, -34a and -504 by targeting the FOXP1 gene), cell cycle progression (miR-221, -222, -160b, -93 and -25, by targeting the p57, p21 and p27 genes) and invasion/metastasis (miR-21, by targeting the RECK gene) were detected. Ueda *et al*[121] analyzed 160 paired tumor and non-tumor mucosa samples and detected 22 miRNAs that were upregulated and 13 that were downregulated in gastric cancer. Interestingly, for intestinal and diffuse types of gastric cancer two different miRNA signatures were identified. Importantly, the miRNAs (miR-105, miR-100, miR-125b, miR-199a, miR-99a, miR-143, miR-145 and miR-133a) and (miR-373*, miR-498, miR-202*, and miR-494) were upregulated in diffuse-type and intestinal-type gastric cancer, respectively. Differential analysis of miRNA expression also revealed that progression in TNM staging correlated with higher expression levels of miR-125b, miR-199a, and miR-100. Also, reduced expression of let-7g and miR-433 and elevated expression of miR-214 were identified as independent predictors of poor survivial that were not related to depth of invasion, lymph-node metastasis or TNM stage. These findings show that miRNAs are differentially expressed in histological gastric cancer subtypes and that these are characterized by specific miRNA signatures.

Moreover, the expression of particular miRNAs is also linked to gastric cancer progression and prognosis. Ueda *et al*[121] identified miRNA expression profiles for gastric cancer by RT-PCR in 100 patients and identified a group of seven miRNAs as independent predictors of overall survival and relapse-free survival. In this study, hazard ratios (HRs) assessed by Cox regression analysis, demonstrated that miRNA expression levels correlated directly or indirectly with death probability: 3 miRNAs (let-7a, miR-126, miR-30a-5p) were protective (HR < 1) and 4 miRNAs (miR-10b, miR-21, miR-223, miR-338) were associated with higher risk of relapse and poorer survival (HR > 1).

The role of miRNAs in the precancerous cascade of gastric cancer was evaluated by Wang *et al*[122]. Using an *in silico* approach with Gene Expression Omnibus (GEO) datasets, these authors identified 20 differentially expressed miRNAs between *H. pylori*-related atrophic gastritis and intestinal metaplasia samples. These miRNAs modulated a variety of cell functions, including signal transduction, cell proliferation and death, as well as metabolite transport and catabolism. Among the target genes, RAB22A, SOX4, IKZF2, PLAG1 and BTBD7 were found to be simultaneously regulated by several differentially expressed miRNAs. On the other hand, miR-204 was decreased in *H. pylori*-associated atrophic gastritis[123]. Knockdown of this miRNA enhanced gastric cancer cell proliferation and invasion *in vitro*. Alternatively, miR-204 down-regulation and overexpression of SOX4 promoted the EMT process[123]. Additionally, the potential of microRNAs as biomarkers for early gastric detection was recently assessed[124-126]. In a population-based study in Linqu, a high-risk area of gastric cancer in China, Song *et al*[124] investigated the relevance of serum miRNAs as biomarkers. Serum pools from GC control and validated gastric cancer and dysplasia subjects were correctly identified by several differentially expressed miRNAs. A signature of 16 miRNAs that were upregulated in gastric cancer patients was identified. miR-221, miR-744, and miR-376c were subsequently validated as non-invasive biomarkers for gastric cancer detection with 82.4% sensitivity and 58.8% specificity. Fu *et al*[127] identified an additional miRNA, miR-222, for which circulating plasma levels increased in atrophic gastritis patients compared to healthy controls ($P < 0.001$) with a diagnostic accuracy of 0.850 and 66.1% sensitivity as well as 88.3% specificity. Also, the expression patterns of serum let-7 microRNA (miRNA) and its target gene pepsinogen C (PGC) were correlated with different stages of the multistage cascade of gastric cancer[126]. Finally, the feasibility of using gastric juice as test material was examined by Yu *et al*[125]. Significantly lower levels of gastric juice miR-129-1-3p and miR-129-2-3p with AUC values of 0.639 and 0.65 to distinguish gastric cancer from healthy controls.

### *Deregulation of miRNAs expression during H. pylori infection*

*H. pylori* infection *in vivo* and *in vitro* alters the expression of many miRNAs[128]. Several of these miRNAs were also found to be deregulated in gastric cancer. On the one hand, down-regulation of let7a, mir-31, mir-101, miR-141, miR-203, miR-210, miR-218, miR-375, miR-449 is observed, while miR-17, miR-20a, miR-21, miR-146a, miR-155 and miR-223 are up-regulated[129]. Interestingly, it has been suggested that these miRNAs may also be relevant to understanding *H. pylori* induced inflammation and carcinogenesis[129]. These miRNAs target mRNAs involved in biological processes, such as invasion and metastasis, pro-

liferation, cell cycle progression, apoptosis, epithelial mesenchymal transition and the immune response[129].

MiR-155-5p, generically referred to as miR-155[130] (Figure 1). It is a typical, multifunctional miRNA involved in several biological processes, such as hematopoiesis, inflammation, immunity and carcinogenesis[130]. This miRNA is considered an oncogenic microRNA (oncomiR), given that it is up-regulated in different types of cancer, such as lymphoma, breast, cervical, lung, colon, pancreatic and thyroid cancer[130]. The miR155HG/miR-155 gene is induced upon exposure to lipopolysaccharide exposure *via* Toll-like receptor activation[130]. This characteristic was ascribed to the presence of two relevant NF-κB binding sequences present in the BIC/miR-155 promoter at -178 and -1150[131]. Similar to other pathogens, *H. pylori* infection elevates miR-155 levels *in vitro* and *in vivo*[132]. Several studies have focused on hematological and inflammation-associated malignances linked to this miRNA. For instance, gastric MALT lymphoma can be reverted by 60%-80% following antibiotic eradication of bacteria; however, resistant MALT lymphomas maintain miR-155 overexpression even after bacterial eradication[133]. Other studies show that miR-155 is a negative regulator of pro-inflammatory responses by targeting the receptor-associated adapter MyD88, thereby reducing NF-κB activation and IL-8 secretion[134]. In another study, *H. pylori*-dependent miR-155 induction reduced DNA-damage and induced apoptosis in macrophages, in a manner dependent on the *cag*PAI secretory system and TLRs, but independent of CagA[135]. In summary, miR-155 represents a multifunctional miRNA, whose potential role in *H. pylori*-associated pathologies is apparent, but still poorly understood.

As mentioned previously, several miRNAs are known to be down-regulated during gastric cancer progression and, similar to tumor suppressor genes, they can be also silenced by promoter hypermethylation. For instance, the expression of several microRNAs, such as miR-10a, -10b, -127, -148a, -181c, -195, -219-2-3p, -224, -338-3p, -340, -378, -433 and -452 among others was recently reported to be silenced by aberrant methylation in gastric cancer[136-143]. Interestingly, Kiga *et al*[144] demonstrated that *H. pylori*-associated inflammation promoted DNA methylation of the miR-210 locus and enhanced proliferation of gastric cells as a consequence of augmented STMN1 and DIMT1 expression. MIR-210 (Figure 1) is an hypoxia responsive multifunctional microRNA whose promoter contains Hypoxia Inducible factor (HIF1α and 2α) response elements (HRE) and the target mRNAs are implicated in the regulation of cell growth arrest, repression of mitochondrial metabolism, stem cell survival under hypoxic conditions, stalling the DNA repair system (promoting genetic instability), angiogenesis induction and cellular differentiation[145]. Again, as for miR-155, silencing of miR-210 following *H. pylori* infection is likely to contribute to disease, but further studies are required.

Taken together, the data presented here suggest that miRNAs play essential roles in gastric cancer by modulating cell proliferation, cell cycle and invasion/metastasis. In the gastric precancerous condition, miRNAs are linked to *H. pylori*-related gastritis and intestinal metaplasia. From a biomarker point of view, miRNAs have a strong potential as novel molecular tools for non-invasive risk assessment of gastric cancer and precancerous lesions.

## REPRIMO AS A MODEL FOR EPIGENETIC CHANGES IN TUMOR SUPPRESSOR GENES IN GASTRIC CANCER

The Reprimo gene (official symbol RPRM, MIM 612171, GeneID 56475) is located in chromosome 2q23.3[146]. Sequence analysis indicates that RPRM is 303 bp long and does not have introns. The protein sequence includes 109 aa and has two glycosylation sites (AA 7-10 and 18-21), one phosphorylation site (AA 95-98) and one myristylation site (AA 13-18) (http://hits.isb-sib.ch/cgi-bin/motif_scan). Computational analysis shows a possible transmembrane domain between aa 56-78 (www.ensembl.org). According to Ohki and coworkers, RPRM is an intracellular cytoplasmic protein[147] that is induced after X-ray-irradiation in a p53-dependent manner. Ectopic p53 expression also results in the induction of the expression of RPRM. In both scenarios, RPRM causes G2 arrest of the cell cycle in association with the inhibition of nuclear translocation of cyclin B1 and Cdc2 activity[147]. An inverse association between RPRM gene expression and promoter methylation (Spearman rank R = -1; $P$ = 0.042) was recently identified. Additionally, RPRM overexpression robustly inhibited colony formation and anchorage-independent growth. Furthermore, in primary gastric cancer cases, RPRM protein was not detected in tumor tissues but was found to be present in non-tumor adjacent mucosa ($P$ = 0.001). Furthermore, the loss of expression is associated with invasive stages GC (stage Ⅰ to Ⅱ-Ⅳ, $P$ = 0.006). Interestingly, these findings correlated with p73 ($P$ < 0.0001 and kappa value = 0,363) but not p53 expression, suggesting a potential link to other members of the p53 family[148]. In a quantitative analysis of RPRM DNA promoter methylation, the *H. pylori* virulence factors cagA (including segments of the 3 end, encoding EPIYA polymorphisms) and vacA s1 and m1 regions were associated with increased RPRM promoter methylation[149]. These results favor the notion that *H. pylori* regulates RPRM gene expression[149]. Bernal *et al*[150] reported that methylated RPRM promoter sequences are not only a common finding in primary tissues of gastric cancer patients, but also that they can be detected in the plasma of these patients and rarely in healthy donors. A translational potentiality of this observation is the possibility that methylated circulating cell-free DNA

may be employed as a non-invasive biomarker for detection of gastric cancer and precancerous lesions.

## THE *H. PYLORI* γ-GLUTAMYL TRANSPEPTIDASE CONNECTION TO ADDITIONAL MODES OF REGULATION IN GASTRIC CANCER: SURVIVIN AS A MODEL

GGT is an enzyme that catalyzes the transpeptidation and hydrolysis of the γ-glutamyl moiety from glutathione and glutathione-conjugated compounds, to amino acids[151]. *H. pylori* γ-glutamyl transpeptidase (HpGGT) is constitutively expressed and detectable in all *H. pylori* strains[152], suggesting it is relevant to bacterial physiology. Among the many effects observed in gastric cells, GGT induces apoptosis[153], cell cycle arrest[154], and generates ROS, in particular $H_2O_2$, that potentially damages DNA[155]. Importantly, significantly higher GGT activity has been observed in *H. pylori* isolates obtained from patients with peptic ulcer disease than in those from patients with non-ulcer dyspepsia[155]. Gastric ulcers are associated with an elevated risk of developing gastric cancer[156] and these observations implicate HpGGT as being clinically relevant to the progression of precancerous gastric lesions.

Beyond the indicated alterations in gastric cells induced by GGT, recent evidence suggests that this virulence factor may also enhance proteasome-mediated degradation of proteins required for cell viability, as is the case for survivin, a member of the inhibitor of apoptosis (IAP) family of proteins. Survivin expression levels are controlled at the transcriptional level by several transcription factors and at the post-transcriptional level by phosphorylation and proteasome-mediated degradation[157]. Survivin is generally not expressed in normal differentiated tissues, except in proliferating stem cell populations, but is strongly upregulated in most human cancer cells where it enhances proliferation, viability, metastasis and angiogenesis[157-159]. Interestingly, the gastric mucosa apparently represents an exception to this general rule, because normal mucosa cells express high levels of survivin and it has been suggested that presence of the protein may favor mucosa cell survival in the adverse gastric environment. Importantly in biopsies from gastritis patients with *H. pylori* infection, survivin protein levels are decreased as compared to control samples from gastritis patients without infection[160]. In the same study, infection *in vitro* of cells of the gastrointestinal lineage with *H. pylori* was shown to trigger the loss of survivin protein and as a consequence reduces cell viability. Subsequently, this ability of *H. pylori* was linked to the secretion of HpGGT and enhanced turnover of the protein *via* the proteasome pathway, possibly *via* a mechanism involving the generation of ROS[161]. It is interesting to note here that this mechanism specifically affected survivin but not other anti-apoptotic proteins, like Bcl2 or Bcl-xL, and one may speculate that related events might control turnover of a number of yet to be identified components in infected cells. Finally, this GGT-proteasome connection may explain how early in disease development *H. pylori* damages the gastric tissue in zones with high levels of bacterial infection, favoring the genesis of a pro-inflammatory environment and potentially subsequent invasion by bone marrow-derived cells and intestinal metaplasia[162]. Further experimentation is required to substantiate this intriguing hypothesis that could also open up new avenues for the treatment of *H. pylori* positive gastritis patients.

## CONCLUSION

In summary, this review highlights how in addition to *H. pylori*-associated virulence factors, epigenetic changes in infected host cells, such as DNA methylation and miRNAs, are likely to play a significant role in gastric cancer development and the progression of the precancerous cascade. Moreover, recent results revealing how HpGGT modulates the turnover of specific protective host proteins underscores the tremendous variability and selectivity of changes that may be attributed to *H. pylori* infection. Importantly, from the clinical perspective, some of the epigenetic factors discussed can be determined by analyzing blood and other body fluid samples, thus opening up a new avenue for non-invasive risk assessment of gastric cancer. In doing so, we may anticipate that earlier diagnosis and, as a consequence, more effective treatment of this still very deadly cancer will become possible.

## REFERENCES


1 **Peleteiro B**, Bastos A, Ferro A, Lunet N. Prevalence of Helicobacter pylori infection worldwide: a systematic review of studies with national coverage. *Dig Dis Sci* 2014; **59**: 1698-1709 [PMID: 24563236]

2 **Watari J**, Chen N, Amenta PS, Fukui H, Oshima T, Tomita T, Miwa H, Lim KJ, Das KM. Helicobacter pylori associated chronic gastritis, clinical syndromes, precancerous lesions, and pathogenesis of gastric cancer development. *World J Gastroenterol* 2014; **20**: 5461-5473 [PMID: 24833876 DOI: 10.3748/wjg.v20.i18.5461]

3 **Liu JB**, Wu XM, Cai J, Zhang JY, Zhang JL, Zhou SH, Shi MX, Qiang FL. CpG island methylator phenotype and Helicobacter pylori infection associated with gastric cancer. *World J Gastroenterol* 2012; **18**: 5129-5134 [PMID: 23049225 DOI: 10.3748/wjg.v18.i36.5129]

4 **Lauren P**. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. an attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* 1965; **64**: 31-49 [PMID: 14320675]

5 **Handa Y**, Saitoh T, Kawaguchi M, Misaka R, Ohno H, Tsai CR, Tani Y, Tsurui M, Yoshida H, Morita S, Midorikawa S, Sanji T. Association of Helicobacter pylori and diffuse type gastric cancer. *J Gastroenterol* 1996; **31** Suppl 9: 29-32 [PMID: 8959514]

6 **Nardone G**, Rocco A, Malfertheiner P. Review article: helicobacter pylori and molecular events in precancerous gastric lesions. *Aliment Pharmacol Ther* 2004; **20**: 261-270 [PMID: 15274662 DOI: 10.1111/j.1365-2036.2004.02075.x]

7 **Correa P**, Houghton J. Carcinogenesis of Helicobacter pylori. *Gastroenterology* 2007; **133**: 659-672 [PMID: 17681184 DOI:

10.1053/j.gastro.2007.06.026]
8 **Murata-Kamiya N**, Kurashima Y, Teishikata Y, Yamahashi Y, Saito Y, Higashi H, Aburatani H, Akiyama T, Peek RM, Azuma T, Hatakeyama M. Helicobacter pylori CagA interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. *Oncogene* 2007; **26**: 4617-4626 [PMID: 17237808 DOI: 10.1038/sj.onc.1210251]
9 **Kaise M**, Yamasaki T, Yonezawa J, Miwa J, Ohta Y, Tajiri H. CpG island hypermethylation of tumor-suppressor genes in H. pylori-infected non-neoplastic gastric mucosa is linked with gastric cancer risk. *Helicobacter* 2008; **13**: 35-41 [PMID: 18205664 DOI: 10.1111/j.1523-5378.2008.00572.x]
10 **Nardone G**, Compare D, De Colibus P, de Nucci G, Rocco A. Helicobacter pylori and epigenetic mechanisms underlying gastric carcinogenesis. *Dig Dis* 2007; **25**: 225-229 [PMID: 17827945 DOI: 10.1159/000103890]
11 **Peek RM**, Crabtree JE. Helicobacter infection and gastric neoplasia. *J Pathol* 2006; **208**: 233-248 [PMID: 16362989 DOI: 10.1002/path.1868]
12 **Hirahara K**, Poholek A, Vahedi G, Laurence A, Kanno Y, Milner JD, O'Shea JJ. Mechanisms underlying helper T-cell plasticity: implications for immune-mediated disease. *J Allergy Clin Immunol* 2013; **131**: 1276-1287 [PMID: 23622118 DOI: 10.1016/j.jaci.2013.03.015]
13 **Yasmin R**, Siraj S, Hassan A, Khan AR, Abbasi R, Ahmad N. Epigenetic regulation of inflammatory cytokines and associated genes in human malignancies. *Mediators Inflamm* 2015; **2015**: 201703 [PMID: 25814785 DOI: 10.1155/2015/201703]
14 **Kawanishi S**, Hiraku Y, Pinlaor S, Ma N. Oxidative and nitrative DNA damage in animals and patients with inflammatory diseases in relation to inflammation-related carcinogenesis. *Biol Chem* 2006; **387**: 365-372 [PMID: 16606333 DOI: 10.1515/BC.2006.049]
15 **Fernandes JV**, Cobucci RN, Jatobá CA, Fernandes TA, de Azevedo JW, de Araújo JM. The role of the mediators of inflammation in cancer development. *Pathol Oncol Res* 2015; **21**: 527-534 [PMID: 25740073 DOI: 10.1007/s12253-015-9913-z]
16 **El-Omar EM**, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF, Rabkin CS. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 2000; **404**: 398-402 [PMID: 10746728 DOI: 10.1038/35006081]
17 **Sugimoto M**, Furuta T, Shirai N, Nakamura A, Xiao F, Kajimura M, Sugimura H, Hishida A. Different effects of polymorphisms of tumor necrosis factor-alpha and interleukin-1 beta on development of peptic ulcer and gastric cancer. *J Gastroenterol Hepatol* 2007; **22**: 51-59 [PMID: 17201881 DOI: 10.1111/j.1440-1746.2006.04442.x]
18 **Taguchi A**, Ohmiya N, Shirai K, Mabuchi N, Itoh A, Hirooka Y, Niwa Y, Goto H. Interleukin-8 promoter polymorphism increases the risk of atrophic gastritis and gastric cancer in Japan. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 2487-2493 [PMID: 16284368 DOI: 10.1158/1055-9965.EPI-05-0326]
19 **Zabaleta J**, Camargo MC, Piazuelo MB, Fontham E, Schneider BG, Sicinschi LA, Ferrante W, Balart L, Correa P, Ochoa AC. Association of interleukin-1beta gene polymorphisms with precancerous gastric lesions in African Americans and Caucasians. *Am J Gastroenterol* 2006; **101**: 163-171 [PMID: 16405550 DOI: 10.1111/j.1572-0241.2006.00387.x]
20 **Na HK**, Woo JH. Helicobacter pylori Induces Hypermethylation of CpG Islands Through Upregulation of DNA Methyltransferase: Possible Involvement of Reactive Oxygen/Nitrogen Species. *J Cancer Prev* 2014; **19**: 259-264 [PMID: 25574460 DOI: 10.15430/JCP.2014.19.4.259]
21 **Niwa T**, Tsukamoto T, Toyoda T, Mori A, Tanaka H, Maekita T, Ichinose M, Tatematsu M, Ushijima T. Inflammatory processes triggered by Helicobacter pylori infection cause aberrant DNA methylation in gastric epithelial cells. *Cancer Res* 2010; **70**: 1430-1440 [PMID: 20124475 DOI: 10.1158/0008-5472.CAN-09-2755]
22 **Niwa T**, Toyoda T, Tsukamoto T, Mori A, Tatematsu M, Ushijima T. Prevention of Helicobacter pylori-induced gastric cancers in gerbils by a DNA demethylating agent. *Cancer Prev Res* (Phila) 2013; **6**: 263-270 [PMID: 23559452 DOI: 10.1158/1940-6207.CAPR-12-0369]
23 **Schneider BG**, Piazuelo MB, Sicinschi LA, Mera R, Peng DF, Roa JC, Romero-Gallo J, Delgado AG, de Sablet T, Bravo LE, Wilson KT, El-Rifai W, Peek RM, Correa P. Virulence of infecting Helicobacter pylori strains and intensity of mononuclear cell infiltration are associated with levels of DNA hypermethylation in gastric mucosae. *Epigenetics* 2013; **8**: 1153-1161 [PMID: 24128875 DOI: 10.4161/epi.26072]
24 **Katayama Y**, Takahashi M, Kuwayama H. Helicobacter pylori causes runx3 gene methylation and its loss of expression in gastric epithelial cells, which is mediated by nitric oxide produced by macrophages. *Biochem Biophys Res Commun* 2009; **388**: 496-500 [PMID: 19665002 DOI: 10.1016/j.bbrc.2009.08.003]
25 **Huang FY**, Chan AO, Rashid A, Wong DK, Cho CH, Yuen MF. Helicobacter pylori induces promoter methylation of E-cadherin via interleukin-1β activation of nitric oxide production in gastric cancer cells. *Cancer* 2012; **118**: 4969-4980 [PMID: 22415887 DOI: 10.1002/cncr.27519]
26 **Huang FY**, Chan AO, Lo RC, Rashid A, Wong DK, Cho CH, Lai CL, Yuen MF. Characterization of interleukin-1β in Helicobacter pylori-induced gastric inflammation and DNA methylation in interleukin-1 receptor type 1 knockout (IL-1R1(-/-)) mice. *Eur J Cancer* 2013; **49**: 2760-2770 [PMID: 23664095 DOI: 10.1016/j.ejca.2013.03.031]
27 **Nam KT**, Oh SY, Ahn B, Kim YB, Jang DD, Yang KH, Hahm KB, Kim DY. Decreased Helicobacter pylori associated gastric carcinogenesis in mice lacking inducible nitric oxide synthase. *Gut* 2004; **53**: 1250-1255 [PMID: 15306579 DOI: 10.1136/gut.2003.030684]
28 **Ushijima T**, Hattori N. Molecular pathways: involvement of Helicobacter pylori-triggered inflammation in the formation of an epigenetic field defect, and its usefulness as cancer risk and exposure markers. *Clin Cancer Res* 2012; **18**: 923-929 [PMID: 22205689 DOI: 10.1158/1078-0432.CCR-11-2011]
29 **Gonda TA**, Kim YI, Salas MC, Gamble MV, Shibata W, Muthupalani S, Sohn KJ, Abrams JA, Fox JG, Wang TC, Tycko B. Folic acid increases global DNA methylation and reduces inflammation to prevent Helicobacter-associated gastric cancer in mice. *Gastroenterology* 2012; **142**: 824-833.e7 [PMID: 22248660 DOI: 10.1053/j.gastro.2011.12.058]
30 **Akhavan-Niaki H**, Samadani AA. Molecular insight in gastric cancer induction: an overview of cancer stemness genes. *Cell Biochem Biophys* 2014; **68**: 463-473 [PMID: 24078401 DOI: 10.1007/s12013-013-9749-7]
31 **Handa O**, Naito Y, Yoshikawa T. Helicobacter pylori: a ROS-inducing bacterial species in the stomach. *Inflamm Res* 2010; **59**: 997-1003 [PMID: 20820854 DOI: 10.1007/s00011-010-0245-x]
32 **Davies GR**, Simmonds NJ, Stevens TR, Sheaff MT, Banatvala N, Laurenson IF, Blake DR, Rampton DS. Helicobacter pylori stimulates antral mucosal reactive oxygen metabolite production in vivo. *Gut* 1994; **35**: 179-185 [PMID: 8307467]
33 **Touati E**, Michel V, Thiberge JM, Wuscher N, Huerre M, Labigne A. Chronic Helicobacter pylori infections induce gastric mutations in mice. *Gastroenterology* 2003; **124**: 1408-1419 [PMID: 12730880]
34 **Ma N**, Adachi Y, Hiraku Y, Horiki N, Horiike S, Imoto I, Pinlaor S, Murata M, Semba R, Kawanishi S. Accumulation of 8-nitroguanine in human gastric epithelium induced by Helicobacter pylori infection. *Biochem Biophys Res Commun* 2004; **319**: 506-510 [PMID: 15178435 DOI: 10.1016/j.bbrc.2004.04.193]
35 **Matsumoto Y**, Marusawa H, Kinoshita K, Endo Y, Kou T, Morisawa T, Azuma T, Okazaki IM, Honjo T, Chiba T. Helicobacter pylori infection triggers aberrant expression of activation-induced cytidine deaminase in gastric epithelium. *Nat Med* 2007; **13**: 470-476 [PMID: 17401375 DOI: 10.1038/nm1566]
36 **Shimizu T**, Marusawa H, Matsumoto Y, Inuzuka T, Ikeda A, Fujii Y, Minamiguchi S, Miyamoto S, Kou T, Sakai Y, Crabtree JE, Chiba T. Accumulation of somatic mutations in TP53 in gastric epithelium with Helicobacter pylori infection. *Gastroenterology* 2014; **147**: 407-417.e3 [PMID: 24786892 DOI: 10.1053/j.gastro.2014.04.036]
37 **Yanaka A**. Sulforaphane enhances protection and repair of gastric

mucosa against oxidative stress in vitro, and demonstrates anti-inflammatory effects on Helicobacter pylori-infected gastric mucosae in mice and human subjects. *Curr Pharm Des* 2011; **17**: 1532-1540 [PMID: 21548875]

38 **Drake IM**, Davies MJ, Mapstone NP, Dixon MF, Schorah CJ, White KL, Chalmers DM, Axon AT. Ascorbic acid may protect against human gastric cancer by scavenging mucosal oxygen radicals. *Carcinogenesis* 1996; **17**: 559-562 [PMID: 8631145]

39 **Weitzman SA**, Turk PW, Milkowski DH, Kozlowski K. Free radical adducts induce alterations in DNA cytosine methylation. *Proc Natl Acad Sci USA* 1994; **91**: 1261-1264 [PMID: 8108398]

40 **Sitaraman R**. Helicobacter pylori DNA methyltransferases and the epigenetic field effect in cancerization. *Front Microbiol* 2014; **5**: 115 [PMID: 24723914 DOI: 10.3389/fmicb.2014.00115]

41 **Krebes J**, Morgan RD, Bunk B, Spröer C, Luong K, Parusel R, Anton BP, König C, Josenhans C, Overmann J, Roberts RJ, Korlach J, Suerbaum S. The complex methylome of the human gastric pathogen Helicobacter pylori. *Nucleic Acids Res* 2014; **42**: 2415-2432 [PMID: 24302578 DOI: 10.1093/nar/gkt1201]

42 **Terradot L**, Waksman G. Architecture of the Helicobacter pylori Cag-type IV secretion system. *FEBS J* 2011; **278**: 1213-1222 [PMID: 21352491 DOI: 10.1111/j.1742-4658.2011.08037.x]

43 **Backert S**, Schwarz T, Miehlke S, Kirsch C, Sommer C, Kwok T, Gerhard M, Goebel UB, Lehn N, Koenig W, Meyer TF. Functional analysis of the cag pathogenicity island in Helicobacter pylori isolates from patients with gastritis, peptic ulcer, and gastric cancer. *Infect Immun* 2004; **72**: 1043-1056 [PMID: 14742552]

44 **Glocker E**, Lange C, Covacci A, Bereswill S, Kist M, Pahl HL. Proteins encoded by the cag pathogenicity island of Helicobacter pylori are required for NF-kappaB activation. *Infect Immun* 1998; **66**: 2346-2348 [PMID: 9573128]

45 **Viala J**, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, Athman R, Mémet S, Huerre MR, Coyle AJ, DiStefano PS, Sansonetti PJ, Labigne A, Bertin J, Philpott DJ, Ferrero RL. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. *Nat Immunol* 2004; **5**: 1166-1174 [PMID: 15489856 DOI: 10.1038/ni1131]

46 **Watanabe T**, Tada M, Nagai H, Sasaki S, Nakao M. Helicobacter pylori infection induces gastric cancer in mongolian gerbils. *Gastroenterology* 1998; **115**: 642-648 [PMID: 9721161]

47 **Franco AT**, Israel DA, Washington MK, Krishna U, Fox JG, Rogers AB, Neish AS, Collier-Hyams L, Perez-Perez GI, Hatakeyama M, Whitehead R, Gaus K, O'Brien DP, Romero-Gallo J, Peek RM. Activation of beta-catenin by carcinogenic Helicobacter pylori. *Proc Natl Acad Sci USA* 2005; **102**: 10646-10651 [PMID: 16027366 DOI: 10.1073/pnas.0504927102]

48 **Suarez G**, Romero-Gallo J, Piazuelo MB, Wang G, Maier RJ, Forsberg LS, Azadi P, Gomez MA, Correa P, Peek RM. Modification of Helicobacter pylori Peptidoglycan Enhances NOD1 Activation and Promotes Cancer of the Stomach. *Cancer Res* 2015; **75**: 1749-1759 [PMID: 25732381 DOI: 10.1158/0008-5472.CAN-14-2291]

49 **Wang G**, Maier SE, Lo LF, Maier G, Dosi S, Maier RJ. Peptidoglycan deacetylation in Helicobacter pylori contributes to bacterial survival by mitigating host immune responses. *Infect Immun* 2010; **78**: 4660-4666 [PMID: 20805339 DOI: 10.1128/IAI.00307-10]

50 **Perez-Perez GI**, Peek RM, Legath AJ, Heine PR, Graff LB. The role of CagA status in gastric and extragastric complications of Helicobacter pylori. *J Physiol Pharmacol* 1999; **50**: 833-845 [PMID: 10695563]

51 **Yamaoka Y**, Kita M, Kodama T, Sawai N, Kashima K, Imanishi J. Induction of various cytokines and development of severe mucosal inflammation by cagA gene positive Helicobacter pylori strains. *Gut* 1997; **41**: 442-451 [PMID: 9391240]

52 **Wu J**, Xu S, Zhu Y. Helicobacter pylori CagA: a critical destroyer of the gastric epithelial barrier. *Dig Dis Sci* 2013; **58**: 1830-1837 [PMID: 23423500 DOI: 10.1007/s10620-013-2589-x]

53 **Hatakeyama M**. Oncogenic mechanisms of the Helicobacter pylori CagA protein. *Nat Rev Cancer* 2004; **4**: 688-694 [PMID: 15343275 DOI: 10.1038/nrc1433]

54 **Azuma T**, Yamazaki S, Yamakawa A, Ohtani M, Muramatsu A, Suto H, Ito Y, Dojo M, Yamazaki Y, Kuriyama M, Keida Y, Higashi H, Hatakeyama M. Association between diversity in the Src homology 2 domain--containing tyrosine phosphatase binding site of Helicobacter pylori CagA protein and gastric atrophy and cancer. *J Infect Dis* 2004; **189**: 820-827 [PMID: 14976598 DOI: 10.1086/381782]

55 **Zhu Y**, Zhong X, Zheng S, Du Q, Xu W. Transformed immortalized gastric epithelial cells by virulence factor CagA of Helicobacter pylori through Erk mitogen-activated protein kinase pathway. *Oncogene* 2005; **24**: 3886-3895 [PMID: 15856031 DOI: 10.1038/sj.onc.1208551]

56 **Bessède E**, Staedel C, Acuña Amador LA, Nguyen PH, Chambonnier L, Hatakeyama M, Belleannée G, Mégraud F, Varon C. Helicobacter pylori generates cells with cancer stem cell properties via epithelial-mesenchymal transition-like changes. *Oncogene* 2014; **33**: 4123-4131 [PMID: 24096479 DOI: 10.1038/onc.2013.380]

57 **Amieva MR**, Vogelmann R, Covacci A, Tompkins LS, Nelson WJ, Falkow S. Disruption of the epithelial apical-junctional complex by Helicobacter pylori CagA. *Science* 2003; **300**: 1430-1434 [PMID: 12775840 DOI: 10.1126/science.1081919]

58 **Iwamoto J**, Mizokami Y, Takahashi K, Nakajima K, Ohtsubo T, Miura S, Narasaka T, Takeyama H, Omata T, Shimokobe K, Ito M, Takehara H, Matsuoka T. Expressions of urokinase-type plasminogen activator, its receptor and plasminogen activator inhibitor-1 in gastric cancer cells and effects of Helicobacter pylori. *Scand J Gastroenterol* 2005; **40**: 783-793 [PMID: 16109653 DOI: 10.1080/00365520510015665]

59 **Wandler AM**, Guillemin K. Transgenic expression of the Helicobacter pylori virulence factor CagA promotes apoptosis or tumorigenesis through JNK activation in Drosophila. *PLoS Pathog* 2012; **8**: e1002939 [PMID: 23093933 DOI: 10.1371/journal.ppat.1002939]

60 **Ohnishi N**, Yuasa H, Tanaka S, Sawa H, Miura M, Matsui A, Higashi H, Musashi M, Iwabuchi K, Suzuki M, Yamada G, Azuma T, Hatakeyama M. Transgenic expression of Helicobacter pylori CagA induces gastrointestinal and hematopoietic neoplasms in mouse. *Proc Natl Acad Sci USA* 2008; **105**: 1003-1008 [PMID: 18192401 DOI: 10.1073/pnas.0711183105]

61 **Hatakeyama M**, Higashi H. Helicobacter pylori CagA: a new paradigm for bacterial carcinogenesis. *Cancer Sci* 2005; **96**: 835-843 [PMID: 16367902 DOI: 10.1111/j.1349-7006.2005.00130.x]

62 **Backert S**, Tegtmeyer N, Selbach M. The versatility of Helicobacter pylori CagA effector protein functions: The master key hypothesis. *Helicobacter* 2010; **15**: 163-176 [PMID: 20557357 DOI: 10.1111/j.1523-5378.2010.00759.x]

63 **Backert S**, Moese S, Selbach M, Brinkmann V, Meyer TF. Phosphorylation of tyrosine 972 of the Helicobacter pylori CagA protein is essential for induction of a scattering phenotype in gastric epithelial cells. *Mol Microbiol* 2001; **42**: 631-644 [PMID: 11722731]

64 **Botham CM**, Wandler AM, Guillemin K. A transgenic Drosophila model demonstrates that the Helicobacter pylori CagA protein functions as a eukaryotic Gab adaptor. *PLoS Pathog* 2008; **4**: e1000064 [PMID: 18483552 DOI: 10.1371/journal.ppat.1000064]

65 **Hatakeyama M**. Helicobacter pylori and gastric carcinogenesis. *J Gastroenterol* 2009; **44**: 239-248 [PMID: 19271114 DOI: 10.1007/s00535-009-0014-1]

66 **Brandt S**, Kwok T, Hartig R, König W, Backert S. NF-kappaB activation and potentiation of proinflammatory responses by the Helicobacter pylori CagA protein. *Proc Natl Acad Sci USA* 2005; **102**: 9300-9305 [PMID: 15972330 DOI: 10.1073/pnas.0409873102]

67 **Lamb A**, Yang XD, Tsang YH, Li JD, Higashi H, Hatakeyama M, Peek RM, Blanke SR, Chen LF. Helicobacter pylori CagA activates NF-kappaB by targeting TAK1 for TRAF6-mediated Lys 63 ubiquitination. *EMBO Rep* 2009; **10**: 1242-1249 [PMID: 19820695 DOI: 10.1038/embor.2009.210]

68 **Sepulveda AR**, Yao Y, Yan W, Park DI, Kim JJ, Gooding W, Abudayyeh S, Graham DY. CpG methylation and reduced expression of O6-methylguanine DNA methyltransferase is associated with Helicobacter pylori infection. *Gastroenterology* 2010; **138**: 1836-1844 [PMID: 20044995 DOI: 10.1053/j.gastro.2009.12.042]

69 **Hayashi Y**, Tsujii M, Wang J, Kondo J, Akasaka T, Jin Y, Li W, Nakamura T, Nishida T, Iijima H, Tsuji S, Kawano S, Hayashi N, Takehara T. CagA mediates epigenetic regulation to attenuate let-7 expression in Helicobacter pylori-related carcinogenesis. *Gut* 2013; **62**: 1536-1546 [PMID: 22936674 DOI: 10.1136/gutjnl-2011-301625]

70 **de Bernard M**, Moschioni M, Papini E, Telford J, Rappuoli R, Montecucco C. Cell vacuolization induced by Helicobacter pylori VacA toxin: cell line sensitivity and quantitative estimation. *Toxicol Lett* 1998; **99**: 109-115 [PMID: 9817082]

71 **de Bernard M**, Arico B, Papini E, Rizzuto R, Grandi G, Rappuoli R, Montecucco C. Helicobacter pylori toxin VacA induces vacuole formation by acting in the cell cytosol. *Mol Microbiol* 1997; **26**: 665-674 [PMID: 9427397]

72 **Galmiche A**, Rassow J, Doye A, Cagnol S, Chambard JC, Contamin S, de Thillot V, Just I, Ricci V, Solcia E, Van Obberghen E, Boquet P. The N-terminal 34 kDa fragment of Helicobacter pylori vacuolating cytotoxin targets mitochondria and induces cytochrome c release. *EMBO J* 2000; **19**: 6361-6370 [PMID: 11101509 DOI: 10.1093/emboj/19.23.6361]

73 **Torres VJ**, VanCompernolle SE, Sundrud MS, Unutmaz D, Cover TL. Helicobacter pylori vacuolating cytotoxin inhibits activation-induced proliferation of human T and B lymphocyte subsets. *J Immunol* 2007; **179**: 5433-5440 [PMID: 17911630]

74 **Terebiznik MR**, Raju D, Vázquez CL, Torbricki K, Kulkarni R, Blanke SR, Yoshimori T, Colombo MI, Jones NL. Effect of Helicobacter pylori's vacuolating cytotoxin on the autophagy pathway in gastric epithelial cells. *Autophagy* 2009; **5**: 370-379 [PMID: 19164948]

75 **Lu H**, Yamaoka Y, Graham DY. Helicobacter pylori virulence factors: facts and fantasies. *Curr Opin Gastroenterol* 2005; **21**: 653-659 [PMID: 16220040]

76 **Supajatura V**, Ushio H, Wada A, Yahiro K, Okumura K, Ogawa H, Hirayama T, Ra C. Cutting edge: VacA, a vacuolating cytotoxin of Helicobacter pylori, directly activates mast cells for migration and production of proinflammatory cytokines. *J Immunol* 2002; **168**: 2603-2607 [PMID: 11884423]

77 **Salama NR**, Otto G, Tompkins L, Falkow S. Vacuolating cytotoxin of Helicobacter pylori plays a role during colonization in a mouse model of infection. *Infect Immun* 2001; **69**: 730-736 [PMID: 11159961 DOI: 10.1128/IAI.69.2.730-736.2001]

78 **Atherton JC**, Cao P, Peek RM, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific vacA types with cytotoxin production and peptic ulceration. *J Biol Chem* 1995; **270**: 17771-17777 [PMID: 7629077]

79 **Rhead JL**, Letley DP, Mohammadi M, Hussein N, Mohagheghi MA, Eshagh Hosseini M, Atherton JC. A new Helicobacter pylori vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. *Gastroenterology* 2007; **133**: 926-936 [PMID: 17854597 DOI: 10.1053/j.gastro.2007.06.056]

80 **Winter JA**, Letley DP, Cook KW, Rhead JL, Zaitoun AA, Ingram RJ, Amilon KR, Croxall NJ, Kaye PV, Robinson K, Atherton JC. A role for the vacuolating cytotoxin, VacA, in colonization and Helicobacter pylori-induced metaplasia in the stomach. *J Infect Dis* 2014; **210**: 954-963 [PMID: 24625807 DOI: 10.1093/infdis/jiu154]

81 **Sun J**, Aoki K, Zheng JX, Su BZ, Ouyang XH, Misumi J. Effect of NaCl and Helicobacter pylori vacuolating cytotoxin on cytokine expression and viability. *World J Gastroenterol* 2006; **12**: 2174-2180 [PMID: 16610017]

82 **Sugimoto M**, Furuta T, Yamaoka Y. Influence of inflammatory cytokine polymorphisms on eradication rates of Helicobacter pylori. *J Gastroenterol Hepatol* 2009; **24**: 1725-1732 [PMID: 20136959 DOI: 10.1111/j.1440-1746.2009.06047.x]

83 **Cech TR**, Steitz JA. The noncoding RNA revolution-trashing old rules to forge new ones. *Cell* 2014; **157**: 77-94 [PMID: 24679528 DOI: 10.1016/j.cell.2014.03.008]

84 **Mikeska T**, Craig JM. DNA methylation biomarkers: cancer and beyond. *Genes* (Basel) 2014; **5**: 821-864 [PMID: 25229548 DOI: 10.3390/genes5030821]

85 **Qu Y**, Dang S, Hou P. Gene methylation in gastric cancer. *Clin Chim Acta* 2013; **424**: 53-65 [PMID: 23669186 DOI: 10.1016/j.cca.2013.05.002]

86 **Cancer Genome Atlas Research Network.** Comprehensive molecular characterization of gastric adenocarcinoma. *Nature* 2014; **513**: 202-209 [PMID: 25079317 DOI: 10.1038/nature13480]

87 **Kang GH**, Shim YH, Jung HY, Kim WH, Ro JY, Rhyu MG. CpG island methylation in premalignant stages of gastric carcinoma. *Cancer Res* 2001; **61**: 2847-2851 [PMID: 11306456]

88 **Park SY**, Yoo EJ, Cho NY, Kim N, Kang GH. Comparison of CpG island hypermethylation and repetitive DNA hypomethylation in premalignant stages of gastric cancer, stratified for Helicobacter pylori infection. *J Pathol* 2009; **219**: 410-416 [PMID: 19639607 DOI: 10.1002/path.2596]

89 **Chan AO**, Lam SK, Wong BC, Wong WM, Yuen MF, Yeung YH, Hui WM, Rashid A, Kwong YL. Promoter methylation of E-cadherin gene in gastric mucosa associated with Helicobacter pylori infection and in gastric cancer. *Gut* 2003; **52**: 502-506 [PMID: 12631658]

90 **Maekita T**, Nakazawa K, Mihara M, Nakajima T, Yanaoka K, Iguchi M, Arii K, Kaneda A, Tsukamoto T, Tatematsu M, Tamura G, Saito D, Sugimura T, Ichinose M, Ushijima T. High levels of aberrant DNA methylation in Helicobacter pylori-infected gastric mucosae and its possible association with gastric cancer risk. *Clin Cancer Res* 2006; **12**: 989-995 [PMID: 16467114 DOI: 10.1158/1078-0432.CCR-05-2096]

91 **Leung WK**, Man EP, Yu J, Go MY, To KF, Yamaoka Y, Cheng VY, Ng EK, Sung JJ. Effects of Helicobacter pylori eradication on methylation status of E-cadherin gene in noncancerous stomach. *Clin Cancer Res* 2006; **12**: 3216-3221 [PMID: 16707623 DOI: 10.1158/1078-0432.CCR-05-2442]

92 **Ushijima T**, Nakajima T, Maekita T. DNA methylation as a marker for the past and future. *J Gastroenterol* 2006; **41**: 401-407 [PMID: 16799880 DOI: 10.1007/s00535-006-1846-6]

93 **Perri F**, Cotugno R, Piepoli A, Merla A, Quitadamo M, Gentile A, Pilotto A, Annese V, Andriulli A. Aberrant DNA methylation in non-neoplastic gastric mucosa of H. Pylori infected patients and effect of eradication. *Am J Gastroenterol* 2007; **102**: 1361-1371 [PMID: 17509026 DOI: 10.1111/j.1572-0241.2007.01284.x]

94 **Miao R**, Guo X, Zhi Q, Shi Y, Li L, Mao X, Zhang L, Li C. VEZT, a novel putative tumor suppressor, suppresses the growth and tumorigenicity of gastric cancer. *PLoS One* 2013; **8**: e74409 [PMID: 24069310 DOI: 10.1371/journal.pone.0074409]

95 **Meşe G**, Richard G, White TW. Gap junctions: basic structure and function. *J Invest Dermatol* 2007; **127**: 2516-2524 [PMID: 17934503 DOI: 10.1038/sj.jid.5700770]

96 **Wang Y**, Huang LH, Xu CX, Xiao J, Zhou L, Cao D, Liu XM, Qi Y. Connexin 32 and 43 promoter methylation in Helicobacter pylori-associated gastric tumorigenesis. *World J Gastroenterol* 2014; **20**: 11770-11779 [PMID: 25206281 DOI: 10.3748/wjg.v20.i33.11770]

97 **Alvarez MC**, Santos JC, Maniezzo N, Ladeira MS, da Silva AL, Scaletsky IC, Pedrazzoli J, Ribeiro ML. MGMT and MLH1 methylation in Helicobacter pylori-infected children and adults. *World J Gastroenterol* 2013; **19**: 3043-3051 [PMID: 23716983 DOI: 10.3748/wjg.v19.i20.3043]

98 **Farrell JJ**, Taupin D, Koh TJ, Chen D, Zhao CM, Podolsky DK, Wang TC. TFF2/SP-deficient mice show decreased gastric proliferation, increased acid secretion, and increased susceptibility to NSAID injury. *J Clin Invest* 2002; **109**: 193-204 [PMID: 11805131 DOI: 10.1172/JCI12529]

99 **Kurt-Jones EA**, Cao L, Sandor F, Rogers AB, Whary MT, Nambiar PR, Cerny A, Bowen G, Yan J, Takaishi S, Chi AL, Reed G, Houghton J, Fox JG, Wang TC. Trefoil family factor 2 is expressed in murine gastric and immune cells and controls both gastrointestinal inflammation and systemic immune responses. *Infect Immun* 2007; **75**: 471-480 [PMID: 17101660 DOI: 10.1128/IAI.02039-05]

100 **Peterson AJ**, Menheniott TR, O'Connor L, Walduck AK, Fox JG, Kawakami K, Minamoto T, Ong EK, Wang TC, Judd LM, Giraud AS. Helicobacter pylori infection promotes methylation and silencing of trefoil factor 2, leading to gastric tumor development in mice and humans. *Gastroenterology* 2010; **139**: 2005-2017 [PMID: 20801119 DOI: 10.1053/j.gastro.2010.08.043]

101 **Yamada C**, Ozaki T, Ando K, Suenaga Y, Inoue K, Ito Y, Okoshi

R, Kageyama H, Kimura H, Miyazaki M, Nakagawara A. RUNX3 modulates DNA damage-mediated phosphorylation of tumor suppressor p53 at Ser-15 and acts as a co-activator for p53. *J Biol Chem* 2010; **285**: 16693-16703 [PMID: 20353948 DOI: 10.1074/jbc.M109.055525]

102 **Chen W**, Gao N, Shen Y, Cen JN. Hypermethylation downregulates Runx3 gene expression and its restoration suppresses gastric epithelial cell growth by inducing p27 and caspase3 in human gastric cancer. *J Gastroenterol Hepatol* 2010; **25**: 823-831 [PMID: 20492341 DOI: 10.1111/j.1440-1746.2009.06191.x]

103 **Lu XX**, Yu JL, Ying LS, Han J, Wang S, Yu QM, Wang XB, Fang XH, Ling ZQ. Stepwise cumulation of RUNX3 methylation mediated by Helicobacter pylori infection contributes to gastric carcinoma progression. *Cancer* 2012; **118**: 5507-5517 [PMID: 22576578 DOI: 10.1002/cncr.27604]

104 **Cheng AS**, Li MS, Kang W, Cheng VY, Chou JL, Lau SS, Go MY, Lee CC, Ling TK, Ng EK, Yu J, Huang TH, To KF, Chan MW, Sung JJ, Chan FK. Helicobacter pylori causes epigenetic dysregulation of FOXD3 to promote gastric carcinogenesis. *Gastroenterology* 2013; **144**: 122-133.e9 [PMID: 23058321 DOI: 10.1053/j.gastro.2012.10.002]

105 **Corre S**, Galibert MD. Upstream stimulating factors: highly versatile stress-responsive transcription factors. *Pigment Cell Res* 2005; **18**: 337-348 [PMID: 16162174 DOI: 10.1111/j.1600-0749.2005.00262.x]

106 **Bussière FI**, Michel V, Mémet S, Avé P, Vivas JR, Huerre M, Touati E. H. pylori-induced promoter hypermethylation downregulates USF1 and USF2 transcription factor gene expression. *Cell Microbiol* 2010; **12**: 1124-1133 [PMID: 20180799 DOI: 10.1111/j.1462-5822.2010.01457.x]

107 **Wen XZ**, Akiyama Y, Pan KF, Liu ZJ, Lu ZM, Zhou J, Gu LK, Dong CX, Zhu BD, Ji JF, You WC, Deng DJ. Methylation of GATA-4 and GATA-5 and development of sporadic gastric carcinomas. *World J Gastroenterol* 2010; **16**: 1201-1208 [PMID: 20222162]

108 **Vadlamudi RK**, Li F, Barnes CJ, Bagheri-Yarmand R, Kumar R. p41-Arc subunit of human Arp2/3 complex is a p21-activated kinase-1-interacting substrate. *EMBO Rep* 2004; **5**: 154-160 [PMID: 14749719 DOI: 10.1038/sj.embor.7400079]

109 **Yan J**, Zhang M, Zhang J, Chen X, Zhang X. Helicobacter pylori infection promotes methylation of WWOX gene in human gastric cancer. *Biochem Biophys Res Commun* 2011; **408**: 99-102 [PMID: 21466786 DOI: 10.1016/j.bbrc.2011.03.127]

110 **Kang C**, Song JJ, Lee J, Kim MY. Epigenetics: an emerging player in gastric cancer. *World J Gastroenterol* 2014; **20**: 6433-6447 [PMID: 24914365 DOI: 10.3748/wjg.v20.i21.6433]

111 **Krol J**, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet* 2010; **11**: 597-610 [PMID: 20661255 DOI: 10.1038/nrg2843]

112 **Stahlhut Espinosa CE**, Slack FJ. The role of microRNAs in cancer. *Yale J Biol Med* 2006; **79**: 131-140 [PMID: 17940623]

113 **Lu J**, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. *Nature* 2005; **435**: 834-838 [PMID: 15944708 DOI: 10.1038/nature03702]

114 **Vickers KC**, Rye KA, Tabet F. MicroRNAs in the onset and development of cardiovascular disease. *Clin Sci* (Lond) 2014; **126**: 183-194 [PMID: 24102098 DOI: 10.1042/CS20130203]

115 **Calin GA**, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, Croce CM. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA* 2004; **101**: 2999-3004 [PMID: 14973191 DOI: 10.1073/pnas.0307323101]

116 **Raisch J**, Darfeuille-Michaud A, Nguyen HT. Role of microRNAs in the immune system, inflammation and cancer. *World J Gastroenterol* 2013; **19**: 2985-2996 [PMID: 23716978 DOI: 10.3748/wjg.v19.i20.2985]

117 **Zhai H**, Fesler A, Ju J. MicroRNA: a third dimension in autophagy. *Cell Cycle* 2013; **12**: 246-250 [PMID: 23255136 DOI: 10.4161/cc.23273]

118 **Zhang J**, Ma L. MicroRNA control of epithelial-mesenchymal transition and metastasis. *Cancer Metastasis Rev* 2012; **31**: 653-662 [PMID: 22684369 DOI: 10.1007/s10555-012-9368-6]

119 **Eulalio A**, Schulte L, Vogel J. The mammalian microRNA response to bacterial infections. *RNA Biol* 2012; **9**: 742-750 [PMID: 22664920 DOI: 10.4161/rna.20018]

120 **Tong F**, Cao P, Yin Y, Xia S, Lai R, Liu S. MicroRNAs in gastric cancer: from benchtop to bedside. *Dig Dis Sci* 2014; **59**: 24-30 [PMID: 24114043 DOI: 10.1007/s10620-013-2887-3]

121 **Ueda T**, Volinia S, Okumura H, Shimizu M, Taccioli C, Rossi S, Alder H, Liu CG, Oue N, Yasui W, Yoshida K, Sasaki H, Nomura S, Seto Y, Kaminishi M, Calin GA, Croce CM. Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. *Lancet Oncol* 2010; **11**: 136-146 [PMID: 20022810 DOI: 10.1016/S1470-2045(09)70343-2]

122 **Wang XW**, Wu Y, Wang D, Qin ZF. MicroRNA network analysis identifies key microRNAs and genes associated with precancerous lesions of gastric cancer. *Genet Mol Res* 2014; **13**: 8695-8703 [PMID: 25366760 DOI: 10.4238/2014.October.27.10]

123 **Zhou X**, Li L, Su J, Zhang G. Decreased miR-204 in H. pylori-associated gastric cancer promotes cancer cell proliferation and invasion by targeting SOX4. *PLoS One* 2014; **9**: e101457 [PMID: 24984017 DOI: 10.1371/journal.pone.0101457]

124 **Song MY**, Pan KF, Su HJ, Zhang L, Ma JL, Li JY, Yuasa Y, Kang D, Kim YS, You WC. Identification of serum microRNAs as novel non-invasive biomarkers for early detection of gastric cancer. *PLoS One* 2012; **7**: e33608 [PMID: 22432036 DOI: 10.1371/journal.pone.0033608]

125 **Yu X**, Luo L, Wu Y, Yu X, Liu Y, Yu X, Zhao X, Zhang X, Cui L, Ye G, Le Y, Guo J. Gastric juice miR-129 as a potential biomarker for screening gastric cancer. *Med Oncol* 2013; **30**: 365 [PMID: 23307240 DOI: 10.1007/s12032-012-0365-y]

126 **Liu WJ**, Xu Q, Sun LP, Dong QG, He CY, Yuan Y. Expression of serum let-7c, let-7i, and let-7f microRNA with its target gene, pepsinogen C, in gastric cancer and precancerous disease. *Tumour Biol* 2015; **36**: 3337-3343 [PMID: 25549793 DOI: 10.1007/s13277-014-2967-9]

127 **Fu Z**, Qian F, Yang X, Jiang H, Chen Y, Liu S. Circulating miR-222 in plasma and its potential diagnostic and prognostic value in gastric cancer. *Med Oncol* 2014; **31**: 164 [PMID: 25129310 DOI: 10.1007/s12032-014-0164-8]

128 **Matsushima K**, Isomoto H, Inoue N, Nakayama T, Hayashi T, Nakayama M, Nakao K, Hirayama T, Kohno S. MicroRNA signatures in Helicobacter pylori-infected gastric mucosa. *Int J Cancer* 2011; **128**: 361-370 [PMID: 20333682 DOI: 10.1002/ijc.25348]

129 **Noto JM**, Peek RM. The role of microRNAs in Helicobacter pylori pathogenesis and gastric carcinogenesis. *Front Cell Infect Microbiol* 2011; **1**: 21 [PMID: 22919587 DOI: 10.3389/fcimb.2011.00021]

130 **Elton TS**, Selemon H, Elton SM, Parinandi NL. Regulation of the MIR155 host gene in physiological and pathological processes. *Gene* 2013; **532**: 1-12 [PMID: 23246696 DOI: 10.1016/j.gene.2012.12.009]

131 **Thompson RC**, Vardinogiannis I, Gilmore TD. Identification of an NF-κB p50/p65-responsive site in the human MIR155HG promoter. *BMC Mol Biol* 2013; **14**: 24 [PMID: 24059932 DOI: 10.1186/1471-2199-14-24]

132 **Xiao B**, Liu Z, Li BS, Tang B, Li W, Guo G, Shi Y, Wang F, Wu Y, Tong WD, Guo H, Mao XH, Zou QM. Induction of microRNA-155 during Helicobacter pylori infection and its negative regulatory role in the inflammatory response. *J Infect Dis* 2009; **200**: 916-925 [PMID: 19650740 DOI: 10.1086/605443]

133 **Saito Y**, Suzuki H, Tsugawa H, Imaeda H, Matsuzaki J, Hirata K, Hosoe N, Nakamura M, Mukai M, Saito H, Hibi T. Overexpression of miR-142-5p and miR-155 in gastric mucosa-associated lymphoid tissue (MALT) lymphoma resistant to Helicobacter pylori eradication. *PLoS One* 2012; **7**: e47396 [PMID: 23209550 DOI: 10.1371/journal.pone.0047396]

134 **Tang B**, Xiao B, Liu Z, Li N, Zhu ED, Li BS, Xie QH, Zhuang Y, Zou QM, Mao XH. Identification of MyD88 as a novel target of miR-155, involved in negative regulation of Helicobacter pylori-induced inflammation. *FEBS Lett* 2010; **584**: 1481-1486 [PMID: 20219467 DOI: 10.1016/j.febslet.2010.02.063]

135 **Koch M**, Mollenkopf HJ, Klemm U, Meyer TF. Induction of microRNA-155 is TLR- and type IV secretion system-dependent in macrophages and inhibits DNA-damage induced apoptosis. *Proc Natl Acad Sci USA* 2012; **109**: E1153-E1162 [PMID: 22509021 DOI: 10.1073/pnas.1116125109]

136 **Deng H**, Guo Y, Song H, Xiao B, Sun W, Liu Z, Yu X, Xia T, Cui L, Guo J. MicroRNA-195 and microRNA-378 mediate tumor growth suppression by epigenetical regulation in gastric cancer. *Gene* 2013; **518**: 351-359 [PMID: 23333942 DOI: 10.1016/j.gene.2012.12.103]

137 **Jia H**, Zhang Z, Zou D, Wang B, Yan Y, Luo M, Dong L, Yin H, Gong B, Li Z, Wang F, Song W, Liu C, Ma Y, Zhang J, Zhao H, Li J, Yu J. MicroRNA-10a is down-regulated by DNA methylation and functions as a tumor suppressor in gastric cancer cells. *PLoS One* 2014; **9**: e88057 [PMID: 24498243 DOI: 10.1371/journal.pone.0088057]

138 **Lei H**, Zou D, Li Z, Luo M, Dong L, Wang B, Yin H, Ma Y, Liu C, Wang F, Zhang J, Yu J, Li Y. MicroRNA-219-2-3p functions as a tumor suppressor in gastric cancer and is regulated by DNA methylation. *PLoS One* 2013; **8**: e60369 [PMID: 23637748 DOI: 10.1371/journal.pone.0060369]

139 **Guo LH**, Li H, Wang F, Yu J, He JS. The Tumor Suppressor Roles of miR-433 and miR-127 in Gastric Cancer. *Int J Mol Sci* 2013; **14**: 14171-14184 [PMID: 23880861 DOI: 10.3390/ijms140714171]

140 **Li P**, Chen X, Su L, Li C, Zhi Q, Yu B, Sheng H, Wang J, Feng R, Cai Q, Li J, Yu Y, Yan M, Liu B, Zhu Z. Epigenetic silencing of miR-338-3p contributes to tumorigenicity in gastric cancer by targeting SSX2IP. *PLoS One* 2013; **8**: e66782 [PMID: 23826132 DOI: 10.1371/journal.pone.0066782]

141 **Sakamoto N**, Naito Y, Oue N, Sentani K, Uraoka N, Zarni Oo H, Yanagihara K, Aoyagi K, Sasaki H, Yasui W. MicroRNA-148a is downregulated in gastric cancer, targets MMP7, and indicates tumor invasiveness and poor prognosis. *Cancer Sci* 2014; **105**: 236-243 [PMID: 24283384 DOI: 10.1111/cas.12330]

142 **Hashimoto Y**, Akiyama Y, Yuasa Y. Multiple-to-multiple relationships between microRNAs and target genes in gastric cancer. *PLoS One* 2013; **8**: e62589 [PMID: 23667495 DOI: 10.1371/journal.pone.0062589]

143 **Li Z**, Lei H, Luo M, Wang Y, Dong L, Ma Y, Liu C, Song W, Wang F, Zhang J, Shen J, Yu J. DNA methylation downregulated mir-10b acts as a tumor suppressor in gastric cancer. *Gastric Cancer* 2015; **18**: 43-54 [PMID: 24481854 DOI: 10.1007/s10120-014-0340-8]

144 **Kiga K**, Mimuro H, Suzuki M, Shinozaki-Ushiku A, Kobayashi T, Sanada T, Kim M, Ogawa M, Iwasaki YW, Kayo H, Fukuda-Yuzawa Y, Yashiro M, Fukayama M, Fukao T, Sasakawa C. Epigenetic silencing of miR-210 increases the proliferation of gastric epithelium during chronic Helicobacter pylori infection. *Nat Commun* 2014; **5**: 4497 [PMID: 25187177 DOI: 10.1038/ncomms5497]

145 **Chan YC**, Banerjee J, Choi SY, Sen CK. miR-210: the master hypoxamir. *Microcirculation* 2012; **19**: 215-223 [PMID: 22171547 DOI: 10.1111/j.1549-8719.2011.00154.x]

146 **Corvalán AH**, Torres VA. RPRM (reprimo, TP53 dependent G2 arrest mediator candidate). *Atlas Genet Cytogenet Oncol Haematol* 2012; **16**: 223-224

147 **Ohki R**, Nemoto J, Murasawa H, Oda E, Inazawa J, Tanaka N, Taniguchi T. Reprimo, a new candidate mediator of the p53-mediated cell cycle arrest at the G2 phase. *J Biol Chem* 2000; **275**: 22627-22630 [PMID: 10930422 DOI: 10.1074/jbc.C000235200]

148 **Saavedra K**, Valbuena J, Olivares W, Marchant MJ, Rodríguez A, Torres-Estay V, Carrasco-Avino G, Guzmán L, Aguayo F, Roa JC, Corvalán AH. Loss of Expression of Reprimo, a p53-induced Cell Cycle Arrest Gene, Correlates with Invasive Stage of Tumor Progression and p73 Expression in Gastric Cancer. *PLoS One* 2015; **10**: e0125834 [PMID: 25954972 DOI: 10.1371/journal.pone.0125834]

149 **Schneider BG**, Peng DF, Camargo MC, Piazuelo MB, Sicinschi LA, Mera R, Romero-Gallo J, Delgado AG, Bravo LE, Wilson KT, Peek RM, Correa P, El-Rifai W. Promoter DNA hypermethylation in gastric biopsies from subjects at high and low risk for gastric cancer. *Int J Cancer* 2010; **127**: 2588-2597 [PMID: 20178103 DOI: 10.1002/ijc.25274]

150 **Bernal C**, Aguayo F, Villarroel C, Vargas M, Díaz I, Ossandon FJ, Santibáñez E, Palma M, Aravena E, Barrientos C, Corvalan AH. Reprimo as a potential biomarker for early detection in gastric cancer. *Clin Cancer Res* 2008; **14**: 6264-6269 [PMID: 18829507 DOI: 10.1158/1078-0432.CCR-07-4522]

151 **Hanigan MH**. gamma-Glutamyl transpeptidase, a glutathionase: its expression and function in carcinogenesis. *Chem Biol Interact* 1998; **111-112**: 333-342 [PMID: 9679564]

152 **Chevalier C**, Thiberge JM, Ferrero RL, Labigne A. Essential role of Helicobacter pylori gamma-glutamyltranspeptidase for the colonization of the gastric mucosa of mice. *Mol Microbiol* 1999; **31**: 1359-1372 [PMID: 10200957]

153 **Kim KM**, Lee SG, Park MG, Song JY, Kang HL, Lee WK, Cho MJ, Rhee KH, Youn HS, Baik SC. Gamma-glutamyltranspeptidase of Helicobacter pylori induces mitochondria-mediated apoptosis in AGS cells. *Biochem Biophys Res Commun* 2007; **355**: 562-567 [PMID: 17307146 DOI: 10.1016/j.bbrc.2007.02.021]

154 **Kim KM**, Lee SG, Kim JM, Kim DS, Song JY, Kang HL, Lee WK, Cho MJ, Rhee KH, Youn HS, Baik SC. Helicobacter pylori gamma-glutamyltranspeptidase induces cell cycle arrest at the G1-S phase transition. *J Microbiol* 2010; **48**: 372-377 [PMID: 20571956 DOI: 10.1007/s12275-010-9293-8]

155 **Gong M**, Ling SS, Lui SY, Yeoh KG, Ho B. Helicobacter pylori gamma-glutamyl transpeptidase is a pathogenic factor in the development of peptic ulcer disease. *Gastroenterology* 2010; **139**: 564-573 [PMID: 20347814 DOI: 10.1053/j.gastro.2010.03.050]

156 **Ricci V**, Giannouli M, Romano M, Zarrilli R. Helicobacter pylori gamma-glutamyl transpeptidase and its pathogenic role. *World J Gastroenterol* 2014; **20**: 630-638 [PMID: 24574736 DOI: 10.3748/wjg.v20.i3.630]

157 **Lladser A**, Sanhueza C, Kiessling R, Quest AF. Is survivin the potential Achilles' heel of cancer? *Adv Cancer Res* 2011; **111**: 1-37 [PMID: 21704829 DOI: 10.1016/B978-0-12-385524-4.00001-5]

158 **Mehrotra S**, Languino LR, Raskett CM, Mercurio AM, Dohi T, Altieri DC. IAP regulation of metastasis. *Cancer Cell* 2010; **17**: 53-64 [PMID: 20129247 DOI: 10.1016/j.ccr.2009.11.021]

159 **Fernández JG**, Rodríguez DA, Valenzuela M, Calderon C, Urzúa U, Munroe D, Rosas C, Lemus D, Díaz N, Wright MC, Leyton L, Tapia JC, Quest AF. Survivin expression promotes VEGF-induced tumor angiogenesis via PI3K/Akt enhanced β-catenin/Tcf-Lef dependent transcription. *Mol Cancer* 2014; **13**: 209 [PMID: 25204429 DOI: 10.1186/1476-4598-13-209]

160 **Valenzuela M**, Pérez-Pérez G, Corvalán AH, Carrasco G, Urra H, Bravo D, Toledo H, Quest AF. Helicobacter pylori-induced loss of the inhibitor-of-apoptosis protein survivin is linked to gastritis and death of human gastric cells. *J Infect Dis* 2010; **202**: 1021-1030 [PMID: 20735270 DOI: 10.1086/656143]

161 **Valenzuela M**, Bravo D, Canales J, Sanhueza C, Díaz N, Almarza O, Toledo H, Quest AF. Helicobacter pylori-induced loss of survivin and gastric cell viability is attributable to secreted bacterial gamma-glutamyl transpeptidase activity. *J Infect Dis* 2013; **208**: 1131-1141 [PMID: 23847060 DOI: 10.1093/infdis/jit286]

162 **Houghton J**, Stoicov C, Nomura S, Rogers AB, Carlson J, Li H, Cai X, Fox JG, Goldenring JR, Wang TC. Gastric cancer originating from bone marrow-derived cells. *Science* 2004; **306**: 1568-1571 [PMID: 15567866 DOI: 10.1126/science.1099513]


**P- Reviewer**: El-Zahaby SA, Smith SM **S- Editor**: Yu J
**L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12757

*World J Gastroenterol* 2015 December 7; 21(45): 12757-12766
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


REVIEW

# Fluid management in living donor hepatectomy: Recent issues and perspectives

Seong-Soo Choi, Sung-Hoon Kim, Young-Kug Kim

Seong-Soo Choi, Sung-Hoon Kim, Young-Kug Kim, Department of Anesthesiology and Pain Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul 138-736, South Korea

Author contributions: Choi SS performed the literature review and wrote the manuscript; Kim SH performed the literature review; and Kim YK performed the literature review and provided expertise and critical revision of the manuscript.

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



Correspondence to: Young-Kug Kim, MD, PhD, Professor, Department of Anesthesiology and Pain Medicine, Asan Medical Center, University of Ulsan College of Medicine, 388-1 Pungnap-Dong, Songpa-Gu, Seoul 138-736, South Korea. kyk@amc.seoul.kr
Telephone: +82-2-30105976
Fax: +82-2-30106790

Received: April 28, 2015
Peer-review started: May 7, 2015
First decision: June 2, 2015
Revised: July 28, 2015
Accepted: October 20, 2015
Article in press: October 20, 2015
Published online: December 7, 2015

## Abstract

The importance of the safety of healthy living liver donors is widely recognized during donor hepatectomy which is associated with blood loss, transfusion, and subsequent post-operative morbidity. Although the low central venous pressure (CVP) technique can still be effective, it may not be advantageous concerning the safety of healthy donors undergoing hepatectomy. Emerging evidence suggests that stroke volume variation (SVV), a simple and useful index for fluid responsiveness and preload status in various clinical situations, can be applied as a guide for fluid management to reduce blood loss during living donor hepatectomy. Synthetic colloid solutions are also associated with serious adverse events such as the use of renal replacement therapy and transfusion in critically ill or septic patients. However, it is uncertain whether the intra-operative use of colloid solution is associated with similarly adverse effects in patients undergoing living donor hepatectomy. In this review article we discuss the recent issues regarding the low CVP technique and the high SVV method, *i.e.*, maintaining 10%-20% of SVV, for fluid management in order to reduce blood loss during living donor hepatectomy. In addition, we briefly discuss the effects of intra-operative colloid or crystalloid administration for surgical rather than septic or critically ill patients.

**Key words:** Donor hepatectomy; Central venous pressure; Stroke volume variation; Fluid; Synthetic colloid



**Core tip:** The low central venous pressure technique can still be effective for reducing blood loss during hepatectomy. However, it may not be advantageous regarding the safety of healthy donors undergoing hepatectomy. Therefore, to reduce blood loss during donor hepatectomy, we propose an alternative fluid management technique using a high stroke volume variation method. For the type of fluid, the use of a non-lactate-containing crystalloid solution is advisable during donor hepatectomy. Colloid administration

should be carefully determined depending upon each clinical situation of donor hepatectomy, although future studies will be required to elucidate the effect of colloid solutions on donor outcomes.

Choi SS, Kim SH, Kim YK. Fluid management in living donor hepatectomy: Recent issues and perspectives. *World J Gastroenterol* 2015; 21(45): 12757-12766 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12757.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12757

## INTRODUCTION

Living donor liver transplantation (LDLT) is an established treatment modality for end-stage liver disease and it alleviates the shortage of cadaveric donor organs. This approach to organ procurement greatly benefits the recipient population, although the ethical issues associated with donor hepatectomy and the extreme risks of perioperative morbidity and mortality must always be considered[1-3]. The importance of donor safety during hepatectomy is widely recognized, and potential risks during the perioperative period are a major concern of LDLT. The liver transplantation program at Asan Medical Center began in 1992 with deceased donor liver transplantation and now accomplishes more than 300 LDLTs per year (Figure 1)[4]. At our medical institution, perioperative complications associated with general anesthesia and living donor hepatectomy have been reduced and prevented by careful perioperative management as well as by the clinical experience of the anesthesiologists and the surgical staffs[5].

Our article will review the key considerations for the optimal fluid management of living donor hepatectomies for adult-to-adult liver transplantation. These will be based on our clinical experience at Asan Medical Center in Seoul, Korea. We will focus in particular on central venous pressure (CVP) and stroke volume variation (SVV) monitoring and the selection of the appropriate fluid type.

### *CVP-guided fluid management in living donor hepatectomy*

In general, hepatectomy inevitably causes a significant blood loss and transfusion requirement, which are among the major causes of post-operative morbidity and mortality[6-10]. In addition to the many surgical techniques which attempt to reduce blood loss during hepatectomy, fluid management to aid in the reduction of operative bleeding has also been developed. Careful fluid management is believed to be one of the important strategies to minimize blood loss during hepatectomy. Various anesthetic methods to reduce blood loss during living donor hepatectomy include acute normovolemic intraoperative hemodilution with autologous blood donation[11-13], intraoperative cell salvage[13], low CVP technique[14], and the high SVV method[15]. Of these, fluid management, such as lowering CVP and maintaining high SVV, is simpler and easier to perform as it has no requirement for specific devices, equipment or additional personnel.

Following the first prospective report of Jones *et al*[16], maintaining a low CVP is widely used to diminish intraoperative blood loss in some anesthetic protocols of hepatectomy[17-19]. Because CVP is thought to reflect hepatic sinusoid pressure, lowering CVP during liver resection can reduce hepatic parenchymal congestion and subsequent blood loss by helping to control hepatic venous hemorrhage[16,17]. It is known that the target CVP during hepatectomy is 5 mmHg or less, and which has been shown to reduce intraoperative blood loss, the need for blood transfusions, morbidity, and mortality[16-18,20]. A low CVP can be simply produced and maintained by fluid restriction, although there is no study evaluating the lowest safe rate of fluid infusion. For example, Wang *et al*[20] reported that maintenance of CVP $\leqslant$ 4 mmHg by reduction of the fluid infusion rate to near 75 mL/h decreased blood loss during hepatectomy and shortened the length of the patient hospital stay without detrimental effects on hepatic or renal function. A low CVP has also been achieved by elective addition of pharmacologic agents such as nitroglycerin[20-22], morphine[23], milrinone[14], and furosemide[14,20,24]. In addition, the low CVP technique used during the pre-anhepatic phase of liver transplantation surgery reduces intraoperative blood loss as well as protecting the liver function and having no detrimental effects on renal function[25]. On the contrary, a low CVP strategy was associated with higher mortality and morbidity rates among orthotopic liver transplant recipients[23].

However, several lines of evidence have demonstrated that CVP did not correlate with blood loss during hepatectomy of healthy living donors[13,26-28]. For example, CVP during hepatic resection was not associated with intraoperative blood loss in living liver donors[13,27]. In addition, the amount of intraoperative blood loss was not significantly reduced in donors with relatively low CVP during living donor hepatectomy[26,28]. An evaluation of nearly 1000 living liver donors in our medical institution has revealed that factors associated with intraoperative blood loss were patient gender, body weight, and fatty changes in the liver, although not the CVP level during hepatectomy[28]. Furthermore, Niemann *et al*[27] found that blood loss was similar in donors with and without CVP monitoring, and thus suggesting CVP monitoring may not be necessary in a highly selected patient population, such as living donors[27]. It was also reported that CVP did not correlate with the preload status in normal healthy volunteers[29]. Discrepancies regarding the usefulness of the low CVP technique to reduce blood loss may arise from differences in patient

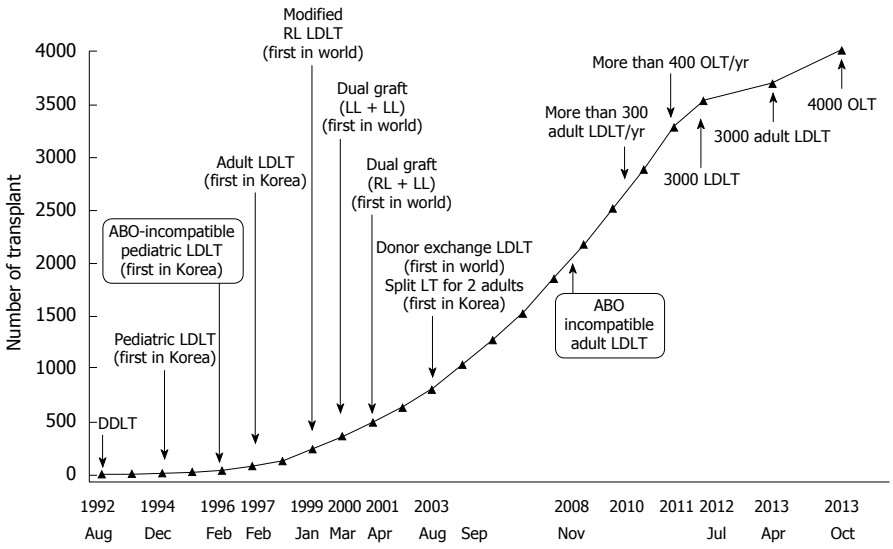



**Figure 1 Evolution of liver transplantation at Asan Medical Center, University of Ulsan College of Medicine.** From February 1997 to April 2013, 3000 ALDLTs were performed without donor mortality. As of 2010, the annual number of ALDLTs exceeded 300 and with increased performance of ABO-incompatible ALDLT. ALDLT: Adult living donor liver transplantation; DDLT: Deceased donor liver transplantation; LDLT: Living donor liver transplantation; RL: Right-lobe; LL: Left-lobe; LT: Liver transplantation; OLT: Orthotopic liver transplantation. Jan: January; Feb: February; Mar: March; Apr: April; Jul: July; Aug: August; Sep: September; Oct: October; Nov: November; Dec: December. Reprinted with permission[4].

populations. The hemorrhagic tendency of liver tissue in patients with benign or malignant hepatic lesions may differ from that in healthy living liver donors.

In various surgical situations, CVP measurements may be inaccurate, although the correct measurement of CVP is crucial for its proper application[30]. A critical review has assessed the correct placement of pressure transducer for CVP measurement in the physiological context[30]. The authors of this review used a questionnaire among intensivists and anesthesiologists in order to determine the correct alignment of the pressure transducer to the phlebostatic axis (a horizontal plane through the tricuspid valve). Surprisingly, only 3.4% of the respondents identified the correct phlebostatic axis for CVP measurement. Furthermore, 45.6% of the respondents mentioned that CVP had no clear-cut relation to the intravascular volume status or to changes in volume. CVP can be affected by factors, including the patient position, surgical liver manipulation, intrathoracic pressure [including positive end-expiratory pressure (PEEP)], pulmonary disease, tricuspid valve disease, pericardial pressure, and intra-abdominal pressure[30-32]. Indeed, during hepatectomy, liver manipulation, occasional clamping of the inferior vena cava (IVC), hepatic veins or even the portal vein by the surgeon, and relatively frequent patient position changes can cause confusion regarding the correct measurement of the CVP value. It has also been noted that head-down tilting is frequently used during maintenance of low CVP[20,21]. Sand *et al*[22,33] reported that there was a dissociation between CVP and hepatic venous pressure in a head-down tilt (Trendelenburg's position) at both 5 cm$H_2O$ PEEP and zero end-expiratory pressure. They suggested that CVP cannot be used as a surrogate for both portal and hepatic venous pressures in the head-down position.

More importantly, potential fatal consequences of the low CVP technique during hepatectomy include air embolism and unnecessary hypoperfusion[21,34], although a head-down tilt position is frequently used to prevent possible air embolism and to improve the venous return[21,22]. It can be reasonably expected that a negative CVP can allow large volumes of air through small or unrecognized, opened hepatic veins during hepatic parenchymal resection. In fact, an earlier report by Jones *et al*[16] also documented that a small air embolism was suspected in two patients (5% of patients maintaining a low CVP). It should be also kept in mind that central venous catheterization is associated with its own morbidity[35,36]. The overall complication rate of central vein catheterization was from 5% to more than 26% in patients who were recommended for treatment or monitoring[36]. Even in living liver donors, central venous catheter-related thrombosis has been observed[37].

Taken together, it is suggested that a low CVP technique used during living donor hepatectomy may not be advantageous regarding the safety of healthy living donors undergoing hepatic resection. Safer, simpler, and more useful fluid management methods are, therefore, required in order to reduce blood loss and subsequent morbidity during living donor hepatectomy.

### *SVV-guided fluid management during living donor hepatectomy*

SVV is one of the dynamic parameters of hemodynamic monitoring used to access the volume status and fluid responsiveness in mechanically ventilated patients.

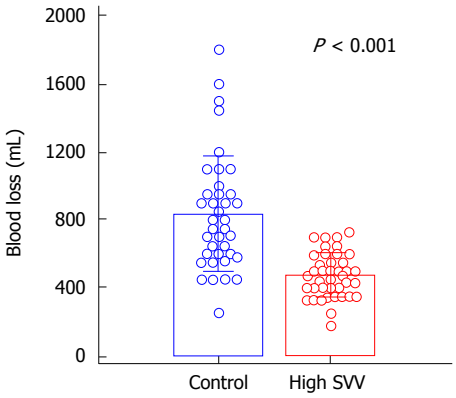



**Figure 2 Effect of stroke volume variation-directed fluid management on blood loss during living donor right hepatectomy.** The control group (< 10% SVV) received usual fluid management (6-10 mL/kg per hour during surgery), whereas the high SVV group underwent fluid restriction (2-4 mL/kg per hour until completion of hepatectomy) with SVV ranging from 10% to 20%. Blood loss was significantly lower in the high SVV than in the control group. SVV: Stroke volume variation. Reproduced data with permission[15].

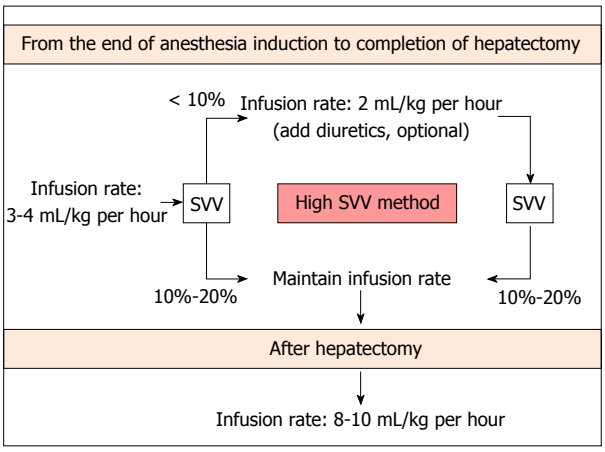



**Figure 3 Proposed algorithm of stroke volume variation-guided fluid management during living donor hepatectomy.** SVV: Stroke volume variation. Modified with permission[15].

SVV is currently accepted as a simple and sensitive indicator for evaluating fluid responsiveness and the preload status in various clinical settings[38-44]. Our group found that SVV may be an independent hemodynamic predictor of intraoperative blood loss ⩾ 700 mL, as well as being a valuable functional preload index during living donor hepatectomy[45]. Dunki-Jacobs *et al*[46] demonstrated that CVP values significantly correlated to SVV value, thus implying that SVV can be used as an alternative to CVP monitoring during hepatic resection in patients with diseased, but not normal, liver. The usefulness of the SVV value was also reported in patients undergoing laparoscopic liver resection[47]. Kitaguchi *et al*[47] found that CVP of -1 to 1 mmHg was significantly correlated to an SVV of 18%-21% ($R^2$ = 0.85, $P$ < 0.001), and thus indicating a safe alternative to CVP monitoring during hepatic resection and with equivalent outcomes in blood loss. In addition, when IVC with or without the portal triad was clamped during hepatic resection, the SVV was significantly higher in patients with lower blood loss than in those with higher blood loss[48]. Therefore, SVV may be used as a guide for fluid management in order to reduce blood loss during living donor hepatectomy.

To demonstrate this concept, we recently performed a randomized controlled study and compared the effects of SVV 10%-20% (high SVV) and < 10% SVV (control) on blood loss during living donor right hepatectomy[15]. The mean blood loss during living donor right hepatectomy was remarkably lower in the high SVV group than in the control group (475.5 ± 131.2 mL *vs* 835.5 ± 341.1 mL, $P$ < 0.001; Figure 2). There was also no significant between-group difference observed in the perioperative laboratory values such as hemoglobin, the lactate and creatinine levels, liver function test, and coagulation profiles, the incidence of intraoperative hypotensive episode and mean arterial blood pressure during hepatectomy. Neither group of donors experienced renal failure or death. All donors were discharged from the hospital without any major complications.

We determined high SVV as 10%-20% based on previous studies[41,44] and our numerous experiences with living donor hepatectomy over 20 years. In our previous study, the median SVV of the high SVV group was 11.7% (11.0%-13.0%) [*vs* 5.3% (4.7%-6.3%) in the control group] and was safely maintained[15]. Therefore, in order to reduce blood loss during living donor hepatectomy, we propose a fluid management technique using a high SVV method.

### *Proposed algorithm of SVV-guided fluid management*

SVV can be safely maintained at 10%-20% with simple fluid restriction and optional administration of a small dose of diuretics during living donor hepatectomy, in accordance with our previous study results[15] and clinical experience. A proposed fluid management algorithm during living donor hepatectomy is shown in Figure 3.

Neither replacement of a volume deficit from fasting nor compensatory intravascular volume expansion is conducted prior to induction of anesthesia when using our protocol. Donors' lungs are mechanically ventilated in order to maintain a constant tidal volume of 8-10 mL/kg. We do not apply PEEP during mechanical ventilation because SVV is obviously influenced by PEEP[49,50]. After anesthesia induction, fluid restriction is begun and is continued until the completion of hepatectomy. The fluid is administered intraoperatively using crystalloid (plasmalyte) and colloid solutions (albumin). Colloid solution is administered only after hepatectomy for volume replacement. At first, fluid administration is restricted with the infusion rate of 3-4 mL/kg per hour until completion of hepatectomy in order to maintain an SVV of 10%-20%. If the donor's SVV was < 10% during this period, the infusion rate was

decreased to 2 mL/kg per hour and furosemide (max. 40 mg) was optionally administered according to the clinicians' decision. After completion of hepatectomy, fluid replacement is maintained with the infusion of a colloid or crystalloid solution at a rate of 8-10 mL/kg per hour. Systolic arterial blood pressure is maintained at ⩾ 90 mmHg. If systolic arterial blood pressure decreased further < 80 mmHg during donor hepatectomy, ephedrine and additional fluid are administered. Urine output is maintained at ⩾ 0.5 mL/kg per hour. Hemoglobin concentration is maintained at ⩾ 7 g/dL; if the hemoglobin concentration is < 7 g/dL, transfusion of packed red blood cells is planned.

Furosemide may be optionally administered in order to achieve high SVV according to our protocol. However, furosemide may induce acute kidney injury. Several reviews in diseased subjects have found that rapid or early excessive fluid removal using diuretics, and which leads to hypovolemia, may be associated with an increased risk for acute kidney injury[51,52]. In addition, furosemide has not shown significant clinical benefits in the prevention or treatment of acute kidney injury[51,52]. In our recent clinical trial, healthy living liver donors showed no significant changes in perioperative serum creatinine levels regardless of furosemide administration[15]. This discrepancy may be related, at least in part, to the investigated population. Although a relatively small dose of adjuvant furosemide may be used with care in order to maintain high SVV in healthy living donors, further studies concerning effective and safe alternative drugs for maintaining high SVV of 10%-20% will be required.

We reported in a large cohort study that the preoperative hemoglobin level and graft-to-donor weight ratio were useful independent predictors of blood transfusion in living donor hepatectomy[53]. Preoperatively moderate anemia (hemoglobin < 11 g/dL, but ⩾ 8 g/dL) was observed in 1.4% of living liver donors[53]. Thus, the high SVV method can be recommended for living liver donors with risk factors such as anemia in order to reduce blood loss during donor hepatectomy as well as subsequent blood transfusion requirements.

However, this parameter has several possible limitations because SVV utilizes cardiorespiratory interaction over a mechanically ventilated respiratory cycle[43,54-56]. First, the patient must be mechanically ventilated without any spontaneous breathing. Second, the tidal volume should be ⩾ 8 mL/kg of the ideal body weight. Third, several clinical conditions, such as cardiac arrhythmia, preclude the use of this parameter.

### *Types of fluids in living donor hepatectomy*

Numerous recent reviews and meta-analyses have reported that synthetic colloids than crystalloids or albumin seemed to increase the need for renal replacement therapy and blood transfusion and resulted in more serious adverse events while providing no overall clinical benefits for critically ill or septic patients[57-63]. However, it is uncertain whether the intraoperative use of colloid solution is associated with similarly adverse effects in patients undergoing surgery, as shown Table 1. A meta-analysis of 17 randomized studies evaluating the renal safety of hydroxyethyl starches (HES) 130/0.40 in surgical patients observed no evidence of renal dysfunction[64]. Another meta-analysis from Gillies *et al*[65] found no difference in the incidence of death or AKI in surgical patients receiving 6% HES. However, they did not recommend the use of 6% HES solution in surgical patients because of the absence of demonstrable benefits[65]. Cytokines and other inflammatory mediators produced in a septic condition can induce gaps between endothelial cells, thus resulting in microvascular leak and tissue edema[66]. Because the endothelial permeability of septic patients differs from that in the healthy population, harmful or no beneficial results from these critically ill patients cannot be extended in living liver donors or elective surgical patients. In trauma patients having similar pathophysiology as that of surgical patients, it has been reported that fluid resuscitation with colloid solution decreased both the morbidity and mortality[67,68]. Currently, there is few report evaluating the outcomes of the intraoperative use of colloid solutions in living donors undergoing hepatectomy. Further studies will be required in order to demonstrate either the significant benefits or harms associated with the administration of synthetic colloid solutions in living liver donors. Therefore, administration of colloid solution should be carefully determined depending upon each clinical situation of living donor hepatectomy.

Crystalloid solutions are extensively used to maintain and replace the intravascular volume in a variety of surgical procedures performed under general anesthesia. When a large amount of normal saline containing no buffer or other electrolytes is administered during surgery, a hyperchloremic metabolic acidosis can result, thus causing increased morbidity and a longer hospital stay following major surgery[69-71]. In living liver donors, normal saline administration during hepatectomy may be detrimental due to its association with hyperchloremic metabolic acidosis[26,72]. Ringer's lactate (RL) solution which contains 28 mmol/L of lactate and has a pH of 6.5, is one of the most preferred crystalloids due to its similar electrolyte composition to extracellular fluid as well as its buffering effect[72]. However, it has been shown that the infusion of a large volume of RL solution during major spine surgery caused postoperative mild hyponatremia and respiratory acidosis, although it did not induce hyperchloremic metabolic acidosis as does normal saline[73]. A recent report conducted following major abdominal surgery found that total infused saline and the lactate level

**Table 1 Controversial effects of intraoperative colloid administration on the outcomes of surgical patients**

| Ref. | Study design | Types of surgery (*n*) | Types of colloid | Clinical outcomes of colloid |
|---|---|---|---|---|
| Mukhtar *et al*[77] | RCT | Living donor liver transplantation (40) | 6% HES 130/0.4 *vs* Alb | No difference in renal outcome |
| Rioux *et al*[78] | Retrospective, propensity score matching | Cardiac surgery (254) | 10% pentastarch 250/0.45 (< 14 mL/kg *vs* ≥ 14 mL/kg) | Dose-dependent renal toxicity |
| Hokema *et al*[79] | Retrospective | Renal transplantation (113) | 6% HES 130/0.4 *vs* NS or RA | No difference in delayed renal graft function |
| Martin *et al*[64] | Meta-analysis | Various surgical procedures (1230) | 6% HES 130/0.4 *vs* comparators[1] | No differences in renal outcome and length of hospital stay |
| Canet *et al*[80] | Prospective observational, propensity score matching, multicenter | General surgery (2462) | Colloids[2] *vs* crystalloids | Increased pulmonary or cardiovascular complications and length of hospital stay<br>No difference in mortality |
| Van der Linden *et al*[81] | RCT, double-blind, two-center | Pediatric cardiac surgery (61) | 6% HES 130/0.4 *vs* Alb | No differences in complications |
| Skhirtladze *et al*[82] | RCT, double-blind | Cardiac surgery (236) | 6% HES 130/0.4 *vs* Alb *vs* RL | Both colloids increased blood transfusion, decreased coagulation and increased the creatinine level |
| Rasmussen *et al*[83] | RCT | Cystectomy (33) | 6% HES 130/0.4 *vs* RL | Increased blood loss and decreased coagulation<br>No differences in re-operation and hospital stay |
| Kancir *et al*[84] | RCT | Hip arthroplasty (38) | 6% HES 130/0.4 *vs* NS | No harmful effect on renal function |
| Lindroos *et al*[85] | RCT | Neurosurgery (30) | 6% HES 130/0.4 *vs* RA | Decreased coagulation<br>No differences in blood loss |
| Yates *et al*[86] | RCT, double-blind | Colorectal surgery (202) | 6% HES 130/0.4 *vs* RL | No differences in complications |
| Gillies *et al*[65] | Meta-analysis | Non-cardiac surgery (1567) | 6% HES *vs* comparators[1] | No differences in mortality or AKI |
| Kashy *et al*[87] | Retrospective, propensity score matching | Non-cardiac and non-urological surgery (29360) | Colloids[3] *vs* crystalloids | Dose-dependent renal toxicity |
| Mercier *et al*[88] | RCT, double-blind, multicenter | Caesarean section (167) | 6% HES 130/0.4 *vs* RL | Same effects in both mother and neonate |
| Hand *et al*[89] | Retrospective, propensity score matching | Deceased donor liver transplantation (174) | 6% HES 130/0.4 *vs* Alb | 6% HES increase risk of AKI |
| Kancir *et al*[90] | RCT, double-blind | Radical prostatectomy (36) | 6% HES 130/0.4 *vs* NS | No difference in renal outcome |
| Choi *et al*[91] | Retrospective | Living donor hepatectomy (1969 ) | 6% HES 130/0.4 *vs* crystalloids | Postoperative delayed recovery of hepatic function |

[1]Comparators included crystalloid solutions, gelatin solutions, and albumin; [2]Mainly 6% HES 130/0.4; [3]Mainly 6% hexastarch 670/0.75. AKI: Acute kidney injury; Alb: 5% human albumin; HES: Hydroxyethyl starch; NS: Normal saline; RA: Ringer's acetate solution; RCT: Randomized controlled trial; RL: Ringer's lactate solution.

were independent factors related to metabolic acidosis[71]. Due to the increased concentration of lactate after living donor hepatectomy[74], the RL solution should be carefully infused in living donors undergoing extensive hepatic dissection. Because hepatic blood flow and the capacity of the liver to metabolize lactate may change during and after hepatectomy[75], additional administration of exogenous lactate may increase the lactate concentration[5]. Shin *et al*[76] reported a prospective study comparing the effects of two, crystalloid solutions with and without lactate on the liver function test data and the serum lactate level in living donors undergoing right hepatectomy. In that study, median lactate concentrations 1 h after hepatectomy were significantly higher in donors with RL solution than with the plasmalyte, a balanced solution of pH 7.4 and containing acetate and gluconate (rather than lactate) as bicarbonate precursors [4.2 (3.2-5.7) mmol/L *vs* 3.3 (2.6-4.6) mmol/L, respectively][76]. Therefore, the use of non-lactate-containing crystalloid solution, such as plasmalyte, is advisable during living donor hepatectomy in order to avoid induction of hyperlactatemia and hyperchloremia[5,76].

## CONCLUSION

The importance of precise fluid management in order to reduce blood loss during hepatectomy in healthy living liver donors cannot be overemphasized. Although a low CVP technique may be still effective, we propose the high SVV method, *i.e.*, maintaining 10%-20% of SVV for fluid management in order to reduce blood loss during living donor hepatectomy. The non-lactate-containing crystalloid solution is also advisable for use during living donor hepatectomy. However, intraoperative administration of synthetic colloid solution should be carefully determined depending on each clinical situation of living donor hepatectomy. Future studies will be required in order to elucidate the effect of colloid solutions on the outcomes of living liver donors undergoing hepatectomy.

## ACKNOWLEDGMENTS

The authors thank Professors, Gyu-Sam Hwang (Department of Anesthesiology and Pain Medicine), Sung-Gyu Lee, Shin Hwang, and Clinical Associate Professor, Tae-Yong Ha (Division of Liver Transplantation and Hepatobiliary Surgery, Department of Surgery, Asan Medical Center, Seoul, South Korea) for preparing this review article.

## REFERENCES

1 **Abecassis MM**, Fisher RA, Olthoff KM, Freise CE, Rodrigo DR, Samstein B, Kam I, Merion RM; A2ALL Study Group. Complications of living donor hepatic lobectomy--a comprehensive report. *Am J Transplant* 2012; **12**: 1208-1217 [PMID: 22335782 DOI: 10.1111/j.1600-6143.2011.03972.x]

2 **Coelho JC**, de Freitas AC, Matias JE, de Godoy JL, Zeni Neto C, Parolin MB, Okawa L. Donor complications including the report of one death in right-lobe living-donor liver transplantation. *Dig Surg* 2007; **24**: 191-196 [PMID: 17522466 DOI: 10.1159/000102898]

3 **Iida T**, Ogura Y, Oike F, Hatano E, Kaido T, Egawa H, Takada Y, Uemoto S. Surgery-related morbidity in living donors for liver transplantation. *Transplantation* 2010; **89**: 1276-1282 [PMID: 20216482 DOI: 10.1097/TP.0b013e3181d66c55]

4 **Lee SG**. A complete treatment of adult living donor liver transplantation: a review of surgical technique and current challenges to expand indication of patients. *Am J Transplant* 2015; **15**: 17-38 [PMID: 25358749 DOI: 10.1111/ajt.12907]

5 **Hwang GS**, McCluskey SA. Anesthesia and outcome after partial hepatectomy for adult-to-adult donor transplantation. *Curr Opin Organ Transplant* 2010; **15**: 377-382 [PMID: 20308895 DOI: 10.1097/MOT.0b013e3283387f75]

6 **Jarnagin WR**, Gonen M, Fong Y, DeMatteo RP, Ben-Porat L, Little S, Corvera C, Weber S, Blumgart LH. Improvement in perioperative outcome after hepatic resection: analysis of 1,803 consecutive cases over the past decade. *Ann Surg* 2002; **236**: 397-406; discussion 406-407 [PMID: 12368667 DOI: 10.1097/01.SLA.0000029003.66466.B3]

7 **Katz SC**, Shia J, Liau KH, Gonen M, Ruo L, Jarnagin WR, Fong Y, D'Angelica MI, Blumgart LH, Dematteo RP. Operative blood loss independently predicts recurrence and survival after resection of hepatocellular carcinoma. *Ann Surg* 2009; **249**: 617-623 [PMID: 19300227 DOI: 10.1097/SLA.0b013e31819ed22f]

8 **Fan ST**, Mau Lo C, Poon RT, Yeung C, Leung Liu C, Yuen WK, Ming Lam C, Ng KK, Ching Chan S. Continuous improvement of survival outcomes of resection of hepatocellular carcinoma: a 20-year experience. *Ann Surg* 2011; **253**: 745-758 [PMID: 21475015 DOI: 10.1097/SLA.0b013e3182111195]

9 **Ibrahim S**, Chen CL, Lin CC, Yang CH, Wang CC, Wang SH, Liu YW, Yong CC, Concejero A, Jawan B, Cheng YF. Intraoperative blood loss is a risk factor for complications in donors after living donor hepatectomy. *Liver Transpl* 2006; **12**: 950-957 [PMID: 16721773 DOI: 10.1002/lt.20746]

10 **Kusano T**, Sasaki A, Kai S, Endo Y, Iwaki K, Shibata K, Ohta M, Kitano S. Predictors and prognostic significance of operative complications in patients with hepatocellular carcinoma who underwent hepatic resection. *Eur J Surg Oncol* 2009; **35**: 1179-1185 [PMID: 19443173 DOI: 10.1016/j.ejso.2009.04.008]

11 **Balci ST**, Pirat A, Torgay A, Cinar O, Sevmis S, Arslan G. Effect of restrictive fluid management and acute normovolemic intraoperative hemodilution on transfusion requirements during living donor hepatectomy. *Transplant Proc* 2008; **40**: 224-227 [PMID: 18261592 DOI: 10.1016/j.transproceed.2007.12.011]

12 **Jawan B**, Cheng YF, Tseng CC, Chen YS, Wang CC, Huang TL, Eng HL, Liu PP, Chiu KW, Wang SH, Lin CC, Lin TS, Liu YW, Chen CL. Effect of autologous blood donation on the central venous pressure, blood loss and blood transfusion during living donor left hepatectomy. *World J Gastroenterol* 2005; **11**: 4233-4236 [PMID: 16015696 DOI: 10.3748/wjg.v11.i27.4233]

13 **Lutz JT**, Valentín-Gamazo C, Görlinger K, Malagó M, Peters J. Blood-transfusion requirements and blood salvage in donors undergoing right hepatectomy for living related liver transplantation. *Anesth Analg* 2003; **96**: 351-355 [PMID: 12538176 DOI: 10.1213/00000539-200302000-00010]

14 **Ryu HG**, Nahm FS, Sohn HM, Jeong EJ, Jung CW. Low central venous pressure with milrinone during living donor hepatectomy. *Am J Transplant* 2010; **10**: 877-882 [PMID: 20420642 DOI: 10.1111/j.1600-6143.2010.03051.x]

15 **Choi SS**, Jun IG, Cho SS, Kim SK, Hwang GS, Kim YK. Effect of stroke volume variation-directed fluid management on blood loss during living-donor right hepatectomy: a randomised controlled study. *Anaesthesia* 2015; **70**: 1250-1258 [PMID: 26215206 DOI: 10.1111/anae.13155]

16 **Jones RM**, Moulton CE, Hardy KJ. Central venous pressure and its effect on blood loss during liver resection. *Br J Surg* 1998; **85**: 1058-1060 [PMID: 9717995 DOI: 10.1046/j.1365-2168.1998.00795.x]

17 **Li Z**, Sun YM, Wu FX, Yang LQ, Lu ZJ, Yu WF. Controlled low central venous pressure reduces blood loss and transfusion requirements in hepatectomy. *World J Gastroenterol* 2014; **20**: 303-309 [PMID: 24415886 DOI: 10.3748/wjg.v20.i1.303]

18 **Huntington JT**, Royall NA, Schmidt CR. Minimizing blood loss during hepatectomy: a literature review. *J Surg Oncol* 2014; **109**: 81-88 [PMID: 24449171 DOI: 10.1002/jso.23455]

19 **Mise Y**, Sakamoto Y, Ishizawa T, Kaneko J, Aoki T, Hasegawa K, Sugawara Y, Kokudo N. A worldwide survey of the current daily practice in liver surgery. *Liver Cancer* 2013; **2**: 55-66 [PMID: 24159597 DOI: 10.1159/000346225]

20 **Wang WD**, Liang LJ, Huang XQ, Yin XY. Low central venous pressure reduces blood loss in hepatectomy. *World J Gastroenterol* 2006; **12**: 935-939 [PMID: 16521223 DOI: 10.3748/wjg.v12.i6.935]

21 **Melendez JA**, Arslan V, Fischer ME, Wuest D, Jarnagin WR, Fong Y, Blumgart LH. Perioperative outcomes of major hepatic resections under low central venous pressure anesthesia: blood loss, blood transfusion, and the risk of postoperative renal dysfunction. *J Am Coll Surg* 1998; **187**: 620-625 [PMID: 9849736 DOI: 10.1016/S1072-7515(98)00240-3]

22 **Sand L**, Lundin S, Rizell M, Wiklund J, Stenqvist O, Houltz E. Nitroglycerine and patient position effect on central, hepatic and portal venous pressures during liver surgery. *Acta Anaesthesiol Scand* 2014; **58**: 961-967 [PMID: 24943197 DOI: 10.1111/aas.12349]

23 **Schroeder RA**, Collins BH, Tuttle-Newhall E, Robertson K, Plotkin J, Johnson LB, Kuo PC. Intraoperative fluid management during orthotopic liver transplantation. *J Cardiothorac Vasc Anesth* 2004; **18**: 438-441 [PMID: 15365923 DOI: 10.1053/j.jvca.2004.05.020]

24 **Kim YK**, Shin WJ, Song JG, Jun IG, Kim HY, Seong SH, Sang BH, Hwang GS. Factors associated with changes in coagulation profiles after living donor hepatectomy. *Transplant Proc* 2010; **42**: 2430-2435 [PMID: 20832521 DOI: 10.1016/j.transproceed.2010.04.069]

25 **Feng ZY**, Xu X, Zhu SM, Bein B, Zheng SS. Effects of low central venous pressure during preanhepatic phase on blood loss and liver and renal function in liver transplantation. *World J Surg* 2010; **34**: 1864-1873 [PMID: 20372900 DOI: 10.1007/s00268-010-0544-y]

26 **Chhibber A**, Dziak J, Kolano J, Norton JR, Lustik S. Anesthesia care for adult live donor hepatectomy: our experiences with 100 cases. *Liver Transpl* 2007; **13**: 537-542 [PMID: 17394151 DOI: 10.1002/lt.21074]

27 **Niemann CU**, Feiner J, Behrends M, Eilers H, Ascher NL, Roberts JP. Central venous pressure monitoring during living right donor hepatectomy. *Liver Transpl* 2007; **13**: 266-271 [PMID: 17256757 DOI: 10.1002/lt.21051]

28 **Kim YK**, Chin JH, Kang SJ, Jun IG, Song JG, Jeong SM, Park JY, Hwang GS. Association between central venous pressure and blood loss during hepatic resection in 984 living donors. *Acta*

*Anaesthesiol Scand* 2009; **53**: 601-606 [PMID: 19419353 DOI: 10.1111/j.1399-6576.2009.01920.x]
29 **Kumar A**, Anel R, Bunnell E, Habet K, Zanotti S, Marshall S, Neumann A, Ali A, Cheang M, Kavinsky C, Parrillo JE. Pulmonary artery occlusion pressure and central venous pressure fail to predict ventricular filling volume, cardiac performance, or the response to volume infusion in normal subjects. *Crit Care Med* 2004; **32**: 691-699 [PMID: 15090949 DOI: 10.1097/01.CCM.0000114996.68110.C9]
30 **Sondergaard S**, Parkin G, Aneman A. Central venous pressure: we need to bring clinical use into physiological context. *Acta Anaesthesiol Scand* 2015; **59**: 552-560 [PMID: 25684176 DOI: 10.1111/aas.12490]
31 **Giordano C**, Deitte LA, Gravenstein N, Rice MJ. What is the preferred central venous pressure zero reference for hepatic resection? *Anesth Analg* 2010; **111**: 660-664 [PMID: 19955507 DOI: 10.1213/ANE.0b013e3181c76d3e]
32 **Gelman S**. Venous function and central venous pressure: a physiologic story. *Anesthesiology* 2008; **108**: 735-748 [PMID: 18362606 DOI: 10.1097/ALN.0b013e3181672607]
33 **Sand L**, Rizell M, Houltz E, Karlsen K, Wiklund J, Odenstedt Hergès H, Stenqvist O, Lundin S. Effect of patient position and PEEP on hepatic, portal and central venous pressures during liver resection. *Acta Anaesthesiol Scand* 2011; **55**: 1106-1112 [PMID: 22092208 DOI: 10.1111/j.1399-6576.2011.02502.x]
34 **Smyrniotis V**, Kostopanagiotou G, Theodoraki K, Tsantoulas D, Contis JC. The role of central venous pressure and type of vascular control in blood loss during major liver resections. *Am J Surg* 2004; **187**: 398-402 [PMID: 15006570 DOI: 10.1016/j.amjsurg.2003.12.001]
35 **Kusminsky RE**. Complications of central venous catheterization. *J Am Coll Surg* 2007; **204**: 681-696 [PMID: 17382229 DOI: 10.1016/j.jamcollsurg.2007.01.039]
36 **McGee DC**, Gould MK. Preventing complications of central venous catheterization. *N Engl J Med* 2003; **348**: 1123-1133 [PMID: 12646670 DOI: 10.1056/NEJMra011883]
37 **Hata T**, Fujimoto Y, Suzuki K, Kim B, Ishigami M, Ogawa H, Arikawa T, Nagai S, Kamei H, Nakamura T, Edamoto Y, Kiuchi T. Two cases of central venous catheter-related thrombosis in living liver donors: how can the risk be minimized? *Clin Transplant* 2009; **23**: 289-293 [PMID: 19191817 DOI: 10.1111/j.1399-0012.2008.00939.x]
38 **Zhang Z**, Lu B, Sheng X, Jin N. Accuracy of stroke volume variation in predicting fluid responsiveness: a systematic review and meta-analysis. *J Anesth* 2011; **25**: 904-916 [PMID: 21892779 DOI: 10.1007/s00540-011-1217-1]
39 **Kim YK**, Shin WJ, Song JG, Jun IG, Kim HY, Seong SH, Hwang GS. Comparison of stroke volume variations derived from radial and femoral arterial pressure waveforms during liver transplantation. *Transplant Proc* 2009; **41**: 4220-4228 [PMID: 20005373 DOI: 10.1016/j.transproceed.2009.09.050]
40 **Xia J**, He Z, Cao X, Che X, Chen L, Zhang J, Liang W. The brain relaxation and cerebral metabolism in stroke volume variation-directed fluid therapy during supratentorial tumors resection: crystalloid solution versus colloid solution. *J Neurosurg Anesthesiol* 2014; **26**: 320-327 [PMID: 24487733 DOI: 10.1097/ANA.0000000000000046]
41 **Biais M**, Nouette-Gaulain K, Cottenceau V, Revel P, Sztark F. Uncalibrated pulse contour-derived stroke volume variation predicts fluid responsiveness in mechanically ventilated patients undergoing liver transplantation. *Br J Anaesth* 2008; **101**: 761-768 [PMID: 18852114 DOI: 10.1093/bja/aen277]
42 **Biais M**, Nouette-Gaulain K, Roullet S, Quinart A, Revel P, Sztark F. A comparison of stroke volume variation measured by Vigileo/FloTrac system and aortic Doppler echocardiography. *Anesth Analg* 2009; **109**: 466-469 [PMID: 19608819 DOI: 10.1213/ane.0b013e3181ac6dac]
43 **Slagt C**, Malagon I, Groeneveld AB. Systematic review of uncalibrated arterial pressure waveform analysis to determine cardiac output and stroke volume variation. *Br J Anaesth* 2014; **112**: 626-637 [PMID: 24431387 DOI: 10.1093/bja/aet429]
44 **Kim SH**, Hwang GS, Kim SO, Kim YK. Is stroke volume variation a useful preload index in liver transplant recipients? A retrospective analysis. *Int J Med Sci* 2013; **10**: 751-757 [PMID: 23630440 DOI: 10.7150/ijms.6074]
45 **Kim YK**, Shin WJ, Song JG, Jun IG, Hwang GS. Does stroke volume variation predict intraoperative blood loss in living right donor hepatectomy? *Transplant Proc* 2011; **43**: 1407-1411 [PMID: 21693206 DOI: 10.1016/j.transproceed.2011.02.056]
46 **Dunki-Jacobs EM**, Philips P, Scoggins CR, McMasters KM, Martin RC. Stroke volume variation in hepatic resection: a replacement for standard central venous pressure monitoring. *Ann Surg Oncol* 2014; **21**: 473-478 [PMID: 24150192 DOI: 10.1245/s10434-013-3323-9]
47 **Kitaguchi K**, Gotohda N, Yamamoto H, Kato Y, Takahashi S, Konishi M, Hayashi R. Intraoperative circulatory management using the FloTrac™ system in laparoscopic liver resection. *Asian J Endosc Surg* 2015; **8**: 164-170 [PMID: 25470208 DOI: 10.1111/ases.12158]
48 **Harimoto N**, Matsuyama H, Kajiyama K, Nagaie T, Ikegami T, Yoshizumi T, Soejima Y, Shirabe K, Ikeda T, Kawanaka H, Uchiyama H, Yamashita Y, Morita M, Oki E, Saeki H, Maehara Y. Significance of stroke volume variation during hepatic resection under infrahepatic inferior vena cava and portal triad clamping. *Fukuoka Igaku Zasshi* 2013; **104**: 362-369 [PMID: 24511667]
49 **Kubitz JC**, Annecke T, Kemming GI, Forkl S, Kronas N, Goetz AE, Reuter DA. The influence of positive end-expiratory pressure on stroke volume variation and central blood volume during open and closed chest conditions. *Eur J Cardiothorac Surg* 2006; **30**: 90-95 [PMID: 16723238 DOI: 10.1016/j.ejcts.2006.04.008]
50 **Michard F**, Chemla D, Richard C, Wysocki M, Pinsky MR, Lecarpentier Y, Teboul JL. Clinical use of respiratory changes in arterial pulse pressure to monitor the hemodynamic effects of PEEP. *Am J Respir Crit Care Med* 1999; **159**: 935-939 [PMID: 10051276 DOI: 10.1164/ajrccm.159.3.9805077]
51 **Ejaz AA**, Mohandas R. Are diuretics harmful in the management of acute kidney injury? *Curr Opin Nephrol Hypertens* 2014; **23**: 155-160 [PMID: 24389731 DOI: 10.1097/01.mnh.0000441150.17202.be]
52 **Prowle JR**, Kirwan CJ, Bellomo R. Fluid management for the prevention and attenuation of acute kidney injury. *Nat Rev Nephrol* 2014; **10**: 37-47 [PMID: 24217464 DOI: 10.1038/nrneph.2013.232]
53 **Choi SS**, Cho SS, Kim SH, Jun IG, Hwang GS, Kim YK. Factors associated with blood transfusion in donor hepatectomy: results from 2344 donors at a large single center. *Transplantation* 2013; **96**: 1000-1007 [PMID: 23985722 DOI: 10.1097/TP.0b013e3182a41937]
54 **Michard F**, Biais M. Rational fluid management: dissecting facts from fiction. *Br J Anaesth* 2012; **108**: 369-371 [PMID: 22337956 DOI: 10.1093/bja/aer511]
55 **Marik PE**, Cavallazzi R, Vasu T, Hirani A. Dynamic changes in arterial waveform derived variables and fluid responsiveness in mechanically ventilated patients: a systematic review of the literature. *Crit Care Med* 2009; **37**: 2642-2647 [PMID: 19602972 DOI: 10.1097/CCM.0b013e3181a590da]
56 **McGee WT**, Raghunathan K. Physiologic goal-directed therapy in the perioperative period: the volume prescription for high-risk patients. *J Cardiothorac Vasc Anesth* 2013; **27**: 1079-1086 [PMID: 24075639 DOI: 10.1053/j.jvca.2013.04.019]
57 **Patel A**, Laffan MA, Waheed U, Brett SJ. Randomised trials of human albumin for adults with sepsis: systematic review and meta-analysis with trial sequential analysis of all-cause mortality. *BMJ* 2014; **349**: g4561 [PMID: 25099709 DOI: 10.1136/bmj.g4561]
58 **Patel A**, Waheed U, Brett SJ. Randomised trials of 6% tetrastarch (hydroxyethyl starch 130/0.4 or 0.42) for severe sepsis reporting mortality: systematic review and meta-analysis. *Intensive Care Med* 2013; **39**: 811-822 [PMID: 23430019 DOI: 10.1007/s00134-013-2863-6]
59 **Perel P**, Roberts I, Ker K. Colloids versus crystalloids for fluid resuscitation in critically ill patients. *Cochrane Database Syst Rev* 2013; **2**: CD000567 [PMID: 23450531 DOI: 10.1002/14651858.

CD000567.pub6]
60 **Serpa Neto A**, Veelo DP, Peireira VG, de Assunção MS, Manetta JA, Espósito DC, Schultz MJ. Fluid resuscitation with hydroxyethyl starches in patients with sepsis is associated with an increased incidence of acute kidney injury and use of renal replacement therapy: a systematic review and meta-analysis of the literature. *J Crit Care* 2014; **29**: 185.e1-185.e7 [PMID: 24262273 DOI: 10.1016/j.jcrc.2013.09.031]
61 **Zarychanski R**, Abou-Setta AM, Turgeon AF, Houston BL, McIntyre L, Marshall JC, Fergusson DA. Association of hydroxyethyl starch administration with mortality and acute kidney injury in critically ill patients requiring volume resuscitation: a systematic review and meta-analysis. *JAMA* 2013; **309**: 678-688 [PMID: 23423413 DOI: 10.1001/jama.2013.430]
62 **Haase N**, Perner A, Hennings LI, Siegemund M, Lauridsen B, Wetterslev M, Wetterslev J. Hydroxyethyl starch 130/0.38-0.45 versus crystalloid or albumin in patients with sepsis: systematic review with meta-analysis and trial sequential analysis. *BMJ* 2013; **346**: f839 [PMID: 23418281 DOI: 10.1136/bmj.f839]
63 **Navickis RJ**, Haynes GR, Wilkes MM. Effect of hydroxyethyl starch on bleeding after cardiopulmonary bypass: a meta-analysis of randomized trials. *J Thorac Cardiovasc Surg* 2012; **144**: 223-230 [PMID: 22578894 DOI: 10.1016/j.jtcvs2012.04.009]
64 **Martin C**, Jacob M, Vicaut E, Guidet B, Van Aken H, Kurz A. Effect of waxy maize-derived hydroxyethyl starch 130/0.4 on renal function in surgical patients. *Anesthesiology* 2013; **118**: 387-394 [PMID: 23340352 DOI: 10.1097/ALN.0b013e31827e5569]
65 **Gillies MA**, Habicher M, Jhanji S, Sander M, Mythen M, Hamilton M, Pearse RM. Incidence of postoperative death and acute kidney injury associated with i.v. 6% hydroxyethyl starch use: systematic review and meta-analysis. *Br J Anaesth* 2014; **112**: 25-34 [PMID: 24046292 DOI: 10.1093/bja/aet303]
66 **Lee WL**, Slutsky AS. Sepsis and endothelial permeability. *N Engl J Med* 2010; **363**: 689-691 [PMID: 20818861 DOI: 10.1056/NEJMcibr1007320]
67 **James MF**, Michell WL, Joubert IA, Nicol AJ, Navsaria PH, Gillespie RS. Resuscitation with hydroxyethyl starch improves renal function and lactate clearance in penetrating trauma in a randomized controlled study: the FIRST trial (Fluids in Resuscitation of Severe Trauma). *Br J Anaesth* 2011; **107**: 693-702 [PMID: 21857015 DOI: 10.1093/bja/aer229]
68 **Ogilvie MP**, Pereira BM, McKenney MG, McMahon PJ, Manning RJ, Namias N, Livingstone AS, Schulman CI, Proctor KG. First report on safety and efficacy of hetastarch solution for initial fluid resuscitation at a level 1 trauma center. *J Am Coll Surg* 2010; **210**: 870-880 [PMID: 20421069 DOI: 10.1016/j.jamcollsurg.2010.01.010]
69 **McCluskey SA**, Karkouti K, Wijeysundera D, Minkovich L, Tait G, Beattie WS. Hyperchloremia after noncardiac surgery is independently associated with increased morbidity and mortality: a propensity-matched cohort study. *Anesth Analg* 2013; **117**: 412-421 [PMID: 23757473 DOI: 10.1213/ANE.0b013e318293d81e]
70 **Waters JH**, Gottlieb A, Schoenwald P, Popovich MJ, Sprung J, Nelson DR. Normal saline versus lactated Ringer's solution for intraoperative fluid management in patients undergoing abdominal aortic aneurysm repair: an outcome study. *Anesth Analg* 2001; **93**: 817-822 [PMID: 11574339 DOI: 10.1097/00000539-200110000-00004]
71 **Park CM**, Chun HK, Jeon K, Suh GY, Choi DW, Kim S. Factors related to post-operative metabolic acidosis following major abdominal surgery. *ANZ J Surg* 2014; **84**: 574-580 [PMID: 22985420 DOI: 10.1111/j.1445-2197.2012.06235.x]
72 **Stephens R**, Mythen M. Optimizing intraoperative fluid therapy. *Curr Opin Anaesthesiol* 2003; **16**: 385-392 [PMID: 17021487 DOI: 10.1097/01.aco.0000084478.59960.76]
73 **Takil A**, Eti Z, Irmak P, Yilmaz Göğüş F. Early postoperative respiratory acidosis after large intravascular volume infusion of lactated ringer's solution during major spine surgery. *Anesth Analg* 2002; **95**: 294-298 [PMID: 12145036 DOI: 10.1213/00000539-200208000-00006]
74 **Khalaf H**, Al-Sofayan M, El-Sheikh Y, Al-Bahili H, Al-Sagheir M, Al-Sebayel M. Donor outcome after living liver donation: a single-center experience. *Transplant Proc* 2007; **39**: 829-834 [PMID: 17524825 DOI: 10.1016/j.transproceed.2007.03.003]
75 **Chioléro R**, Tappy L, Gillet M, Revelly JP, Roth H, Cayeux C, Schneiter P, Leverve X. Effect of major hepatectomy on glucose and lactate metabolism. *Ann Surg* 1999; **229**: 505-513 [PMID: 10203083]
76 **Shin WJ**, Kim YK, Bang JY, Cho SK, Han SM, Hwang GS. Lactate and liver function tests after living donor right hepatectomy: a comparison of solutions with and without lactate. *Acta Anaesthesiol Scand* 2011; **55**: 558-564 [PMID: 21342149 DOI: 10.1111/j.1399-6576.2011.02398.x]
77 **Mukhtar A**, Aboulfetouh F, Obayah G, Salah M, Emam M, Khater Y, Akram R, Hoballah A, Bahaa M, Elmeteini M, Hamza A. The safety of modern hydroxyethyl starch in living donor liver transplantation: a comparison with human albumin. *Anesth Analg* 2009; **109**: 924-930 [PMID: 19690268 DOI: 10.1213/ane.0b013e3181aed54f]
78 **Rioux JP**, Lessard M, De Bortoli B, Roy P, Albert M, Verdant C, Madore F, Troyanov S. Pentastarch 10% (250 kDa/0.45) is an independent risk factor of acute kidney injury following cardiac surgery. *Crit Care Med* 2009; **37**: 1293-1298 [PMID: 19242349 DOI: 10.1097/CCM.0b013e31819cc1a0]
79 **Hokema F**, Ziganshyna S, Bartels M, Pietsch UC, Busch T, Jonas S, Kaisers U. Is perioperative low molecular weight hydroxyethyl starch infusion a risk factor for delayed graft function in renal transplant recipients? *Nephrol Dial Transplant* 2011; **26**: 3373-3378 [PMID: 21427075 DOI: 10.1093/ndt/gfr017]
80 **Canet J**, Sabaté S, Mazo V. Effects of intraoperative colloid administration on outcome in a population-based general surgical cohort: a propensity score analysis. *Minerva Anestesiol* 2013; **79**: 891-905 [PMID: 23652169]
81 **Van der Linden P**, De Villé A, Hofer A, Heschl M, Gombotz H. Six percent hydroxyethyl starch 130/0.4 (Voluven®) versus 5% human serum albumin for volume replacement therapy during elective open-heart surgery in pediatric patients. *Anesthesiology* 2013; **119**: 1296-1309 [PMID: 23934169 DOI: 10.1097/ALN.0b013e3182a6b387]
82 **Skhirtladze K**, Base EM, Lassnigg A, Kaider A, Linke S, Dworschak M, Hiesmayr MJ. Comparison of the effects of albumin 5%, hydroxyethyl starch 130/0.4 6%, and Ringer's lactate on blood loss and coagulation after cardiac surgery. *Br J Anaesth* 2014; **112**: 255-264 [PMID: 24169821 DOI: 10.1093/bja/aet348]
83 **Rasmussen KC**, Johansson PI, Højskov M, Kridina I, Kistorp T, Thind P, Nielsen HB, Ruhnau B, Pedersen T, Secher NH. Hydroxyethyl starch reduces coagulation competence and increases blood loss during major surgery: results from a randomized controlled trial. *Ann Surg* 2014; **259**: 249-254 [PMID: 24100337 DOI: 10.1097/SLA.0000000000000267]
84 **Kancir AS**, Pleckaitiene L, Hansen TB, Ekeløf NP, Pedersen EB. Lack of nephrotoxicity by 6% hydroxyethyl starch 130/0.4 during hip arthroplasty: a randomized controlled trial. *Anesthesiology* 2014; **121**: 948-958 [PMID: 25127210 DOI: 10.1097/ALN.0000000000000413]
85 **Lindroos AC**, Niiya T, Randell T, Niemi TT. Stroke volume-directed administration of hydroxyethyl starch (HES 130/0.4) and Ringer's acetate in prone position during neurosurgery: a randomized controlled trial. *J Anesth* 2014; **28**: 189-197 [PMID: 24077833 DOI: 10.1007/s00540-013-1711-8]
86 **Yates DR**, Davies SJ, Milner HE, Wilson RJ. Crystalloid or colloid for goal-directed fluid therapy in colorectal surgery. *Br J Anaesth* 2014; **112**: 281-289 [PMID: 24056586 DOI: 10.1093/bja/aet307]
87 **Kashy BK**, Podolyak A, Makarova N, Dalton JE, Sessler DI, Kurz A. Effect of hydroxyethyl starch on postoperative kidney function in patients having noncardiac surgery. *Anesthesiology* 2014; **121**: 730-739 [PMID: 25054470 DOI: 10.1097/ALN.0000000000000375]
88 **Mercier FJ**, Diemunsch P, Ducloy-Bouthors AS, Mignon A, Fischler M, Malinovsky JM, Bolandard F, Aya AG, Raucoules-Aimé M, Chassard D, Keita H, Rigouzzo A, Le Gouez A. 6%

Hydroxyethyl starch (130/0.4) vs Ringer's lactate preloading before spinal anaesthesia for Caesarean delivery: the randomized, double-blind, multicentre CAESAR trial. *Br J Anaesth* 2014; **113**: 459-467 [PMID: 24970272 DOI: 10.1093/bja/aeu103]

89 **Hand WR**, Whiteley JR, Epperson TI, Tam L, Crego H, Wolf B, Chavin KD, Taber DJ. Hydroxyethyl starch and acute kidney injury in orthotopic liver transplantation: a single-center retrospective review. *Anesth Analg* 2015; **120**: 619-626 [PMID: 25036375 DOI: 10.1213/ANE.0000000000000374]

90 **Kancir AS**, Johansen JK, Ekeloef NP, Pedersen EB. The effect of 6% hydroxyethyl starch 130/0.4 on renal function, arterial blood pressure, and vasoactive hormones during radical prostatectomy: a randomized controlled trial. *Anesth Analg* 2015; **120**: 608-618 [PMID: 25627076 DOI: 10.1213/ANE.0000000000000596]

91 **Choi SS**, Cho SS, Ha TY, Hwang S, Lee SG, Kim YK. Intraoperative factors associated with delayed recovery of liver function after hepatectomy: analysis of 1969 living donors. *Acta Anaesthesiol Scand* (Epub) 2015 [DOI: 10.1111/aas.12630]

**P- Reviewer**: De Raffele E **S- Editor**: Ma YJ **L- Editor**: A
**E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12767

*World J Gastroenterol* 2015 December 7; 21(45): 12767-12777
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

Basic Study

# Dextran sulfate sodium-induced acute colitis impairs dermal lymphatic function in mice

Germaine D Agollah, Grace Wu, Ho-Lan Peng, Sunkuk Kwon

Germaine D Agollah, Grace Wu, Sunkuk Kwon, Center for Molecular Imaging, The Brown Foundation Institute of Molecular Medicine, The University of Texas Health Science Center, Houston, TX 77030, United States

Germaine D Agollah, The University of Texas Graduate School of Biomedical Sciences at Houston, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, United States

Ho-Lan Peng, The University of Texas Health Science Center at Houston, School of Public Health, Houston, TX 77030, United States

Author contributions: Agollah GD, Wu G and Kwon S contributed to the acquisition and interpretation and statistical analysis of *in vivo* and *ex vivo* data; Peng HL performed statistical analysis; Kwon S designed and supervised the research; Agollah GD and Kwon S wrote the manuscript.

Supported by (in part) A pilot/feasibility grant from NIH/National Institute of Diabetes and Digestive and Kidney Disease, Center Grant P30 DK56338 (for Kwon S); the Schissler Foundation Fellowship for Translational Studies of Common Human Diseases (for Agollah GD).

Institutional review board statement: The study was reviewed and approved by University of Texas Health Science Center Institutional Review Board.

Institutional animal care and use committee statement: All animal experiments were reviewed and approved by University of Texas Health Science Center Institutional Animal Welfare Committee (AWC-14-0034).

Conflict-of-interest statement: The authors declared that they have no conflicts of interest to this work.

Data sharing statement: No additional data are available.



Correspondence to: Sunkuk Kwon, PhD, Assistant Professor, Center for Molecular Imaging, The Brown Foundation Institute of Molecular Medicine, The University of Texas Health Science Center, 1825 Pressler Street, SRB 330F, Houston, TX 77030, United States. sunkuk.kwon@uth.tmc.edu
Telephone: +1-713-5003563
Fax: +1-713-5000319

Received: May 16, 2015
Peer-review started: May 20, 2015
First decision: June 19, 2015
Revised: July 10, 2015
Accepted: August 31, 2015
Article in press: August 31, 2015
Published online: December 7, 2015

## Abstract

**AIM:** To investigate whether dermal lymphatic function and architecture are systemically altered in dextran sulfate sodium (DSS)-induced acute colitis.

**METHODS:** Balb/c mice were administered 4% DSS in lieu of drinking water *ad libitum* for 7 d and monitored to assess disease activity including body weight, diarrhea severity, and fecal bleeding. Control mice received standard drinking water with no DSS. Changes in mesenteric lymphatics were assessed following oral administration of a fluorescently-labelled fatty acid analogue, while dermal lymphatic function and architecture was longitudinally characterized using dynamic near-infrared fluorescence (NIRF) imaging following intradermal injection of indocyanine green (ICG) at the base of the tail or to the dorsal aspect of the left paw prior to, 4, and 7 d after DSS

administration. We also measured dye clearance rate after injection of Alexa680-bovine serum albumin (BSA). NIRF imaging data was analyzed to reveal lymphatic contractile activity after selecting fixed regions of interest (ROIs) of the same size in fluorescent lymphatic vessels on fluorescence images. The averaged fluorescence intensity within the ROI of each fluorescence image was plotted as a function of imaging time and the lymphatic contraction frequency was computed by assessing the number of fluorescent pulses arriving at a ROI.

**RESULTS:** Mice treated with DSS developed acute inflammation with clinical symptoms of loss of body weight, loose feces/watery diarrhea, and fecal blood, all of which were aggravated as disease progressed to 7 d. Histological examination of colons of DSS-treated mice confirmed acute inflammation, characterized by segmental to complete loss of colonic mucosa with an associated chronic inflammatory cell infiltrate that extended into the deeper layers of the wall of the colon, compared to control mice. *In situ* intravital imaging revealed that mice with acute colitis showed significantly fewer fluorescent mesenteric lymphatic vessels, indicating impaired uptake of a lipid tracer within mesenteric lymphatics. Our *in vivo* NIRF imaging data demonstrated dilated dermal lymphatic vessels, which were confirmed by immunohistochemical staining of lymphatic vessels, and significantly reduced lymphatic contractile function in the skin of mice with DSS-induced acute colitis. Quantification of the fluorescent intensity remaining in the depot as a function of time showed that there was significantly higher Alexa680-BSA fluorescence in mice with DSS-induced acute colitis compared to pre-treatment with DSS, indicative of impaired lymphatic drainage.

**CONCLUSION:** The lymphatics are locally and systemically altered in acute colitis, and functional NIRF imaging is useful for noninvasively monitoring systemic lymphatic changes during inflammation.

**Key words:** Dextran sulfate sodium; Colitis; Lymphatic system; Inflammation; Near-infrared fluorescence imaging



**Core tip:** Inflammatory bowel disease (IBD) is a systemic disease, as it is often associated with extra-intestinal manifestations, complications, and other autoimmune disorders. However, it is unknown whether dermal lymphatic function changes systemically in response to IBD. In this study, we employed near-infrared fluorescence imaging to characterize dermal lymphatic function and architecture in mice with dextran sulfate sodium (DSS)-induced acute colitis. Our results demonstrated impaired lymphatic function in mesenteric lymphatics accompanied by dilated lymphatic vessels and reduced lymphatic contractility in the skin of mice with DSS-induced acute colitis, indicating that DSS-induced acute colitis results in systemic lymphatic dysfunction.

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(45): 12767-12777 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12767.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12767

## INTRODUCTION

Inflammatory bowel disease (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD), involves progressive and destructive inflammation of the small and large intestines. Recent evidence suggests that IBD pathogenesis may result from an abnormal immune response to normal gut microbial antigens within the intestinal flora of genetically-susceptible individuals[1]. However, the precise mechanisms of immune and genetic involvement in IBD remain poorly understood.

IBD is a systemic disease, as it is often associated with extra-intestinal manifestations (EIMs), complications, and other autoimmune disorders including psoriasis and rheumatoid arthritis[2]. Cutaneous complications, such as erythema nodosum and pyoderma gangrenosum, are relatively common manifestations of IBD[3,4]. Although dependent on different mechanisms, both IBD and its extra-intestinal complications are characterized by an influx of destructive inflammatory cells with the subsequent secretion of pro-inflammatory cytokines and mediators, which may affect lymphatic function, both locally and systemically. A previous study demonstrated increased lymphatic vessel diameters, reduced number of spontaneously-pumping lymphatic vessels, and lower contraction frequency *in vitro* and *in situ* in the mesenteric lymphatic vessels in the 2,4,6-trinitrobenzene sulfonic acid (TNBS) model of guinea pig ileitis[5]. Impaired lymphatic function during intestinal inflammation may delay immunological responses and thus hinder the resolution of inflammation-associated edema[6], a common condition associated with IBD[7,8]. However, it is unknown whether the lymphatic system changes systemically in response to gut inflammation.

The lymphatic system plays important roles in: (1) removing excess fluid from the tissues and thus maintaining tissue-fluid homeostasis; and (2) transporting activated immune cells into draining lymph nodes (DLNs) *via* afferent lymphatic vessels, thus evoking inflammatory immune response and subsequently resolving inflammation[9,10]. Impaired lymphatic function has been implicated in many pathological conditions, including inflammation[10]. Given the essential role played by the lymphatics in the initiation, progression, and resolution of inflammation,

lymphatic function may be systemically altered during gut inflammation. Additionally, intestinal lymphatic vessels, known as lacteals, within intestinal villi take up dietary lipids for transportation back to the blood vasculature. Thus, it is likely that lymphatics play an important role in the complex etiology of IBD and its EIMs[6]. Herein, we describe lymphatic function in the skin of mice with DSS-induced acute colitis using near-infrared fluorescence (NIRF) lymphatic imaging[11]. Our data demonstrates for the first time, systemically-altered dermal lymphatic function in mice with acute colitis.

## MATERIALS AND METHODS

### Animals

Six to eight week-old female Balb/c mice (Charles River) were housed and fed sterilized pelleted food and sterilized drinking water. Animals were maintained in a pathogen-free mouse facility accredited by the American Association for Laboratory Animal Care (AALAC). All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC). Animal experiments were approved by University of Texas Health Science Center Institutional Animal Welfare Committee (AWC-14-0034).

### Induction of colitis and assessment of disease severity

Experimental colitis was induced by administering 4% (wt/vol) DSS (molecular weight 36-50 kDa, MP Biomedicals)[12] solution to replace drinking water *ad libitum* for 7 d. Control mice received standard drinking water. On day 7, mice were euthanized and tissues harvested for *ex vivo* studies. In all mice, body weight and diarrhea severity (diarrhea score: 0, normal; 1, slightly loose feces; 2, loose feces; 3, watery diarrhea) were monitored[13]. Body weight at day 4 and 7 were normalized to day 0, and body weight change expressed as a percentage. In addition, fecal bleeding (visible fecal blood score: 0, normal; 1, slightly bloody; 2, bloody; 3, blood in whole feces) was scored[13]. Colon length was measured to determine severity of colitis.

### In situ Mesenteric lymphangiography imaging

For imaging mesenteric lymphatic vessels, 1 mL of a long-chain fatty acid, Bodipy-FL-C16 (Life Technologies) was orally administered to control mice and 7-d DSS-treated mice. At 30 min after oral administration, mice were euthanized and fluorescence imaging was performed to visualize fluorescent lymphatic vessels in the mesentery using a stereomicroscope (MZ16 A, Leica Microsystems, Inc.) with excitation light at 493 nm and emission light at 503 nm. The number of fluorescent mesenteric lymphatic vessels was counted.

### In vivo functional NIRF lymphatic imaging

Mice were imaged for baseline lymphatic parameters prior to beginning DSS treatment, at 4, and 7 d after administration. Mice were anesthetized with isoflurane and maintained at 37 ℃ on a warming pad. A volume of either 10 μL or 2 μL of 645 μmol/L ICG (Akorn, Inc.) dissolved in mixture of distilled water and 0.9% sodium chloride in a volume ratio of 1:9 was injected intradermally at either the base of the tail or to the dorsal aspect of left hind paw, respectively, using 31 gauge needles (BD Ultra-FineTM Ⅱ Short Needle, Becton and Dickinson Medical) or 34 gauge needles (Nanofil, World Precision Instruments, Inc.). Fluorescence images were acquired immediately before and for up to 20 min after id injection using a custom-built NIRF imaging system as described previously[14]. The number of fluorescent lymphatics in the dorsum of the foot or the base of the tail near the injection site was counted.

### Quantification of dye clearance

In order to measure dye clearance, 2 μL of Alexa680-BSA was injected intradermally at the dorsal aspect of the left hind paw in an anesthetized mouse prior to DSS treatment for baseline, and 7 d after DSS treatment using 34 gauge needles (Nanofil, World Precision Instruments, Inc.). The injection area in a mouse was imaged every 1 h for up to 6.5 h after injection. Mice regained consciousness between measurements. The region of interest (ROI) was defined as the entire paw and fluorescent intensities within the ROI were measured.

### Immunohistochemical analysis

For histological analysis, tissue samples were fixed in 10% formalin overnight before transfer into 70% ethanol. Tissue samples were embedded in paraffin and 4 μm sections used in all staining procedures. The tissue sections stained with hematoxylin-eosin (HE) were analyzed by Dr. Roger Price at Center for Comparative Medicine Pathology Core at Baylor College of Medicine (BCM). Paraffin-embedded sections were stained for LYVE-1 as follows. Following paraffin removal and antigen retrieval using citrate buffer, tissues were blocked with 5% bovine serum albumin (BSA) and stained with rabbit anti-mouse LYVE-1 antibody (AngioBio) and biotin-anti rabbit secondary antibody (Vector Labs). Vectastain Elite ABC system for peroxidase and DAB or ImmPACT Novared chromagen reagents were used before tissues were counter-stained with hematoxylin (Vector Labs). LYVE-1 expression in three different fields in each section was examined at magnification × 400 (Leica Microsystems Inc.). Lymphatic vessel number and relative lymphatic vessel area, which was defined as the percentage of positively stained lymphatic vessel area were determined as described previously[15] using Image-Pro Plus software (Media Cybernetics, Inc.).

### Lymphatic function analysis

The data was analyzed with Matlab (The MathWorks,

Inc.) and ImageJ (National Institutes of Health). To reveal contractile activity resulting in propulsive lymph flow, fixed regions of interest (ROIs) of equal size in the fluorescent lymphatic vessels were defined on fluorescence images. The averaged fluorescence intensity within each ROI in each fluorescence image was plotted as a function of imaging time. The number of "pulses" of ICG-laden lymph was an indication of lymphatic contractile activity and termed as "contractions".

### Statistical analysis

Data were presented as average values ± standard error. The statistical analysis was performed by Ho-Lan Peng from School of Public Health, The University of Texas Health Science Center at Houston, using SAS Enterprise Guide 5.1 and SigmaPlot 11.0. For the pairwise comparisons, the paired *t* test or Wilcoxon signed rank test were used depending on the type and the normality of the data. For between group comparisons, the two-sample *t* test or Wilcoxon rank sum test were used depending on the normality of the data. The significance level was defined as $P < 0.05$.

## RESULTS

### Characteristics of DSS-induced acute colitis

Mice treated for 7 d with DSS developed acute inflammation. As shown in Figure 1, clinical symptoms in mice with DSS-induced acute colitis included loss of body weight (Figure 1A), loose feces/watery diarrhea (Figure 1B), and fecal blood (Figure 1C), all of which were aggravated as disease progressed to 7 d. In addition, the length of the colon decreased as disease severity increased and was significantly shorter in mice at day 7 (Figure 1D). Histopathological examination of colons of DSS-treated mice confirmed acute inflammation (Figure 1F), characterized by segmental to complete loss of the colonic mucosa with an associated chronic inflammatory cell infiltrate that extended into the deeper layers of the wall of the colon, compared to control mice (Figure 1E). Ulceration of the colon was typically associated with hyperplasia of the colonic mucosa adjacent to the areas of ulceration. Thus, mice treated with DSS for 7 d exhibited acute inflammation as assessed by clinical symptoms.

### DSS treatment induces mesenteric and dermal lymphatic vessel remodeling

To assess whether DSS-induced colitis affected mesenteric lymphatic drainage, we performed intravital fluorescent lymphangiography 30 min after oral gavage administration of Bodipy-FL-C16. Mice with acute colitis (Figure 2B) showed impaired uptake of Bodipy-FL-C16 within mesenteric lymphatics as compared to controls (Figure 2A). We observed significantly fewer fluorescent mesenteric lymphatic vessels in mice treated with DSS for 7 d than those in control mice (Figure 2E). To examine differences in lymphatic vessel architecture within the colons, we performed immunohistochemical (IHC) staining for lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1). Colons of DSS-treated mice exhibited dilated lymphatic vessels (Figure 2D and G) and increased number of vessels (Figure 2F), compared to control mice (Figure 2C).

Given the changes within the mesenteric lymphatics, we next assessed whether DSS-induced colitis affected peripheral lymphatic architecture and function as well. We performed NIRF imaging after id injection of ICG in the dorsal aspect of the hind paw. *In vivo* fluorescence imaging data demonstrated that mice with acute colitis after 7 d of DSS treatment (Figure 3B) showed similar peripheral lymphatic drainage patterns in the paw as compared to control animals (Figure 3A), although dilated lymphatic vessels were observed in mice with DSS-induced colitis. However, the number of fluorescent lymphatic vessels in the foot between baseline (2.2 ± 0.4) and 7 d after DSS treatment (1.8 ± 0.4) was not statistically different. We also assessed the depot clearance of Alexa680-BSA. Fluorescent images from one representative mouse showing clearance of Alexa680-BSA from the depot (dorsal aspect of the left hind paw) over about 6 h are shown in Figure 3C. We found that while the fluorescence rapidly decreased in mice prior to DSS treatment, 7 d DSS treatment delayed the clearance. Quantification of the fluorescent intensity remaining in the depot as a function of time as shown in Figure 3D, showed that there was significantly higher fluorescence in mice with DSS-induced acute colitis than that in mice prior to DSS treatment, indicative of impaired lymphatic drainage.

Next, to examine whether dermal lymphatics were altered in response to DSS-treatment, skin and ear tissues were immunostained with antibody to LYVE-1. We observed no significant differences in the number of lymphatic vessels between control (Figure 4A and E) and mice with DSS-induced colitis (Figure 4B and F) in either skin or ears, whereas a significant increase of the relative area occupied by peripheral lymphatic vessels in mice with DSS-induced colitis was observed as compared to that in control, indicative of dilated dermal lymphatic vessels in response to DSS-induced acute colitis (Figure 4C, D, G and H). However, we did not observe any clinically apparent manifestations in the skin of DSS-treated mice.

### DSS-induced colitis impairs dermal lymphatic contractile function

We examined lymphatic contractile function in the skin of mice with DSS-induced acute colitis to investigate whether lymphatic function was systemically impaired in response to intestinal inflammation. Quantitative analysis of lymphatic contractile function demonstrated

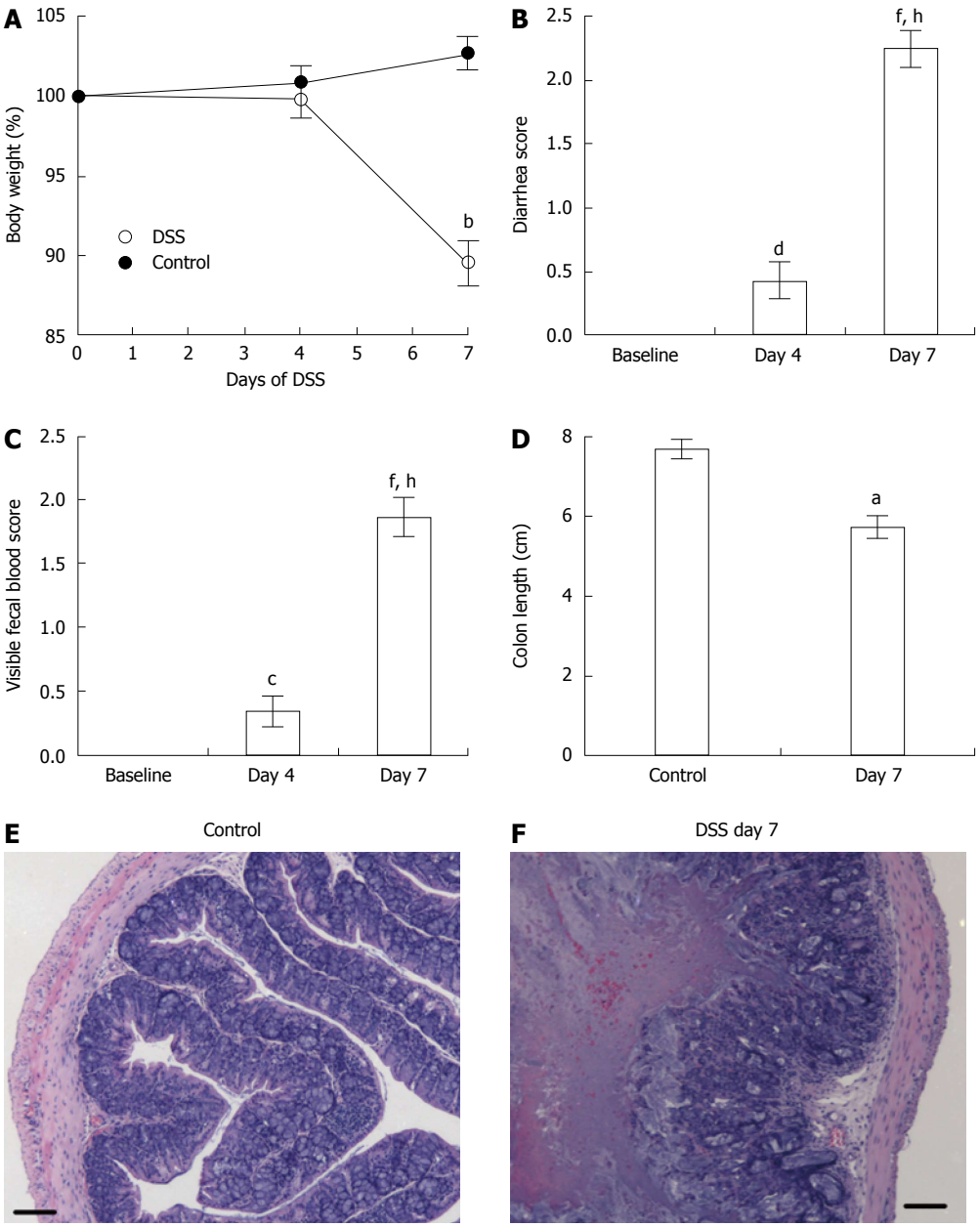



**Figure 1 Clinical and inflammatory symptoms during dextran sulfate sodium-induced acute colitis assessed by changes in body weight (A), diarrhea score (B), visible fecal blood score (C), colon length (D), and histological sections of the colons in control mice (E) and mice that received dextran sulfate sodium for 7 d (F).** Control ($n$ = 9) and dextran sulfate sodium (DSS)-treated ($n$ = 21) mice. Data presented as mean ± SE. [a]$P$ < 0.05, [b]$P$ < 0.001 *vs* control. [c]$P$ < 0.05, [d]$P$ < 0.01, [f]$P$ < 0.001 *vs* baseline. [h]$P$ < 0.001 *vs* day 4. Scale bar = 100 μm.

significant reduction of lymphatic contraction frequency at day 4 in the popliteal afferent pre-nodal lymphatic vessels (Figure 5B) in DSS-treated mice, which further decreased at day 7, which correlated to increased disease severity (Figure 1). In addition, there were significantly decreased lymphatic contraction frequencies at day 4 and 7 in the popliteal post-nodal efferent lymphatic vessel of DSS-treated mice as compared to the baseline data (Figure 5C). On the other hand, both afferent and efferent lymphatic contractility in control mice remained unchanged from baseline to day 7.

## DISCUSSION

The purpose of this study was to characterize changes in lymphatic function and architecture in a chemically-induced murine model of colitis. We utilized *in situ* lymphangiography and non-invasive NIRF imaging techniques to investigate dermal lymphatic response during gut inflammation. As DSS-induced colitis progressed (Figure 1), we observed alterations in mesenteric lymphatics (Figure 2), peripheral lymphatic flow (Figure 3), and dermal lymphatic architecture (Figure 4). Additionally, there was a gradual reduction

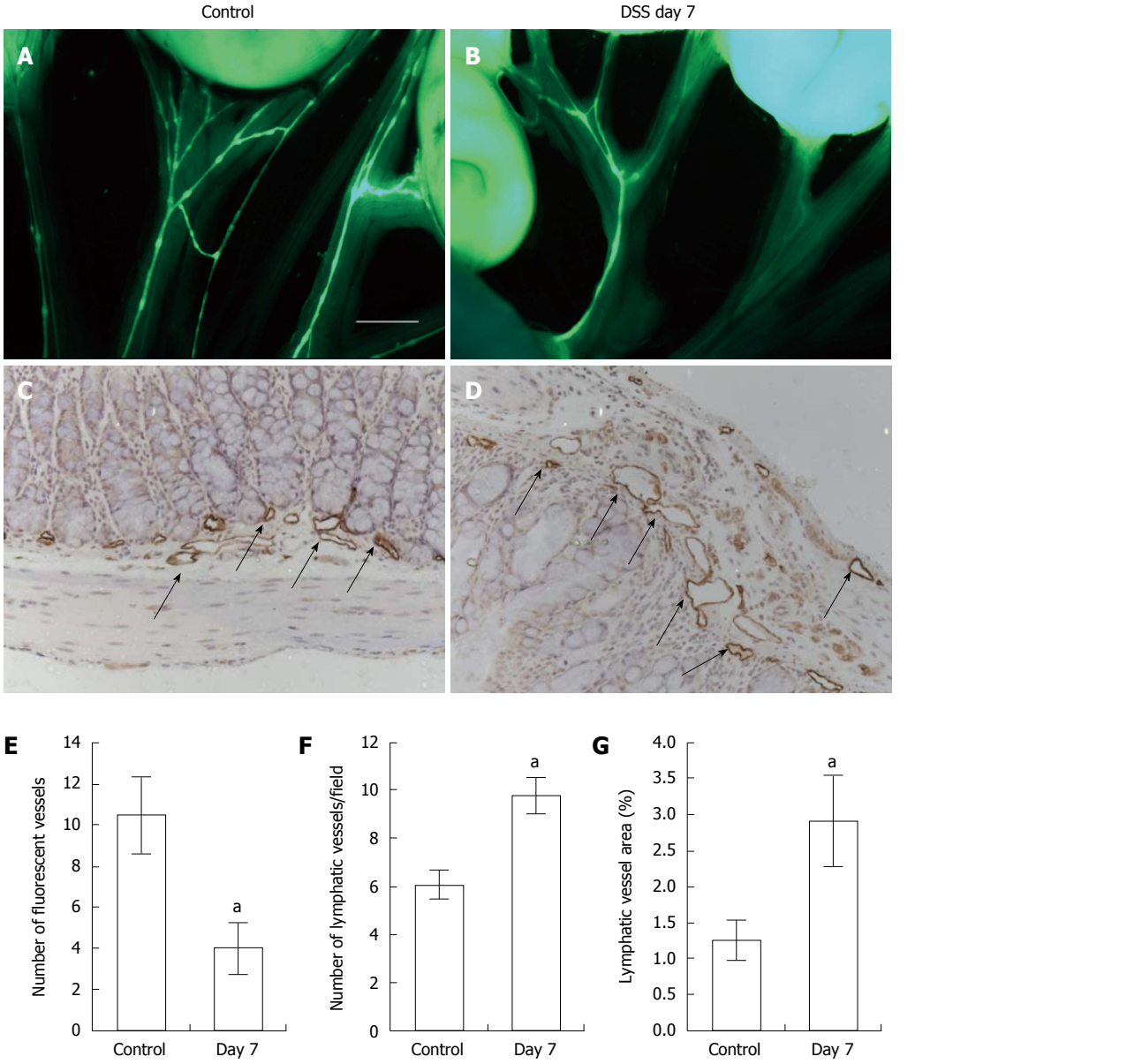



**Figure 2 Intravital fluorescent lymphangiography 30 min after oral gavage administration of Bodipy-FL-C16.** Lymphangiography showing mesenteric lymphatic drainage in control (A, *n* = 4) and mice with dextran sulfate sodium (DSS)-induced colitis (B, *n* = 5) after oral gavage of 1 mL of Bodipy-FL-C16. Significantly reduced number of fluorescent mesenteric lymphatic vessels was observed in mice with DSS-induced colitis as compared to control mice (E). Scale bar = 2 mm. IHC assessment of lymphatic vessels using antibody against LYVE-1 in the colon of control (C) and mice with DSS-induced colitis (D) and quantification of number of lymphatic vessels (F) and vessel area (G) in colons. Data presented as mean ± SE. $^{a}P < 0.05$ control *vs* DSS day 7.

in lymphatic contractility in the skin of DSS-treated mice (Figure 5), suggesting that DSS-induced acute colitis has a significant impact on both local and systemic lymphatic function.

IBD, including UC and CD, is an autoimmune disorder of unknown etiology that mainly involves the intestines[16]. Previous studies showed functional and structural changes in the blood vasculature, such as dilated and tortuous vessels with increased vascularity and changes in intestinal blood flow, in patients with IBD and chemically-induced murine models of colitis[17,18]. In addition to vascular alterations, submucosal edema, possibly due to impaired lymphatic function, has previously been observed in intestinal lymphatics in IBD[6-8]. Increased lymphangiogenesis has also been observed in patients with and experimental models of IBD[6,19] as observed in our study (Figure 1). Previous data demonstrated that mice lacking angiopoietin-2, which exhibit disorganized and hyperplastic lymphatic vasculature, have exaggerated disease activity in the DSS colitis model, indicating that the lymphatic system plays an important role in IBD[20]. Recent studies have also demonstrated that vascular endothelial growth factor receptor (VEGFR)-3 blockade caused inhibition of disease resolution in animal models of colitis and adenoviral induction of VEGF-C provided increased protection against the development of DSS-induced acute and chronic colitis as a result of increased

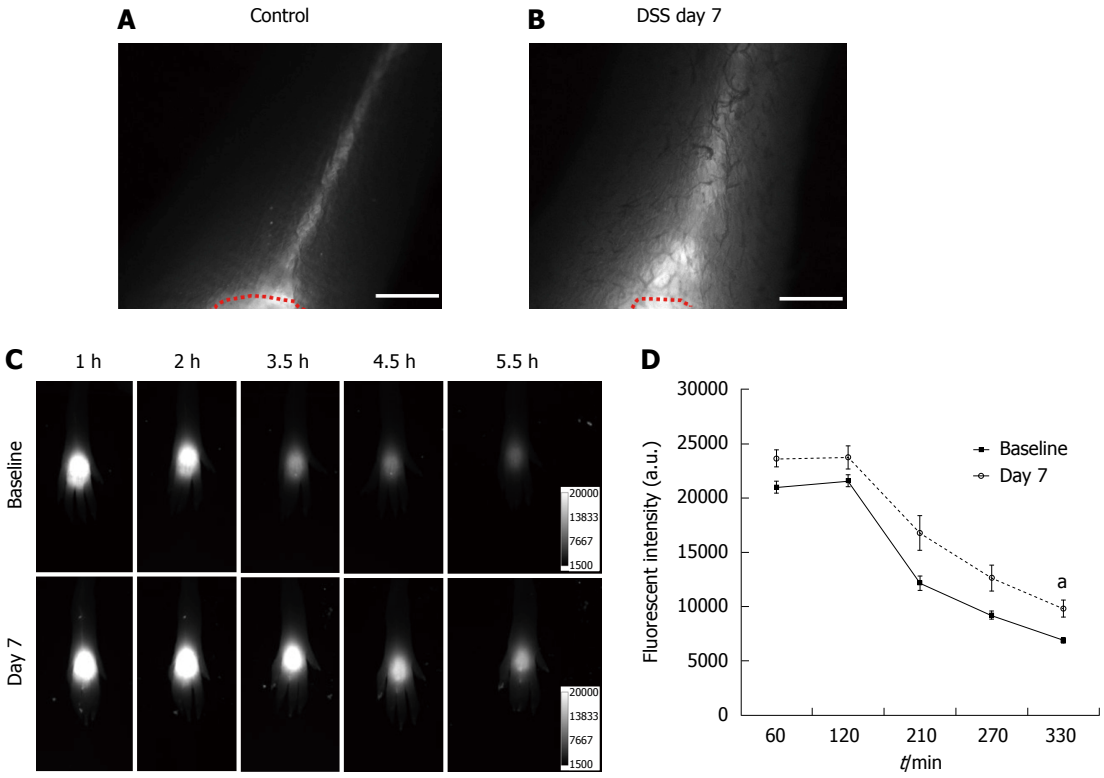



**Figure 3 Near-infrared fluorescence imaging after id injection of indocyanine green in the dorsal aspect of the hind paw.** NIR fluorescent images of the foot in control mice (A) and mice treated with dextran sulfate sodium (DSS) for 7 d (B) after id injection of 2 μL of indocyanine green (ICG) in the dorsal aspect of the foot. A red dotted line delineates the ICG injection area. Representative fluorescent images in the foot of a mouse 1, 2, 3.5, 4.5, and 5.5 h after id injection of 2 μL of Alexa680-BSA prior to (baseline), and 7 d after DSS treatment displayed the clearance of Alexa680-BSA from the depot over time (C). Quantification of the fluorescent intensities (D) remaining in the depot of Alexa680-BSA in the skin of mice treated with DSS for 7 d ($n$ = 7; grey open circle) showed higher fluorescent intensity over time as compared to baseline (filled black square), which was statistically significant at 5.5 h ($P$ = 0.01) in comparison to control mice. [a]$P <$ 0.05. Scale bar = 1 mm.

lymphangiogenesis and lymph flow[21,22], suggesting that stimulation of functional lymphangiogenesis using VEGF-C can provide a novel therapeutic strategy for IBD. It is known that DSS causes damage to the intestinal mucosal barrier, allowing permeability of bacteria and other luminal antigens into the mucosa, thus resulting in gut inflammation[23]. The distal colon is severely damaged with histopathological features including loss of crypts, ulceration/erosion, and edema as well as increased immune cell infiltration in the DSS model[23]. It has also been shown that the small intestine was also damaged in response to DSS[24,25]. Our *in situ* data after oral gavage of Bodipy-FL-C16 showed significantly fewer functional lymphatic vessels were observed in the mesentery of mice with DSS-induced acute colitis (Figure 2B), as detected by fluorescence, when compared to control (Figure 2A), due to DSS-induced disruption of intestinal and lymphatic integrity. A previous study also showed fewer functional lymphatics in TNBS-treated mice than sham animals[5]. Bodipy-FL-C16, a fluorescently-labeled 16-carbon chain fatty acid, has been used as a lipid tracer to invasively study lymphatic architecture and function in mesenteric lymphatics[26-29]. Previous data has demonstrated that mice with DSS-induced colitis had decreased food and water intake and body fat content as well as a disturbance of lipid and energy metabolism as compared to control[30,31]. Therefore, it is likely that normal fat absorption could be impaired as well. Thus, our observed impaired uptake of Bodipy-FL-C16 by mesenteric lymphatics in mice with DSS-induced colitis may also be due to metabolic alterations. A recent report showed significantly increased lymph flow in the acute phase of colitis (*i.e.*, C57BL/6 mice treated with 2.5% DSS for 7 d) as compared to control, by indirectly measuring remaining blue dye in the colon 16 h after an injection of the dye into the colonic mucosa[32]. In our study, we did not measure the extent of lymph flow in the mesenteric lymphatic vessels. Therefore, further studies are needed to assess whether fewer mesenteric lymphatic vessels observed in our study show an increase in lymph flow during DSS-induced acute colitis. Since acute DSS-induced mucosal injury is dependent on not only DSS concentration, but also strain of mouse[23], it is possible that the extent of acute injury due to different DSS concentrations and/or mouse strain may affect uptake of a lipid tracer.

Although the association of EIMs with IBD has been recognized, the pathologic mechanisms of EIMs are largely unknown. Adams *et al*[33] proposed the mechanism by which mucosal T cells are recruited to the liver in response to abnormally expressed endothelial-cell adhesion molecules and chemokines.

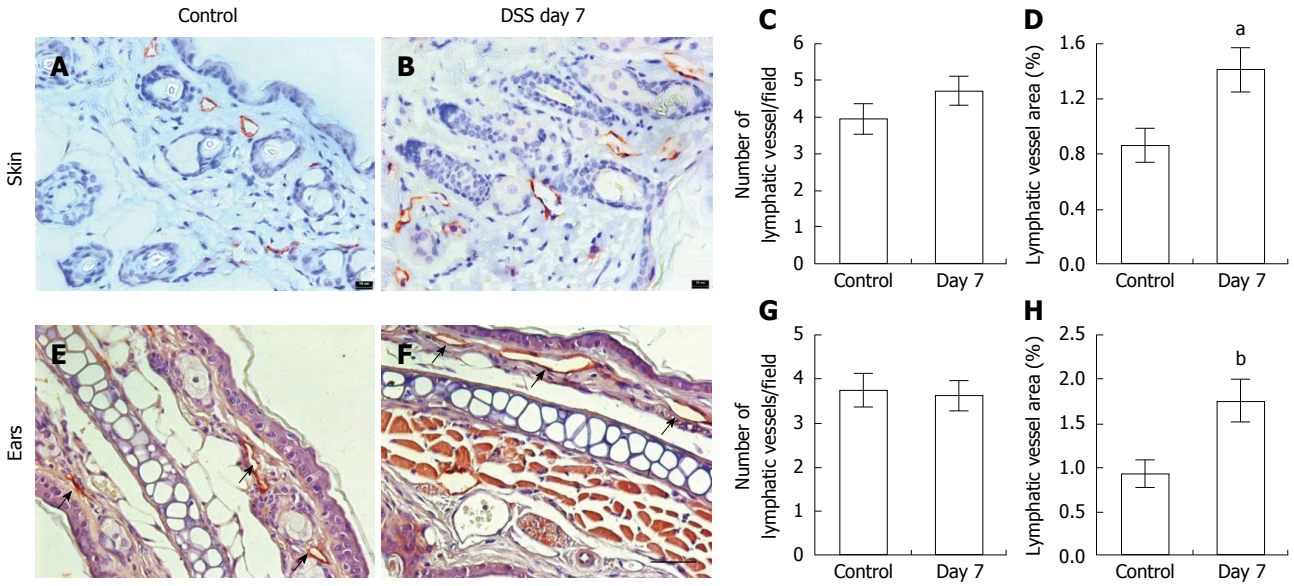



**Figure 4 Immunohistochemical assessment of lymphatic vessels using antibodies to LYVE-1.** In the skin (A) and ears (E, arrows) of control ($n$ = 5 skin; $n$ = 7 ear) and mice with dextran sulfate sodium (DSS)-induced acute colitis (B, F; $n$ = 6 skin; $n$ = 9 ears). Computer-assisted image analysis showed no difference in the number of lymphatic vessels per field (C, G) but increased lymphatic vessel area in both skin and ears (D, H) compared to control mice. Data presented as mean ± SE. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control. Scale bar = 100 μm (C, D).

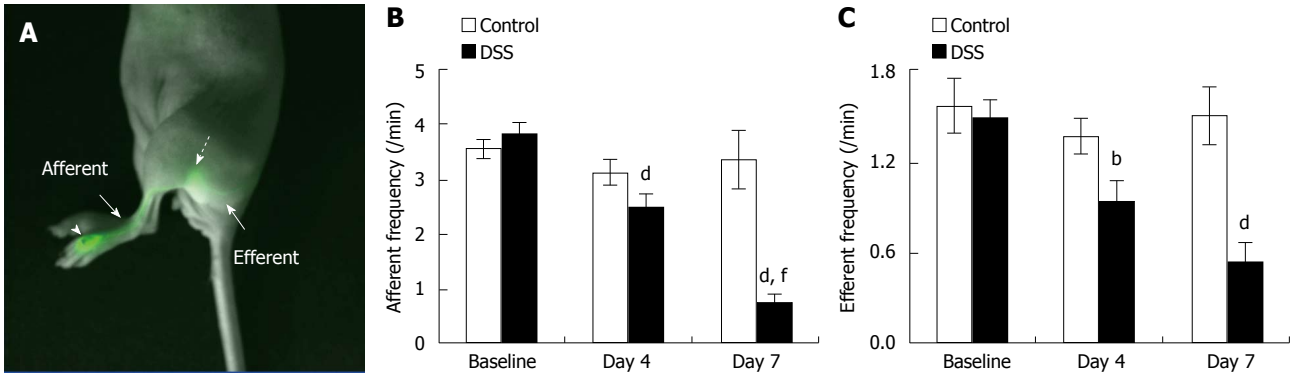



**Figure 5 Lymphatic contractile function in the skin of mice with dextran sulfate sodium-induced acute colitis.** Overlay of fluorescent and white light images showing lymphatic drainage of indocyanine green (ICG) from the foot, where ICG was injected (arrowhead), to the popliteal LN (broken arrow) via the popliteal afferent lymphatic vessel (A). The quantification of lymphatic contractility in the popliteal afferent (B) and efferent (C) lymphatic vessels in the foot of mice prior to 4, and 7 d after DSS alone ($n$ = 21). Data represent mean ± SE. $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* baseline; $^{f}P < 0.001$ *vs* day 4.

Therefore, T cells are exposed to hepatic antigens, and thus liver damage occurs. Aberrant homing of mucosal T cells and excessive secretion of inflammatory cytokines have also been suggested to be responsible for skin and other EIMs of IBD[33]. Inflammatory cytokines, including TNF-α, have an important role in the recruitment of immune cells to the sites of tissue damage[34]. TNF-α has been shown to play an important role in the pathogenesis of IBD[34]. Thus, anti-TNF-α antibodies, such as infliximab, are used to treat IBD as well as skin EIMs, such as erythema nodosum. However, recent studies showed that anti-TNF antibodies, which are also used for the treatment of psoriasis, can cause psoriasiform skin lesions in patients with IBD[35]. It has been demonstrated that IL-17A/IL-22-secreting Th17 cells and interferon (IFN)-γ-secreting Th1 cells are responsible for these lesions and anti-IL-12/IL-23 antibody treatment is an effective therapy for anti-TNF antibody-induced psoriasis[35] IL-23 is known to be one of the major players in the pathogenesis of IBD[34]. IL-23 is highly expressed in pyoderma gangrenosum and treatment with a monoclonal antibody ustekinumab (IL-12/23 IgG1) resulted in clinical resolution of the lesions[36].

Studies have shown that DSS-treatment results in extra-intestinal inflammation[37], thus stimulating inflammation by inducing the secretion of cytokines and inflammatory mediators that are transported to the lymphatic and/or blood circulation[38]. Elevated levels of IL-6, IL-17, TNF-α, and keratinocyte-derived chemokine (KC) have been observed in mice with acute DSS-colitis; however, in chronic DSS colitis after 4 cycles of DSS (3%) for 7 d/cycle and 10 d of normal drinking water in between each cycle, significantly

elevated levels of IL-6, IFN-γ, IL-4, and IL-10 were observed as compared to control mice[38]. We found significantly increased levels of both IL-6 (control *vs* DSS, 12.37 ± 8.73 *vs* 34.77 ± 9.25 in arbitrary unit, $P = 0.035$) and TNF-α (control *vs* DSS, 0.87 ± 0.61 *vs* 24.92 ± 5.71, $P = 0.006$) in skin of DSS-treated mice compared to control mice. We have previously shown that locally administered pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 inhibit systemic lymphatic function[39].

Preclinical and clinical studies showed that NO may be involved in GI inflammation and play a pathologic role in IBD[40]. NO has been implicated as a mediator of tissue injury in the DSS-induced colitis model[40,41]. Significantly increased NO production from inducible NO synthase (iNOS) was observed in the circulation and other systemic organs in mice with DSS-induced acute inflammation[40-43]. We observed a significant increase in serum $NO_x$ levels in mice (19 ± 1.9 μmol/L) with DSS-induced colitis as compared to control mice (12.8 ± 1.2 μmol/L, $P < 0.05$). However, it has also been reported that serum $NO_x$ concentration was significantly increased in iNOS knockout ($iNOS^{-/-}$) mice over a 7 d DSS exposure as compared to untreated $iNOS^{-/-}$ mice, suggesting that other NOS isoforms can also generate $NO_x$[44]. NO has an inhibitory effect on lymphatic pump function under physiological or pathological conditions[29,45,46], although lymphatic responses to NOS inhibition using pharmacological agents differ depending on experimental conditions[47-51]. Liao *et al*[46] showed that under normal conditions endothelial NOS (eNOS) produces NO in lymphatic endothelial cells that maintains lymphatic contractions; however, at the peak of oxazolone-induced skin inflammation on day 4, increased NO production by iNOS expressing $CD11b^+Gr1^+$ cells overwhelms the eNOS-produced NO to inhibit lymphatic contractility. They also found that $iNOS^{-/-}$ mice with oxazolone-induced skin inflammation did not change lymphatic contractility at 4 d after inflammation[46]. Therefore, NO, together with pro-inflammatory cytokines, may affect dermal lymphatic vessel function in DSS-induced acute colitis.

Previous studies demonstrated that Balb/c mice treated with DSS for 5-7 d developed acute colitis as observed in our studies; however, Balb/c mice completely recovered 4 weeks after DSS removal as evidenced by histopathology and cytokine levels[52]. Our preliminary data in mice treated with 2% DSS for 7 d, followed by 7 d of water, showed significantly decreased lymphatic contractility 7 d after DSS treatment as compared to baseline (Baseline *vs* Day 7, 4.2 ± 1.5 *vs* 1.6 ± 0.4, $P < 0.001$); however, we observed recovery of lymphatic contractile function in the popliteal afferent lymphatic vessels at day 14, although it was not significantly different from that on Day 7 (Day 7 *vs* Day 14, 1.6 ± 0.4 *vs* 2.6 ± 0.5, $P = 0.154$). Mice regained their body weight 7 d after DSS removal (changes in body weight; baseline *vs* day 7 *vs* day 14, 100% *vs* 95% ± 1.5% *vs* 100.4% ± 1.3%), indicating that the process of recovery of lymphatic function from acute colitis was underway. Additional work is required to determine whether chronic gut inflammation or other chemically-induced acute models of intestinal inflammation such as TNBS-, oxazolone-mediated colitis, or genetically-modified models of colitis, lead to systemically impaired lymphatic function and/or architecture as assessed by NIRF imaging, and to investigate the effects of dissimilar cytokine/chemokine profiles that have been observed in different animal models[38]. Indeed, a previous study demonstrated distinct cytokine profiles even between acute and chronic DSS colitis[38]. Therefore, this information would provide additional insights in understanding the complex nature of IBD and its EIMs and how alterations to the lymphatics play a role in the pathogenesis of this disease, thus leading toward better disease management.

In conclusion, we have shown that acute inflammation induced by DSS in mice is associated with changes of dermal lymphatic architecture and function. The NIRF imaging technique employed in this study can be used to image altered lymphatic function and architecture in response to other types of systemic diseases with cutaneous involvement and likely assess lymphatic responses during therapy.

## ACKNOWLEDGMENTS

The authors would like to thank Holly Robinson and Gabriel Dickinson for technical assistance with the mice used in these experiments; and Drs. Melissa B. Aldrich and Eva M. Sevick-Muraca for scientific review of this manuscript.

## COMMENTS

### Background

Inflammatory bowel disease (IBD) and extra-intestinal manifestations are characterized by an influx of inflammatory cells which secrete pro-inflammatory cytokines. The lymphatic system is an active and integral part of the immune system as well as a mediator of fluid return to the blood vasculature. Recent data suggests that the lymphatic system plays an important role in the pathogenesis of IBD and extra-intestinal manifestations. However, it is unknown whether gut inflammation systemically affects the lymphatics.

### Research frontiers

Abnormal lymphatic function and architecture are implicated in a number of pathological conditions including edema, obesity, and immune dysfunction. Lymphatic biology has become a hot research topic and the use of near-infrared fluorescence (NIRF) imaging to investigate the role of lymphatics *in vivo* in health and disease allows the interrogation of lymphatic dysfunction in animal and man.

### Innovations and breakthroughs

This is the first study investigating dermal lymphatic function and anatomy in mice with dextran sulfate sodium (DSS)-induced acute colitis. Current data demonstrates that DSS-induced colitis systemically affects the lymphatics, inducing dilation of lymphatic vessels and reducing lymphatic contractile

function in peripheral skin tissues.

### Applications

The authors' observations have implications for the pathophysiology of extra-intestinal manifestations in IBD. They demonstrate that DSS-induced colitis is accompanied by impaired lymphatic function and drainage, both locally and systemically. Noninvasive functional lymphatic imaging can provide early diagnosis, risk stratification, and monitoring of lymphatic response to intervention with the appropriate therapy.

### Terminology

The lymphatics consist of two major types of vessels, the initial lymphatics and the contractile, collecting lymphatics. The collecting lymphatic vessels have a unique, dynamic contractile characteristic critical to lymph flow. The collecting lymphatics have functional units, called lymphangions, which are spontaneous contractile subunits bound by "secondary valves" which prevent backflow and ensure unidirectional flow. Fluid accumulates in lymphangions before being propelled to the next sequential lymphangion, which is generated by the coordinated contractions of lymphatic vascular smooth muscle cells.

### Peer-review

Authors evaluate whether dermal lymphatic function and architecture are systemically altered in DSS-induced acute colitis. The authors demonstrate that lymphatics are locally and systemically altered in acute colitis by the use of NIRF lymphatic imaging. The study is interesting and well structured.

## REFERENCES


1 **Bouma G**, Strober W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* 2003; **3**: 521-533 [PMID: 12876555 DOI: 10.1038/nri1132]

2 **Danese S**, Semeraro S, Papa A, Roberto I, Scaldaferri F, Fedeli G, Gasbarrini G, Gasbarrini A. Extraintestinal manifestations in inflammatory bowel disease. *World J Gastroenterol* 2005; **11**: 7227-7236 [PMID: 16437620 DOI: 10.3748/wjg.v11.i46.7227]

3 **Huang BL**, Chandra S, Shih DQ. Skin manifestations of inflammatory bowel disease. *Front Physiol* 2012; **3**: 13 [PMID: 22347192 DOI: 10.3389/fphys.2012.00013]

4 **Trost LB**, McDonnell JK. Important cutaneous manifestations of inflammatory bowel disease. *Postgrad Med J* 2005; **81**: 580-585 [PMID: 16143688 DOI: 10.1136/pgmj.2004.031633]

5 **Wu TF**, Carati CJ, Macnaughton WK, von der Weid PY. Contractile activity of lymphatic vessels is altered in the TNBS model of guinea pig ileitis. *Am J Physiol Gastrointest Liver Physiol* 2006; **291**: G566-G574 [PMID: 16675748 DOI: 10.1152/ajpgi.00058.2006]

6 **Alexander JS**, Chaitanya GV, Grisham MB, Boktor M. Emerging roles of lymphatics in inflammatory bowel disease. *Ann N Y Acad Sci* 2010; **1207** Suppl 1: E75-E85 [PMID: 20961310 DOI: 10.1111/j.1749-6632.2010.05757.x]

7 **Heatley RV**, Bolton PM, Hughes LE, Owen EW. Mesenteric lymphatic obstruction in Crohn's disease. *Digestion* 1980; **20**: 307-313 [PMID: 7390055]

8 **Kovi J**, Duong HD, Hoang CT. Ultrastructure of intestinal lymphatics in Crohn's disease. *Am J Clin Pathol* 1981; **76**: 385-394 [PMID: 6117198]

9 **Olszewski WL**. The lymphatic system in body homeostasis: physiological conditions. *Lymphat Res Biol* 2003; **1**: 11-21; discussion 21-24 [PMID: 15624317]

10 **Angeli V**, Randolph GJ. Inflammation, lymphatic function, and dendritic cell migration. *Lymphat Res Biol* 2006; **4**: 217-228 [PMID: 17394405 DOI: 10.1089/lrb.2006.4406]

11 **Kwon S**, Sevick-Muraca EM. Noninvasive quantitative imaging of lymph function in mice. *Lymphat Res Biol* 2007; **5**: 219-231 [PMID: 18370912]

12 **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]

13 **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]

14 **Kwon S**, Sevick-Muraca EM. Functional lymphatic imaging in tumor-bearing mice. *J Immunol Methods* 2010; **360**: 167-172 [PMID: 20600076]

15 **Dadras SS**, Lange-Asschenfeldt B, Velasco P, Nguyen L, Vora A, Muzikansky A, Jahnke K, Hauschild A, Hirakawa S, Mihm MC, Detmar M. Tumor lymphangiogenesis predicts melanoma metastasis to sentinel lymph nodes. *Mod Pathol* 2005; **18**: 1232-1242 [PMID: 15803182]

16 **Kaser A**, Zeissig S, Blumberg RS. Inflammatory bowel disease. *Annu Rev Immunol* 2010; **28**: 573-621 [PMID: 20192811 DOI: 10.1146/annurev-immunol-030409-101225]

17 **Danese S**. Role of the vascular and lymphatic endothelium in the pathogenesis of inflammatory bowel disease: 'brothers in arms'. *Gut* 2011; **60**: 998-1008 [PMID: 21212253 DOI: 10.1136/gut.2010.207480]

18 **Deban L**, Correale C, Vetrano S, Malesci A, Danese S. Multiple pathogenic roles of microvasculature in inflammatory bowel disease: a Jack of all trades. *Am J Pathol* 2008; **172**: 1457-1466 [PMID: 18458096 DOI: 10.2353/ajpath.2008.070593]

19 **von der Weid PY**, Rehal S, Ferraz JG. Role of the lymphatic system in the pathogenesis of Crohn's disease. *Curr Opin Gastroenterol* 2011; **27**: 335-341 [PMID: 21543977 DOI: 10.1097/MOG.0b013e3283476e8f]

20 **Ganta VC**, Cromer W, Mills GL, Traylor J, Jennings M, Daley S, Clark B, Mathis JM, Bernas M, Boktor M, Jordan P, Witte M, Alexander JS. Angiopoietin-2 in experimental colitis. *Inflamm Bowel Dis* 2010; **16**: 1029-1039 [PMID: 19902545 DOI: 10.1002/ibd.21150]

21 **D'Alessio S**, Tacconi C, Fiocchi C, Danese S. Advances in therapeutic interventions targeting the vascular and lymphatic endothelium in inflammatory bowel disease. *Curr Opin Gastroenterol* 2013; **29**: 608-613 [PMID: 24100721 DOI: 10.1097/MOG.0b013e328365d37c]

22 **Jurisic G**, Sundberg JP, Detmar M. Blockade of VEGF receptor-3 aggravates inflammatory bowel disease and lymphatic vessel enlargement. *Inflamm Bowel Dis* 2013; **19**: 1983-1989 [PMID: 23835443 DOI: 10.1097/MIB.0b013e31829292f7]

23 **Perše M**, Cerar A. Dextran sodium sulphate colitis mouse model: traps and tricks. *J Biomed Biotechnol* 2012; **2012**: 718617 [PMID: 22665990 DOI: 10.1155/2012/718617]

24 **Takahashi S**, Kawamura T, Kanda Y, Taniguchi T, Nishizawa T, Iiai T, Hatakeyama K, Abo T. Multipotential acceptance of Peyer's patches in the intestine for both thymus-derived T cells and extrathymic T cells in mice. *Immunol Cell Biol* 2005; **83**: 504-510 [PMID: 16174100 DOI: 10.1111/j.1440-1711.2005.01361.x]

25 **Geier MS**, Smith CL, Butler RN, Howarth GS. Small-intestinal manifestations of dextran sulfate sodium consumption in rats and assessment of the effects of Lactobacillus fermentum BR11. *Dig Dis Sci* 2009; **54**: 1222-1228 [PMID: 19005763 DOI: 10.1007/s10620-008-0495-4]

26 **Kassis T**, Kohan AB, Weiler MJ, Nipper ME, Cornelius R, Tso P, Dixon JB. Dual-channel in-situ optical imaging system for quantifying lipid uptake and lymphatic pump function. *J Biomed Opt* 2012; **17**: 086005 [PMID: 23224192 DOI: 10.1117/1.JBO.17.8.086005]

27 **Harvey NL**, Srinivasan RS, Dillard ME, Johnson NC, Witte MH, Boyd K, Sleeman MW, Oliver G. Lymphatic vascular defects promoted by Prox1 haploinsufficiency cause adult-onset obesity. *Nat Genet* 2005; **37**: 1072-1081 [PMID: 16170315]

28 **Jeon BH**, Jang C, Han J, Kataru RP, Piao L, Jung K, Cha HJ, Schwendener RA, Jang KY, Kim KS, Alitalo K, Koh GY. Profound but dysfunctional lymphangiogenesis via vascular endothelial growth factor ligands from CD11b+ macrophages in advanced ovarian cancer. *Cancer Res* 2008; **68**: 1100-1109 [PMID: 18281485 DOI: 10.1158/0008-5472.CAN-07-2572]

29 **Ribera J**, Pauta M, Melgar-Lesmes P, Tugues S, Fernández-Varo

G, Held KF, Soria G, Tudela R, Planas AM, Fernández-Hernando C, Arroyo V, Jiménez W, Morales-Ruiz M. Increased nitric oxide production in lymphatic endothelial cells causes impairment of lymphatic drainage in cirrhotic rats. *Gut* 2013; **62**: 138-145 [PMID: 22267600 DOI: 10.1136/gutjnl-2011-300703]

30 **Dong F**, Zhang L, Hao F, Tang H, Wang Y. Systemic responses of mice to dextran sulfate sodium-induced acute ulcerative colitis using 1H NMR spectroscopy. *J Proteome Res* 2013; **12**: 2958-2966 [PMID: 23651354 DOI: 10.1021/pr4002383]

31 **Melgar S**, Bjursell M, Gerdin AK, Svensson L, Michaëlsson E, Bohlooly-Y M. Mice with experimental colitis show an altered metabolism with decreased metabolic rate. *Am J Physiol Gastrointest Liver Physiol* 2007; **292**: G165-G172 [PMID: 16844678 DOI: 10.1152/ajpgi.00152.2006]

32 **D'Alessio S**, Correale C, Tacconi C, Gandelli A, Pietrogrande G, Vetrano S, Genua M, Arena V, Spinelli A, Peyrin-Biroulet L, Fiocchi C, Danese S. VEGF-C-dependent stimulation of lymphatic function ameliorates experimental inflammatory bowel disease. *J Clin Invest* 2014; **124**: 3863-3878 [PMID: 25105363 DOI: 10.1172/JCI72189]

33 **Adams DH**, Eksteen B. Aberrant homing of mucosal T cells and extra-intestinal manifestations of inflammatory bowel disease. *Nat Rev Immunol* 2006; **6**: 244-251 [PMID: 16498453 DOI: 10.1038/nri1784]

34 **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]

35 **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]

36 **Guenova E**, Teske A, Fehrenbacher B, Hoerber S, Adamczyk A, Schaller M, Hoetzenecker W, Biedermann T. Interleukin 23 expression in pyoderma gangrenosum and targeted therapy with ustekinumab. *Arch Dermatol* 2011; **147**: 1203-1205 [PMID: 21680759 DOI: 10.1001/archdermatol.2011.168]

37 **Kitajima S**, Takuma S, Morimoto M. Tissue distribution of dextran sulfate sodium (DSS) in the acute phase of murine DSS-induced colitis. *J Vet Med Sci* 1999; **61**: 67-70 [PMID: 10027168]

38 **Alex P**, Zachos NC, Nguyen T, Gonzales L, Chen TE, Conklin LS, Centola M, Li X. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. *Inflamm Bowel Dis* 2009; **15**: 341-352 [PMID: 18942757 DOI: 10.1002/ibd.20753]

39 **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]

40 **Kolios G**, Valatas V, Ward SG. Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. *Immunology* 2004; **113**: 427-437 [PMID: 15554920 DOI: 10.1111/j.1365-2567.2004.01984.x]

41 **Cross RK**, Wilson KT. Nitric oxide in inflammatory bowel disease. *Inflamm Bowel Dis* 2003; **9**: 179-189 [PMID: 12792224]

42 **Saijo F**, Milsom AB, Bryan NS, Bauer SM, Vowinkel T, Ivanovic M, Andry C, Granger DN, Rodriguez J, Feelisch M. On the dynamics of nitrite, nitrate and other biomarkers of nitric oxide production in inflammatory bowel disease. *Nitric Oxide* 2010; **22**: 155-167 [PMID: 20005300 DOI: 10.1016/j.niox.2009.11.009]

43 **Kubes P**. Inducible nitric oxide synthase: a little bit of good in all of us. *Gut* 2000; **47**: 6-9 [PMID: 10861252]

44 **Beck PL**, Xavier R, Wong J, Ezedi I, Mashimo H, Mizoguchi A, Mizoguchi E, Bhan AK, Podolsky DK. Paradoxical roles of different nitric oxide synthase isoforms in colonic injury. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G137-G147 [PMID: 14665440 DOI: 10.1152/ajpgi.00309.2003]

45 **Shirasawa Y**, Ikomi F, Ohhashi T. Physiological roles of endogenous nitric oxide in lymphatic pump activity of rat mesentery in vivo. *Am J Physiol Gastrointest Liver Physiol* 2000; **278**: G551-G556 [PMID: 10762608]

46 **Liao S**, Cheng G, Conner DA, Huang Y, Kucherlapati RS, Munn LL, Ruddle NH, Jain RK, Fukumura D, Padera TP. Impaired lymphatic contraction associated with immunosuppression. *Proc Natl Acad Sci USA* 2011; **108**: 18784-18789 [PMID: 22065738 DOI: 10.1073/pnas.1116152108]

47 **Gasheva OY**, Zawieja DC, Gashev AA. Contraction-initiated NO-dependent lymphatic relaxation: a self-regulatory mechanism in rat thoracic duct. *J Physiol* 2006; **575**: 821-832 [PMID: 16809357]

48 **Koller A**, Mizuno R, Kaley G. Flow reduces the amplitude and increases the frequency of lymphatic vasomotion: role of endothelial prostanoids. *Am J Physiol* 1999; **277**: R1683-R1689 [PMID: 10600914]

49 **Mizuno R**, Dörnyei G, Koller A, Kaley G. Myogenic responses of isolated lymphatics: modulation by endothelium. *Microcirculation* 1997; **4**: 413-420 [PMID: 9431509]

50 **Mizuno R**, Koller A, Kaley G. Regulation of the vasomotor activity of lymph microvessels by nitric oxide and prostaglandins. *Am J Physiol* 1998; **274**: R790-R796 [PMID: 9530247]

51 **Bohlen HG**, Wang W, Gashev A, Gasheva O, Zawieja D. Phasic contractions of rat mesenteric lymphatics increase basal and phasic nitric oxide generation in vivo. *Am J Physiol Heart Circ Physiol* 2009; **297**: H1319-H1328 [PMID: 19666850 DOI: 10.1152/ajpheart.00039.2009]

52 **Melgar S**, Karlsson A, Michaëlsson E. Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation. *Am J Physiol Gastrointest Liver Physiol* 2005; **288**: G1328-G1338 [PMID: 15637179 DOI: 10.1152/ajpgi.00467.2004]

**P- Reviewer**: De Minicis S, Hayashi S **S- Editor**: Yu J
**L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12778

*World J Gastroenterol* 2015 December 7; 21(45): 12778-12786
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

Basic Study

# New drug delivery system for liver sinusoidal endothelial cells for ischemia-reperfusion injury

Naoki Sano, Takafumi Tamura, Naoyuki Toriyabe, Takeshi Nowatari, Ken Nakayama, Tomohito Tanoi, Soichiro Murata, Yu Sakurai, Mamoru Hyodo, Kiyoshi Fukunaga, Hideyoshi Harashima, Nobuhiro Ohkohchi

Naoki Sano, Takafumi Tamura, Takeshi Nowatari, Ken Nakayama, Tomohito Tanoi, Soichiro Murata, Kiyoshi Fukunaga, Nobuhiro Ohkohchi, Department of Surgery, Doctoral Program in Clinical Science, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba 305-8575, Japan

Naoyuki Toriyabe, Yu Sakurai, Mamoru Hyodo, Hideyoshi Harashima, Faculty of Pharmaceutical Sciences, Hokkaido University, Hokkaido 060-0812, Japan

Author contributions: Sano N performed the majority of experiments and analyzed the data; Toriyabe N, Sakurai Y and Hyodo M created the reagnt; Tamura T, Harashima H and Ohkohchi N designed and coordinated the research; Sano N, Tamura T, Toriyabe N, Nowatari T, Nakayama K, Tanoi T, Murata S, Sakurai Y, Hyodo M, Fukunaga K, Harashima H and Ohkohchi N wrote the paper.

Supported by Ministry of Education, Culture, Sports, Science, and Technology of Japan, KAKENHI, No. 23390319.

Institutional review board statement: Animal experiments were performed in accordance with the university's Regulations for Animal Experiments and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions, under the jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

Institutional animal care and use committee statement: Animal experiments were performed in a humane manner after receiving approval from the Institutional University Experiment Committee of the University of Tsukuba.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: No additional data are available.



Correspondence to: Nobuhiro Ohkohchi, MD, PhD, Professor, Department of Surgery, Doctoral Program in Clinical Science, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan. nokochi3@md.tsukuba.ac.jp
Telephone: +81-29-8533221
Fax: +81-29-8533222

Received: June 16, 2015
Peer-review started: June 19, 2015
First decision: July 10, 2015
Revised: July 22, 2015
Accepted: September 15, 2015
Article in press: September 15, 2015
Published online: December 7, 2015

## Abstract

**AIM:** To investigate the cytoprotective effects in hepatic ischemia-reperfusion injury, we developed a new formulation of hyaluronic acid (HA) and sphingosine 1-phophate.

**METHODS:** We divided Sprague-Dawley rats into 4 groups: control, HA, sphingosine 1-phosphate (S1P), and HA-S1P. After the administration of each agent, we subjected the rat livers to total ischemia followed by reperfusion. After reperfusion, we performed the following investigations: alanine aminotransferase (ALT), histological findings, TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining, and transmission electron microscopy (TEM). We also investigated the expression

of proteins associated with apoptosis, hepatoprotection, and S1P accumulation.

**RESULTS:** S1P accumulated in the HA-S1P group livers more than S1P group livers. Serum ALT levels, TUNEL-positive hepatocytes, and expression of cleaved caspase-3 expression, were significantly decreased in the HA-S1P group. TEM revealed that the liver sinusoidal endothelial cell (LSEC) lining was preserved in the HA-S1P group. Moreover, the HA-S1P group showed a greater increase in the HO-1 protein levels compared to the S1P group.

**CONCLUSION:** Our results suggest that HA-S1P exhibits cytoprotective effects in the liver through the inhibition of LSEC apoptosis. HA-S1P is an effective agent for hepatic ischemia/reperfusion injury.

**Key words:** Hyaluronic acid; Sphingosine 1-phosphate; Liver sinusoidal endothelial cell; Drug delivery system; Heme oxygenase-1; Stabilin-2



**Core tip:** We have developed a new formulation by directly combining hyaluronic acid and sphingosine 1-phosphate (HA-S1P), which targets liver sinusoidal endothelial cell (LSEC) by binding specifically to HA and HA receptors. This study demonstrated that HA-S1P protects the liver from hepatic ischemia/reperfusion (I/R) injury in rats. The strong protective effect of HA-S1P may be mediated by the anti-apoptotic effect of S1P on LSECs. These data indicate that HA-S1P is an effective agent for preventing hepatic I/R injury.

Sano N, Tamura T, Toriyabe N, Nowatari T, Nakayama K, Tanoi T, Murata S, Sakurai Y, Hyodo M, Fukunaga K, Harashima H, Ohkohchi N. New drug delivery system for liver sinusoidal endothelial cells for ischemia-reperfusion injury. *World J Gastroenterol* 2015; 21(45): 12778-12786 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12778.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12778

## INTRODUCTION

Hepatic ischemia/reperfusion (I/R) injury is a major problem in liver transplantation and liver resection[1]. A critical event during hepatic I/R injury is the death of liver sinusoidal endothelial cells (LSECs), which occurs within a few minutes of reperfusion and precedes hepatocyte death by several hours[2]. The apoptosis of LSECs is a pivotal mechanism of hepatic I/R[3]. Thus, apoptosis plays an important role in hepatic I/R injury and LSEC death[4] and therefore LSECs are the primary treatment target for hepatic I/R injury[5].

Sphingosine-1-phosphate (S1P) is a bioactive lipid that regulates diverse cellular functions, including proliferation, differentiation, migration and survival through interaction with different G protein-coupled S1P receptors[6,7]. S1P exhibits anti-apoptotic effects on human LSECs[8] and protects rat LSECs from ethanol-induced apoptosis[9]. In addition S1P also reduces hepatic I/R-induced acute kidney injury through the attenuation of endothelial injury in mice[10]. However, accumulating S1P (as a single-agent) on the LSECs is difficult, as S1P receptors are widely expressed in various different tissues, such as brain, heart, lung, spleen, kidney, intestine, testis, and liver[11-13].

In this study, we developed a new drug delivery system (DDS) for targeting the LSEC by combining S1P with HA, to make the formula HA-S1P. Circulating HA in the blood is taken up through receptor-mediated endocytosis into the LSECs[14] because the HA receptors, namely Stabilin-2 (STAB2), are highly and specifically expressed on LSECs. STAB2 is a type Ⅰ transmembrane scavenger receptor that is highly expressed in LSECs and is the major clearance receptor for circulating HA[15-19]. We hypothesized that HA-S1P would accumulate in LSECs of liver *via* the STAB2 receptors. The aim of this study was to investigate whether the newly developed HA-S1P protects livers in the case of hepatic I/R injury.

## MATERIALS AND METHODS

### Materials

S1P was purchased from Sigma Chemical Company (St. Louis, MO, United States) and HA [average molecular weight (MW) 8 kDa] was purchased from Food Chemifa (Tokyo, Japan).

### Synthesis of HA-S1P

A mixture was made of 95.85 μL 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (0.1 g/mL), 57.535 μL N-hydroxysuccinimide (NHS) (0.1 g/mL), HA (2 mg/mL, average MW 8 kDa), and water/DMF/$CHCl_3$ (5/4/1). The mixture was stirred well at 55 ℃ with the addition of 67.378 μL S1P (25 mg/mL), and the reaction was allowed to proceed for 24 h. Dialysis was performed to remove the S1P, EDC, and NHS. Integrations of nuclear magnetic resonance (NMR) were used to confirm the amount of S1P introduced [comparison of peaks appeared at 1.2 ppm (methylene group of S1P) and 2.0 ppm (acetyl group of hyaluronic acid)]. Approximately 13.5%-40% of S1P bound to the carboxylic acid of HA.

### Animal model

Male Sprague-Dawley rats, weighing 200 to 250 g, were obtained from CLEA Japan (CLEA Corporation, Tokyo, Japan). Animal experiments were performed in a humane manner after receiving approval from the Institutional University Experiment Committee of the University of Tsukuba, and in accordance with the university's Regulations for Animal Experiments

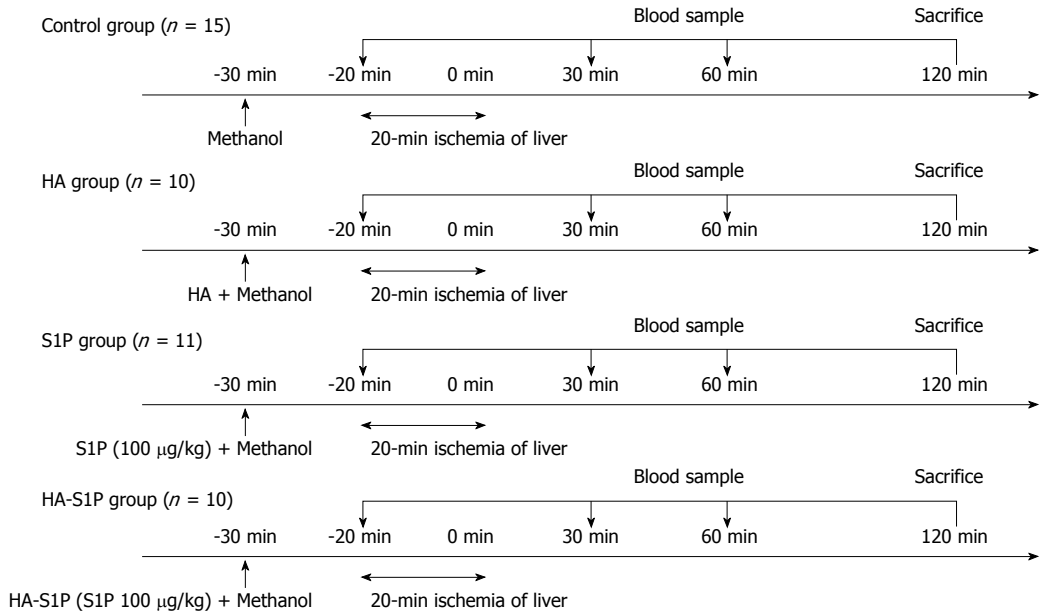



**Figure 1 Experimental groups.** Total hepatic ischemia was established for 20 min by clamping the portal triad in the rat subjects. The rats were divided into 4 groups: (1) control group; (2) HA group; (3) S1P group; and (4) HA-S1P group. Each drug was injected *via* the tail vein 10 min before ischemia. HA: Hyaluronic acid; S1P: Sphingosine 1-phosphate.

and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions, under the jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

### *Experimental groups*

Total warm hepatic ischemia was induced in the rats for 20 min by clamping the portal triad. The rats were divided into 4 groups as follows: (1) methanol injection group (control group; $n$ = 15); (2) HA injection group (HA group; $n$ = 10); (3) single-agent S1P injection group (S1P group; $n$ = 11); and (4) HA-S1P injection group (HA-S1P group; $n$ = 10) (Figure 1). Methanol, S1P, HA, or HA-S1P were injected intravenously *via* the tail vein of the rats.

### *Surgical procedure*

The rats were anesthetized with intraperitoneal injections of sodium pentobarbital (50 mg/kg) and maintained by machine-regulated isoflurane. The rats were placed in a supine position on a heated pad to maintain a rectal temperature of 37 ℃. After transverse laparotomy, the ligaments around the liver were dissected in order to clamp the portal triad. Concurrently, the hepatoduodenal ligament was taped in preparation for the subsequent clamping. Hepatic ischemia was induced *via* clamping of the portal triad, *i.e.*, the hepatic artery, portal vein, and bile duct, by means of a microclip (B. Braun Aesculap Japan Co., Ltd, Tokyo, Japan) for 20 min. Surgical procedures were performed under sterile conditions. Blood samples were taken for the analysis of enzyme activities in serum before ischemia and at 30, 60, and 120 min after reperfusion. At the end of the experiments, liver tissue was obtained for histological examination. Finally, the experimental animals were euthanized by total blood collection *via* the abdominal aorta. During the period from drug administration until sacrificed, respiratory and circulatory dynamics of rats was stable. Moreover, all of rats did not die in this experiment.

### *Serum alanine transaminase levels*

To assess damage to the hepatic parenchyma, serum alanine transaminase (ALT) levels were measured using a Dry-Chem 7000 V autoanalyzer (Fujifilm Co, Tokyo, Japan). Blood samples were taken from the tail vein before the induction of ischemia and up to 120 min after reperfusion.

### *Histological analysis*

After 120 min of reperfusion, liver tissues were obtained from each group, fixed with 10% formaldehyde, and embedded in paraffin. Thin sections (4 μm) were prepared and stained with hematoxylin-eosin (HE). Tissue damage was evaluated in randomly selected high-power fields (× 200).

### *Liver apoptosis*

To detect apoptotic cells in liver tissue, a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (*In situ* Apoptosis Detection Kit; Takara Bio Inc., Otsu, Japan) was performed after 120 min of reperfusion. The ratio of TUNEL-positive/total cells in the microscope field was calculated at × 400.

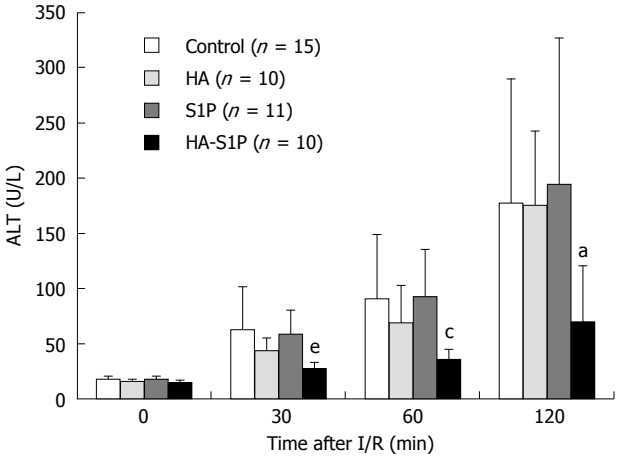



**Figure 2 Serum alanine aminotransferase levels.** Serum ALT levels before ischemia and after reperfusion. Kruskal Wallis *H*-test. $^{a}P < 0.05$ *vs* HA group; $^{c}P < 0.05$ *vs* control and the HA and S1P groups; $^{e}P < 0.05$ *vs* control and S1P groups. ALT: Alanine aminotransferase; HA: Hyaluronic acid; S1P: Sphingosine 1-phosphate; I/R: Ischemia/reperfusion.

### *Protein extraction and Western blot analysis*

Liver tissues were kept at -80 ℃ and were homogenized in a buffer consisting of 150 mmol/L NaCl, 50 mmol/L TrisCl, 1% NP-40, and proteinase inhibitors. The samples were centrifuged, and the supernatants collected for analysis. The samples were separated on 10% and 15% SDS-PAGE gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA, United States). Anti-cleaved caspase-3 (9661), anti-heme oxygenase 1 (HO-1) (5141) (Cell Signaling Technology, Beverly, MA, United States) and anti-S1P antibodies (140592) (Abcam Ltd., Cambridge, United Kingdom) were used as primary antibodies. A secondary goat anti-rabbit antibody conjugated to horseradish peroxidase was purchased from Zymed Laboratories (San Francisco, CA, United States).

### *Transmission electron microscopy*

To assess the LSECs after hepatic I/R, we investigated the samples using transmission electron microscopy (TEM). After 120 min of reperfusion, the livers were resected. Tissue samples from the left hepatic lobe were cut into 1 $mm^3$ cubes and stored in 2.5% glutaraldehyde. The specimens were postfixed with osmium tetroxide, dehydrated in graded alcohol series and embedded in Epon mixture. Ultrathin sections were prepared using an Ultracut S microtome (Leica Aktiengesellschaft, Vienna, Austria) and placed on copper grids. Sections were treated using uranyl acetate and lead citrate to enhance contrast. Specimens were examined using a Hitachi H-7000 transmission electron microscope (Hitachi Co., Tokyo, Japan).

### *Statistical analysis*

All data are expressed as the mean ± SD. The Mann-Whitney *U*-test and Kruskal-Wallis *H*-test were used for statistical analysis, followed by the Mann-Whitney *U*-test with Bonferroni correction. *P* values of $< 0.05$ were considered statistically significant.

## RESULTS

### *Serum ALT levels*

In the control, HA, and S1P groups, serum ALT (which reflects the degree of hepatic parenchymal injury) immediately increased after reperfusion. However, in the HA-S1P group serum ALT levels were unchanged up to 120 min (Figure 2).

### *Histological findings*

The light microscopy findings from the liver tissue, stained with HE, are shown in Figure 3A. In the control, HA and S1P groups, vacuolation of the hepatocytes and sinusoidal narrowing were observed after 120 min of reperfusion. These findings were more severely localized near the portal vein. On the other hand, no histological alteration was observed in the HA-S1P group.

### *Liver apoptosis and western blot analysis*

In the control, HA, and S1P groups, TUNEL-positive cells were observed after 120 min of reperfusion near the portal vein. However, TUNEL-positive cells were not observed in the HA-S1P group (Figure 3B). The ratio of TUNEL-positive to total hepatocytes was significantly lower in the HA-S1P group (Figure 3C). Western blot analysis of the liver tissue is presented in Figure 4. The expression of cleaved caspase-3 in the control, HA, and S1P groups was extremely higher than that in the HA-S1P group (Figure 4A). The expression of the HO-1 (a cytoprotective enzyme against hepatic I/R injury) in the HA-S1P group was higher than that in the control, HA, and S1P groups (Figure 4B).

### *Drug delivery system*

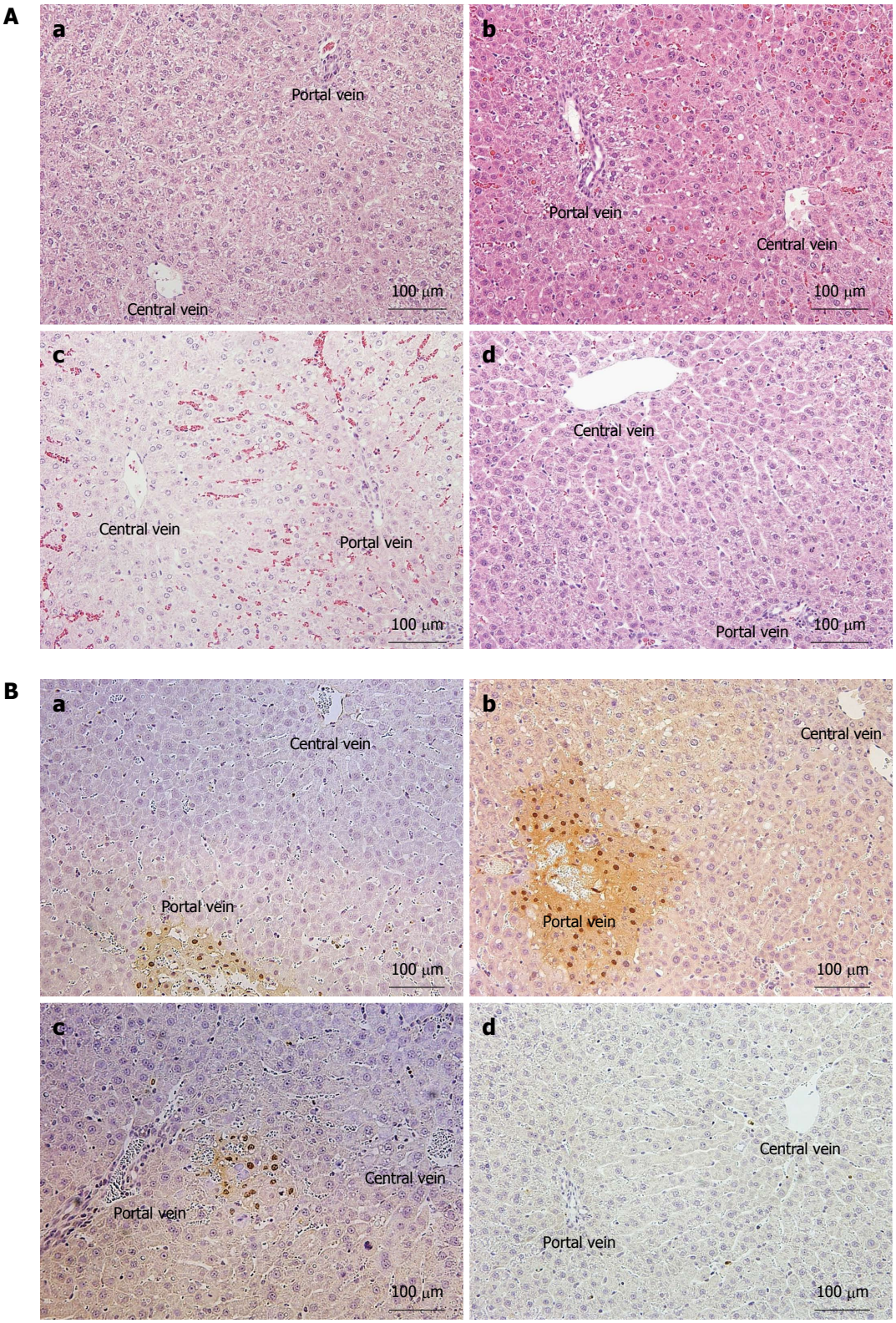
In the control and HA groups, S1P did not accumulate in the liver. In the HA-S1P group, S1P accumulated in the liver to a greater degree than it did in the S1P group (Figure 4C).

### *Transmission electron microscopy*

Deterioration of the sinusoidal endothelial lining was observed and severe destruction of the perisinusoidal space structures were observed in the control, HA, and S1P groups (Figure 5). In the HA group vacuolation induced by hypoxia was observed. However, the structure of the endothelial lining in the HA-S1P group was well preserved. Moreover, it was no obvious mitochondrial disorders in the HA-S1P group.

## DISCUSSION

LSECs play important functional roles in hepatic I/R injury, being particularly vulnerable to it[20,21]. S1P has an anti-apoptotic effect on LSECs[8,9], but S1P receptors


A
a
Portal vein
Central vein
100 μm
b
Portal vein
Central vein
100 μm
c
Central vein
Portal vein
100 μm
d
Central vein
Portal vein
100 μm
B
a
Central vein
Portal vein
100 μm
b
Central vein
Portal vein
100 μm
c
Central vein
Portal vein
100 μm
d
Central vein
Portal vein
100 μm

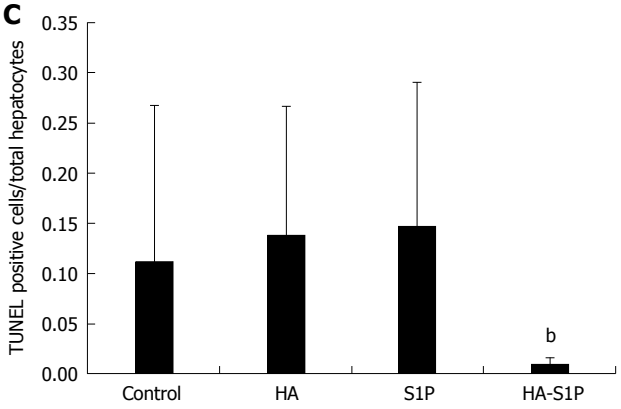



**Figure 3 Histological findings.** A: HE staining In the control (a), HA (b), and S1P group (c) histologic examination revealed vacuolation of the hepatocytes and loss of palisade arrangement after 120 min of reperfusion. These findings were not observed in the HA-S1P group (d). (hematoxylin-eosin stain, magnification × 200); B: TUNEL (TdT-mediated dUTP-biotin nick end labeling) assay of the liver tissue and (C) the ratio of TUNEL-positive/total hepatocytes In the control (a), HA (b), and S1P groups (c), TUNEL-positive cells were observed in zone 1 after 120 min of reperfusion. These findings were not observed in the HA-S1P group (d). $^{b}P < 0.01$ *vs* the control, HA, and S1P groups. HA: Hyaluronic acid; S1P: Sphingosine 1-phosphate.

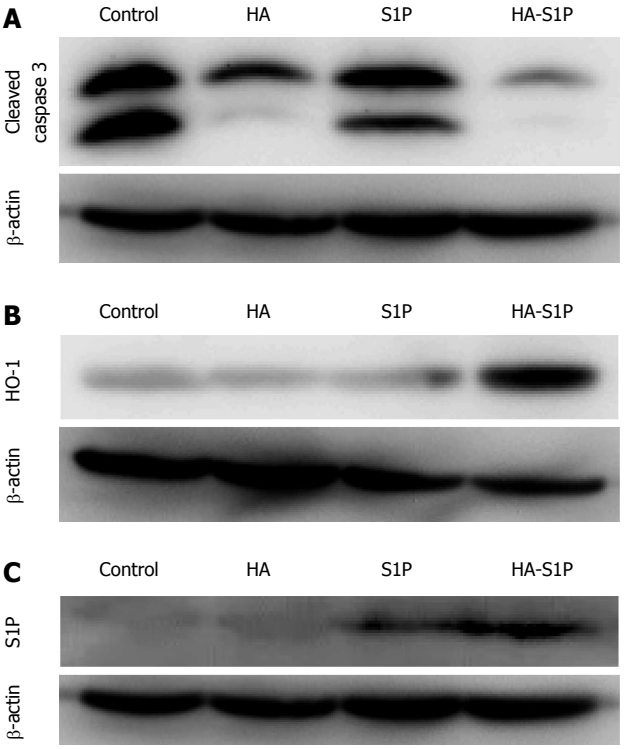



**Figure 4 Western blot analysis of cleaved caspase-3 (A), heme oxygenase-1 (B) and sphingosine 1-phosphate (C) in liver tissues.** The expression of cleaved caspase-3 in the control, HA, and S1P groups, was higher than that in the HA-S1P group. The expression of HO-1 in the HA-S1P group was higher than that in the control, HA, and S1P group. The expression of S1P in the HA-S1P group was higher than in the control, HA, or S1P groups. HO-1: Heme oxygenase 1; HA: Hyaluronic acid; S1P: Sphingosine 1-phosphate.

are widely expressed in many organs[11-13]. Thus, it is difficult to deliver agents specifically to LSECs through a single-agent administration of S1P. However, HA receptors of STAB2 are specifically expressed on LSECs[15-19]. We have developed a new formulation of HA-S1P that targets LSECs by binding specifically to HA and HA receptors. This study demonstrates that HA-S1P protects the liver from hepatic I/R injury in rats and that the strong protective effect of HA-S1P may be mediated by the antiapoptotic effect of S1P on LSECs. Therefore, the results of this study strongly suggest that HA-S1P could be a useful agent for suppressing hepatic I/R injury.

In hepatic I/R injury, LSECs are injured and become apoptotic soon after reperfusion and this phenomenon occurs much earlier than it does in hepatocytes[22]. In addition, LSECs have important functional roles throughout hepatic injury in acute hepatitis[23] and hepatectomy[24]. In general, LSECs are known to be a septum between blood and hepatocytes and are involved in the stabilization of hepatocytes. In previously research we determined that LSEC injury results in microcirculatory blood flow disturbances[25]. In I/R injury, hepatocytes become seriously impaired after disorder of the LSECs, which subsequently induces liver dysfunction[26]. As such, LSECs are primary therapeutic targets of hepatic I/R injury[26]. In the present study we performed total hepatic I/R injury in rats and we found that serum alanine aminotransferase levels were also significantly relieved by HA-S1P administration. TEM findings supported our hypothesis that HA-S1P has a positive effect on LSEC injury. After HA-S1P administration there were no observations of apoptotic cells in the liver tissue thus preventing hepatic I/R injury. These results indicate that HA-S1P administration inhibits the deterioration of the LSEC lining structure and that this phenomenon leads to the suppression of hepatic injury.

S1P is a lipid mediator contained in platelets and is reported to regulate a broad variety of cellular processes, such as cell proliferation, apoptosis, calcium homeostasis, vascular maturation, or angiogenesis[27,28]. S1P is excreted in large amounts from activated platelets[29]. Zheng *et al*[9] reported that S1P protects LSECs from ethanol-induced injury through inhibition of apoptosis and we have previously reported that S1P prevents the apoptosis of LSECs by inhibiting

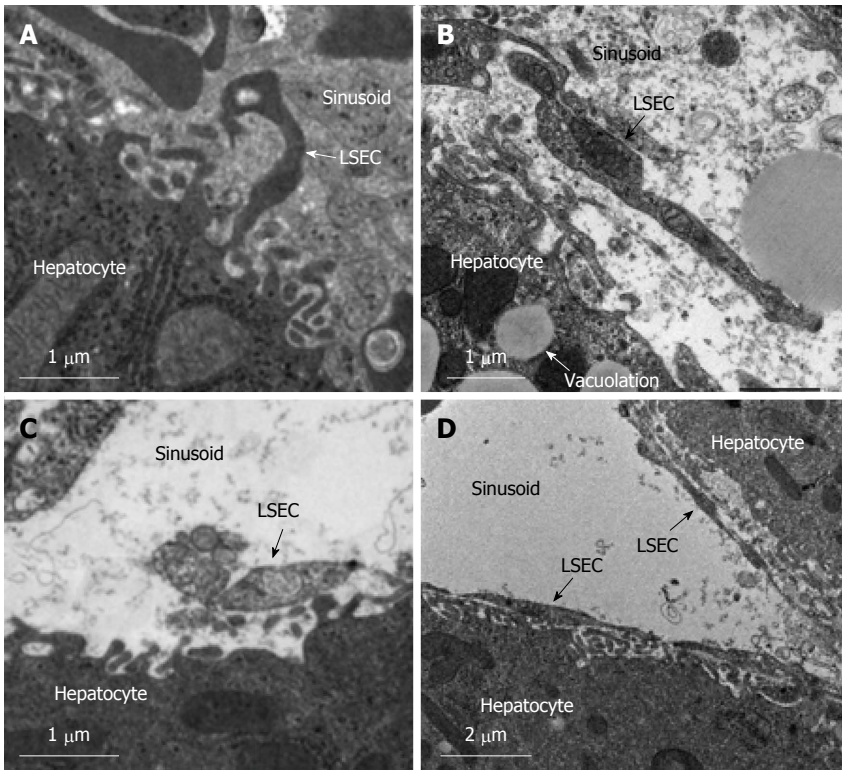



**Figure 5 Transmission electron microscopic findings after 120 min of hepatic ischemia/reperfusion.** In the control (A), HA (B), and S1P groups (C), the sinusoidal endothelial linings were destroyed and detached into the sinusoidal space. In contrast, in the HA-S1P group (D), the sinusoidal endothelial cells were well preserved. HA: Hyaluronic acid; S1P: Sphingosine 1-phosphate; LSEC: Liver sinusoidal endothelial cell.

the cleavage of caspase-3[8]. From these reports, we hypothesized that S1P is the most effective agent for inhibiting hepatic I/R injury, because apoptosis of LSEC is the primary mechanism underlying hepatic I/R injury[30,31]. S1P acts directly either on intracellular targets or activates G protein-coupled receptors[32]. Five S1P receptors have been identified, namely S1PR1-5[33]. S1PR1-3 are widely expressed in various tissues (including liver), whereas the expression of S1PR4 and S1PR5 are confined to lymphoid/hematopoietic tissue and to the central nervous system, respectively[33].

Recent investigations revealed that LSECs possess unique HA receptors of STAB2 that recognize and internalize HA[34]. We tried to utilize this feature of LSECs in the development of a novel DDS to achieve the accumulation of S1P[35]. Exogenous HA has been reported to rapidly integrate into the liver after *in vivo* administration[15,17]. HA has been widely used as a targeted delivery material for LSECs[36]. However, up to now, there has been no specific research focused on LSECs that has lead to the treatment of liver disease. To this end we developed HA-S1P formulation that is prepared by direct combination of HA and S1P. In the present study, we revealed that HA-S1P selectively accumulates in the liver to a greater extent than dose a single-agent administration of S1P.

HO-1 is widely expressed among the different liver cell populations, including LSECs, hepatocytes, Kupffer cells and hepatic stellate cells[37]. The cytoprotective effects of HO-1 have been shown to ameliorate hepatic I/R injury in various experimental models[38]. We have previously demonstrated that HO-1 overexpression exerts cytoprotective effects and improves hepatic I/R injury[39]. HO-1, also known as heat shock protein 32, is an inducible enzyme that converts heme into carbon monoxide (CO), billiverdin and free iron[40]. Moreover, induction of CO in the hepatic sinusoids before hepatic I/R may have prominent effects[39]. In the present study, we found that HA-S1P resulted in greater expression of HO-1 than single-agent administration of S1P. Consequently, these data suggest that by inducing HO-1 to maintain normal cellular function HA-S1P protects LSECs from hepatic I/R injury.

In conclusion, we have succeeded in specific delivery of S1P to LSECs for the first time by using HA as a carrier. Furthermore, HA-S1P exhibits a cytoprotective effect on the liver through the inhibition of LSEC apoptosis. HA-S1P attenuates hepatic I/R injury by protecting LSECs, which represent the primary therapeutic targets for treating hepatic I/R injury. Thus, HA-S1P is a promising new agent for hepatic I/R injury. Further investigations are needed to support our results and elucidate the molecular mechanisms.

## ACKNOWLEDGMENTS

The authors thank Ako Takahashi for providing technical assistance.

## COMMENTS

### Background

The apoptosis of liver sinusoidal endothelial cells (LSECs) is a pivotal mechanism of hepatic ischemia/reperfusion (I/R). Sphingosine 1-phosphate (S1P) exhibits antiapoptotic effects on human LSECs. However, accumulating S1P (as a single-agent) on the LSECs is difficult, as S1P receptors are widely expressed in various different tissues. The authors developed a new drug delivery system for targeting the LSEC by combining S1P with HA, to make the formula hyaluronic acid-S1P (HA-S1P). The aim of this study was to investigate whether the newly developed HA-S1P protects livers in the case of hepatic I/R injury.

### Research frontiers

LSECs play important functional roles in hepatic I/R injury. It is difficult to deliver protective agents specifically to LSECs through a single-agent administration. However, HA receptors of STAB2 are specifically expressed on LSECs. The authors have developed a new formulation of HA-S1P that targets LSECs by binding specifically to HA and HA receptors to protect the liver from hepatic I/R injury.

### Innovations and breakthroughs

This is the first study to succeed in specific delivery of S1P to LSECs for the first time by using HA as a carrier. There has been no specific research focused on LSECs that has lead to the treatment of liver disease. HA-S1P attenuates hepatic I/R injury by protecting LSECs.

### Applications

Hepatic I/R injury is a major problem in liver transplantation and liver resection. The authors suggest that HA-S1P is a promising new agent for hepatic I/R injury.

### Terminology

S1P is a bioactive lipid that regulates diverse cellular functions, including proliferation, differentiation, migration and survival through interaction with different G protein-coupled S1P receptors. The HA receptors, namely STAB2, are highly and specifically expressed on LSECs. STAB2 is a type I transmembrane scavenger receptor that is highly expressed in LSECs and is the major clearance receptor for circulating HA.

### Peer-review

The authors have developed a new formulation of HA-S1P that targets LSECs by binding specifically to HA and HA receptors. This study demonstrates that HA-S1P protects the liver from hepatic I/R injury in rats and that the strong protective effect of HA-S1P may be mediated by the antiapoptotic effect of S1P on LSECs. Therefore, the results of this study strongly suggest that HA-S1P could be a useful agent for suppressing hepatic I/R injury.

## REFERENCES


1 **Man K**, Fan ST, Ng IO, Lo CM, Liu CL, Wong J. Prospective evaluation of Pringle maneuver in hepatectomy for liver tumors by a randomized study. *Ann Surg* 1997; **226**: 704-711; discussion 711-713 [PMID: 9409569]

2 **Selzner N**, Liu H, Boehnert MU, Adeyi OA, Shalev I, Bartczak AM, Xue-Zhong M, Manuel J, Rotstein OD, McGilvray ID, Grant DR, Phillips MJ, Levy GA, Selzner M. FGL2/fibroleukin mediates hepatic reperfusion injury by induction of sinusoidal endothelial cell and hepatocyte apoptosis in mice. *J Hepatol* 2012; **56**: 153-159 [PMID: 21756857 DOI: 10.1016/j.jhep.2011.05.033]

3 **Gao W**, Bentley RC, Madden JF, Clavien PA. Apoptosis of sinusoidal endothelial cells is a critical mechanism of preservation injury in rat liver transplantation. *Hepatology* 1998; **27**: 1652-1660 [PMID: 9620339 DOI: 10.1002/hep.510270626]

4 **Banga NR**, Prasad KR, Burn JL, Homer-Vanniasinkam S, Graham A. An in vitro model of warm hypoxia-reoxygenation injury in human liver endothelial cells. *J Surg Res* 2012; **178**: e35-e41 [PMID: 22472696 DOI: 10.1016/j.jss.2011.12.036]

5 **Enomoto K**, Nishikawa Y, Omori Y, Tokairin T, Yoshida M, Ohi N, Nishimura T, Yamamoto Y, Li Q. Cell biology and pathology of liver sinusoidal endothelial cells. *Med Electron Microsc* 2004; **37**: 208-215 [PMID: 15614445 DOI: 10.1007/s00795-004-0261-4]

6 **Hla T**, Lee MJ, Ancellin N, Liu CH, Thangada S, Thompson BD, Kluk M. Sphingosine-1-phosphate: extracellular mediator or intracellular second messenger? *Biochem Pharmacol* 1999; **58**: 201-207 [PMID: 10423159]

7 **Igarashi Y**, Yatomi Y. Sphingosine 1-phosphate is a blood constituent released from activated platelets, possibly playing a variety of physiological and pathophysiological roles. *Acta Biochim Pol* 1998; **45**: 299-309 [PMID: 9821862]

8 **Nowatari T**, Murata S, Nakayama K, Sano N, Maruyama T, Nozaki R, Ikeda N, Fukunaga K, Ohkohchi N. Sphingosine 1-phosphate has anti-apoptotic effect on liver sinusoidal endothelial cells and proliferative effect on hepatocytes in a paracrine manner in human. *Hepatol Res* 2014; Epub ahead of print [PMID: 25371278 DOI: 10.1111/hepr.12446]

9 **Zheng DM**, Kitamura T, Ikejima K, Enomoto N, Yamashina S, Suzuki S, Takei Y, Sato N. Sphingosine 1-phosphate protects rat liver sinusoidal endothelial cells from ethanol-induced apoptosis: Role of intracellular calcium and nitric oxide. *Hepatology* 2006; **44**: 1278-1287 [PMID: 17058266 DOI: 10.1002/hep.21384]

10 **Lee SY**, Kim DH, Sung SA, Kim MG, Cho WY, Kim HK, Jo SK. Sphingosine-1-phosphate reduces hepatic ischaemia/reperfusion-induced acute kidney injury through attenuation of endothelial injury in mice. *Nephrology* (Carlton) 2011; **16**: 163-173 [PMID: 21272128 DOI: 10.1111/j.1440-1797.2010.01386.x]

11 **McVerry BJ**, Garcia JG. In vitro and in vivo modulation of vascular barrier integrity by sphingosine 1-phosphate: mechanistic insights. *Cell Signal* 2005; **17**: 131-139 [PMID: 15494205 DOI: 10.1016/j.cellsig.2004.08.006]

12 **Rosen H**, Goetzl EJ. Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. *Nat Rev Immunol* 2005; **5**: 560-570 [PMID: 15999095 DOI: 10.1038/nri1650]

13 **Pyne S**, Pyne NJ. Sphingosine 1-phosphate signalling in mammalian cells. *Biochem J* 2000; **349**: 385-402 [PMID: 10880336]

14 **Shimizu H**, He W, Guo P, Dziadkoviec I, Miyazaki M, Falk RE. Serum hyaluronate in the assessment of liver endothelial cell function after orthotopic liver transplantation in the rat. *Hepatology* 1994; **20**: 1323-1329 [PMID: 7927268]

15 **Fraser JR**, Alcorn D, Laurent TC, Robinson AD, Ryan GB. Uptake of circulating hyaluronic acid by the rat liver. Cellular localization in situ. *Cell Tissue Res* 1985; **242**: 505-510 [PMID: 4075374]

16 **McCourt PA**, Smedsrød BH, Melkko J, Johansson S. Characterization of a hyaluronan receptor on rat sinusoidal liver endothelial cells and its functional relationship to scavenger receptors. *Hepatology* 1999; **30**: 1276-1286 [PMID: 10534350 DOI: 10.1002/hep.510300521]

17 **Smedsrød B**, Pertoft H, Eriksson S, Fraser JR, Laurent TC. Studies in vitro on the uptake and degradation of sodium hyaluronate in rat liver endothelial cells. *Biochem J* 1984; **223**: 617-626 [PMID: 6508733]

18 **Weigel JA**, Raymond RC, McGary C, Singh A, Weigel PH. A blocking antibody to the hyaluronan receptor for endocytosis (HARE) inhibits hyaluronan clearance by perfused liver. *J Biol Chem* 2003; **278**: 9808-9812 [PMID: 12645574 DOI: 10.1074/jbc.M211462200]

19 **Simpson MA**, Weigel JA, Weigel PH. Systemic blockade of the hyaluronan receptor for endocytosis prevents lymph node metastasis of prostate cancer. *Int J Cancer* 2012; **131**: E836-E840 [PMID: 22234863 DOI: 10.1002/ijc.27427]

20 **Schmidt R**, Tritschler E, Hoetzel A, Loop T, Humar M, Halverscheid L, Geiger KK, Pannen BH. Heme oxygenase-1 induction by the clinically used anesthetic isoflurane protects rat livers from ischemia/reperfusion injury. *Ann Surg* 2007; **245**: 931-942 [PMID: 17522519 DOI: 10.1097/01.sla.0000256891.45790.4d]

21 **Peralta C**, Jiménez-Castro MB, Gracia-Sancho J. Hepatic ischemia and reperfusion injury: effects on the liver sinusoidal milieu. *J Hepatol* 2013; **59**: 1094-1106 [PMID: 23811302 DOI: 10.1016/j.jhep.2013.06.017]

22 **Kohli V**, Selzner M, Madden JF, Bentley RC, Clavien PA. Endothelial cell and hepatocyte deaths occur by apoptosis after ischemia-reperfusion injury in the rat liver. *Transplantation* 1999; **67**: 1099-1105 [PMID: 10232558]

23 **Hisakura K**, Murata S, Takahashi K, Matsuo R, Pak S, Ikeda N, Kawasaki T, Kohno K, Myronovych A, Nakano Y, Ikeda O, Watanabe M, Ohkohchi N. Platelets prevent acute hepatitis induced by anti-fas antibody. *J Gastroenterol Hepatol* 2011; **26**: 348-355 [PMID: 21261726 DOI: 10.1111/j.1440-1746.2010.06334.x]

24 **Hisakura K**, Murata S, Fukunaga K, Myronovych A, Tadano S, Kawasaki T, Kohno K, Ikeda O, Pak S, Ikeda N, Nakano Y, Matsuo R, Konno K, Kobayashi E, Saito T, Yasue H, Ohkohchi N. Platelets prevent acute liver damage after extended hepatectomy in pigs. *J Hepatobiliary Pancreat Sci* 2010; **17**: 855-864 [PMID: 20734209 DOI: 10.1007/s00534-010-0276-2]

25 **Tamura T**, Kondo T, Pak S, Nakano Y, Murata S, Fukunaga K, Ohkohchi N. Interaction between Kupffer cells and platelets in the early period of hepatic ischemia-reperfusion injury--an in vivo study. *J Surg Res* 2012; **178**: 443-451 [PMID: 22480836 DOI: 10.1016/j.jss.2011.12.010]

26 **Natori S**, Selzner M, Valentino KL, Fritz LC, Srinivasan A, Clavien PA, Gores GJ. Apoptosis of sinusoidal endothelial cells occurs during liver preservation injury by a caspase-dependent mechanism. *Transplantation* 1999; **68**: 89-96 [PMID: 10428274]

27 **Kawasaki T**, Murata S, Takahashi K, Nozaki R, Ohshiro Y, Ikeda N, Pak S, Myronovych A, Hisakura K, Fukunaga K, Oda T, Sasaki R, Ohkohchi N. Activation of human liver sinusoidal endothelial cell by human platelets induces hepatocyte proliferation. *J Hepatol* 2010; **53**: 648-654 [PMID: 20615569 DOI: 10.1016/j.jhep.2010.04.021]

28 **Spiegel S**, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* 2003; **4**: 397-407 [PMID: 12728273 DOI: 10.1038/nrm1103]

29 **Yatomi Y**, Ohmori T, Rile G, Kazama F, Okamoto H, Sano T, Satoh K, Kume S, Tigyi G, Igarashi Y, Ozaki Y. Sphingosine 1-phosphate as a major bioactive lysophospholipid that is released from platelets and interacts with endothelial cells. *Blood* 2000; **96**: 3431-3438 [PMID: 11071638]

30 **Fondevila C**, Busuttil RW, Kupiec-Weglinski JW. Hepatic ischemia/reperfusion injury--a fresh look. *Exp Mol Pathol* 2003; **74**: 86-93 [PMID: 12710939]

31 **Selzner N**, Rudiger H, Graf R, Clavien PA. Protective strategies against ischemic injury of the liver. *Gastroenterology* 2003; **125**: 917-936 [PMID: 12949736]

32 **Adada M**, Canals D, Hannun YA, Obeid LM. Sphingosine-1-phosphate receptor 2. *FEBS J* 2013; **280**: 6354-6366 [PMID: 23879641 DOI: 10.1111/febs.12446]

33 **Sanchez T**, Hla T. Structural and functional characteristics of S1P receptors. *J Cell Biochem* 2004; **92**: 913-922 [PMID: 15258915 DOI: 10.1002/jcb.20127]

34 **Zhou B**, Weigel JA, Fauss L, Weigel PH. Identification of the hyaluronan receptor for endocytosis (HARE). *J Biol Chem* 2000; **275**: 37733-37741 [PMID: 10952975 DOI: 10.1074/jbc.M003030200]

35 **Takei Y**, Maruyama A, Ferdous A, Nishimura Y, Kawano S, Ikejima K, Okumura S, Asayama S, Nogawa M, Hashimoto M, Makino Y, Kinoshita M, Watanabe S, Akaike T, Lemasters JJ, Sato N. Targeted gene delivery to sinusoidal endothelial cells: DNA nanoassociate bearing hyaluronan-glycocalyx. *FASEB J* 2004; **18**: 699-701 [PMID: 14977882 DOI: 10.1096/fj.03-0494fje]

36 **Yamada Y**, Hashida M, Hayashi Y, Tabata M, Hyodo M, Ara MN, Ohga N, Hida K, Harashima H. An approach to transgene expression in liver endothelial cells using a liposome-based gene vector coated with hyaluronic acid. *J Pharm Sci* 2013; **102**: 3119-3127 [PMID: 23471825 DOI: 10.1002/jps.23480]

37 **Bauer I**, Wanner GA, Rensing H, Alte C, Miescher EA, Wolf B, Pannen BH, Clemens MG, Bauer M. Expression pattern of heme oxygenase isoenzymes 1 and 2 in normal and stress-exposed rat liver. *Hepatology* 1998; **27**: 829-838 [PMID: 9500714 DOI: 10.1002/hep.510270327]

38 **Sun L**, Shi T, Qiao H, Jiang X, Jiang H, Krissansen GW, Sun X. Hepatic overexpression of heme oxygenase-1 improves liver allograft survival by expanding T regulatory cells. *J Surg Res* 2011; **166**: e187-e194 [PMID: 21227452 DOI: 10.1016/j.jss.2010.11.917]

39 **Tamura T**, Kondo T, Ogawa K, Fukunaga K, Ohkohchi N. Protective effect of heme oxygenase-1 on hepatic ischemia-reperfusion injury through inhibition of platelet adhesion to the sinusoids. *J Gastroenterol Hepatol* 2013; **28**: 700-706 [PMID: 23215739 DOI: 10.1111/jgh.12075]

40 **Katori M**, Busuttil RW, Kupiec-Weglinski JW. Heme oxygenase-1 system in organ transplantation. *Transplantation* 2002; **74**: 905-912 [PMID: 12394829 DOI: 10.1097/01.TP.0000031935.23826.27]


**P- Reviewer:** Corrales FJ, Wu SL **S- Editor**: Ma YJ **L- Editor**: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12787

*World J Gastroenterol* 2015 December 7; 21(45): 12787-12799
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

Basic Study

# Peroxisome proliferator-activated receptor-delta agonist ameliorated inflammasome activation in nonalcoholic fatty liver disease

Hyun Jung Lee, Jong Eun Yeon, Eun Jung Ko, Eileen L Yoon, Sang Jun Suh, Keunhee Kang, Hae Rim Kim, Seoung Hee Kang, Yang Jae Yoo, Jihye Je, Beom Jae Lee, Ji Hoon Kim, Yeon Seok Seo, Hyung Joon Yim, Kwan Soo Byun

Hyun Jung Lee, Jong Eun Yeon, Eun Jung Ko, Eileen L Yoon, Sang Jun Suh, Keunhee Kang, Hae Rim Kim, Seoung Hee Kang, Yang Jae Yoo, Jihye Je, Beom Jae Lee, Ji Hoon Kim, Yeon Seok Seo, Hyung Joon Yim, Kwan Soo Byun, Department of Internal Medicine, Korea University College of Medicine, Seoul 152-703, South Korea

Author contributions: Lee HJ and Yeon JE designed the research; Lee HJ and Ko EJ performed the research; Yoon EL, Suh SJ, Kang K, Lee BJ, Kim JH, Seo YS, Yim HJ and Byun KS contributed reagents/analytic tools; Kim HR, Kang SH, Yoo YJ and Je J anlyazed the data; Lee HJ and Yeon JE wrote the paper.

Institutional review board statement: This study was approved by the institutional review board Korea University and conducted in compliance with the Guide for the care and Use of Laboratory Animals.

Institutional animal care and use committee statement: All procedure involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Korea University, Seoul, Korea, KUIACUC-2013-66 and carried out according to the Guide for the Care and Use of Laboratory Animals.

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

Data sharing statement: No additional data are available.



Correspondence to: Jong Eun Yeon, MD, PhD, Department of Internal Medicine, Korea University College of Medicine, 97 Guro-Dong Gil, Guro-Dong, Guro-Ku, Seoul 152-703, South Korea. jeyyeon@hotmail.com
Telephone: +82-2-26263010
Fax: +82-2-26261038

Received: May 7, 2015
Peer-review started: May 11, 2015
First decision: June 2, 2015
Revised: July 24, 2015
Accepted: October 13, 2015
Article in press: October 13, 2015
Published online: December 7, 2015

## Abstract

**AIM:** To evaluate the inflammasome activation and the effect of peroxisome proliferator-activated receptors (PPAR)-δ agonist treatment in nonalcoholic fatty liver disease (NAFLD) models.

**METHODS:** Male C57BL/6J mice were classified according to control or high fat diet (HFD) with or without PPAR-δ agonist (GW) over period of 12 wk [control, HFD, HFD + lipopolysaccharide (LPS), HFD + LPS + GW group]. HepG2 cells were exposed to palmitic acid (PA) and/or LPS in the absence or presence of GW.

**RESULTS:** HFD caused glucose intolerance and hepatic steatosis. In mice fed an HFD with LPS, caspase-1 and interleukin (IL)-1β in the liver were significantly increased. Treatment with GW ameliorated the steatosis and inhibited overexpression of pro-inflammatory cytokines. In HepG2 cells, PA and LPS treatment markedly increased mRNA of several nucleotide-binding and

oligomerization domain-like receptor family members (NLRP3, NLRP6, and NLRP10), caspase-1 and IL-1β. PA and LPS also exaggerated reactive oxygen species production. All of the above effects of PA and LPS were reduced by GW. GW also enhanced the phosphorylation of AMPK-α.

**CONCLUSION:** PPAR-δ agonist reduces fatty acid-induced inflammation and steatosis by suppressing inflammasome activation. Targeting the inflammasome by the PPAR-δ agonist may have therapeutic implication for NAFLD.

**Key words:** Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Inflammasome; Nucleotide-binding and oligomerization domain-like receptor; Peroxisome proliferator-activated receptors delta



**Core tip:** Until now, the underlying mechanisms of disease progression and therapeutic targets were uncertain in nonalcoholic fatty liver disease (NAFLD). Our study were to evaluate the inflammasome activation and the effect of peroxisome proliferator-activated receptors (PPAR)-δ agonist treatment in NAFLD models. In our NAFLD models, mRNA of several NOD-like receptor family members, caspase-1 and interleukin-1β were markedly increased. All of those effects were reduced by PPAR-δ agonist treatment. It also ameliorated the steatosis and inhibited overexpression of pro-inflammatory cytokines. In conclusion, PPAR-δ agonist reduces fatty acid-induced inflammation and steatosis by suppressing inflammasome activation.

Lee HJ, Yeon JE, Ko EJ, Yoon EL, Suh SJ, Kang K, Kim HR, Kang SH, Yoo YJ, Je J, Lee BJ, Kim JH, Seo YS, Yim HJ, Byun KS. Peroxisome proliferator-activated receptor-delta agonist ameliorated inflammasome activation in nonalcoholic fatty liver disease. *World J Gastroenterol* 2015; 21(45): 12787-12799 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12787.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12787

## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases, occurring in 10%-35% of the general population, and its prevalence has increased in parallel with the worldwide epidemic of obesity and its related insulin-resistant state[1,2]. The spectrum of NAFLD ranges from simple steatosis to nonalcoholic steatohepatitis (NASH) and fibrosis. Certain portion of NAFLD eventually progressed to liver cirrhosis and hepatocellular carcinoma[1,3]. Recently, the two-hit hypothesis was been reported in the literature[4], but the precise mechanism involved in the development and progression of NAFLD is not entirely understood.

Regarding the pathogenesis of NAFLD[5,6], steatosis sensitizes the liver and makes it susceptible to additional insults. Factors such as increased oxidative stress, pro-inflammatory cytokines and impaired adenosine triphosphate (ATP) production[7,8] could trigger necroinflammation and lead to the progression of steatohepatitis. Although many kind of drugs such as thiazolidinediones, vitamin E, losartan, and silybin have been evaluated in several studies, few pharmacological treatments can be recommended at present[9-11].

Inflammasome is a large, intracellular multiprotein complex that is a sensor of the exogenous and endogenous danger signals, such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), that promotes the cleavage and maturation of pro-inflammatory cytokines such as pro-interleukin (IL)-1β and pro-IL-18[12,13]. Most DAMPs induce the production of reactive oxygen species (ROS), which results in nucleotide-binding and oligomerization domain (NOD)-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome activation[14]. The NLRP3 inflammasome is known to be related to the pathogenesis of obesity, insulin resistance, and development of diabetes[15-18]. But, the role of inflammasome in the pathogenesis of NAFLD/NASH has not been elucidated.

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins, and three types have been identified in mammals: PPAR-α, PPAR-γ, and PPAR-δ. Pharmacological targets of PPAR-α and PPAR-γ are relatively well-known in the treatment of dyslipidemia and diabetes[19]. It has become increasingly apparent that PPAR-δ also play important roles in the regulation of metabolism, as its activation increases fatty acid (FA) oxidation, ameliorates glucose homeostasis, and attenuates macrophage inflammatory responses[19,20]. In the mouse model of NASH, PPAR-δ improves hepatic steatosis and inflammation by regulation of lipid metabolism and inhibition of inflammatory response[21].

In this study, we determined whether treatment with the PPAR-δ agonist could ameliorate high fat-induced inflammasome activation and the inflammatory response using *in vivo* and *in vitro* NAFLD models.

## MATERIALS AND METHODS

### Materials

PPAR-δ agonist, GW501516 (GW), was obtained from Enzo Life Sciences (Farmingdale, NY, United States). Lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4) and palmitic acid (PA) were purchased from Sigma-Aldrich (St Louis, MO, United States). Solutions and reagents used for cell culture were obtained from Invitrogen (Carlsbad, CA, United States) unless otherwise noted. Antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, United States).

### Animals and experimental design

The animal protocol was designed to minimize pain or

**Table 1 Sequence of primers employed for quantitative real-time polymerase chain reaction**

| mRNA | Forward (5'-, -3') | Reverse (5'-, -3') |
|---|---|---|
| Mouse | | |
| Caspase-1 | CACAGCTCTGGAGATGGTGA | GGTCCCACATATTCCCTCC |
| IL-1β | GCTGCTTCCAAACCTTTGAC | AGCTTCTCCACAGCCACAAT |
| GAPDH | CAGCCTCAAGATCATCAGCA | GTCTTCTGGGTGGCAGTGAT |
| Human | | |
| NLRP1 | TCTCAAGGGGACCTGCATAC | AGCCAGCTACAGGGAAGTGA |
| NLRP3 | AAGGAAGTGGACTGCGAGA | AACGTTCGTCCTTCCTTCCT |
| NLRP6 | TTCATCACCAGCGTTCTGAG | GTTGCTCCAGTTCCTTCTCG |
| NLRP10 | GGATGAGAAGCAAGCTGACC | CTTTGCCTCTCTCCATCTGC |
| NLRC4 | GCAAGGCTCTGACCAAGTTC | TGTCTGCTTCCTGATTGTGC |
| Caspase-1 | CCACAATGGGCTCTGTTTTT | CATCTGGCTGCTCAAATGAA |
| IL-1β | GGACAAGCTGAGGAAGATGC | TCGTTATCCCATGTGTCGAA |
| GAPDH | CAGCCTCAAGATCATCAGCA | GTCTTCTGGGTGGCAGTGAT |

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

discomfort to the animals. Male 4-5-wk-old C57BL/6J mice were obtained from Japan SLC Inc. (Shizuoka, Japan). They were acclimatized to laboratory conditions (22-24 ℃ and 37%-64% humidity, with a 12-h dark-light cycle, free access to food and water) for one week prior to experimentation. Reflecting endotoxemia in NAFLD, we were decided to inject non-lethal very low dosage of LPS. Mice were randomly divided into four groups, which were treated for 12 wk as follows: standard diet (control, *n* = 5); HFD (HFD, 60% kcal from fat; D12492, Research Diets; New Brunswick, NJ, United States, *n* = 5); HFD plus one daily oral gavage of vehicle (0.5% carboxymethyl cellulose solution) with one weekly intraperitoneal (IP) injection of LPS (1 mg/kg per week) (HFD + LPS, *n* = 5); and HFD plus one daily oral dose of 3 mg/kg per day of GW501516, which was dissolved in the vehicle, with IP injection of LPS (HFD + LPS + GW, *n* = 6). Vehicle and GW501516 were administered for the last 3 wk. Body weight and food intake were recorded weekly. Body length (from nose to anus) was measured before sacrifice. Body mass index (BMI) was calculated by dividing body weight by the square of the body length (g/cm$^2$)[22].

The study was reviewed and approved by the Institutional Review Board of Korea University and it was approved by the institutional animal review board of Korea University, Seoul, Korea, KUIACUC-2013-66 and conducted in compliance with the Guide for the Care and Use of Laboratory Animals.

### Glucose tolerance test

The glucose tolerance test was conducted in all animals at 11 wk after dietary manipulation. After overnight fasting, 2 g/kg glucose was injected intraperitoneally, and blood samples were taken from the tail vein at 0, 15, 30, 60, 90, and 120 min. Blood glucose was measured using an Accu-Check Compact kit (Roche Diagnostics GmbH; Mannheim, Germany).

### Blood biochemistry

At 12 wk, the mice were intraperitoneally anesthetized with a mixture of tiletamine/zolazepam (30 mg/kg, Zoletil; Yuhan Corp.; Seoul, Korea) and xylazine (10 mg/kg, Rompun; Bayer, Inc.; Frankfurt, Germany), and sacrificed by exsanguination. Blood samples were extracted and the serum was isolated. The livers were rapidly excised and weighed. Serum alanine transaminase (ALT), aspartate transaminase (AST), triglyceride (TG), and total cholesterol (TC) levels were measured using common biochemical kits (Mindray Medical International Ltd.; Shenzhen, China).

### Liver histological analysis

The right liver lobe was stored at -80 ℃ until analysis of mRNA and protein. The left liver lobe was immediately fixed in 10% neutral-buffered formalin, paraffin-embedded, sectioned, and sections were stained with hematoxylin and eosin (HE). To visualize the neutral lipids, some of the frozen sections of fresh liver were stained using Oil Red O reagent. The liver samples were examined histologically in a blind manner by an experienced pathologist using the histological scoring system for NAFLD[23].

### Cell culture and treatment

The human hepatoma HepG2 cell line (ATCC; Manassas, VA, United States) cells were cultured as manufacture's instruction. In all experiments, PA concentration of 0.2 mmol/L which had no influence on cell viability was selected. The cells were stimulated with PA-BSA (0.2 μmol/L), LPS (1 μg/mL), or both, with or without GW501516 (1 or 10 μmol/L).

### RNA preparation and analysis

Total RNA was extracted from mouse liver tissues and HepG2 cells with TRIzol reagent according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Foster City, CA, United States). Gene expressions of NLRP1, NLRP3, NLRP6, NLRP10, NLRC4, caspase-1, and IL-1β were quantified by real-time polymerase chain

**Table 2 Clinical and biochemical characteristics**

| | Control ($n$ = 5) | HFD ($n$ = 5) | HFD + LPS ($n$ = 5) | HFD + LPS + GW ($n$ = 6) |
|---|---|---|---|---|
| Food/wk (g) | 20.78 ± 1.41 | 17.33 ± 0.82[a] | 14.08 ± 1.28[a,c] | 13.92 ± 1.23[a,c] |
| BW (g) | 26.70 ± 0.69 | 41.04 ± 4.69[a] | 30.98 ± 3.86[c] | 29.87 ± 3.31[c] |
| BMI (g/cm$^2$) | 0.33 ± 0.02 | 0.41 ± 0.04[a] | 0.36 ± 0.02[c] | 0.35 ± 0.02[c] |
| Liver/BW (%) | 3.50 ± 0.30 | 3.04 ± 0.46 | 2.77 ± 0.27 | 3.08 ± 0.94 |
| Liver/BL (g/cm) | 0.10 ± 0.01 | 0.13 ± 0.03 | 0.09 ± 0.01 | 0.10 ± 0.03 |
| AST (IU/L) | 52.6 ± 10.33 | 123.8 ± 30.54[a] | 62.0 ± 13.71[c] | 45.67 ± 11.11[c] |
| ALT (IU/L) | 23.4 ± 4.04 | 136.6 ± 69.43[a] | 49.6 ± 31.09[c] | 20.5 ± 6.12[c] |
| TG (mg/dL) | 97.8 ± 19.33 | 69.6 ± 21.55 | 65.2 ± 13.16 | 79.2 ± 23.82 |
| TC (mg/dL) | 86.6 ± 10.92 | 118.4 ± 37.16 | 116.0 ± 9.43 | 135.3 ± 34.09[a] |

Data are presented as mean ± SD. $P$ values are presented as: [a]$P$ < 0.05 *vs* control; [c]$P$ < 0.05 *vs* HFD. BW: Body weight; BMI: Body mass index; BL: Body length; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TG: Triglyceride; TC: Total cholesterol; HFD: High fat diet.

reaction (PCR) with a 7000 Real-time PCR system (Applied Biosystems). PCR reactions were prepared with the Power SYBR Green PCR Master mix (Applied Biosystems) in 20 μL. The cycling parameters were 10 min at 95 ℃ followed by 40 cycles of 95 ℃ (15 s) and 30 s at 60 ℃, followed by melting curve analysis. Gene expression by real-time PCR was presented relative to glyceraldehyde 3-phosphate dehydrogenase. The PCR primers used are listed in the Table 1.

### *Western blot analysis*

Mouse liver tissues and HepG2 cell were lysed using M-PER Mammalian Protein Extraction Reagent (Pierce Chemical; Rockford, IL, United States). The protein concentration was determined using the bicinchoninic acid (BCA) method. Equal amounts (20 μg) of total proteins were boiled for 5 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrotransfer to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% BSA in Tris-buffered saline (TBS) for 1 h at room temperature, followed by incubation with antibodies: anti-capase-1 (D7F10), anti-IL-1β (3A6), anti-p-AMPK-α (Thr172;40H9), anti-AMPK-α (23A3), and anti-β-actin (AC-15). The bands were visualized with an enhanced chemiluminescent direct labeling (ECL) system. The Gel Pro Analyzer 4.5, 2000 software (Media Cybernetics; Silver Spring, MD, United States) was used to determine the band density.

### *Measurement of lipid peroxidation in the liver and intracellular ROS production*

Liver was perfused with saline and homogenized in Tris-HCl buffer (20 mmol/L, pH 7.4). The homogenates were centrifuged at 2500 × *g* for 10 min at 4 ℃. Two hundred microliters of homogenates was analyzed for malondialdehyde (MDA) levels using a kit (Cell Biolabs, Inc.; San Diego, CA, United States), and the value was read at 532 nm.

Intracellular ROS levels in HepG2 cells were determined using 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). Cells were seeded on a 96-well bottom dishes. After the treatment described above in *Cell culture and treatment*, the cells were collected, and incubated with 10 μmol/L DCFDA (Molecular Probes; CA, United States) for 30 min at 37 ℃. DCFDA fluorescence intensity was measured at 530 nm with an excitation wavelength of 488 nm using flow cytometry (Becton Dickinson; Heidelberg, Germany). The percentage of ROS-producing cells was determined by counting only those cells that produced high levels of ROS.

### *Statistical analysis*

Data are presented as mean ± SD. Statistical significance was determined with one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons using the GraphPad Instant program (GraphPad Software version 5.00; GraphPad Software Inc.; San Diego, CA). $P$ < 0.05 was considered statistically significant. The statistical methods of this study were reviewed by a biomedical statistician.

## RESULTS

### *Effects of HFD and GW501516 on body weight, blood glucose, and hepatic function in mice fed the HFD*

The body weight and BMI of the HFD group were significantly higher compared with those of control mice (all $P$ < 0.05) (Table 2). The food intake was greater in the HFD group compared with the HFD + LPS and HFD + LPS + GW501516 groups. The proportion of liver weight to body weight or body length was similar among the four groups. Serum AST and ALT levels were significantly increased in the HFD group compared with the control group (AST, 123.8 ± 30.54 IU/L *vs* 52.6 ± 10.33 IU/L; ALT, 136.6 ± 69.43 IU/L *vs* 23.4 ± 4.04 IU/L, all $P$ < 0.05). GW treatment in HFD+LPS group significantly reduced serum AST and ALT compared with HFD (AST, 45.67 ± 11.11 IU/L *vs* 123.8 ± 30.54 IU/L; ALT, 20.5 ± 6.12 IU/L *vs* 136.6 ± 69.43 IU/L, all $P$ < 0.05). HFD and/or LPS injection had no significant effect on TG or TC levels.

When subjected to a glucose tolerance test, glucose levels of control mice peaked at 30 min and returned to the basal level (Figure 1A). As expected, mice fed the HFD with or without LPS infection were more glucose-intolerant compared with controls, as demonstrated

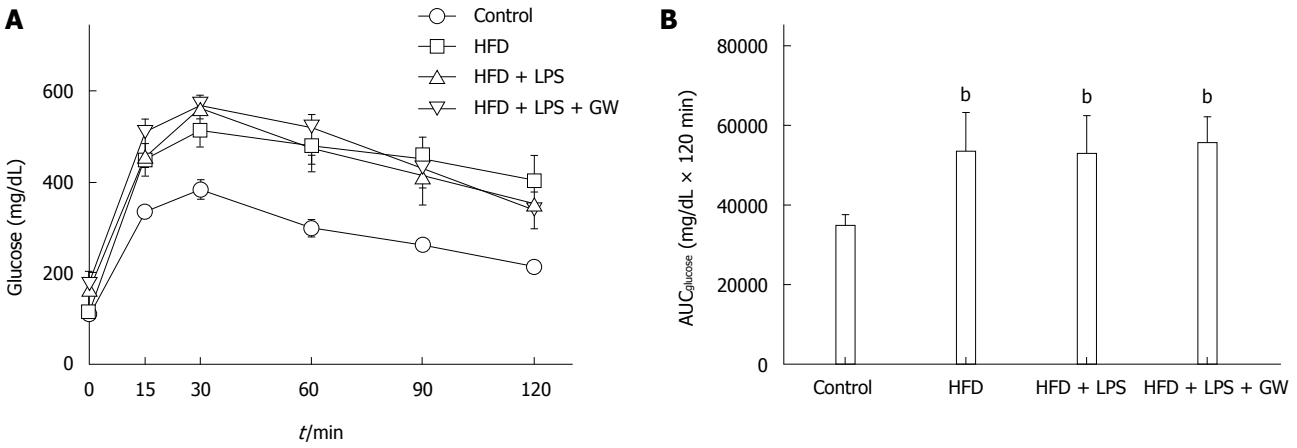



**Figure 1 Effects of high-fat diet for 12 wk on glucose tolerance in male mice treated with GW501516 (GW, 3 mg/kg per day for 3 wk).** Intraperitoneal glucose tolerance test (A) and area under the curve for glucose ($AUC_{glucose}$) (B) showed glucose intolerance in mice fed the HFD with or without lipopolysaccharide (LPS) injection compared with the control group. GW treatment did not improve glucose intolerance. Data are expressed as the mean ± SD (5-6 mice per group). $^{b}P < 0.01$ *vs* control group.

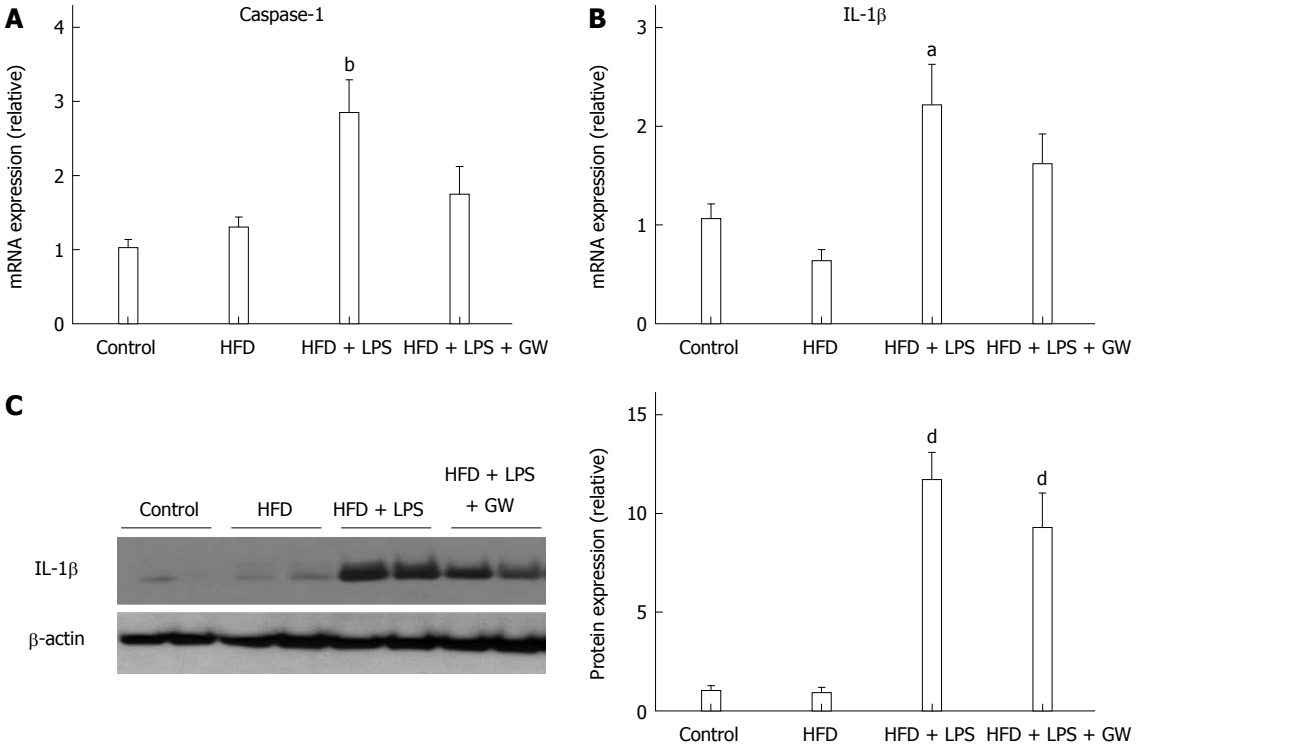



**Figure 2 Effects of GW501516 on caspase-1 and IL-1β levels in mice fed a high fat diet.** Hepatic mRNA levels of caspase-1 (A) and IL-1β (B) at 12 wk showed a significant increase in the HFD + lipopolysaccharide (LPS) group compared with the control group. GW501516 treatment tended to ameliorate this increase. Representative immunoblot and graphic presentation show hepatic protein levels of IL-1β (C) in different groups of mice as indicated. Mice fed an HFD with LPS injection with or without GW501516 treatment had increased protein levels of IL-1β. Data are expressed as the mean ± SD (5-6 mice per group). $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{d}P < 0.001$ *vs* control group.

by the significant increase in the area under the curve (AUC) (53108 ± 9750 mg/dL and 52653 ± 9425 mg/dL *vs* 34545 ± 2776 mg/dL × 120 min, all $P < 0.01$) (Figure 1B). However, this glucose intolerance was not abolished by GW501516 treatment.

### *Effects of GW501516 on IL-1β levels and inflammasome activation in mice and HepG2 cells*

In addition to steatosis, chronic inflammation is an important contributing factor in NASH pathogenesis[5]. To investigate the inflammatory response in this model, we measured hepatic caspase-1 and IL-1β levels. The gene expression level of caspase-1, which is known to be activated by the NLRP3 inflammasome complex and to cleave pro-IL-1β to the active form, was increased in the HFD + LPS group relative to the control group ($P < 0.01$) (Figure 2A). The hepatic gene expression and protein levels of IL-1β were also increased in the HFD

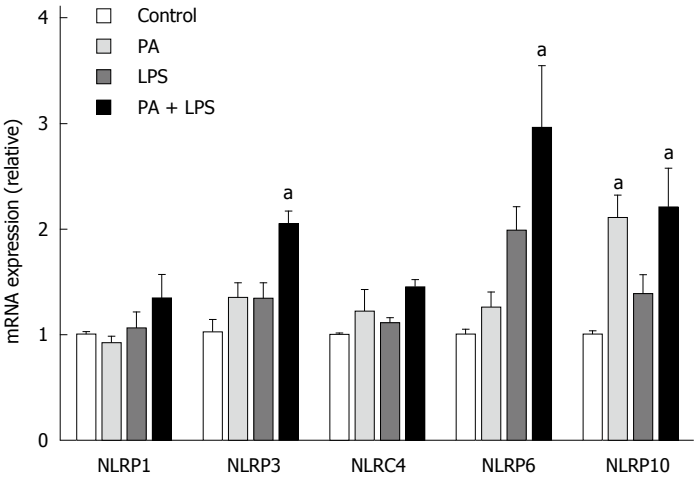



**Figure 3 Expression of inflammasome components in HepG2 cells after stimulation with palmitic acid and lipopolysaccharide.** Relative mRNA levels of NLRP1, NLRP3, NLRC4, NLRP6, and NLRP10 in HepG2 cells were analyzed by RT-PCR. Compared with the control group, significantly increased expression of NLPR3, NLRP6, and NLRP10 was observed in the PA and LPS stimulation group. Data are expressed as the mean ± SD. $^{a}P < 0.05$ *vs* control cells.

+ LPS group relative to the control group ($P < 0.05$ for mRNA expression and $P < 0.001$ for protein-level expression) (Figure 2B and C). When compared with HFD+LPS, PPAR-delta agonist GW treatment reduced hepatic caspase-1 mRNA (2.8-fold *vs* 1.7-fold) and IL-1β mRNA (2.2-fold *vs* 1.6-fold).

We also investigated the effect of PPAR-δ activator on inflammasome induced by PA and LPS in HepG2 cells. First of all, we analyzed the PPAR-δ protein expression itself in various stimuli. *In vitro* exposure with- or without stimuli such as LPS only, PA only or GW only, or combined with it, PPAR-delta protein expressions were not different. After then, we analyzed the expression of several inflammasome-related mRNAs upon activation with PA and/or LPS. PA and LPS together elicited the mRNA expression of NLRP3, NRLP6, and NLRP10 (all $P < 0.05$) (Figure 3). We next tested whether GW501516 could prevent the overexpression of such inflammasome components and the downstream pro-inflammatory signals. As shown in Figure 4, treatment with 10 μmol/L GW501516 reduced the PA and LPS-induced mRNA expression of NLRP3, NRLP6, and NLRP10 (all $P < 0.05$). GW501516 also reduced the expression of caspase-1 and IL-1β mRNA in a dose-dependent manner (all $P < 0.05$) (Figure 4D and E). In concordance with mRNA expression, the presence of GW501516 reduced the PA and LPS-induced production of caspase-1 and IL-1β protein expression (Figure 4F).

### *Effects of GW501516 on hepatic steatosis in mice fed the HFD*

Histological images of liver pathology were obtained. Liver sections were stained with HE and Oil Red O. In mice fed the standard diet, there was no detectable fatty change in the microscopic image. By contrast, relative to the control group, mice on a 12-wk HFD with or without LPS injection developed extensive macrovesicular steatosis (0.0 *vs* 17.0% ± 5.7% and 16.4% ± 14.7%, all $P < 0.05$) and inflammation around the perisinusoidal area. Ballooned hepatocytes and fibrosis were not observed in any study group. In mice that were administrated GW501516, the intensity of fat accumulation (2.7% ± 2.6%) and NAS (0.5) were significantly decreased ($P < 0.05$) compared to those of the HFD + LPS group (16.4% ± 14.7% and 1.8%, respectively) (Figure 5A, B and C).

### *Effects of GW501516 on phosphorylation of AMPKα and ROS generation in mice and HepG2 cells*

Oxidative stress is thought to play a role in the pathogenesis of NASH, and ROS are essential for inflammasome activation[7,14]. A large body of evidence indicates that AMP-activated protein kinase (AMPK) is an essential regulator of fatty acid metabolism[21], and suppresses ROS generation by regulating intracellular nicotinamide adenine dinucleotide phosphate (NADPH) production[18,24]. In the *in vivo* study, levels of MDA, a product of lipid peroxidation, in liver homogenates, there was no statistically significant difference in four groups (Figure 6A). We also examined the effect of GW501516 on AMPK activation. As the activity of AMPK correlates with phosphorylation at Thr-172, the activation of AMPK was assessed by determining phosphorylation of AMPKα. As shown in Figure 6B, treatment with GW501516 increased the levels of AMPKα phosphorylation in mice fed the HFD with LPS injection by 1.6-fold compared to the control group.

In the *in vitro* study, the effect of GW501516 on PA and LPS-induced ROS production was investigated using DCFDA to detect cellular ROS levels. As illustrated in Figure 7A, incubating HepG2 cells with LPS and LPS + PA induced a significant increase in the cellular ROS level at 16 h (all $P < 0.05$ *vs* control group). Treatment with GW501516 (1 μmol/L and 10 μmol/L) attenuated the ROS generation in the PA- and LPS-stimulated HepG2 cells (all $P < 0.05$ *vs* PA + LPS group). In addition, we assessed AMPK

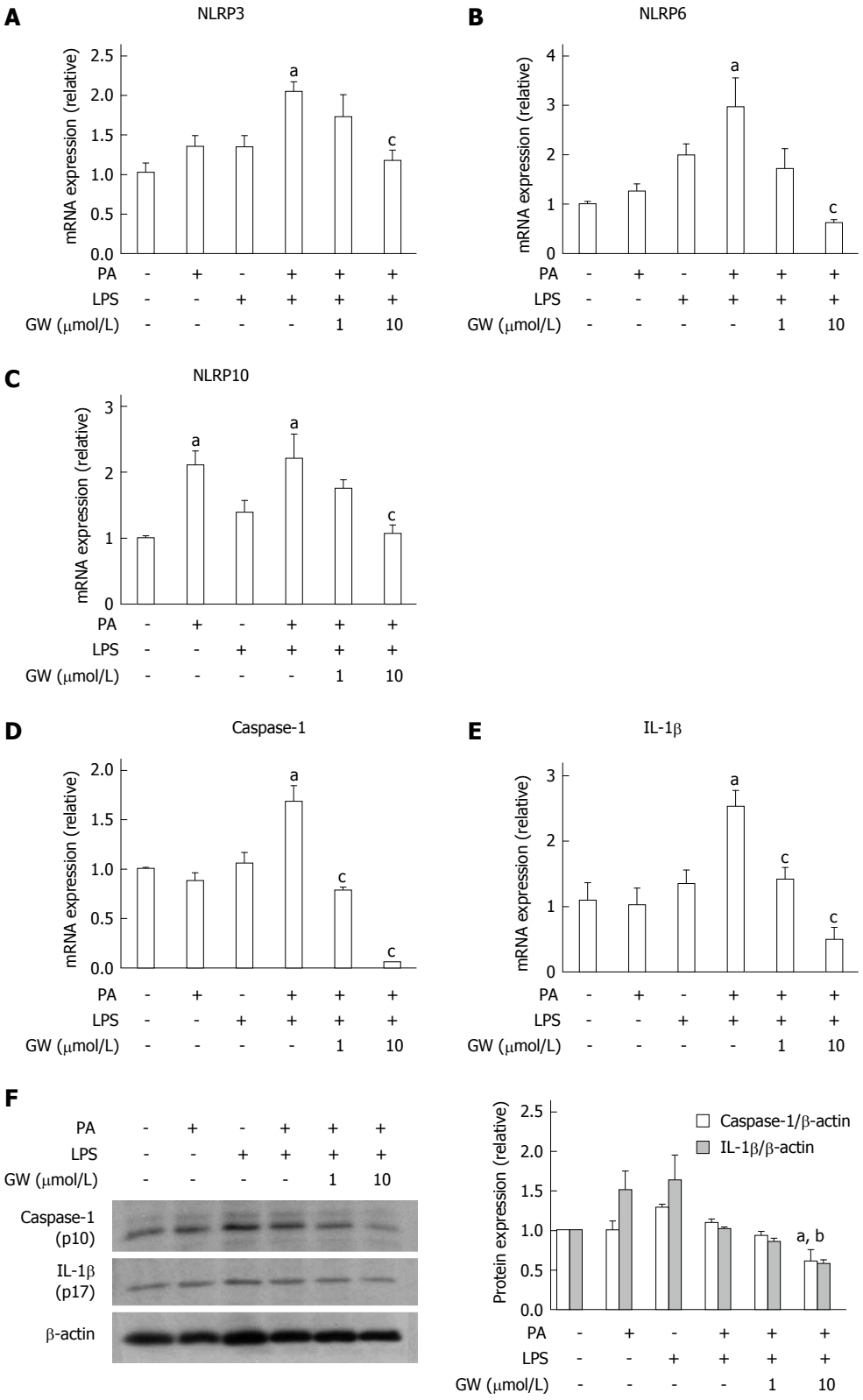
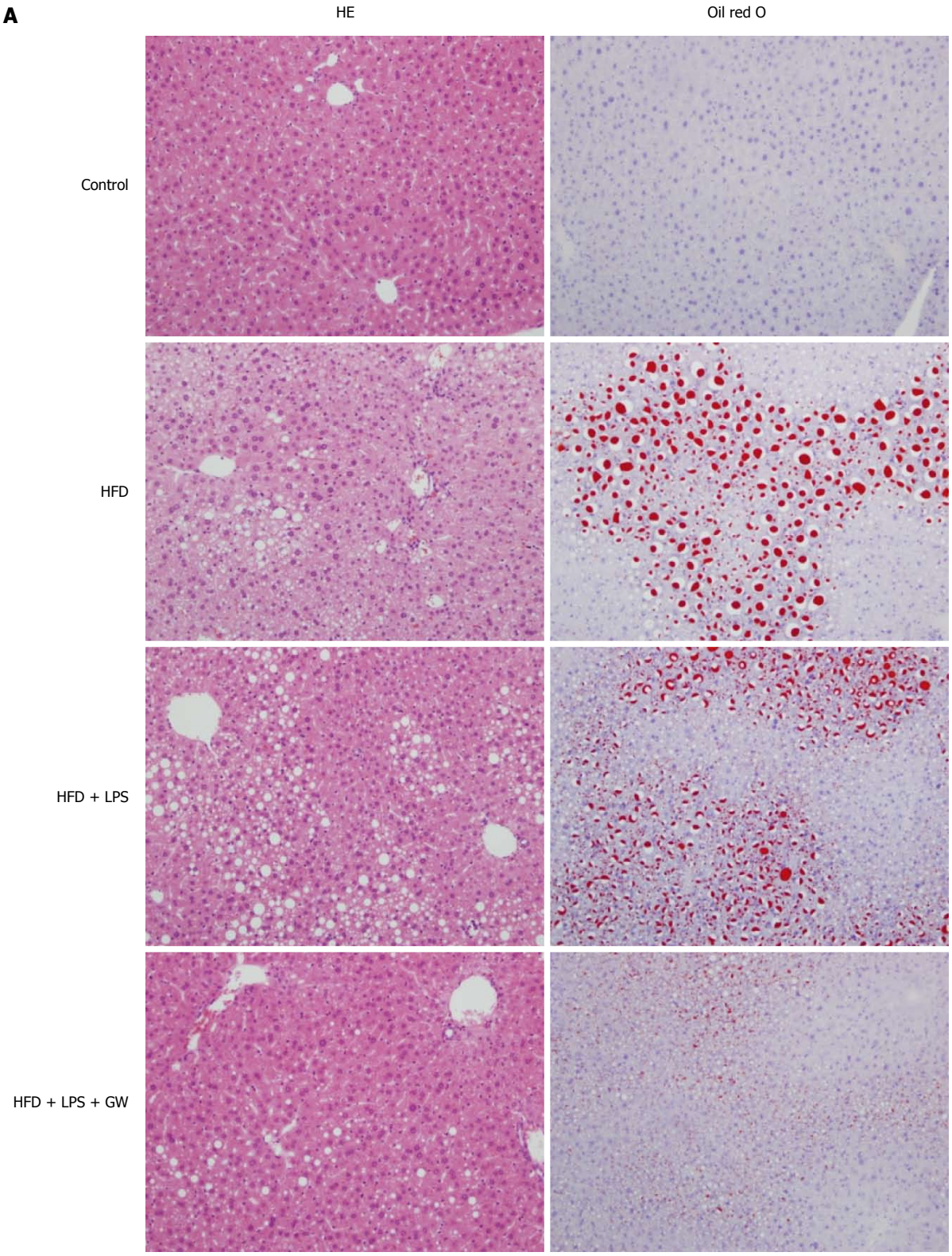



**Figure 4 Effects of GW501516 on palmitic acid- and lipopolysaccharide-induced inflammasome and pro-inflammatory cytokine in HepG2 cells.** Relative mRNA levels of NLRP3 (A), NLRP6 (B), NLRP10 (C), caspase-1 (D), and IL-1β (E) in HepG2 cells were analyzed by RT-PCR. GW501516 inhibited PA- and LPS-induced mRNA expression of several inflammasome components, caspase-1, and IL-1β. Representative immunoblot and graphic presentation show protein levels of caspase-1 and IL-1β (F) in HepG2 cells. PA and LPS tended to elicit caspase-1 and IL-1β release, and GW501516 reduced this processing significantly. Data are expressed as the mean ± SD. $^{a}P < 0.05$ *vs* control cells, $^{c}P < 0.05$ and $^{b}P < 0.01$ *vs* PA + LPS-treated cells.


A
HE
Oil red O
Control
HFD
HFD + LPS
HFD + LPS + GW

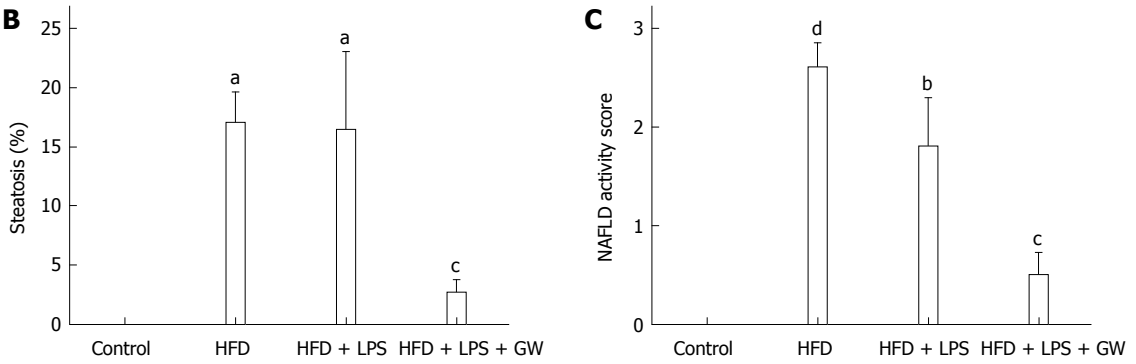



**Figure 5 Histopathological features of livers in mice fed a high fat diet with or without lipopolysaccharide injection and GW501516 treatment.** A: Hematoxylin and eosin (HE) staining and Oil Red O staining of hepatic lipid accumulation (magnification × 100). In mice fed an HFD, moderate macrovesicular steatosis (17.0%) and inflammatory cell infiltration were observed compared with the control group. The macrovesicular steatosis was improved to 2.7% following GW501516 treatment; B: Histogram of the percentage of hepatocytes showing macrovesicular fatty change; C: Nonalcoholic fatty liver disease (NAFLD) activity score. The NAFLD activity score in the GW501516-treated group was significantly lower than that in the HFD + LPS group (1.8 *vs* 0.5, $P < 0.05$). Data are expressed as the mean ± standard deviation (SD) (5-6 mice per group). $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{d}P < 0.001$ *vs* control group. $^{c}P < 0.05$ *vs* the HFD + LPS group.

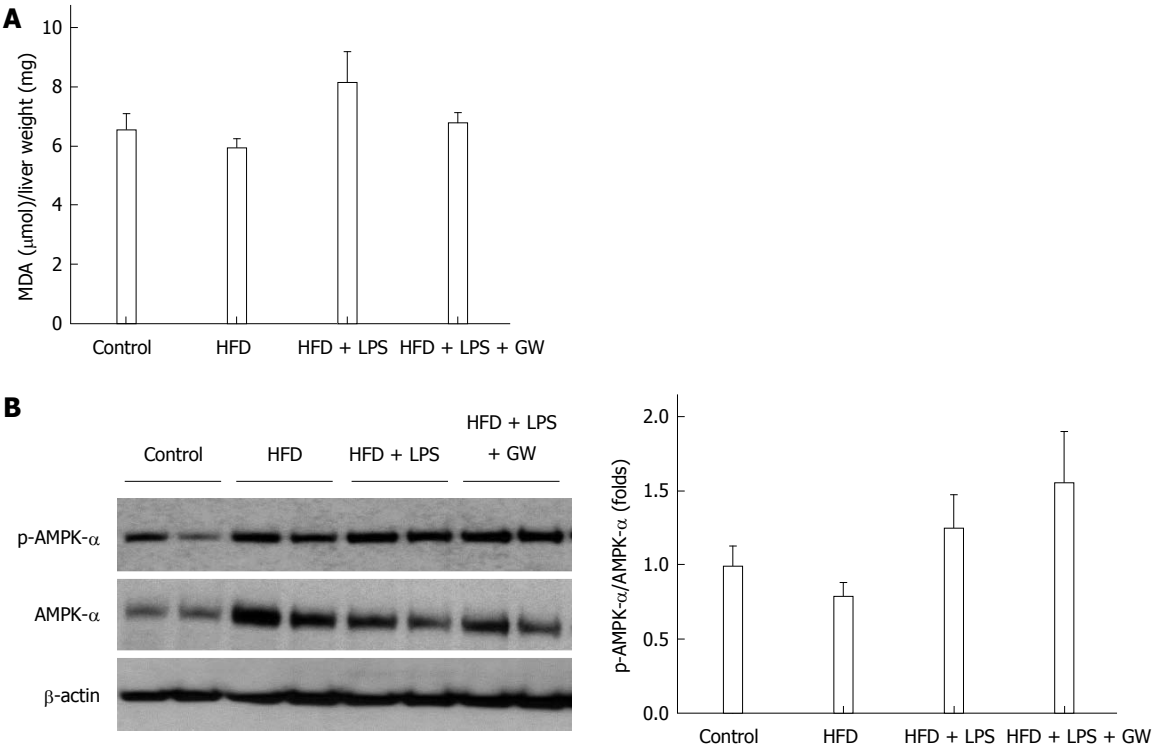



**Figure 6 Effects of GW501516 on lipid peroxidation and associated molecular pattern-activated protein kinase-α phosphorylation in mice fed a high fat diet.** Liver malondialdehyde (MDA) levels (A) showed a slight increase in the HFD + lipopolysaccharide (LPS) group without statistical significance; B: Representative immunoblot and graphic presentation of p-AMPK-α and total AMPK-α protein levels in the livers of mice are shown. Treatment of GW501516 enhanced phosphorylation of AMPK-α in mice fed an HFD with LPS injection. Data are expressed as the mean ± SD (5-6 mice per group).

activation with PA and LPS. PA or LPS alone had little effect on phosphorylation of the AMPKα subunit. However, adding GW501516 (1 μmol/L and 10 μmol/L) substantially increased phosphorylation of the AMPKα subunit in HepG2 cells (2.3- and 2.2-fold *vs* control group) (Figure 7B). These *in vivo* and *in vitro* results demonstrated that GW501516 could suppress hepatic oxidative stress and enhance phosphorylation of the AMPKα under high fat conditions.

## DISCUSSION

Although inflammasome is known to have an important role in metabolic syndrome such as obesity and diabetes, there is few study to assess its role in the pathogenesis of NAFLD/NASH and the therapeutic implication of the PPAR-δ activator on inflammasome activation in NAFLD model.

The results of our study suggested that IL-1β,

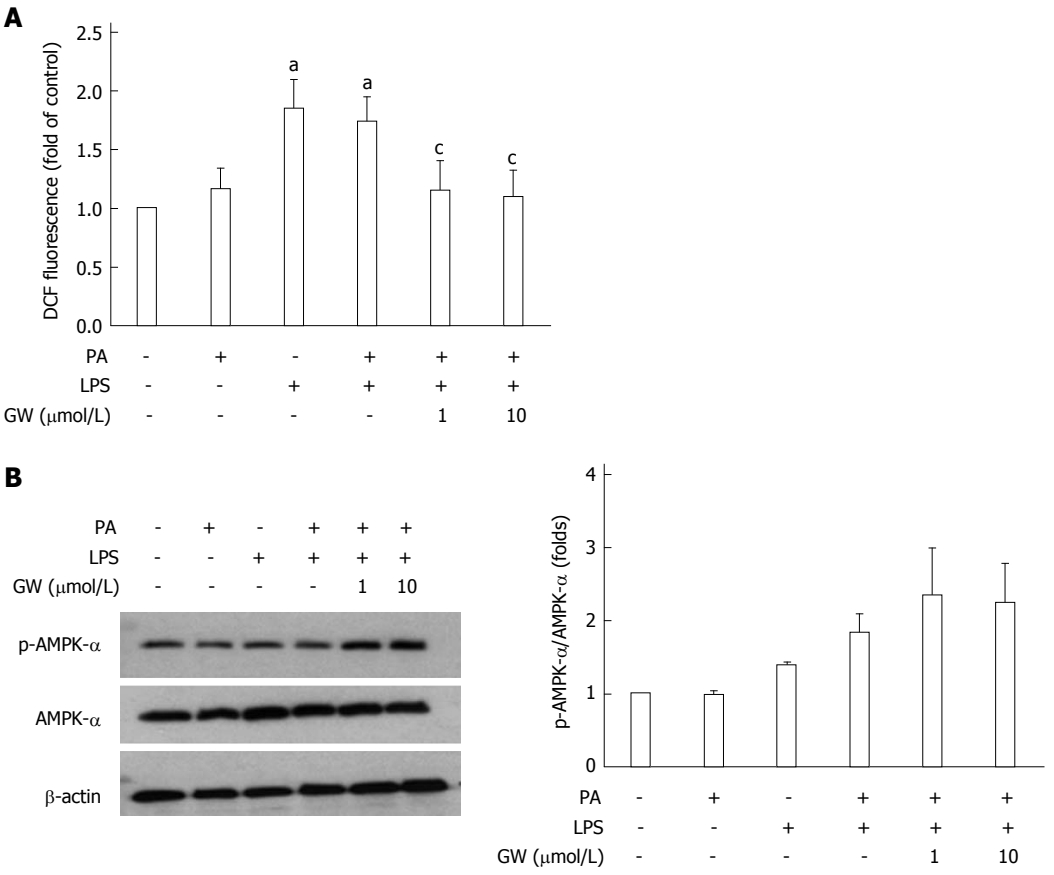



**Figure 7 Effects of GW501516 on reactive oxygen species production and associated molecular pattern-activated protein kinase-α phosphorylation in HepG2 cells.** Intracellular ROS production was quantified using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) (A). GW501516 inhibited lipopolysaccharide (LPS)- and palmitic acid (PA)-induced ROS generation. Representative immunoblot and graphic presentation of p-AMPK-α and total AMPK-α protein levels in HepG2 cells are shown (B). GW501516 tended to increase the phosphorylation of AMPK-α in PA- and LPS-treated cells. Data are expressed as the mean ± SD (5-6 mice per group). $^{a}P < 0.05$ *vs* control cells, $^{c}P < 0.05$ *vs* PA + LPS-treated cells.

caspase, NLRP6 and NLRP10, as well as NLRP3, were activated in NAFLD models and PPAR-δ activator GW501516 has the ability to reduce hepatic steatosis, inflammation, and oxidative stress in mice fed with HFD. Also, these results were confirmed in the *in vitro* models.

A recent study demonstrated that NLRP6 is important in the self-renewal and integrity of the intestinal epithelium[25]. NLRP6 seemed to negatively regulate the progression from NAFLD to NASH by preventing the increase in colitogenic bacteria, indicating a role for NLRP6 in the pathogenesis of NAFLD[17,26]. On the other hand, the function of NLRP10 remains largely uncharacterized. With the exception of the present study, few studies have described the association between NLRP10 inflammasome and NAFLD. Further research is required into the exact mechanisms of how NLRP6 or NLRP10 functions in NAFLD.

To date, NLRP3 is the most widely studied inflammasome linked to obesity, insulin resistance, and atherosclerosis[15-18]. Excessive formation of ROS, which results in oxidative stress, is the central and common element for activating the NLRP3 inflammasome[14]. In addition, IL-1β, which is stimulated by islet amyloid polypeptide, promotes β-cell dysfunction and cell death, linking NLPR3 activation to insulin resistance[27]. Therefore, the role of inflammasome in the pathogenesis of NAFLD, the hepatic manifestation of metabolic syndrome, has been receiving increased attention.

In the present study, we showed that several inflammasomes, including NLRP3, NRLP6, and NLRP10, were activated in parallel with the production of ROS and the overexpression of caspase-1 and IL-1β in HepG2 cells after stimulation with PA and LPS. Overproduction of these pro-inflammatory cytokines was also confirmed in mice fed the HFD with LPS injection. These results are consistent with those from previous diet-induced NASH models including the methionine-choline-deficient (MCD) diet[28], the HFD [18], and the choline-deficient amino acid-defined (CDAA) diet[29].

PPAR-δ is the only subtype in the PPAR subfamily of nuclear receptors that is not a target for currently used drugs. Several studies have revealed that PPAR-δ activation exerts many metabolic effects, including reducing hepatic glucose production, increasing fatty acid catabolism in adipose tissue and muscle, and lowering the inflammatory status[19-21,30,31]. Until now,

studies on the relationship between PPAR-δ activation and inflammasome have been scarce. In a recent study, the PPAR-δ agonist GW0742 was shown to attenuate the renal dysfunction and inflammation caused by chronic high-fructose corn syrup (HFCS-55) exposure by preventing activation of the NLRP3 inflammasome in the kidney[32]. In the present study, the PPAR-δ agonist GW501516 was found to reduce the activation of inflammasome as well as the overproduction of pro-inflammatory cytokines in HepG2 cells and the livers of mice. Moreover, GW501516 alleviated hepatic steatosis *in vivo.*

AMPK is a key metabolic regulator in cellular and organismal survival owing to its ability to maintain metabolic homeostasis; it is an essential mediator for fatty acid metabolism[18,21,33]. Several studies have demonstrated that PPAR-δ prevents the downregulation of AMPK[21,30]. In this study, although GW501516 treatment did not have an influence on the lipid profile in mice, it did increase phosphorylation of the AMPK-α subunit, both *in vivo* and *in vitro*. AMPK also has an anti-inflammatory effect and is associated with oxidative stress *via* suppression of ROS production[18,24]. The current study also demonstrated, for the first time, that the PPAR-δ agonist suppressed the ROS production induced by PA and LPS in the hepatocyte cell line. In immune cells such as macrophages, the AMPK-ROS signaling pathway is known to be associated with the activation of the NLRP3 inflammasome[18]. The current study may provide a clue into the complex role of PPAR-δ in metabolism, and suggests that the negative effect of PPAR-δ agonist on inflammasome activation in hepatocytes may be associated with the AMPK-ROS signaling pathway.

In the present study, we could not demonstrate statistical significant positive effect of PPAR-δ activator on insulin resistance and hyperlipidemia. Consistent with these findings, PPAR-δ protein expression were not different in controls and PA treatment with or without GW501516. The lipid-lowering and anti-diabetic effects of PPAR-δ, as well as its liver-protective effect, are already well documented[21,34-36]. In recent human clinical trials, 8 wk of therapy with MBX-8025 and GFT505, which are a novel PPAR-δ agonist and a dual PPAR-α/PPAR-δ agonist, respectively, significantly improved the plasma lipid profile, insulin resistance, and, interestingly, also decreased liver enzyme levels[34,37]. In our study, the treatment duration of GW501516 in mice was relatively short (3 wk), which may explain the insufficient effect observed on hyperlipidemia and glucose intolerance. Also, increasing oxidative capacity of muscle cells without provoking insulin sensitivity might supports our findings because PPAR-δ is highly expressed in the skeletal muscle, heart, and pancreatic β-cells, as well as in the liver[38]. Future challenges in determining the complex mechanism and cross-talk of PPAR-δ in different tissues and cell types are expected.

Recently, accumulating evidence has shown that bacterial endotoxins play a key role in the pathogenesis of NASH[28,39,40]. LPS, as an exogenous ligand for Toll-like receptor 4 (TLR4), may be capable of stimulating inflammasome expression, cytokine production, and the accumulation of inflammatory cells[28,39]. Therefore, we expected that the chronic exposure of low-dose LPS would lead to liver inflammation and fibrosis in mice fed an HFD. As for our study, we were decided to inject non-lethal dose of LPS which can reflect low level of endotoxemia of NAFLD. In our animal model, mice fed the HFD with LPS injection developed hepatic steatosis, although it failed to produce the severe form of NASH characterized by balloon degeneration and severe inflammation. However, unlike the MCD diet model[41], HFD with LPS injection resulted in weight gain and glucose intolerance, and therefore might more closely reflect the characteristics of human NAFLD. Future studies using endotoxins as HFD-enhancing factors in the murine NAFLD model are required to support the results of this study.

In conclusion, the results from our *in vivo* and *in vitro* studies demonstrate that the PPAR-δ agonist GW501516 suppresses the activation of inflammasome and reduces IL-1β levels, possibly by modulation of AMPK phosphorylation and decreased production of ROS. This anti-inflammatory effect might be associated with improvement of hepatic steatosis in mice. The targeting of inflammasome by the PPAR-δ agonist may have therapeutic implications for the treatment of NAFLD.

## COMMENTS

### Background

Nonalcoholic fatty liver disease (NAFLD) is one of the most common disease of the liver. Although thiazolidinediones and antioxidants such as vitamin E have been evaluated in several clinical trials, few pharmacological treatments can be recommended at present. Inflammasome is a large, intracellular multi-protein complex that is a sensor of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) that govern the cleavage of pro-inflammatory cytokines such as pro-interleukin (IL)-1β and pro-IL-18. Previous studies have shown that NLRP3 inflammasome is involved in the pathogenesis of obesity-induced inflammation, insulin resistance, and development of type 2 diabetes. However, the role of inflammasome in the pathogenesis of NAFLD/NASH has not been elucidated.

### Research frontiers

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily, and three isotypes exist in mammals. It has become increasingly evident that PPAR-δ is also an important metabolic regulator, as its activation enhances fatty acid oxidation, improves glucose homeostasis, and attenuates macrophage inflammatory responses. Current research hot spot is to whether the treatment with the PPAR-δ agonist could ameliorate high fat-induced inflammasome activation and the inflammatory response using *in vivo* and *in vitro* NAFLD models.

### Innovative and breakthroughs

Until now, studies on the relationship between PPAR-δ activation and inflammasome have been scarce. In a recent study, the PPAR-δ agonist GW0742 was shown to attenuate the renal dysfunction and inflammation caused by chronic high-fructose corn syrup-55 exposure by preventing activation of the NLRP3 inflammasome in the kidney. To investigate whether

the treatment with the PPAR-δ agonist could ameliorate high fat-induced inflammasome activation and the inflammatory response in the liver, the authors were using *in vivo* and *in vitro* NAFLD models. This study showed that PPAR-δ agonist GW501516 was found to reduce the activation of inflammasome as well as the overproduction of pro-inflammatory cytokines in HepG2 cells and the livers of mice. Moreover, GW501516 alleviated hepatic steatosis *in vivo*.

### Applications

The study results suggested that the PPAR-δ agonist GW501516 suppresses the activation of inflammasome and reduces IL-1β levels in the liver, possibly by modulation of AMPK phosphorylation and decreased production of ROS. This anti-inflammatory effect might be associated with improvement of hepatic steatosis in mice. The targeting of inflammasome by the PPAR-δ agonist may have therapeutic implications for the treatment of NAFLD.

### Terminology

NAFLD is a spectrum of disease entity including simple steatosis, steatosis with inflammation, fibrosis and cirrhosis. Inflammasome is a large, intracellular multi-protein complex that is a sensor of PAMPs or DAMPs that govern the cleavage of pro-inflammatory cytokines such as pro-IL-1β and pro-IL-18.

### Peer-review

The authors studied effects of PPAR-δ activator; GW501516 on inflamasome pathway in mouse model of NASH (high fat diet, LPS/PA) and HepG2 cells culture. They show that the beneficial role in mouse liver was through increased NLRP3-10 in HepG2 cells.

## REFERENCES


1 **Vernon G**, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharmacol Ther* 2011; **34**: 274-285 [PMID: 21623852 DOI: 10.1111/j.1365-2036.2011.04724.x]

2 **Vuppalanchi R**, Chalasani N. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: Selected practical issues in their evaluation and management. *Hepatology* 2009; **49**: 306-317 [PMID: 19065650 DOI: 10.1002/hep.22603]

3 **Musso G**, Gambino R, Cassader M, Pagano G. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Ann Med* 2011; **43**: 617-649 [PMID: 21039302 DOI: 10.3109/07853890.2010.518623]

4 **Abenavoli L**, Milic N, Peta V, Alfieri F, De Lorenzo A, Bellentani S. Alimentary regimen in non-alcoholic fatty liver disease: Mediterranean diet. *World J Gastroenterol* 2014; **20**: 16831-16840 [PMID: 25492997 DOI: 10.3748/wjg.v20.i45.16831]

5 **Farrell GC**, van Rooyen D, Gan L, Chitturi S. NASH is an Inflammatory Disorder: Pathogenic, Prognostic and Therapeutic Implications. *Gut Liver* 2012; **6**: 149-171 [PMID: 22570745 DOI: 10.5009/gnl.2012.6.2.149]

6 **Neuschwander-Tetri BA**. Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. *Hepatology* 2010; **52**: 774-788 [PMID: 20683968 DOI: 10.1002/hep.23719]

7 **Rolo AP**, Teodoro JS, Palmeira CM. Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. *Free Radic Biol Med* 2012; **52**: 59-69 [PMID: 22064361 DOI: 10.1016/j.freeradbiomed.2011.10.003]

8 **Carabelli J**, Burgueño AL, Rosselli MS, Gianotti TF, Lago NR, Pirola CJ, Sookoian S. High fat diet-induced liver steatosis promotes an increase in liver mitochondrial biogenesis in response to hypoxia. *J Cell Mol Med* 2011; **15**: 1329-1338 [PMID: 20629985 DOI: 10.1111/j.1582-4934.2010.01128.x]

9 **Musso G**, Gambino R, Cassader M, Pagano G. A meta-analysis of randomized trials for the treatment of nonalcoholic fatty liver disease. *Hepatology* 2010; **52**: 79-104 [PMID: 20578268 DOI: 10.1002/hep.23623]

10 **Abenavoli L**, Greco M, Nazionale I, Peta V, Milic N, Accattato F, Foti D, Gulletta E, Luzza F. Effects of Mediterranean diet supplemented with silybin-vitamin E-phospholipid complex in overweight patients with non-alcoholic fatty liver disease. *Expert Rev Gastroenterol Hepatol* 2015; **9**: 519-527 [PMID: 25617046 DOI: 10.1586/17474124.2015.1004312]

11 **Rosselli MS**, Burgueño AL, Carabelli J, Schuman M, Pirola CJ, Sookoian S. Losartan reduces liver expression of plasminogen activator inhibitor-1 (PAI-1) in a high fat-induced rat nonalcoholic fatty liver disease model. *Atherosclerosis* 2009; **206**: 119-126 [PMID: 19230890 DOI: 10.1016/j.atherosclerosis.2009.01.026]

12 **Szabo G**, Csak T. Inflammasomes in liver diseases. *J Hepatol* 2012; **57**: 642-654 [PMID: 22634126 DOI: 10.1016/j.jhep.2012.03.035]

13 **Kubes P**, Mehal WZ. Sterile inflammation in the liver. *Gastroenterology* 2012; **143**: 1158-1172 [PMID: 22982943 DOI: 10.1053/j.gastro.2012.09.008]

14 **Martinon F**. Signaling by ROS drives inflammasome activation. *Eur J Immunol* 2010; **40**: 616-619 [PMID: 20201014 DOI: 10.1002/eji.200940168]

15 **Stienstra R**, Tack CJ, Kanneganti TD, Joosten LA, Netea MG. The inflammasome puts obesity in the danger zone. *Cell Metab* 2012; **15**: 10-18 [PMID: 22225872 DOI: 10.1016/j.cmet.2011.10.011]

16 **Vandanmagsar B**, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL, Ravussin E, Stephens JM, Dixit VD. The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nat Med* 2011; **17**: 179-188 [PMID: 21217695 DOI: 10.1038/nm.2279]

17 **Henao-Mejia J**, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, Thaiss CA, Kau AL, Eisenbarth SC, Jurczak MJ, Camporez JP, Shulman GI, Gordon JI, Hoffman HM, Flavell RA. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature* 2012; **482**: 179-185 [PMID: 22297845 DOI: 10.1038/nature10809]

18 **Wen H**, Gris D, Lei Y, Jha S, Zhang L, Huang MT, Brickey WJ, Ting JP. Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. *Nat Immunol* 2011; **12**: 408-415 [PMID: 21478880 DOI: 10.1038/ni.2022]

19 **Poulsen LI**, Siersbæk M, Mandrup S. PPARs: fatty acid sensors controlling metabolism. *Semin Cell Dev Biol* 2012; **23**: 631-639 [PMID: 22273692 DOI: 10.1016/j.semcdb.2012.01.003]

20 **Tailleux A**, Wouters K, Staels B. Roles of PPARs in NAFLD: potential therapeutic targets. *Biochim Biophys Acta* 2012; **1821**: 809-818 [PMID: 22056763 DOI: 10.1016/j.bbalip.2011.10.016]

21 **Barroso E**, Rodríguez-Calvo R, Serrano-Marco L, Astudillo AM, Balsinde J, Palomer X, Vázquez-Carrera M. The PPARβ/δ activator GW501516 prevents the down-regulation of AMPK caused by a high-fat diet in liver and amplifies the PGC-1α-Lipin 1-PPARα pathway leading to increased fatty acid oxidation. *Endocrinology* 2011; **152**: 1848-1859 [PMID: 21363937 DOI: 10.1210/en.2010-1468]

22 **Buchner DA**, Burrage LC, Hill AE, Yazbek SN, O'Brien WE, Croniger CM, Nadeau JH. Resistance to diet-induced obesity in mice with a single substituted chromosome. *Physiol Genomics* 2008; **35**: 116-122 [PMID: 18628339 DOI: 10.1152/physiolgenomics.00033.2008]

23 **Kleiner DE**, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313-1321 [PMID: 15915461 DOI: 10.1002/hep.20701]

24 **Wang S**, Zhang M, Liang B, Xu J, Xie Z, Liu C, Viollet B, Yan D, Zou MH. AMPKalpha2 deletion causes aberrant expression and activation of NAD(P)H oxidase and consequent endothelial dysfunction in vivo: role of 26S proteasomes. *Circ Res* 2010; **106**: 1117-1128 [PMID: 20167927 DOI: 10.1161/CIRCRESAHA.109.212530]

25 **Normand S**, Delanoye-Crespin A, Bressenot A, Huot L, Grandjean T, Peyrin-Biroulet L, Lemoine Y, Hot D, Chamaillard M. Nod-

like receptor pyrin domain-containing protein 6 (NLRP6) controls epithelial self-renewal and colorectal carcinogenesis upon injury. *Proc Natl Acad Sci USA* 2011; **108**: 9601-9606 [PMID: 21593405 DOI: 10.1073/pnas.1100981108]

26 **Kempster SL**, Belteki G, Forhead AJ, Fowden AL, Catalano RD, Lam BY, McFarlane I, Charnock-Jones DS, Smith GC. Developmental control of the Nlrp6 inflammasome and a substrate, IL-18, in mammalian intestine. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G253-G263 [PMID: 21088234 DOI: 10.1152/ajpgi.00397.2010]

27 **Masters SL**, Dunne A, Subramanian SL, Hull RL, Tannahill GM, Sharp FA, Becker C, Franchi L, Yoshihara E, Chen Z, Mullooly N, Mielke LA, Harris J, Coll RC, Mills KH, Mok KH, Newsholme P, Nuñez G, Yodoi J, Kahn SE, Lavelle EC, O'Neill LA. Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1β in type 2 diabetes. *Nat Immunol* 2010; **11**: 897-904 [PMID: 20835230 DOI: 10.1038/ni.1935]

28 **Csak T**, Ganz M, Pespisa J, Kodys K, Dolganiuc A, Szabo G. Fatty acid and endotoxin activate inflammasomes in mouse hepatocytes that release danger signals to stimulate immune cells. *Hepatology* 2011; **54**: 133-144 [PMID: 21488066 DOI: 10.1002/hep.24341]

29 **Miura K**, Yang L, van Rooijen N, Brenner DA, Ohnishi H, Seki E. Toll-like receptor 2 and palmitic acid cooperatively contribute to the development of nonalcoholic steatohepatitis through inflammasome activation in mice. *Hepatology* 2013; **57**: 577-589 [PMID: 22987396 DOI: 10.1002/hep.26081]

30 **Coll T**, Alvarez-Guardia D, Barroso E, Gómez-Foix AM, Palomer X, Laguna JC, Vázquez-Carrera M. Activation of peroxisome proliferator-activated receptor-{delta} by GW501516 prevents fatty acid-induced nuclear factor-{kappa}B activation and insulin resistance in skeletal muscle cells. *Endocrinology* 2010; **151**: 1560-1569 [PMID: 20185762 DOI: 10.1210/en.2009-1211]

31 **Lee CH**, Olson P, Hevener A, Mehl I, Chong LW, Olefsky JM, Gonzalez FJ, Ham J, Kang H, Peters JM, Evans RM. PPARdelta regulates glucose metabolism and insulin sensitivity. *Proc Natl Acad Sci USA* 2006; **103**: 3444-3449 [PMID: 16492734 DOI: 10.1073/pnas.0511253103]

32 **Collino M**, Benetti E, Rogazzo M, Mastrocola R, Yaqoob MM, Aragno M, Thiemermann C, Fantozzi R. Reversal of the deleterious effects of chronic dietary HFCS-55 intake by PPAR-δ agonism correlates with impaired NLRP3 inflammasome activation. *Biochem Pharmacol* 2013; **85**: 257-264 [PMID: 23103566 DOI: 10.1016/j.bcp.2012.10.014]

33 **Sid B**, Verrax J, Calderon PB. Role of AMPK activation in oxidative cell damage: Implications for alcohol-induced liver disease. *Biochem Pharmacol* 2013; **86**: 200-209 [PMID: 23688501 DOI: 10.1016/j.bcp.2013.05.007]

34 **Bays HE**, Schwartz S, Littlejohn T, Kerzner B, Krauss RM, Karpf DB, Choi YJ, Wang X, Naim S, Roberts BK. MBX-8025, a novel peroxisome proliferator receptor-delta agonist: lipid and other metabolic effects in dyslipidemic overweight patients treated with and without atorvastatin. *J Clin Endocrinol Metab* 2011; **96**: 2889-2897 [PMID: 21752880 DOI: 10.1210/jc.2011-1061]

35 **Cariou B**, Zaïr Y, Staels B, Bruckert E. Effects of the new dual PPAR α/δ agonist GFT505 on lipid and glucose homeostasis in abdominally obese patients with combined dyslipidemia or impaired glucose metabolism. *Diabetes Care* 2011; **34**: 2008-2014 [PMID: 21816979 DOI: 10.2337/dc11-0093]

36 **Nagasawa T**, Inada Y, Nakano S, Tamura T, Takahashi T, Maruyama K, Yamazaki Y, Kuroda J, Shibata N. Effects of bezafibrate, PPAR pan-agonist, and GW501516, PPARdelta agonist, on development of steatohepatitis in mice fed a methionine- and choline-deficient diet. *Eur J Pharmacol* 2006; **536**: 182-191 [PMID: 16574099 DOI: 10.1016/j.ejphar.2006.02.028]

37 **Cariou B**, Hanf R, Lambert-Porcheron S, Zaïr Y, Sauvinet V, Noël B, Flet L, Vidal H, Staels B, Laville M. Dual peroxisome proliferator-activated receptor α/δ agonist GFT505 improves hepatic and peripheral insulin sensitivity in abdominally obese subjects. *Diabetes Care* 2013; **36**: 2923-2930 [PMID: 23715754 DOI: 10.2337/dc12-2012]

38 **de Lange P**, Lombardi A, Silvestri E, Goglia F, Lanni A, Moreno M. Peroxisome Proliferator-Activated Receptor Delta: A Conserved Director of Lipid Homeostasis through Regulation of the Oxidative Capacity of Muscle. *PPAR Res* 2008; **2008**: 172676 [PMID: 18815630 DOI: 10.1155/2008/172676]

39 **Kudo H**, Takahara T, Yata Y, Kawai K, Zhang W, Sugiyama T. Lipopolysaccharide triggered TNF-alpha-induced hepatocyte apoptosis in a murine non-alcoholic steatohepatitis model. *J Hepatol* 2009; **51**: 168-175 [PMID: 19446916 DOI: 10.1016/j.jhep.2009.02.032]

40 **Shanab AA**, Scully P, Crosbie O, Buckley M, O'Mahony L, Shanahan F, Gazareen S, Murphy E, Quigley EM. Small intestinal bacterial overgrowth in nonalcoholic steatohepatitis: association with toll-like receptor 4 expression and plasma levels of interleukin 8. *Dig Dis Sci* 2011; **56**: 1524-1534 [PMID: 21046243 DOI: 10.1007/s10620-010-1447-3]

41 **Takahashi Y**, Soejima Y, Fukusato T. Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World J Gastroenterol* 2012; **18**: 2300-2308 [PMID: 22654421 DOI: 10.3748/wjg.v18.i19.2300]

**P- Reviewer**: Abenavoli L, Balaban YH, Laguna JC, Pirola CJ
**S- Editor**: Qi Y **L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12800

*World J Gastroenterol* 2015 December 7; 21(45): 12800-12813
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

Basic Study

# Biliary tract external drainage protects against intestinal barrier injury in hemorrhagic shock rats

Lu Wang, Bing Zhao, Ying Chen, Li Ma, Er-Zhen Chen, En-Qiang Mao

Lu Wang, Bing Zhao, Ying Chen, Li Ma, Er-Zhen Chen, En-Qiang Mao, Department of Emergency Intensive Care Unit, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

Author contributions: Chen EZ and Mao EQ contributed equally to this work; Wang L, Chen EZ and Mao EQ wrote the paper; Wang L, Zhao B and Chen Y participated in the surgical procedure; Wang L designed the protocol and performed enzyme-linked immunosorbent assay and immunohistochemistry; Wang L and Ma L performed the statistical analysis; Zhao B and Chen Y carried out the Western blotting; Zhao B and Ma L performed the histologic analysis; Chen EZ and Mao EQ conceived and designed the study.

Supported by National Natural Science Foundation of China, No. 81171789.

Institutional review board statement: This study has been reviewed and approved by Institutional Review Board of Ruijin Hospital, Shanghai Jiao Tong University School of 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 Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China [IACUC protocol number: SYXK (Shanghai) 2011-0113].

Conflict-of-interest statement: We declare no conflicts of interest regarding this study.

Data sharing statement: No additional data are available.



Correspondence to: En-Qiang Mao, MD, PhD, Professor of Medicine, Department of Emergency Intensive Care Unit, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, 197 Ruijin Road Number Two, Shanghai 200025, China. maoeq@yeah.net
Telephone: +86-21-64370045
Fax: +86-21-64370045

Received: June 4, 2015
Peer-review started: June 6, 2015
First decision: July 10, 2015
Revised: July 20, 2015
Accepted: September 15, 2015
Article in press: September 15, 2015
Published online: December 7, 2015

## Abstract

**AIM:** To investigate the effects of biliary tract external drainage (BTED) on intestinal barrier injury in rats with hemorrhagic shock (HS).

**METHODS:** BTED was performed *via* cannula insertion into the bile duct of rats. HS was induced by drawing blood from the femoral artery at a rate of 1 mL/min until a mean arterial pressure (MAP) of 40 ± 5 mmHg was achieved. That MAP was maintained for 60 min. A total of 99 Sprague-Dawley rats were randomized into a sham group, an HS group and an HS + BTED group. Nine rats in the sham group were sacrificed 0.5 h after surgery. Nine rats in each of the HS and HS + BTED groups were sacrificed 0.5 h, 1 h, 2 h, 4 h and 6 h after resuscitation. Plasma tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and lipopolysaccharide (LPS) levels were analyzed using enzyme-linked immunosorbent assay. Plasma D-lactate levels were analyzed using colorimetry. The expression levels of occludin and claudin-1 in the ileum were analyzed using Western blot and immunohistochemistry. Histology of the ileum

was evaluated by hematoxylin and eosin staining.

**RESULTS:** Plasma TNF-α levels in the HS + BTED group decreased significantly compared with the HS group at 1 h and 6 h after resuscitation ($P < 0.05$). Plasma IL-6 levels in the HS + BTED group decreased significantly compared with the HS group at 0.5 h, 1 h and 2 h after resuscitation ($P < 0.05$). Plasma D-lactate and LPS levels in the HS + BTED group decreased significantly compared with the HS group at 6 h after resuscitation ($P < 0.05$). The expression levels of occludin in the HS + BTED group increased significantly compared with the HS group at 4 h and 6 h after resuscitation ($P < 0.05$). The expression levels of claudin-1 in the HS + BTED group increased significantly compared with the HS group at 6 h after resuscitation ($P < 0.05$). Phenomena of putrescence and desquamation of epithelial cells in the ileal mucosa were attenuated in the HS + BTED group. Ileal histopathologic scores in the HS + BTED group decreased significantly compared with the HS group at 2 h, 4 h and 6 h after resuscitation ($P < 0.05$).

**CONCLUSION:** BTED protects against intestinal barrier injury in HS rats.

**Key words:** Hemorrhagic shock; Biliary tract external drainage; Occludin; Claudin-1; D-lactate



**Core tip:** Our previous studies demonstrated that biliary tract external drainage decreased proinflammatory cytokine production and relieved tissue damage in rat models of hemorrhagic shock. In this research, we found that biliary tract external drainage increased the expression levels of occludin and claudin-1 and decreased plasma D-lactate and lipopolysaccharide levels under hemorrhagic shock conditions. These results demonstrate that biliary tract external drainage protects against intestinal barrier injury in hemorrhagic shock rats.

Wang L, Zhao B, Chen Y, Ma L, Chen EZ, Mao EQ. Biliary tract external drainage protects against intestinal barrier injury in hemorrhagic shock rats. *World J Gastroenterol* 2015; 21(45): 12800-12813 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12800.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12800

## INTRODUCTION

Hemorrhagic shock (HS) induces gut barrier failure, which initiates a systemic inflammatory response[1]. Bile full of proinflammatory mediators enters into the gut following HS, which contributes to tissue injury in the intestine. On one hand, injured gut cells release a large number of inflammatory mediators that cause endothelial dysfunction and activate neutrophils. On the other hand, a large amount of lipopolysaccharide (LPS) is released by gut bacteria through the damaged intestinal barrier into the peripheral blood and may cause distant organ injury[2].

A widespread normal bacterial flora resides in the ileum. D-lactate is the end product of intestinal bacteria. It is neither produced nor metabolized by mammalian cells. During ischemia, as the normal mucosal barrier is damaged and permeability increases, a large amount of D-lactate is released through the damaged intestinal mucosa into the peripheral blood. Thus, D-lactate in peripheral blood can indicate damage situation of the intestinal barrier[3-6]. LPS produced by gut bacteria also enters into the bloodstream and spreads to the entire body under HS conditions. Therefore, the level of LPS in the blood also can reflect the degree of intestinal barrier damage[7-9].

Tight junction (TJ) proteins, including occludin, claudins, and cytoskeleton proteins, play critical roles in the maintenance of the intestinal barrier integrity[10]. Occludin was the first transmembrane TJ protein discovered[11]. Occludin plays a crucial role in the maintenance of epithelial TJs[12-14]. The absence of occludin increases the ion permeability of TJs and causes intestinal barrier dysfunction[15,16]. The claudin family confers barrier functions as constituents of TJ strands, and these proteins directly participate in the transport of materials across epithelia through paracellular pathways by adjusting the tightness and selectivity of TJ strands[17,18]. Claudins determine the paracellular ionic selectivity of the TJ because these proteins have two extracellular loops that display variability in the distribution and number of charged residues[19]. We examined the expression levels of claudin-1 in the ileum for claudin-1 is strongly expressed in rat ileum[20].

Organ function in shock patients with severe acute pancreatitis who accept biliary tract external drainage (BTED) (endoscopic naso-biliary drainage, cholecystostomy or gallbladder percutaneous catheter drainage) rapidly improves in clinical practice. Infection incidence and morbidity of multiple organ dysfunction syndrome (MODS) also significantly decrease. The amelioration of intestinal barrier function may play a vital role in this process. Previous studies also indicated that BTED eased damage of vital organs and improved the survival rate of shock rats[21,22]. However, studies on the relationship between BTED and intestinal barrier function are limited, and most of these studies focused on obstructive jaundice[23-26]. Therefore, we designed this study to observe changes in occludin and claudin-1 in the ileum and D-lactate, LPS, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels in plasma. We explored the effects of BTED on intestinal barrier in HS.

## MATERIALS AND METHODS

### *Ethics statement*

This study was carried out in strict accordance with the guidelines for the care and use of laboratory animals established by the Animal Use and Care Committee of the Shanghai Committee on Animal Care. Animal surgical procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Ruijin Hospital, Shanghai Jiao Tong University, Shanghai, China. The animal protocol was designed to minimize pain or discomfort to the animals.

### *Animal model*

Ninety-nine adult male Sprague-Dawley rats (250-300 g) were purchased from the Experimental Animal Center of Ruijin Hospital. The animals were acclimatized to laboratory conditions (25 ℃, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for one week prior to experimentation. Rats were randomly divided into 3 groups after adaptation: sham group, HS group, and HS + BTED group. Rats were fasted overnight before the experiment, but rats were allowed to drink water. Rats in the HS + BTED group were intraperitoneally anesthetized with 3% sodium pentobarbital (0.2 mL/100 g), and laparotomies were performed after shaving and sterilization. Catheters were placed in both femoral arteries for blood pressure measurement and blood withdrawal. The bile duct was exposed long enough for BTED. Rats were subjected to HS by slowly withdrawing blood at a rate of 1 mL/min until a mean arterial pressure (MAP) of 40 ± 5 mmHg was achieved. A catheter (inner diameter, 0.4 mm; outer diameter, 0.8 mm; length, 20 cm) was inserted into the bile duct. The distal end of the bile duct was ligated, and the catheter was passed through the rat flank to avoid bile passage into the gut and allow the external collection of bile. The abdomen was closed subsequently. An MAP of 40 ± 5 mmHg was maintained for 1 h. Rats were resuscitated using their shed blood and an equal volume of normal saline at the end of shock period. HS rats underwent pentobarbital anesthesia, laparotomy, vascular cannulation, blood withdrawal and suturing but no BTED. Sham rats underwent pentobarbital anesthesia, laparotomy, vascular cannulation and suturing, but no blood withdrawal or BTED. Nine rats in the sham group were sacrificed 0.5 h after surgery. Nine rats in the HS group and the HS + BTED group were sacrificed 0.5 h, 1 h, 2 h, 4 h and 6 h after resuscitation.

### *Enzyme-linked immunosorbent assay*

Plasma TNF-α, IL-6, and LPS levels were quantified using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. The absorbance from each sample was normalized for the actual concentration.

### *D-lactate colorimetric assay*

Plasma D-lactate levels were quantified using a D-lactate colorimetric assay kit according to the manufacturer's instructions. The absorbance from each sample was normalized for the D-lactate concentration.

### *Western blot analysis*

Intestinal mucosal scrapings from all animals were stored at -80 ℃ for Western blot analysis. RIPA lysis buffer and 5 × loading buffer were prepared. Briefly, samples were homogenized in RIPA lysis buffer. Tissues were frozen immediately in liquid nitrogen and placed in a mortar for pulverization. Total protein was extracted using tissue total protein lysis buffer, and protein concentration was measured using a BCA Protein Assay Kit.

Proteins were separated using SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidenedifluofide (PVDF) membranes. The blot was immune probed using primary antibody overnight at 4 ℃. Primary antibodies for Western blotting were a rabbit polyclonal antibody to occludin (1:250), a rabbit polyclonal antibody to claudin-1 (1:500) and a mouse monoclonal antibody to GAPDH (1:2000). The blots were incubated with an HRP-conjugated secondary antibody for 1 h at room temperature and reacted with an enhanced chemiluminescence substrate. The resulting chemiluminescence was recorded using an imaging system (Imagequant LAS 400, GE, United States). The enhanced chemiluminescence signals were digitized using Photoshop CS6 software (Adobe, United States) to quantify the expression levels of occludin and claudin-1. Relative occludin and claudin-1 protein expression was normalized to respective values for GAPDH, and the results are described as fold increases relative to baseline levels in negative control.

### *Immunohistochemistry*

The samples were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 4 μm. Sections were mounted onto APES-coated slides, deparaffinized, rehydrated, incubated in 3% hydrogen peroxide to quench any endogenous peroxidase activity, and washed with distilled water and PBS for 5 min. Sections were placed in 3% citrate buffer to repair antigens. The buffer was heated to a temperature of 92 ℃-98 ℃ using microwave, and the temperature was maintained for 10 min. Sections were cooled to room temperature. A 10% nonimmune goat serum was applied to eliminate nonspecific staining. Sections were incubated overnight at 4 ℃ with an optimally diluted rabbit polyclonal anti-rat occludin antibody (1:100) or rabbit polyclonal anti-rat claudin-1 antibody (1:100). The sections were washed with PBS and incubated with a broad-spectrum secondary antibody for 30 min, rewashed, and incubated with peroxidase-conjugated streptavidin for 15 min. Peroxidation activity was visualized by incubation with DAB solution.

The sections were counterstained with hematoxylin.

### *Hematoxylin and eosin staining*

The samples were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 4 μm. Sections were mounted onto APES-coated slides. After de-paraffinization and dehydration, the sections were stained with hematoxylin and eosin for microscopic examination. The severity of ileal injury was scored from 0 to 3 as follows: 0, normal (no damage); 1, mild (focal epithelial edema and necrosis); 2, moderate (diffuse swelling or necrosis of the villi); 3, severe (diffuse necrosis of the villi with evidence of neutrophil infiltration in the submucosa and/or hemorrhage). All evaluations were made on six fields per section and six sections under 100 × magnification[27,28].

### *Reagents*

The rat TNF-α ELISA kit was purchased from the MaibioCompany (MHK0008, Shanghai, China). The rat IL-6 ELISA kit was purchased from the MaibioCompany (MRK0004, Shanghai, China). The rat LPS ELISA kit was purchased from the CusabioCompany (CSB-E14247r, Wuhan, China). The D-lactate Colorimetric Assay Kit was purchased from the BioVision Company (K667-100, Milpitas, United States). RIPA lysis buffer, BCA Protein Assay Kit and 5 × loading buffer were purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). The rabbit polyclonal antibody to occludin was purchased from the Abcam Company (ab31721, Cambridge, MA, United States). The rabbit polyclonal antibody to claudin-1 was purchased from the Biorbyt Company (Ab-210, Cambridge, Cambridgeshire, United Kingdom). The mouse monoclonal antibody to GAPDH was purchased from the Abcam Company (ab8245, Cambridge, MA, United States). The enhanced chemiluminescence substrate was purchased from the ComWin Biotechnology Company (Beijing, China). The immunohistochemistry kit was purchased from the InvitrogenCompany (Frederick, United States).

### *Statistical analysis*

Data were analyzed using SPSS 16.0 software. All data are expressed as mean ± SE and compared using the unpaired Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

## RESULTS

### *Effect of BTED on plasma TNF-α levels*

Plasma TNF-α levels in the HS + BTED group showed no significant differences compared with the HS group at 0.5 h and 2 h after resuscitation. Plasma TNF-α levels in the HS + BTED group decreased significantly compared with the HS group at 1 h and 6 h after resuscitation ($P < 0.05$). Plasma TNF-α levels in the HS + BTED group increased significantly compared with the HS group at 4 h after resuscitation ($P < 0.05$) (Figure 1A).

### *Effect of BTED on plasma IL-6 levels*

Plasma IL-6 levels in the HS + BTED group showed no significant differences compared with the HS group at 6 h after resuscitation. Plasma IL-6 levels in the HS + BTED group decreased significantly compared with the HS group at 0.5 h, 1 h and 2 h after resuscitation ($P < 0.05$). Plasma IL-6 levels in the HS + BTED group increased significantly compared with the HS group at 4 h after resuscitation ($P < 0.05$) (Figure 1B).

### *Effect of BTED on plasma LPS levels*

Plasma LPS levels in the HS + BTED group showed no significant differences compared with the HS group at 0.5 h, 1 h and 4 h after resuscitation. Plasma LPS levels in the HS + BTED group increased significantly compared with the HS group at 2 h after resuscitation ($P < 0.05$). Plasma LPS levels in the HS + BTED group decreased significantly compared with the HS group at 6 h after resuscitation ($P < 0.05$) (Figure 1C).

### *Effect of BTED on plasma D-lactate levels*

Plasma D-lactate levels in the HS + BTED group showed no significant differences compared with the HS group at 0.5 h after resuscitation. Plasma D-lactate levels in the HS + BTED group increased significantly compared with the HS group at 1 h, 2 h and 4 h after resuscitation ($P < 0.05$). Plasma D-lactate levels in the HS + BTED group decreased significantly compared with the HS group at 6 h after resuscitation ($P < 0.05$) (Figure 1D).

### *Western blot analysis of expression of occludin and claudin-1 in the ileum*

The expression levels of occludin in the ileum of the HS + BTED group did not show significant differences compared with the HS group at 0.5 h, 1 h and 2 h after resuscitation. The expression levels of occludin in the ileum in the HS + BTED group increased significantly compared with the HS group at 4 h and 6 h after resuscitation ($P < 0.05$) (Figure 2A and B). The expression levels of claudin-1 in the ileum in the HS + BTED group showed no significant differences compared with the HS group at 0.5 h, 1 h, 2 h and 4 h after resuscitation. The expression levels of claudin-1 in the ileum in the HS + BTED group increased significantly compared with the HS group at 6 h after resuscitation ($P < 0.05$) (Figure 2A and C).

### *Immunohistochemical analysis of expression of occludin in the ileum*

Occludin in the sham group was expressed as cyto-plasmic granules located mostly at the apical part of epithelial cells. The total epithelial cells lining the villi exhibited positive immunostaining for occludin in the sham group (Figure 3A). There was loss of occludin

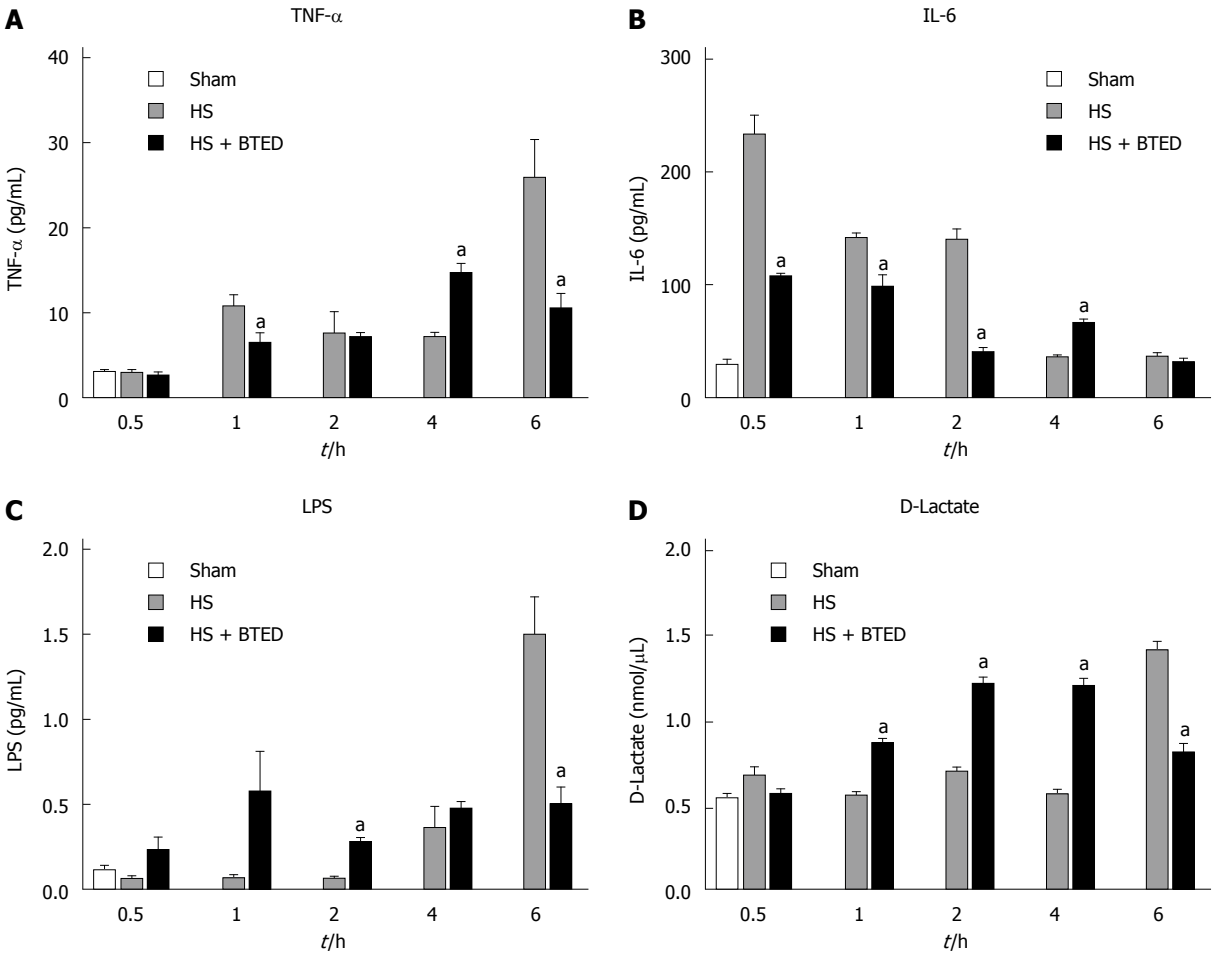



**Figure 1 Plasma tumor necrosis factor-α (A), interleukin-6 (B), lipopolysaccharide (C), and D-lactate levels (D).** Results are presented as mean ± SE ($n$ = 6). [a]$P$ < 0.05, *vs* the HS group at the same time point. HS: Hemorrhagic shock; BTED: Biliary tract external drainage; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; LPS: Lipopolysaccharide.

expression by most epithelial cells lining the villi in the HS group (Figure 3B-F). Occludin expression in the HS + BTED group showed no significant differences compared with the HS group at 0.5 h, 1 h and 2 h after resuscitation (Figure 3B-D and G-I). Occludin expression in the epithelial cells lining the villi was enhanced significantly in the HS + BTED group compared with the HS group at 4 h and 6 h after resuscitation (Figure 3 E, F, J and K).

### *Immunohistochemical analysis of expression of claudin-1 in the ileum*

The epithelial cells lining the villi exhibited positive immunostaining for claudin-1 in the sham group (Figure 4A). There was loss of claudin-1 expression by most epithelial cells lining the villi in the HS group (Figure 4B-F). Claudin-1 expression in the HS + BTED group showed no significant differences compared with the HS group at 0.5 h, 1 h, 2 h and 4 h after resuscitation (Figure 4B-E and G-J). Claudin-1 expression was enhanced significantly in the HS + BTED group compared with the HS group at 6 h after resuscitation (Figure 4F and K).

### *Histomorphology of the ileum*

No obvious tissue damage of the ileum was shown in the sham group (Figure 5Aa). Epithelial cells of small intestinal villi of rats in the HS group showed necrosis and exfoliation. Inflammatory cell infiltration was observed (Figure 5Ab-f). The tissue damage in the ileum of the HS + BTED group was significantly alleviated compared with the HS group. Phenomena of putrescence and desquamation of epithelial cells in the intestinal mucosa were attenuated (Figure 5Ag-k). Histopathologic scores in the HS + BTED group showed no significant differences compared with the HS group at 0.5 h and 1 h after resuscitation. Histopathologic scores in the HS + BTED group decreased significantly compared with the HS group at 2 h, 4 h and 6 h after resuscitation ($P$ < 0.05) (Figure 5B).

## DISCUSSION

This study demonstrated that plasma TNF-α, IL-6, LPS, and D-lactate levels decreased significantly after BTED under HS conditions. The expression levels of occludin and claudin-1 in the ileum increased significantly

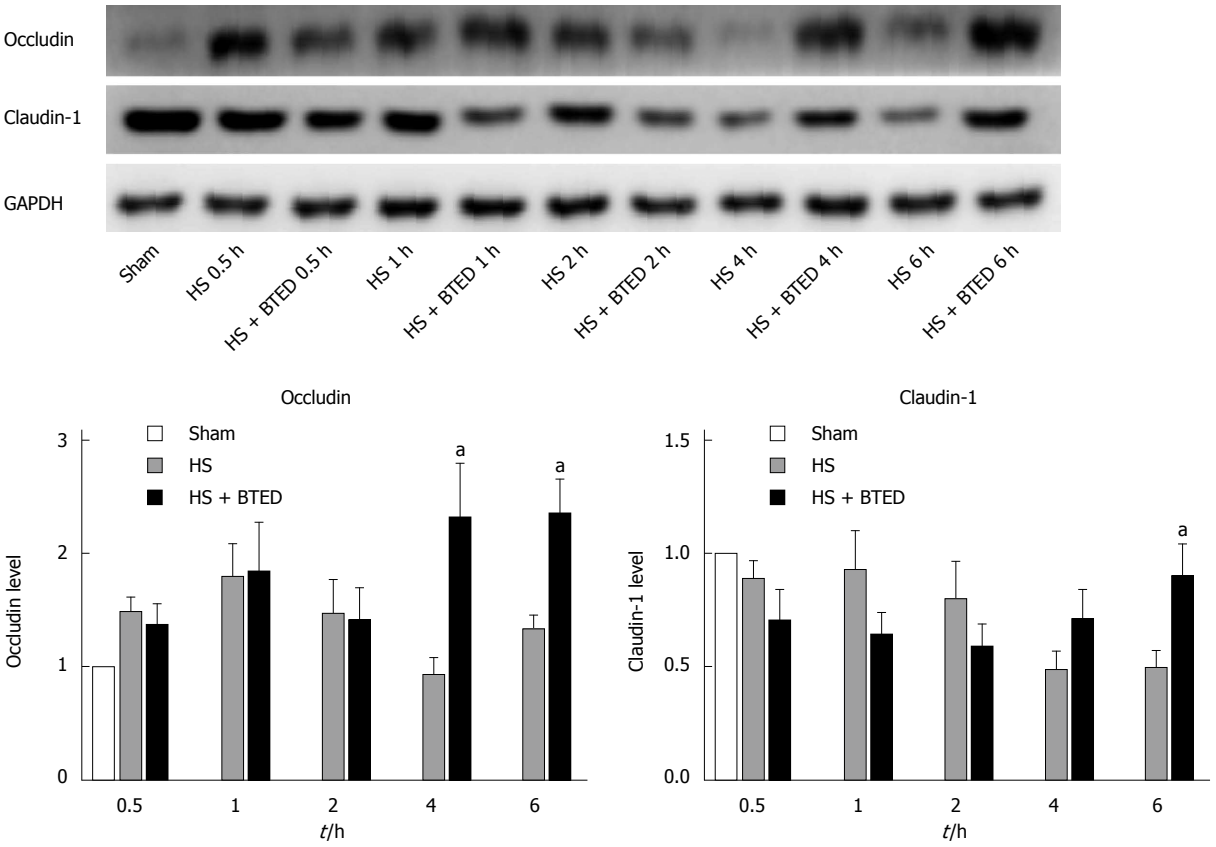
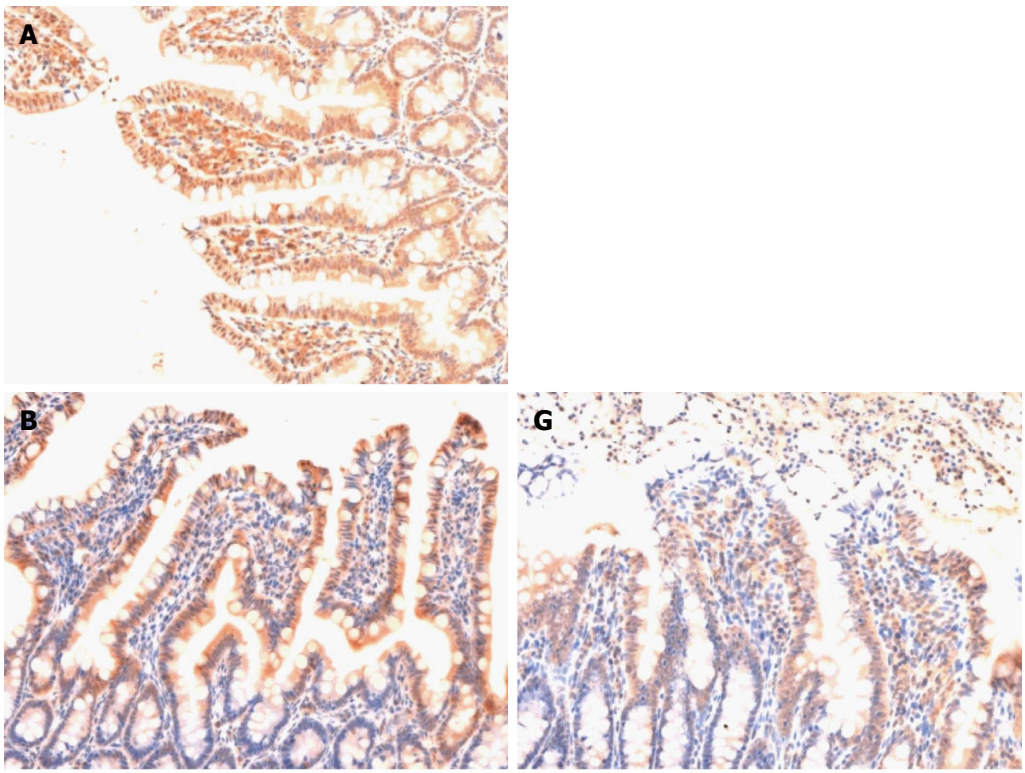



**Figure 2 Western blot analysis of expression of occludin (A and B) and claudin-1 (A and C) in the ileum.** Results are presented as mean ± SE ($n$ = 9). $^{a}P < 0.05$ *vs* the HS group at the same time point. HS: Hemorrhagic shock; BTED: Biliary tract external drainage.



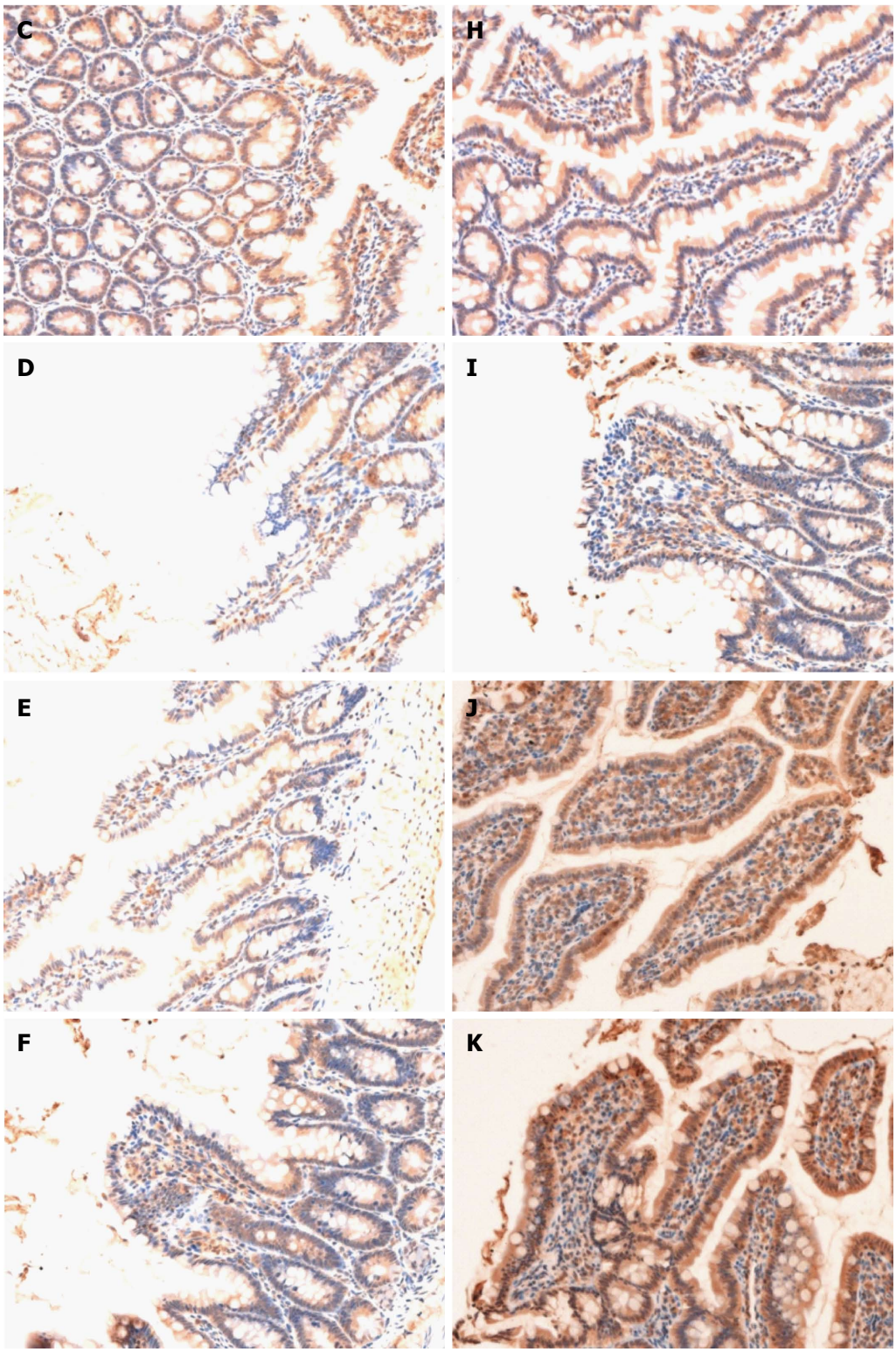
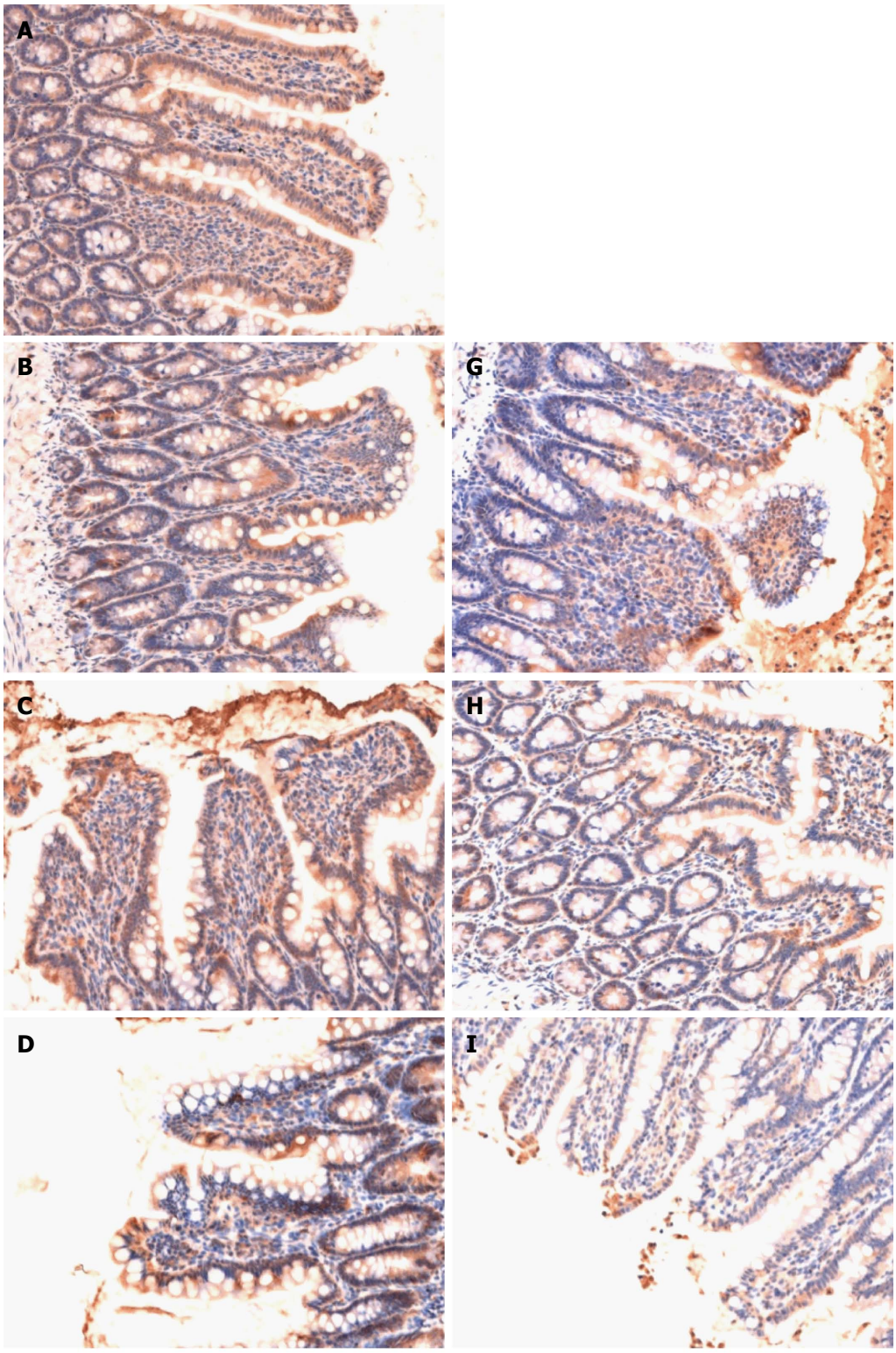



**Figure 3 Immunohistochemical analysis of expression of occludin in the ileum.** Sham group (A), HS group (B: 0.5 h after resuscitation; C: 1 h after resuscitation; D: 2 h after resuscitation; E: 4 h after resuscitation; F: 6 h after resuscitation) and HS + BTED group (G: 0.5 h after resuscitation; H: 1 h after resuscitation; I: 2 h after resuscitation; J: 4 h after resuscitation; K: 6 h after resuscitation). The expression levels of occludin in the ileum in the HS + BTED group were enhanced significantly compared with the HS group at 4 h and 6 h after resuscitation. HS: Hemorrhagic shock; BTED: Biliary tract external drainage.

after BTED under HS conditions. Phenomena of putrescence and desquamation of epithelial cells in the intestinal mucosa were attenuated after BTED. Ileal histopathologic scores decreased significantly after BTED under HS conditions.

Activated Kupffer cells produce proinflammatory TNF-$\alpha$ and IL-6 during the pathogenesis of HS, which induces the release of additional proinflammatory


A
B
G
C
H
D
I

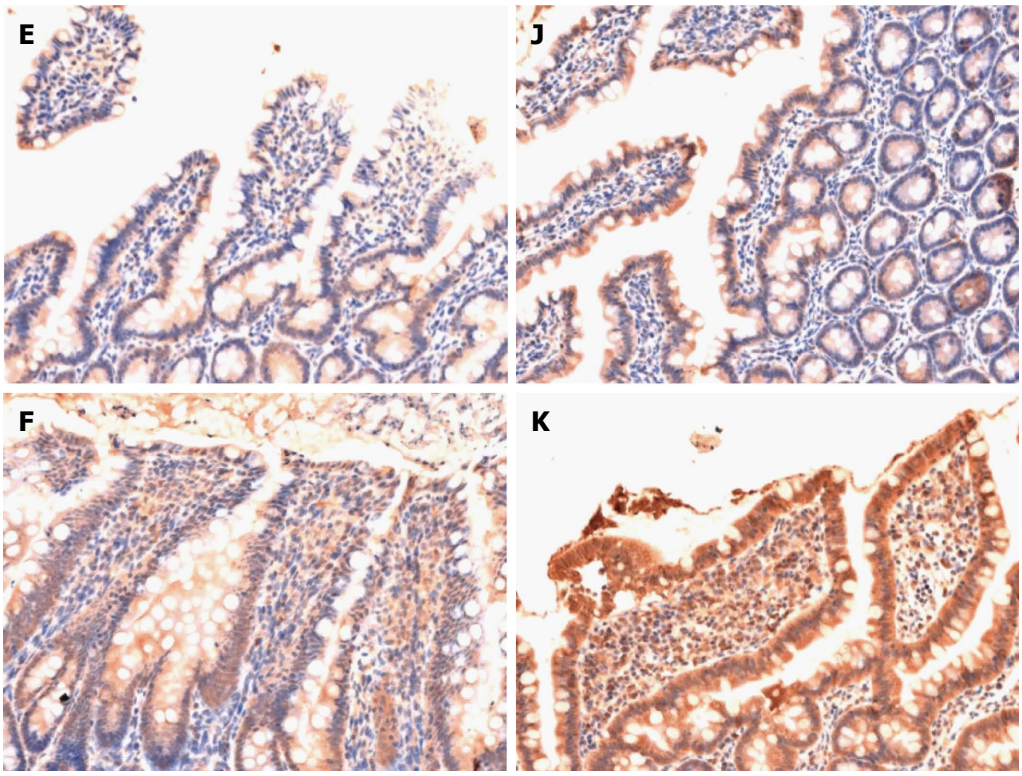
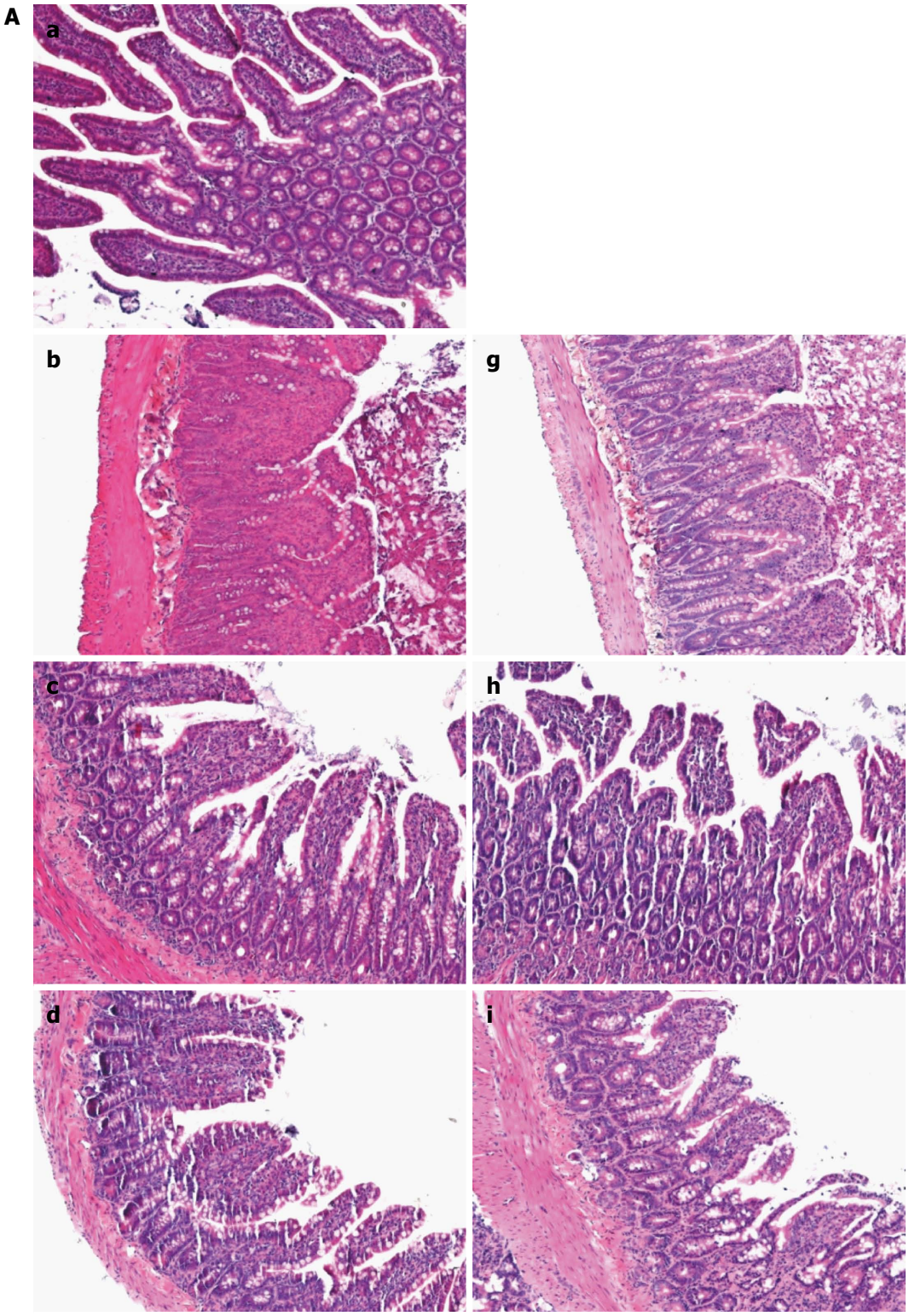



**Figure 4 Immunohistochemical analysis of expression of claudin-1 in the ileum.** Sham group (A), HS group (B: 0.5 h after resuscitation; C: 1 h after resuscitation; D: 2 h after resuscitation; E: 4 h after resuscitation; F: 6 h after resuscitation) and HS + BTED group (G: 0.5 h after resuscitation; H: 1 h after resuscitation; I: 2 h after resuscitation; J: 4 h after resuscitation; K: 6 h after resuscitation). The expression levels of claudin-1 in the ileum in the HS + BTED group were enhanced significantly compared with the HS group at 6 h after resuscitation. HS: Hemorrhagic shock; BTED: Biliary tract external drainage.

mediators from hepatocytes[29,30]. Bile full of pro-inflammatory mediators enters into the gut, which aggravates tissue injury in the ileum and induces the release of additional proinflammatory mediators from intestinal cells. Gut-derived cytokines, such as TNF-α and IL-6, enter into the liver *via* the portal vein, which aggravates liver injury and induces the release of more proinflammatory mediators from Kupffer cells to complete the inflammatory loop of the gut-liver axis[31-34]. This vicious cycle eventually leads to MODS.

The initial application of biliary drainage was to temporarily relief patient's biliary obstruction[35,36]. This technique is widely used clinically because it is less invasive and exhibits fewer complications. Many methods are used, such as nasal biliary drainage, percutaneous transhepaticcholangial drainage, gal-lbladder fistula and so on, with the development of this technology[37,38]. Previous studies demonstrated that the inflammatory cytokine TNF-α in bile increased significantly in HS rats[22]. BTED blocks the entry of inflammatory cytokines into the ileum with bile, which reduces inflammatory cytokines that enter into the blood through the gut and avoids intestinal cell release of more inflammatory cytokines after stimulation by inflammatory cytokines. BTED blockade of the vicious cycle of the gut-liver axis may play an important role in the prevention and treatment of MODS. TNF-α and IL-6 are key mediators involved in many physiologic processes including immunity, inflammation, and metabolism. Plasma TNF-α levels rose within 10 min after hemorrhage, and peaked at 30 min after hemorrhage during HS. Higher concentrations of TNF-α and IL-6 were associated, not only with an increased mortality rate, but also with an increased risk for subsequent adult respiratory distress syndrome and multiple organ failure in HS patients. In our study, plasma TNF-α levels in the HS + BTED group decreased significantly compared with the HS group at 1 h and 6 h after resuscitation. Plasma IL-6 levels in the HS + BTED group decreased significantly compared with the HS group at 0.5 h, 1 h and 2 h after resuscitation. These results showed that BTED reduces the body's inflammatory reaction in HS. However, plasma TNF-α and IL-6 levels in the HS + BTED group increased significantly compared with the HS group at 4 h after resuscitation. The increases of plasma TNF-α and IL-6 levels may be because BTED reduced inflammation of intestinal villi and retained more capillaries. Blood supply in the HS + BTED group was better after resuscitation. TNF-α and IL-6 accumulated in the ileum and were released into the bloodstream faster.

In our earlier study, necrosis and exfoliation of epithelial cells of small intestinal villi of rats with severe acute pancreatitis were attenuated after BTED, which suggests that BTED plays a protective role on the ileum of rats with severe acute pancreatitis. BTED attenuated the phenomena of putrescence


A
a
b
g
c
h
d
i

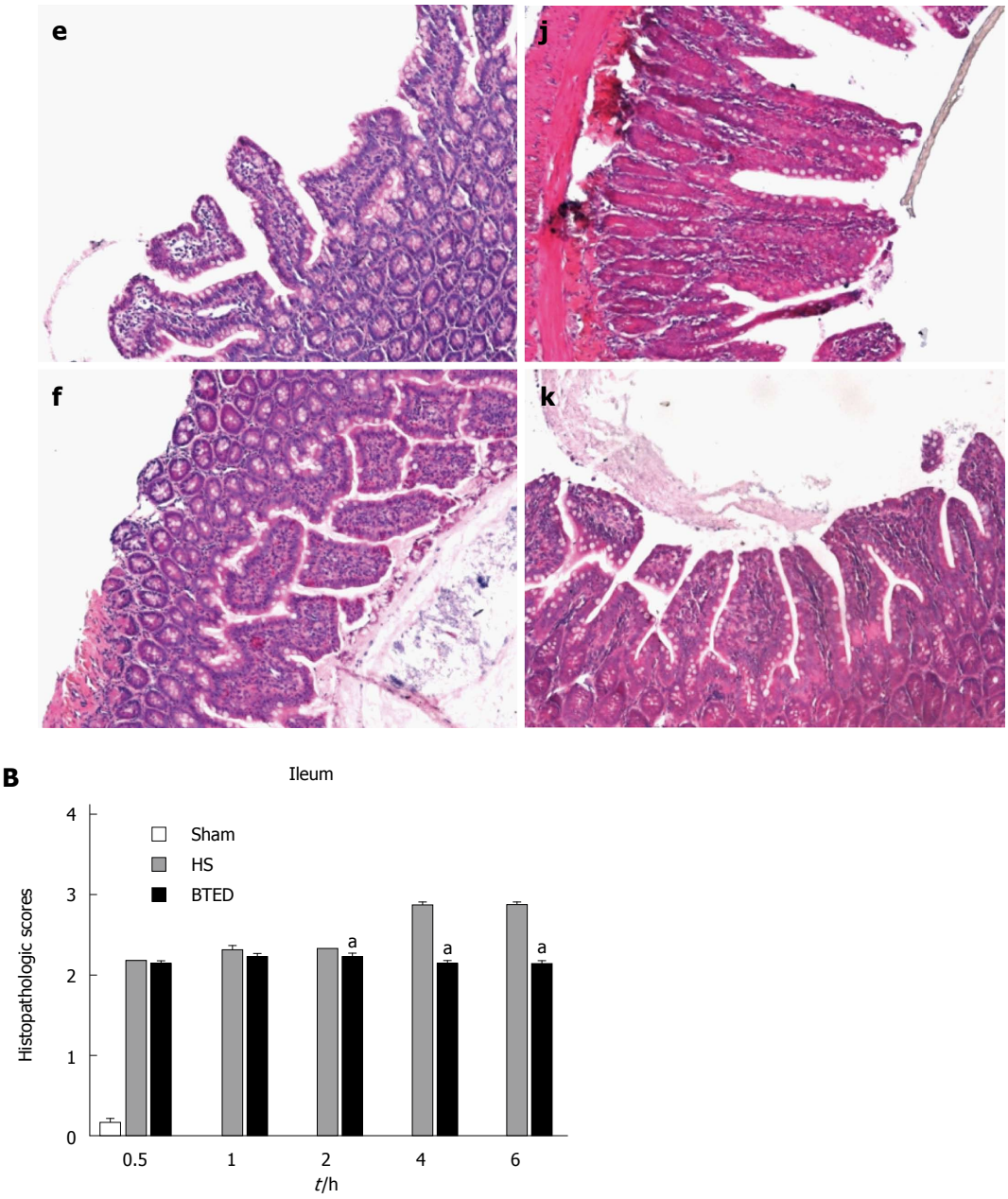



**Figure 5 Hematoxylin and eosin staining (A) and histopathologic scores (B) of the ileum.** Sham group (a), HS group (b: 0.5 h after resuscitation; c: 1 h after resuscitation; d: 2 h after resuscitation; e: 4 h after resuscitation; f: 6 h after resuscitation) and HS + BTED group (g: 0.5 h after resuscitation; h: 1 h after resuscitation; i: 2 h after resuscitation; j: 4 h after resuscitation; k: 6 h after resuscitation). Histopathologic scores are presented as mean ± SE ($n$ = 6). $^{a}P < 0.05$ *vs* the HS group at the same time point. HS: Hemorrhagic shock; BTED: Biliary tract external drainage.

and desquamation of epithelial cells in the intestinal mucosa of HS rats[22]. BTED reduced neutrophil infiltration, superficial necrosis and sloughing of epithelium of intestinal villus and improved survival rates of the LPS treated rats[21]. BTED improved intestinal barrier function in obstructive jaundice models[24]. The tissue damage to the ileum in the HS + BTED group was significantly relieved compared with the HS group in this study. Phenomena of putrescence and desquamation of epithelial cells in the intestinal mucosa were attenuated. Ileal histopathologic scores decreased significantly after BTED under HS conditions in this study. These results showed that BTED protects against intestinal injury in HS.

The severity of hemorrhagic/traumatic shock affected plasma D-lactate concentrations in rats[16]. Increased plasma D-lactate levels predict an increased risk of mortality after hemorrhage and trauma[39]. A rapid decrease in plasma D-lactate could indicate reduced 28-d mortality in critically ill septic shock patients[40]. Ethyl pyruvate can lessen intestinal permeability and protect intestinal barrier function in dogs with septic shock *via* decreasing the levels of plasma D-lactate and reducing inflammation of the small intestinal mucosa[41]. Plasma D-lactate levels in the HS + BTED group decreased significantly compared

with the HS group at 6 h after resuscitation in this study. Plasma LPS levels showed the same variation trend. These results showed that BTED protects against intestinal barrier injury in HS. However, plasma D-lactate levels in the HS + BTED group increased significantly compared with the HS group at 1 h, 2 h and 4 h after resuscitation. Plasma LPS levels in the HS + BTED group increased significantly compared with the HS group at 2 h after resuscitation. These results may be caused by the following reasons. On one hand, BTED reduced inflammation of intestinal villi and retained more capillaries. Intestinal villus blood supply was better in the HS + BTED group. Therefore, more D-lactate and LPS were absorbed through the damaged intestinal mucosa into the peripheral blood. On the other hand, BTED may aggravate intestinal barrier damage as an invasive operation in early stages.

Increasing occludin content in the small intestine enhances the intestinal barrier[42-44]. In our study, the expression levels of occludin in the ileum of the HS + BTED group increased significantly compared with the HS group at 4 h after resuscitation. Fish oil enhanced intestinal integrity by increasing protein expression of the intestinal TJ protein claudin-1 in weaned pigs after LPS challenge[45]. In our study, the expression levels of claudin-1 in the ileum of the HS + BTED group increased significantly compared with the HS group at 6 h after resuscitation. These results showed that BTED increases the expression of occludin and claudin-1 under HS conditions.

There are some limitations in our study. First, the observation time was relatively short so that we are not sure how long the effect of BTED lasts. Second, there are many kinds of TJ proteins expressed in the ileal epithelium. Changes of occludin and claudin-1 may not reflect the real changes of all TJ proteins.

In summary, BTED decreases plasma TNF-α and IL-6 levels and ileal histopathologic scores under HS conditions. BTED increases the expression levels of occludin and claudin-1 and decreases plasma D-lactate and LPS levels under HS conditions. These results show that BTED protects against intestinal barrier injury in HS. Specific mechanisms require further research.

## ACKNOWLEDGMENTS

We thank the staff of Shanghai Institute of Traumatology and Orthopaedics for their technical support. We also thank Lin Zhang from Department of Epidemiology and Biostatistics, Shanghai Jiaotong University School of Public Health, for her statistical support.

## COMMENTS

### Background

Hemorrhagic shock (HS) induces gut barrier failure, which initiates a systemic inflammatory response. Bile full of proinflammatory mediators enters into the gut following HS, which contributes to tissue injury in the intestine. A large amount of lipopolysaccharide (LPS) is released by gut bacteria through the damaged intestinal barrier into the peripheral blood and may cause distant organ injury. Studies on the relationship between biliary tract external drainage (BTED) and intestinal barrier function are limited, and most of these studies focused on obstructive jaundice.

### Research frontiers

D-lactate is the end product of intestinal bacteria. It is neither produced nor metabolized by mammalian cells. During ischemia, as the normal mucosal barrier is damaged and permeability increases, a large amount of D-lactate is released through the damaged intestinal mucosa into the peripheral blood. Thus, D-lactate in peripheral blood can indicate damage situation of intestinal barrier. Tight junction (TJ) proteins, including occludin, claudins, and cytoskeleton proteins, play critical roles in the maintenance of the intestinal barrier integrity.

### Innovations and breakthroughs

BTED significantly decreased plasma TNF-α, IL-6, LPS, and D-lactate levels and increased the expression levels of occludin and claudin-1 in the ileum under HS conditions. Phenomena of putrescence and desquamation of epithelial cells in the intestinal mucosa were attenuated after BTED. Ileum histopathologic scores decreased significantly after BTED under HS conditions.

### Applications

These results show that BTED protects against intestinal barrier injury in HS and provide a new choice for the treatment of HS.

### Terminology

The initial application of BTED was to temporarily relief patient's biliary obstruction. This technique is widely used clinically because it is less invasive and exhibits fewer complications. Many methods are used, such as nasal biliary drainage, percutaneous transhepaticcholangial drainage, gallbladder fistula and so on, with the development of this technology. BTED blocks the entry of inflammatory cytokines into the ileum with bile, which reduces inflammatory cytokines that enter into the blood through the gut and avoids intestinal cell release of more inflammatory cytokines after stimulation by inflammatory cytokines.

### Peer-review

This manuscript is quite well written.

## REFERENCES

1 **Reys LG**, Ortiz-Pomales YT, Lopez N, Cheadle G, de Oliveira PG, Eliceiri B, Bansal V, Costantini TW, Coimbra R. Uncovering the neuroenteric-pulmonary axis: vagal nerve stimulation prevents acute lung injury following hemorrhagic shock. *Life Sci* 2013; **92**: 783-792 [PMID: 23439327 DOI: 10.1016/j.lfs.2013.02.009]

2 **Deitch EA**, Xu D, Franko L, Ayala A, Chaudry IH. Evidence favoring the role of the gut as a cytokine-generating organ in rats subjected to hemorrhagic shock. *Shock* 1994; **1**: 141-145 [PMID: 7749933]

3 **Welch M**. D-lactate as an early marker of intestinal ischaemia after ruptured abdominal aortic aneurysm repair. *Br J Surg* 1999; **86**: 712 [PMID: 10361218 DOI: 10.1046/j.1365-2168.1999.1104a.x]

4 **Ruh J**, Vogel F, Schmidt E, Werner M, Klar E, Secchi A, Gebhard MM, Glaser F, Herfarth C. Effects of hydrogen peroxide scavenger Catalase on villous microcirculation in the rat small intestine in a model of inflammatory bowel disease. *Microvasc Res* 2000; **59**: 329-337 [PMID: 10792963 DOI: 10.1006/mvre.1999.2201]

5 **Szalay L**, Umar F, Khadem A, Jafarmadar M, Fürst W, Ohlinger W, Redl H, Bahrami S. Increased plasma D-lactate is associated with the severity of hemorrhagic/traumatic shock in rats. *Shock* 2003; **20**: 245-250 [PMID: 12923496]

6 **Murray MJ**, Barbose JJ, Cobb CF. Serum D(-)-lactate levels as

a predictor of acute intestinal ischemia in a rat model. *J Surg Res* 1993; **54**: 507-509 [PMID: 8361176 DOI: 10.1006/jsre.1993.1078]
7 **Ding LA**, Li JS, Li YS, Zhu NT, Liu FN, Tan L. Intestinal barrier damage caused by trauma and lipopolysaccharide. *World J Gastroenterol* 2004; **10**: 2373-2378 [PMID: 15285022]
8 **Moore FA**. The role of the gastrointestinal tract in postinjury multiple organ failure. *Am J Surg* 1999; **178**: 449-453 [PMID: 10670850 DOI: 10.1016/S0002-9610(99)00231-7]
9 **Velloso LA**, Folli F, Saad MJ. TLR4 at the Crossroads of Nutrients, Gut Microbiota, and Metabolic Inflammation. *Endocr Rev* 2015; **36**: 245-271 [PMID: 25811237 DOI: 10.1210/er.2014-1100]
10 **Liu Z**, Li N, Neu J. Tight junctions, leaky intestines, and pediatric diseases. *Acta Paediatr* 2005; **94**: 386-393 [PMID: 16092447]
11 **Furuse M**, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S. Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol* 1993; **123**: 1777-1788 [PMID: 8276896]
12 **Schneeberger EE**, Lynch RD. The tight junction: a multifunctional complex. *Am J Physiol Cell Physiol* 2004; **286**: C1213-C1228 [PMID: 15151915 DOI: 10.1152/ajpcell.00558.2003]
13 **Salim SY**, Söderholm JD. Importance of disrupted intestinal barrier in inflammatory bowel diseases. *Inflamm Bowel Dis* 2011; **17**: 362-381 [PMID: 20725949 DOI: 10.1002/ibd.21403]
14 **McCarthy KM**, Skare IB, Stankewich MC, Furuse M, Tsukita S, Rogers RA, Lynch RD, Schneeberger EE. Occludin is a functional component of the tight junction. *J Cell Sci* 1996; **109** (Pt 9): 2287-2298 [PMID: 8886979]
15 **Krug SM**, Schulzke JD, Fromm M. Tight junction, selective permeability, and related diseases. *Semin Cell Dev Biol* 2014; **36**: 166-176 [PMID: 25220018 DOI: 10.1016/j.semcdb.2014.09.002]
16 **Rao R**. Occludin phosphorylation in regulation of epithelial tight junctions. *Ann N Y Acad Sci* 2009; **1165**: 62-68 [PMID: 19538289 DOI: 10.1111/j.1749-6632.2009.04054.x]
17 **Nakao T**, Kurita N, Komatsu M, Yoshikawa K, Iwata T, Utusnomiya T, Shimada M. Irinotecan injures tight junction and causes bacterial translocation in rat. *J Surg Res* 2012; **173**: 341-347 [PMID: 21176921 DOI: 10.1016/j.jss.2010.10.003]
18 **Angelow S**, Ahlstrom R, Yu AS. Biology of claudins. *Am J Physiol Renal Physiol* 2008; **295**: F867-F876 [PMID: 18480174 DOI: 10.1152/ajprenal.90264.2008]
19 **González-Mariscal L**, Tapia R, Chamorro D. Crosstalk of tight junction components with signaling pathways. *Biochim Biophys Acta* 2008; **1778**: 729-756 [PMID: 17950242 DOI: 10.1016/j.bbamem.2007.08.018]
20 **Takizawa Y**, Kishimoto H, Kitazato T, Tomita M, Hayashi M. Changes in protein and mRNA expression levels of claudin family after mucosal lesion by intestinal ischemia/reperfusion. *Int J Pharm* 2012; **426**: 82-89 [PMID: 22285474 DOI: 10.1016/j.ijpharm.2012.01.023]
21 **Jackson GD**, Dai Y, Sewell WA. Bile mediates intestinal pathology in endotoxemia in rats. *Infect Immun* 2000; **68**: 4714-4719 [PMID: 10899877]
22 **Liu YJ**, Mao EQ, Ouyang B, Chen J, Tang YQ, Huang SW, Guan XD. Effect of biliary tract external drainage on cytokine expression and histomorphology of intestine, liver, and lung in rats with hemorrhagic shock. *Crit Care Med* 2009; **37**: 2800-2806 [PMID: 19707134 DOI: 10.1097/CCM.0b013e3181a59469]
23 **Yoshida Y**, Ajiki T, Ueno K, Shinozaki K, Murakami S, Okazaki T, Matsumoto T, Matsumoto I, Fukumoto T, Usami M, Ku Y. Preoperative bile replacement improves immune function for jaundiced patients treated with external biliary drainage. *J Gastrointest Surg* 2014; **18**: 2095-2104 [PMID: 25326124 DOI: 10.1007/s11605-014-2674-2]
24 **Wang SZ**, Wang XB. Effects of biliary drainage on the intestinal barrier function in obstructive jaundice. *Hepatogastroenterology* 2013; **60**: 1284-1288 [PMID: 23933919 DOI: 10.5754/hge13236]
25 **Kamiya S**, Nagino M, Kanazawa H, Komatsu S, Mayumi T, Takagi K, Asahara T, Nomoto K, Tanaka R, Nimura Y. The value of bile replacement during external biliary drainage: an analysis of intestinal permeability, integrity, and microflora. *Ann Surg* 2004; **239**: 510-517 [PMID: 15024312 DOI: 10.1097/01.sla.0000118594.23874.89]
26 **Parks RW**, Clements WD, Smye MG, Pope C, Rowlands BJ, Diamond T. Intestinal barrier dysfunction in clinical and experimental obstructive jaundice and its reversal by internal biliary drainage. *Br J Surg* 1996; **83**: 1345-1349 [PMID: 8944448]
27 **Yang FL**, Subeq YM, Lee CJ, Lee RP, Peng TC, Hsu BG. Melatonin ameliorates hemorrhagic shock-induced organ damage in rats. *J Surg Res* 2011; **167**: e315-e321 [PMID: 19932901 DOI: 10.1016/j.jss.2009.07.026]
28 **Lee CC**, Lee RP, Subeq YM, Lee CJ, Chen TM, Hsu BG. Fluvastatin attenuates severe hemorrhagic shock-induced organ damage in rats. *Resuscitation* 2009; **80**: 372-378 [PMID: 19150166 DOI: 10.1016/j.resuscitation.2008.12.003]
29 **Hsu JT**, Chen TH, Chiang KC, Kuo CJ, Lin CJ, Yeh TS. Role of p38 MAPK pathway in 17β-estradiol-mediated attenuation of hemorrhagic shock-induced hepatic injury. *J Appl Physiol* (1985) 2015; **118**: 187-192 [PMID: 25593217 DOI: 10.1152/japplphysiol.00464.2014]
30 **Jiang JX**, Diao YF, Tian KL, Chen HS, Zhu PF, Wang ZG. Effect of hemorrhagic shock on endotoxin-inducing TNF production and intra-tissue lipopolysaccharide-binding protein mRNA expression and their relationship. *Shock* 1997; **7**: 206-210 [PMID: 9068087]
31 **Wang HJ**, Zakhari S, Jung MK. Alcohol, inflammation, and gut-liver-brain interactions in tissue damage and disease development. *World J Gastroenterol* 2010; **16**: 1304-1313 [PMID: 20238396 DOI: 10.3748/wjg.v16.i11.1304]
32 **Navaneethan U**. Hepatobiliary manifestations of ulcerative colitis: an example of gut-liver crosstalk. *Gastroenterol Rep* (Oxf) 2014; **2**: 193-200 [PMID: 24951514 DOI: 10.1093/gastro/gou036]
33 **Miura K**, Ohnishi H. Role of gut microbiota and Toll-like receptors in nonalcoholic fatty liver disease. *World J Gastroenterol* 2014; **20**: 7381-7391 [PMID: 24966608 DOI: 10.3748/wjg.v20.i23.7381]
34 **Visschers RG**, Luyer MD, Schaap FG, Olde Damink SW, Soeters PB. The gut-liver axis. *Curr Opin Clin Nutr Metab Care* 2013; **16**: 576-581 [PMID: 23873346 DOI: 10.1097/MCO.0b013e32836410a4]
35 **Wurbs D**, Phillip J, Classen M. Experiences with the long standing nasobiliary tube in biliary diseases. *Endoscopy* 1980; **12**: 219-223 [PMID: 7428726 DOI: 10.1055/s-2007-1021747]
36 **Nagai N**, Toli F, Oi I, Suzuki H, Kozu T. Continuous endoscopic pancreatocholedochal catheterization. *Gastrointest Endosc* 1976; **23**: 78-81 [PMID: 992286]
37 **Kawakami H**, Kuwatani M, Abe Y, Kubota Y, Kawakubo K, Kubo K, Kawahata S, Sakamoto N. Direct peroral ultraslim endoscopy-guided biliary drainage in a patient with cystic duct carcinoma and an occluded self-expandable metallic stent. *Endoscopy* 2015; **47** Suppl 1 UCTN: E43-E44 [PMID: 25603523 DOI: 10.1055/s-0034-1391258]
38 **Prachayakul V**, Aswakul P. Endoscopic ultrasound-guided biliary drainage as an alternative to percutaneous drainage and surgical bypass. *World J Gastrointest Endosc* 2015; **7**: 37-44 [PMID: 25610532 DOI: 10.4253/wjge.v7.i1.37]
39 **Xu CL**, Sun R, Qiao XJ, Xu CC, Shang XY, Niu WN. Protective effect of glutamine on intestinal injury and bacterial community in rats exposed to hypobaric hypoxia environment. *World J Gastroenterol* 2014; **20**: 4662-4674 [PMID: 24782618 DOI: 10.3748/wjg.v20.i16.4662]
40 **Huygelen V**, De Vos M, Willemen S, Tambuyzer B, Casteleyn C, Knapen D, Van Cruchten S, Van Ginneken C. Increased intestinal barrier function in the small intestine of formula-fed neonatal piglets. *J Anim Sci* 2012; **90** Suppl 4: 315-317 [PMID: 23365365 DOI: 10.2527/jas.53731]
41 **Liu Y**, Chen F, Odle J, Lin X, Jacobi SK, Zhu H, Wu Z, Hou Y. Fish oil enhances intestinal integrity and inhibits TLR4 and NOD2 signaling pathways in weaned pigs after LPS challenge. *J Nutr* 2012; **142**: 2017-2024 [PMID: 23014495 DOI: 10.3945/jn.112.164947]
42 **Sobhian B**, Kröpfl A, Hölzenbein T, Khadem A, Redl H, Bahrami S. Increased circulating D-lactate levels predict risk

of mortality after hemorrhage and surgical trauma in baboons. *Shock* 2012; **37**: 473-477 [PMID: 22266971 DOI: 10.1097/SHK.0b013e318249cb96]

43 **Sapin V**, Nicolet L, Aublet-Cuvelier B, Sangline F, Roszyk L, Dastugue B, Gazuy N, Deteix P, Souweine B. Rapid decrease in plasma D-lactate as an early potential predictor of diminished 28-day mortality in critically ill septic shock patients. *Clin Chem Lab Med* 2006; **44**: 492-496 [PMID: 16599846 DOI: 10.1515/CCLM.2006.086]

44 **Kou QY**, Guan XD. [Protective effect of ethyl pyruvate on barrier function of intestinal mucosa in dogs with septic shock]. *Zhonghua Weichang Waike Zazhi* 2008; **11**: 177-180 [PMID: 18344089]

45 **De Vos M**, Huygelen V, Van Raemdonck G, Willemen S, Fransen E, Van Ostade X, Casteleyn C, Van Cruchten S, Van Ginneken C. Supplementing formula-fed piglets with a low molecular weight fraction of bovine colostrum whey results in an improved intestinal barrier. *J Anim Sci* 2014; **92**: 3491-3501 [PMID: 25012977 DOI: 10.2527/jas.2013-6437]

**P- Reviewer:** Cerwenka HR **S- Editor**: Ma YJ **L- Editor**: Wang TQ **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12814

*World J Gastroenterol* 2015 December 7; 21(45): 12814-12821
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

Basic Study

# Chemosensitization of HepG2 cells by suppression of NF-κB/p65 gene transcription with specific-siRNA

Yun Shi, Si-Ye Wang, Min Yao, Wen-Li Sai, Wei Wu, Jun-Ling Yang, Yin Cai, Wen-Jie Zheng, Deng-Fu Yao

Yun Shi, Si-Ye Wang, Min Yao, Wen-Li Sai, Wei Wu, Jun-Ling Yang, Wen-Jie Zheng, Deng-Fu Yao, Research Center of Clinical Medicine, Affiliated Hospital of Nantong University, Nantong 226001, Jiangsu Province, China

Min Yao, Department of Immunology, Medical School of Nantong University, Nantong 226001, Jiangsu Province, China

Yin Cai, Department of Oncology, Affiliated Hospital of Nantong University, Nantong 226001, Jiangsu Province, China

Author contributions: Shi Y, Wang SY, and Yao M contributed equally to this work, designed the research, analyzed the data, and wrote the first draft; Sai WL, Wu W, and Qiu LW performed quantitative real time PCR; Yang JL and Cai Y analyzed the data by Western blotting and ELISA; Zhang HJ and Zheng WJ performed immunohistochemistry, transfection, and cell proliferation, survival, and apoptosis assays; Yao DF is the guarantor and contributed to the design and interpretation of the study.

Supported by Grants from the Jiangsu Provincial Special Programs of Medical Science, BL2012053, HK201102; the Nantong Undertaking and Technological Innovation, HS2013007, BK2013048 and HS2014078; the Priority Academic Program Development of Higher Education Institution of Jiangsu Province, the National Natural Science Foundation, No. 81200634; and the international S &T Cooperation Program (2013DFA32150) of China.

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



Correspondence to: Deng-Fu Yao, MD, PhD, Professor, Research Center of Clinical Medicine, Affiliated Hospital of Nantong University, No. 20 West Temple Road, Nantong 226001, Jiangsu Province, China. yaodf@ahnmc.com
Telephone: +86-513-85052297
Fax: +86-513-85052523

Received: March 28, 2015
Peer-review started: March 29, 2015
First decision: April 24, 2015
Revised: July 4, 2015
Accepted: September 15, 2015
Article in press: September 15, 2015
Published online: December 7, 2015

## Abstract

**AIM:** To investigate small interfering RNA (siRNA)-mediated inhibition of nuclear factor-kappa B (NF-κB) activation and multidrug-resistant (MDR) phenotype formation in human HepG2 cells.

**METHODS:** Total RNA was extracted from human HepG2 or LO2 cells. NF-κB/p65 mRNA was amplified by nested reverse transcription polymerase chain reaction and confirmed by sequencing. NF-κB/p65 was analyzed by immunohistochemistry. Specific-siRNA was transfected to HepG2 cells to knock down NF-κB/p65 expression. The effects on cell proliferation, survival, and apoptosis were assessed, and the level of NF-κB/p65 or P-glycoprotein (P-gp) was quantitatively analyzed by enzyme-linked immunosorbent assay.

**RESULTS:** HepG2 cells express NF-κB/p65 and express relatively less phosphorylated p65 (P-p65) and little P-gp. After treatment of HepG2 cells with different doses of doxorubicin, the expression of NF-κB/p65, P-p65, and especially P-gp were dose-dependently upregulated. After HepG2 cells were transfected with NF-κB/p65 siRNA (100 nmol/L), the expression of NF-κB/p65, P-p65, and P-gp were downregulated

significantly and dose-dependently. The viability of HepG2 cells was decreased to 23% in the combination NF-κB/p65 siRNA (100 nmol/L) and doxorubicin (0.5 μmol/L) group and 47% in the doxorubicin (0.5 μmol/L) group ($t$ = 7.043, $P$ < 0.001).

**CONCLUSION:** Knockdown of NF-κB/p65 with siRNA is an effective strategy for inhibiting HepG2 cell growth by downregulating P-gp expression associated chemosensitization and apoptosis induction.

**Key words:** Hepatocellular carcinoma; Nuclear factor-κB; Multidrug-resistant; Chemosensitization; Small interference RNA; P-glycoprotein



**Core tip:** Hepatic nuclear factor-kappa B (NF-κB) signaling pathway could be a potential target for designing highly effective therapeutic agents for the chemoprevention of hepatocellular carcinoma (HCC). Specific siRNAs used in combination with doxorubicin could enhance doxorubicin cytotoxicity in human HepG2 cells by downregulating NF-κB and P-glycoprotein expression. Stable NF-κB inhibition and chemosensitization could significantly inhibit tumor cell proliferation. Thus, the modulation of NF-κB might represent an advance in HCC therapy efficacy and is worthy of further research and investigation.

Shi Y, Wang SY, Yao M, Sai WL, Wu W, Yang JL, Cai Y, Zheng WJ, Yao DF. Chemosensitization of HepG2 cells by suppression of NF-κB/p65 gene transcription with specific-siRNA. *World J Gastroenterol* 2015; 21(45): 12814-12821 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12814.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12814

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers and causes of mortality in China[1,2]. A great deal of progress in understanding the mechanisms of hepatocarcinogenesis has been achieved in recent years[3-5]. Many genes, such as protooncogenes, tumor suppressor genes, and growth factor genes, have been suggested to play an important role in this process[6]. Infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) is involved in HCC development and progression. Tumorigenic protein (HBx or core protein of HCV) activates a variety of signaling pathways, including nuclear- transcription factor kappa B (NF-κB) and tumor necrosis factor (TNF)-α[7,8]. Abnormal activation of NF-κB has been shown to modulate the transcription and expression of many genes in hepatocarcinogenesis[9,10]. NF-κB is formed by five subunits, p65, RelB, C-Rel, p50, and p52. The p50/p65 dimers play the common biological role and can be activated by chemotherapy drugs, cytokines, and viruses induced into nucleus[11-13].

HCC is one of the most resistant tumors to systemic chemotherapy, and doxorubicin is one of the chemotherapy drugs used in HCC[14,15]. Both NF-κB and P-glycoprotein (P-gp) have been described as important mediators of chemotherapy-induced cell death[16-18]. Because the first exon of multidrug resistance (MDR) gene promoter contains the NF-κB binding sequence, it may be one of the downstream target genes of NF-κB[19,20]. A variety of chemotherapeutic drugs can induce formation of the MDR phenotype in malignant cells, thereby restricting the efficacy of these drugs. P-gp is encoded by the MDR gene and is a membrane bound ATP-dependent flow pump. It can pump drugs out of the cell with the energy provided by ATP, and this is the main mechanism underlying the MDR phenomenon[21,22]. However, the role of NF-κB signaling pathway in chemotherapy-induced MDR remains to be elucidated. In this study, we used small interference RNA (siRNA)-mediated inhibition of NF-κB expression in combination with chemotherapy drugs to explore the role of NF-κB in human HepG2 cell growth.

## MATERIALS AND METHODS

### *Cell line and cultures*

Human hepatoma cell (HepG2) and normal liver cell (LO2) lines were purchased from the Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China) and cultured at 37 ℃ with 5% $CO_2$ in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 2 mmol/L L-glutamine and 10% fetal calf serum (FCS). The cells were digested by parenzyme. Recombinant human TNF-α was purchased from the Prepro Tech, Inc. (Rocky Hill, NJ, United States). Doxorubicin and all chemicals not otherwise specified below were purchased from Sigma (St Louis, MI, United States).

### *Synthesis of NF-κB complementary DNA*

To the cells ($5 \times 10^5$), 1.0 ml of Trizol reagent (Promega, Madison, WI, United States) was added. Total RNA was isolated according to standard procedures and the protocols outlined by the manufacturer. RNA purity was estimated from the ratio of absorbance (A) readings at 260 and 280 nm, with an $A_{260/280}$ ratio between 1.8 and 2.0 indicating sufficient purity. The RNA samples were kept frozen at -85 ℃ until required. For synthesis of NF-κB complementary DNA (cDNA), 2 μg of total RNA was denatured in the presence of random hexamers (100 pMol/L, Promega) and reverse-transcriptase (Gibco, Carlsbad, CA, United States) at 23 ℃ for 10 min, 42 ℃ for 60 min, 95 ℃ for 10 min, on ice for 5 min, and then stored at -20 ℃ for polymerase chain reaction (PCR) amplification.

### *PCR amplification of the NF-κB gene*

A set of primers, NF-κB-P1 (sense), 5'-AGCACA

GATACCACCAAGAC-3′ (nt398-417) and NF-κB-P2 (antisense), 5′-TGGTCCCGTGAAATACACCT-3′ (nt523-542) were designed according to NF-κB/p65 sequences obtained from Genebank (NM-021975) and synthesized in the Shanghai Institute of Cell Biology, Chinese Academy Sciences, China, and the size of amplified fragment was 145 bp. The PCR amplification consisted of initial denaturation at 94 ℃ for 5 min, followed by 94 ℃ for 25 s, 55 ℃ for 30 s, and 72 ℃ for 90 s for 30 cycles. The glyceraldehyde-3- phosphate dehydrogenase (GAPDH) genome was used as a control. A set of primer sequences was GAPDH-1 (sense), 5′-AGAAGGCTGGGGCTCATTTG-3′ and GAPDH-2 (antisense), 5′-AGGGGCCATCCACAGTCTTC-3′, and the size of designed fragment was 258 bp. The amplified products were separated by electrophoresis on 2% agarose gels with ethidium bromide staining. The fragment sizes were evaluated using DNA markers (Promega) as molecular weight standards, and Molecular Imager Gel Doc™ System (Biorad, Hercules, CA, United States).

### *Synthesis NF-κB/p65 siRNA and cell transfections*

The human NF-κB/p65 siRNA was purchased from the Biomics Biotechnologies Co. (Nantong, China). The sequences of siRNA were 5′-GA UGAGAUCUUCCUACUGUdTdT-3′ for p65 and 5′-UUCUCCGAACGUGU CACGUTTdTdT-3′ for negative control. Cells at 50% confluence were transfected with 100 nmol/L NF-κB/p65 siRNA using Lipofectamine™ 2000 Transfection Reagent (Invitrogen), according to the manufacturer's specifications. The cells were seeded the day before transfection, using RPMI-1640 with 10% FCS without antibiotics. After 2 d, the cells were subjected to Western blotting analysis or 3-(4,5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay in the presence or absence of doxorubicin.

### *MTT assays*

The effect of doxorubicin on the viability of HepG2 cells was studied using the MTT Cell Proliferation and Cytotoxicity Assay Kit purchased from the Nanjing KeyGen Biotech CO., China. MTT assay was performed in 96-well flat-bottomed plates (Nunc, Rochester, NY, United States). Approximately 5 × $10^4$ cells were seeded in 100 μL of drug free media and incubated for 24 h before drug treatment or siRNA transfection. Different concentrations of doxorubicin were added for 24 h. Then, 10 μL of MTT solution was added and incubated for 4 h, and, subsequently, 100 μL formanzan solution was added and incubated for 4 h. The amount of soluble formanzan produced, by cellular reduction of MTT, was measured at 570 nm. Approximate $IC_{50}$ values were determined from a dose response curve. Data were derived from at least three independent experiments ($n$ = 3). The effects of doxorubicin with or without NF-κB/p65 siRNA on the viability of HepG2 cells were studied using MTT assay. Twenty four hours after seeding, the cells were transfected with NF-κB/p65 siRNA or negative control siRNA. After 24 h, doxorubicin was added for an additional 24 h. The MTT assays were then performed, as described above.

### *Western blotting*

For Western blotting, the cytoplasmic proteins were purified from cells cultured in 6-wells plates and lysed with a hypotonic buffer (20 mmol/L Tris-buffer, pH 8.0, 150 mmol/L NaCl, 100 mMol/L NaF, 10% of glycerol, 1% of Nonidet P-40, 1 mmol/L PMSF, 40 μg/mL leupeptin, and 20 μg/mL arotinin) for 30 min at 4 ℃. After centrifuged, equal amounts of protein (25 μg/lane) were resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. Membranes were blocked in Tris-buffered saline (TBS), containing 2% glycine and 3% non-fat dried milk overnight at 4 ℃, and then incubated with specific primary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, United States) to NF-κB/p65, phosphorylated p65, P-gp, and β-actin for 2 h at 37 ℃. Membranes were them incubated with horseradish peroxidase-labeled secondary antibody for 1.5 h at 37 ℃. The reaction was developed using a chemiluminescence detection system.

### *Immunohistochemistry*

The cells were fixed with 10% formaldehyde, and then the streptavidin-peroxidase (S-P) method with empirical procedure directions was performed. Phosphate buffered saline (PBS) was used to substitute for the primary antibody and served as a negative control. The positive material of NF-κB/p65 was a brown-yellow fine particle layer localized in the nucleus or cytoplasm. NF-κB/p65 staining was evaluated semi-quantitatively according to the percentage of positive cells.

### *Enzyme-linked immunosorbent assay*

The nuclear protein was extracted after cell transfection, according to the instructions for the nuclear and cytoplasmic protein extraction kit, and quantified spectro- photometrically using the BCA assay kit (Beyotime, Haimen, China). The level of NF-κB/p65 was detected according to the human NF-κB/p65 enzyme-linked immunosorbent assay kit (Cusabio Biotech, Wuhan, China), with 30 μL of complete combining buffer, 10 μL of nuclear protein extraction agent, and 20 μL of complete lysis buffer (CLB). The positive control consisted of 2.5 μg of provided nuclear extract diluted in 20 μL of CLB per well; the blank well contained only 20 μL of CLB. Twenty microliters of the appropriate standard diluted in the CLB was added to each well. Solutions were incubated with mild agitation for 1 h at room temperature. Each well was washed three times with 200 μL of washing buffer, and then 100 μL of diluted NF-κB antibody was added. The plate

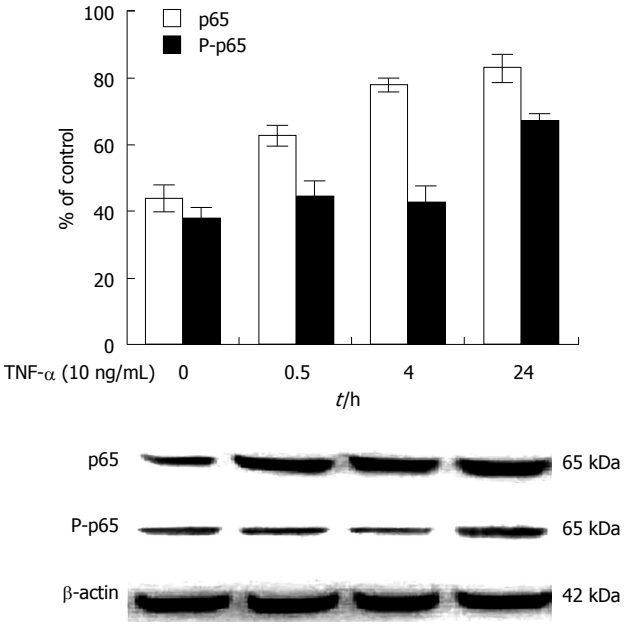



**Figure 1 Comparative analysis of nuclear factor-κB/p65 and P-p65 expression in human HepG2 cells induced with tumor necrosis factor-α.** A: The ratios of nuclear factor-κB (NF-κB) p65 and P-p65 expression to β-actin in HepG2 cells with 10 ng/mL of tumor necrosis factor (TNF)-α at time points, data are means of independent triplicate experiments; B: The expression of NF-κB/p65 and P-p65 by Western blot analysis, NF-κB/p65 and P-p65 were 65 kDa, and β-actin, the control, was 42 kDa.

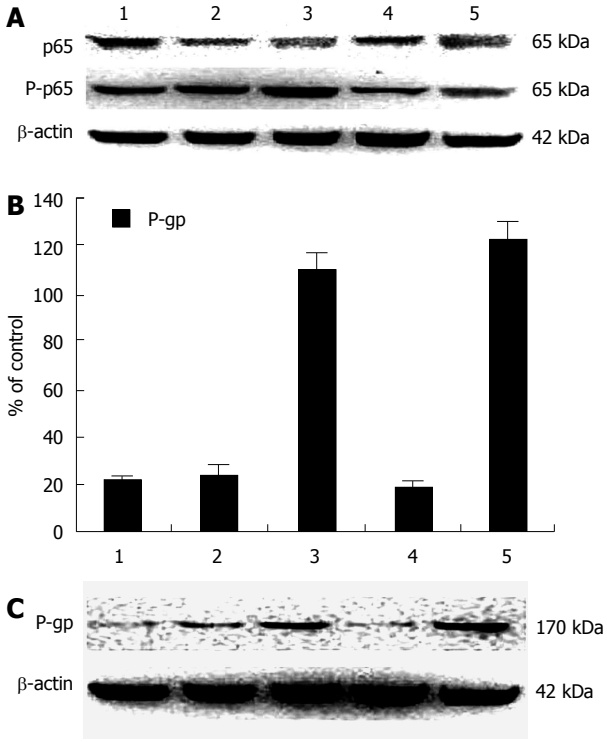



**Figure 2 Expressions of nuclear factor-κB/p65, P-p65, and P-gp induced by different doses of doxorubicin in HepG2 cells.** Lane 1, control without doxorubicin; Lane 2, 0.5 μmol/L of doxorubicin for 4 h; Lane 3, 0.5 μmol/L of doxorubicin for 24 h; Lane 4, 1.0 μmol/L of doxorubicin for 4 h; and Lane 5, 1.0 μmol/L of doxorubicin for 24 h. A: The expressions of NF-κB/p65 and P-p65 in HepG2 cells with different doses of doxorubicin were analyzed by western blotting. NF-κB/p65 and P-p65 shows a 65 kDa band, and β-actin shows a 42 kDa band as control; B: The relative levels of P-glycoprotein (P-gp) expression to β-actin in HepG2 cells with different doses of doxorubicin at different times, data are means of independent triplicate experiments; C: the analysis of P-gp expression in HepG2 cells with different doses of doxorubicin by western blotting. P-gp, 170 kDa; β-actin, 42 kDa as control protein. The action time of HepG2 cells with different doses of doxorubicin.

was covered and incubated for 1 h with mild agitation, washed four times, and 100 μL of Developing Solution was added. After 10 min incubation in the dark, 100 μL of stop solution was added, and within 5 min, the A450 was measured with a spectrophotometer and reference wavelength at 655 nm. NF-κB level was calculated according to a standard curve.

### *Statistical analysis*

Data was expressed as the mean ± standard deviation (SD). Statistical analyses were done using the SPSS 10.0 software package (Chicago, IL, United States). Differences between groups were assessed using Fisher's exact test or the $\chi^2$ test. $P \leqslant 0.05$ was regarded as statistically significant.

## RESULTS

### *Expression of NF-κB/p65 and P-p65 in HepG2 cells with TNF-α*

The comparative analysis of NF-κB/p65 and P-p65 expression in human HepG2 cells induced with TNF-α are shown in Figure 1. The ratio of NF-κB/p65 and the relative expression of P-p65 to β-actin were increased in HepG2 cells treated with a time course of 10 ng/mL of TNF-α (Figure 1A), as shown with Western blotting (Figure 1B). The expression of NF-κB/p65 or P-p65 was slight in HepG2 cells. Incubation of HepG2 cells with TNF-α (10 ng/mL) significantly increased the expression of NF-κB/p65 at 0.5, 4, and 24 h relative to that at 0 h ($P < 0.01$). The expression of P-p65 was significantly higher ($P < 0.01$) at 24 h than at 0, 0.5, and 4 h[23].

### *Expression of p65, P-p65 and P-gp in HepG2 cells with doxorubicin*

The expression of NF-κB/p65, P-p65, and P-gp induced by different doses of doxorubicin in HepG2 cells are shown in Figure 2. The expression of NF-κB/p65 and P-p65 with different doses of doxorubicin was analyzed using Western blotting (Figure 2A). There were no significant changes between NF-κB/p65 or P-p65 with various doses or incubation times of doxorubicin. The ratio of P-gp expression to β-actin in human hepG2 cells with different doses of doxorubicin at different times (Figure 2B) was analyzed by Western blotting (Figure 2C). P-gp expression in cells without doxorubicin was minimal, with no significant change found in the cells with doxorubicin at 4 h. P-p65 expression at 24 h was 6- or 7-fold higher than that at 4 h.

### *Specific siRNA downregulated NF-κB/p65, P-p65, and P-gp expression*

The downregulation of NF-κB/p65, P-p65, and P-gp expression in HepG2 cells after siRNA transfection

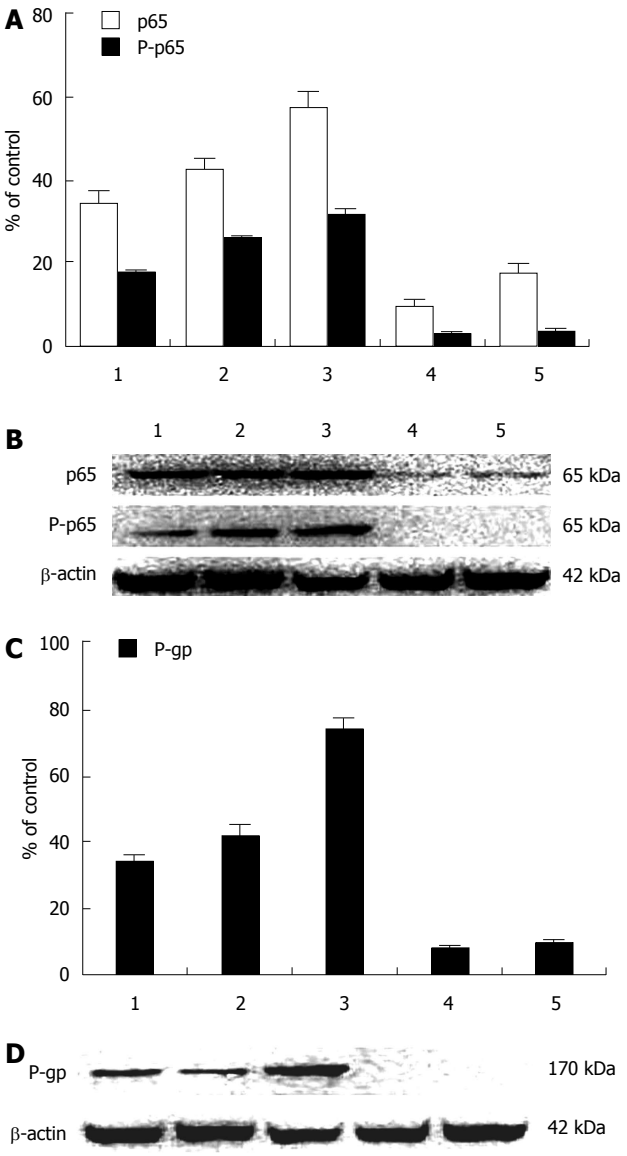



**Figure 3 Downregulation of nuclear factor-κB/p65, P-p65, and P-gp expression in HepG2 cells after siRNA transfection.** 1, control; 2, negative-siRNA for 48 h; 3, 0.5 μmol/L of doxorubicin for 48 h; 4, nuclear factor-κB (NF-κB)/p65 siRNA for 48 h; 5, 100 nmol/L of NF-κB/p65 siRNA for 24 h, then added 0.5 μmol/L of doxorubicin for another 24 h. A: The ratios of NF-κB/p65 and P-p65 expression in HepG2 cells after NF-κB/p65 siRNA transfection, data are means of independent triplicate experiments; B: The analysis of NF-κB/p65 and P-p65 expression in HepG2 cells after NF-κB/p65 siRNA transfection by western blotting; C: The relative levels of P-gp expression in HepG2 cells after NF-κB/p65 siRNA transfection, all values are means of independent triplicate experiments; D: The analysis of P-gp expression in HepG2 cells after NF-κB/p65 siRNA transfection by western blotting. The molecular weight NF-κB/p65, P-p65, and P-gp were 65 kDa, 65 kDa, and 170 kDa, respectively; and β-actin, molecular weight 42 kDa, was the control protein.

is shown in Figure 3. In HepG2 cells transfected with siRNA, the ratios of NF-κB/p65, P-p65, or P-gp expression to β-actin (Figure 3A and C) were decreased significantly, and this was confirmed by Western blotting (Figure 3B and D). The expression of NF-κB/p65, P-p65, and P-gp was inhibited significantly ($P < 0.001$) in compared with the control or doxorubicin group.

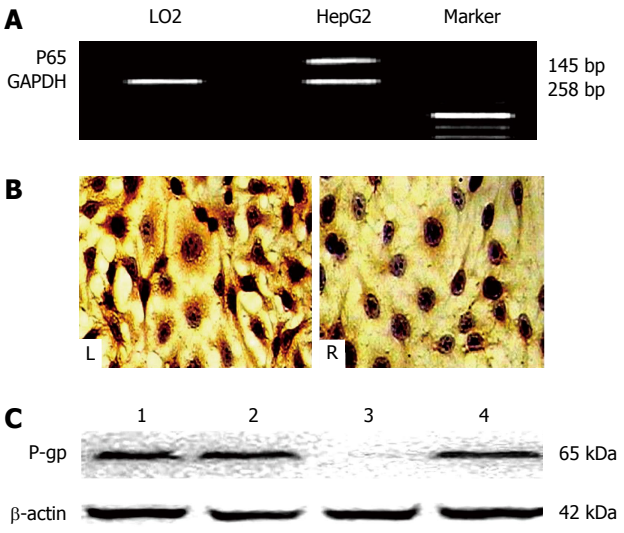



**Figure 4 Expression of nuclear factor-κB or nuclear factor-κB/p65 mRNA in HepG2 cells before and after siRNA transfection.** A: The expression of nuclear factor (NF)-κB/p65 mRNA was higher in HepG2 cells than LO2 cells before siRNA transfection. The fragments (145 bp) of NF-κB/p65 mRNA were amplified by reverse transcription polymerase chain reaction, separated on 2% agarose gel, and stained with ethidium bromide; B: Immunohistochemical staining with anti-NF-κB/p65 (streptavidin-peroxidase, original magnification × 40); NF-κB/p65 positive material was a brown-yellow fine particle layer and localized in the cytoplasm and nucleus of HepG2 cells (L); the expression of NF-κB/p65 positive material in the cytoplasm and nucleus of HepG2 cells with siRNA transfection (R); C: Western-blotting of NF-κB/p65 in cytoplasm and nucleus of HepG2 cells with siRNA transfection. Lanes 1, cytoplasm of HepG2 cells; Lanes 2, nucleus of HepG2 cells; Lanes 3, cytoplasm of HepG2 cells with 100 nmol/L of siRNA transfection; and Lanes 4, nucleus of HepG2 cells with 100 nmol/L of siRNA transfection. p65, 65 kDa; β-actin, 42 kDa, as the control protein.

### *Expression alteration of NF-κB in HepG2 cells with siRNA*

The expression of NF-κB/p65 and NF-κB/p65 mRNA in HepG2 cells before and after siRNA transfection is shown in Figure 4. The expression of NF-κB/p65 mRNA was significantly higher in HepG2 cells than LO2 cells ($P < 0.001$, Figure 4A) before siRNA transfection. The relative ratio of NF-κB/p65 mRNA to GAPDH was 1.13 ± 0.03 in HepG2 cells and 0.29 ± 0.07 in LO2 cells. NF-κB/p65 positive material was a brown-yellow fine particle layer and localized in cytoplasm and nucleus of HepG2 cells; and this expression pattern was different in the cytoplasm and nucleus of HepG2 cells transfected with siRNA [$P < 0.001$, Figure 4B(L) *vs* Figure 4B(R)]. The incidence of NF-κB/p65 expression decreased significantly in the cytoplasm of HepG2 cells with siRNA transfection but not in nucleus of HepG2 cells (Figure 4C), as confirmed by Western blotting.

### *HepG2 cells with siRNA sensitized to doxorubicin*

The effects of human HepG2 cell viability with doxorubicin are shown in Figure 5A. The viability of HepG2 cells was analyzed after the cells were incubated with different doses of doxorubicin for 24 h. Drug cytotoxicity in HepG2 cells was dose-dependent, and the $IC_{50}$ was between 0.5-1.0 μmol/L during 24 h. The viability of HepG2 cells with combination siRNA

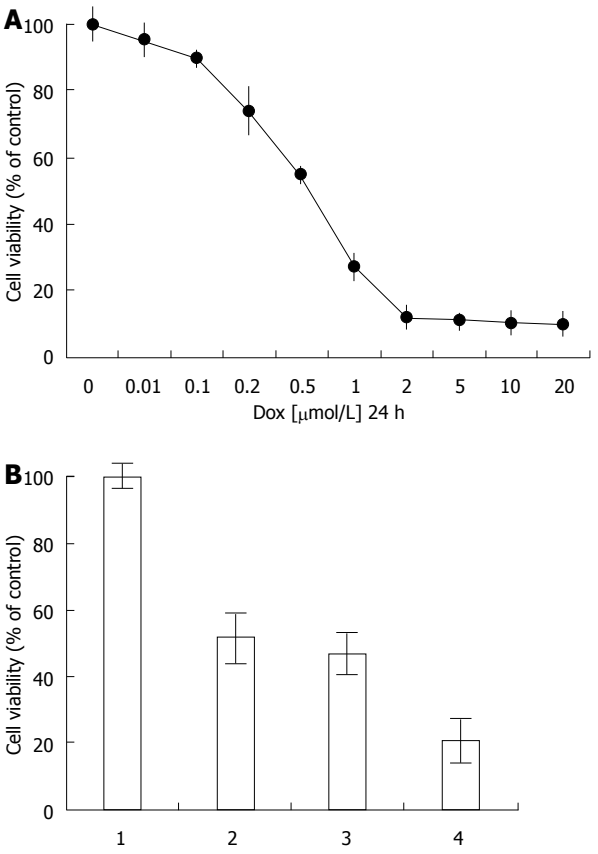



**Figure 5 The viability of human HepG2 cells with doxorubicin after nuclear factor-κB/p65 siRNA transfection.** A: The effects of doxorubicin on HepG2 cells by MTT assay during a 24 h period. All values are means of independent triplicate experiments. Dox, doxorubicin; B: Nuclear factor-κB (NF-κB)/ siRNA inhibition of NF-κB/p65 sensitized the HepG2 cells to doxorubicin, 1, control; 2, 0.5 μmol/L of doxorubicin for 48 h; 3, negtive siRNA for 24 h, then added 0.5 μmol/L of doxorubicin for another 24 h; 4, 100 nmol/L of NF-κB/p65 siRNA for 24 h, then added 0.5 μmol/L of doxorubicin for another 24 h. Data were derived from three independent experiments ($n$ = 3).

transfection and doxorubicin is shown in Figure 5B. Viability in cells treated with doxorubicin (0.5 μmol/L) for 24 h was reduced to 51% compared with the control group. In cells transfected with negative siRNA for 1 d and then treated with doxorubicin for 1 d, the cell viability was reduced to 47% compared with control. There was no significant difference between the doxorubicin only group and the combination negative-siRNA and doxorubicin group. In cells transfected with siRNA for 1 d and then treated with doxorubicin for 1 d, the cell viability was reduced to 23%. The cell viability with siRNA and doxorubicin was significantly lower than that in the doxorubicin only group ($P$ < 0.001). In HepG2 cells transfected with siRNA and treated with doxorubicin, the expression of NF-κB/p65, P-p65, and P-gp was decreased relative to doxorubicin treatment alone.

## DISCUSSION

HCC is one of the most common cancers and one of the most resistant tumors to systemic chemotherapy[24,25]. Chronic infections with HBV and HCV are etiologically linked to hepatitis, liver cirrhosis, and HCC. Both viruses may induce activation of NF-κB in hepatocytes, which plays a crucial role in the regulation of cell growth and apoptosis[26-28]. NF-κB signaling complexes in both viruses in HCC tumor and non-tumor tissues may disclose possible common mechanisms in hepatocarcinogenesis[29,30]. NF-κB and P-gp are important mediators of chemotherapy-induced cell death. In the present study, we evaluated siRNA-mediated inhibition of NF-κB expression and application of chemotherapy to explore the anti-cancer effect on HepG2 cells.

Doxorubicin is a topoisomerase Ⅱ inhibitor and can destruct the synthesis of DNA, induce NF-κB activation, and inhibit cell apoptosis[31,32]. The viability of HepG2 cells was analyzed in cells incubated with different doses of doxorubicin for 1 d. However, extracellular stimulation, including antineoplastic agents, mitogens, hormones, cytokines, and growth factors, can upregulate the expression of the MDR gene. We showed TNF-α can induce NF-κB expression, as shown by the time-dependent increase of NF-κB/p65 in HepG2 cells incubated with TNF-α (10 ng/mL) (Figure 1). In addition, P-p65, the active form of NF-κB/p65 that is phosphorylated at $Ser^{536}$ of the C-terminal transactivation domain during the phosphorylation and degradation of IκB, was increased with 24 h of TNF-α treatment[13]. Our results indicated that HCC is a tumor with high NF-κB expression.

In the process of liver formation or liver regeneration, activity of MDR1 is abnormal. The human MDR gene family includes two members, MDR1 and MDR2, and abnormally regulated MDR1 is closely linked to the MDR phenotype[15,17]. NF-κB was moderately expressed in resting HepG2 cells. In cells treated with doxorubicin, NF-κB/p65 and P-p65 expression was significantly and dose-dependently increased (Figure 2A). However, the P-gp expression in HepG2 cells was related to drug dose and treatment duration. Interestingly, the expression of P-p65 at 24 h was 6-fold to 7-fold higher than that at 4 h (Figure 2B and C). Doxorubicin induced P-gp expression in HepG2 cells, whereas the control HepG2 cells expressed very low P-gp. The first exon of MDR gene promoter contains the NF-κB binding sequence, and a variety of chemotherapeutic drugs can induce formation of MDR phenotype in malignant cells that restrict the efficacy of drugs. These data suggest that activated P-gp is involved in the formation of the MDR phenotype.

NF-κB activation in tumor cells was resistant to doxorubicin-based chemotherapy[16]. The NF-κB/p65 pathway acts as a bridge in the process of doxorubicin-mediated upregulation of P-gp expression. The expression of NF-κB/p65, P-p65, and P-gp was significantly downregulated in HepG2 cells transfected with siRNA (Figure 3) compared with cells in the control or doxorubicin alone groups. The mRNA

expression of NF-κB/p65 and NF-κB/p65 in HepG2 cells before and after siRNA transfection was also significantly different (Figure 4). The NF-κB/p65 mRNA expression or the ratio of NF-κB/p65 mRNA to GAPDH was significantly higher in HepG2 cells than that in LO2 cells before siRNA transfection. After HepG2 cells were transfected with siRNA, positive NF-κB/p65 expression decreased significantly in cytoplasm but not in nucleus, suggesting that inhibiting NF-κB activity can enhance the cytotoxicity of drugs in liver cancer cells.

It is speculated that doxorubicin-induced upregulation of MDR expression is mediated by activation of the NF-κB signaling pathway[33,34]. The drug cytotoxicity on HepG2 cells was dose-dependent, with an $IC_{50}$ between 0.5-1.0 μmol/L (Figure 5A). In order to confirm this conjecture, p65-specific siRNA was designed and transfected in HepG2 cells. The cell viability of HepG2 cells with negative-siRNA treated with doxorubicin was reduced to 47% (Figure 5B), whereas viability of cells with NF-κB/p65-siRNA treated with doxorubicin was reduced to 23%, suggesting that siRNA-mediated inhibition of NF-κB/p65 downregulated P-gp expression, decreased formation of MDR phenotype, and sensitized tumor cells to drug[3].

NF-κB signaling pathway could be a potential target for the design of highly effective therapeutic agents for the chemoprevention of HCC[35,36]. Specific siRNA combined with doxorubicin can enhance doxorubicin cytotoxicity to HepG2 cells by downregulating NF-κB and P-gp expression. Furthermore, stable NF-κB inhibition and chemosensitization can significantly inhibit tumor cell proliferation. Thus, the modulation of NF-κB may represent an advance in HCC therapy efficacy and is worthy of further research and investigation.

## ACKNOWLEDGMENTS

The authors thank Dr. T. FitzGibbon for comments on earlier drafts of the manuscript.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is one of the most resistant tumors to systemic chemotherapy, and doxorubicin is one of the chemotherapy drugs used in the treatment of HCC. Nuclear factor-kappa B (NF-κB) has been described as an important mediator of chemotherapy-induced cell death. Because the first exon of the multi-drug resistance (MDR) gene promoter contains the NF-κB binding sequence, it may be one of the downstream target genes of NF-κB. A variety of chemotherapeutic drugs can induce formation of MDR phenotype in malignant cells, thereby restricting the efficacy of drugs. However, the role of NF-κB signaling pathway in chemotherapy-induced MDR remains to be elucidated.

### Research frontiers

Recently, Gu *et al* investigated the inhibitory effects of intervention of the tumor necrosis factor (TNF)-α/NF-κB signaling pathway on HCC cell proliferation. HepG2 cells were cultured *in vitro* and treated with anti-TNFa mAb to downregulate its expression or transfected with NF-κBp65 siRNA to inhibit its activation, and suggesting that the proliferation of hepatoma cells might be significantly inhibited by intervening in NF-κB signaling pathway activation, which promotes cell apoptosis and blocks cell cycling.

### Innovations and breakthroughs

Hepatic NF-κB signaling pathway could be a potential target for designing highly effective therapeutic agents and chemoprevention of HCC. Specific siRNA combined with doxorubicin could enhance the doxorubicin cytotoxicity to human HepG2 cells by downregulating NF-κB and P-glycoprotein (P-gp) expression. Stable NF-κB inhibition and chemo- sensitization could significantly inhibit tumor cell proliferation. Thus, the modulation of NF-κB might represent an improvement in HCC therapy efficacy, and it is worthy of further research and investigation.

### Applications

Tumorigenic protein (HBx or core protein of HCV) activates a variety of signaling pathways, including NF-κB activation and TNF-α. Abnormal activation of NF-κB modulates the transcription and expression of many genes in hepatocarcinogenesis. Understanding the role of NF-κB, a master regulator of inflammation and cell death, in the development of hepatocellular injury, liver fibrosis, and HCC, with a particular focus on the role of NF-κB in different cellular compartments of the liver is important. The application of NF-κB/p65 siRNA is an effective strategy for inhibiting HepG2 cell growth by down-regulating P-gp expression towards chemosensitization and apoptosis induction.

### Terminology

NF-κB is formed by five subunits, p65, Rel B, C-Rel, p50, and p52. The p50/p65 dimers play the common biological role, and it can be activated by chemotherapy drugs, cytokines, and viruses induced into nucleus. NF-κB acts as a central link between hepatic injury, fibrosis, and HCC, and it may represent a target for the prevention or treatment of liver fibrosis and HCC. NF-κB acts as a two-edged sword, and its inhibition may not only exert beneficial effects but also negatively impact hepatocyte viability. Finding appropriate targets or identifying drugs that either exert only a moderate effect on its activity or that can be specifically delivered to nonparenchymal cells will be essential to avoid the potential increase in liver injury associated with NF-κB blockade in hepatocytes.

### Peer-review

Authors have done excellent work in this study. They have investigated small interference RNA (siRNA)-mediated inhibition of NF-κB activation in human HepG2 cells exposed to an anti-cancer drug. The application of NF-κB/p65 siRNA is an effective strategy for inhibiting HepG2 cell growth by down-regulating P-gp expression towards chemosensitization and apoptosis induction

## REFERENCES

1 **Bruix J**, Gores GJ, Mazzaferro V. Hepatocellular carcinoma: clinical frontiers and perspectives. *Gut* 2014; **63**: 844-855 [PMID: 24531850]
2 **Wu L**, Tang ZY, Li Y. Experimental models of hepatocellular carcinoma: developments and evolution. *J Cancer Res Clin Oncol* 2009; **135**: 969-981 [PMID: 19399516]
3 **Llovet JM**, Bruix J. Molecular targeted therapies in hepatocellular carcinoma. *Hepatology* 2008; **48**: 1312-1327 [PMID: 18821591]
4 **Yao DF**, Dong ZZ, Yao M. Specific molecular markers in hepatocellular carcinoma. *Hepatobiliary Pancreat Dis Int* 2007; **6**: 241-247 [PMID: 17548245]
5 **Frau M**, Biasi F, Feo F, Pascale RM. Prognostic markers and putative therapeutic targets for hepatocellular carcinoma. *Mol Aspects Med* 2010; **31**: 179-193 [PMID: 20176048]
6 **Feo F**, Frau M, Tomasi ML, Brozzetti S, Pascale RM. Genetic and epigenetic control of molecular alterations in hepatocellular carcinoma. *Exp Biol Med* (Maywood) 2009; **234**: 726-736 [PMID: 19429855]
7 **Liu LP**, Liang HF, Chen XP, Zhang WG, Yang SL, Xu T, Ren L. The role of NF-kappaB in Hepatitis b virus X protein-mediated upregulation of VEGF and MMPs. *Cancer Invest* 2010; **28**: 443-451 [PMID: 20073580]

8 **Kim HR**, Lee SH, Jung G. The hepatitis B viral X protein activates NF-kappaB signaling pathway through the up-regulation of TBK1. *FEBS Lett* 2010; **584**: 525-530 [PMID: 19958770]

9 **Dong ZZ**, Yao DF, Wu W, Yao M, Yu HB, Shen JJ, Qiu LW, Yao NH, Sai WL, Yang JL. Delayed hepatocarcinogenesis through antiangiogenic intervention in the nuclear factor-kappa B activation pathway in rats. *Hepatobiliary Pancreat Dis Int* 2010; **9**: 169-174 [PMID: 20382589]

10 **Wu W**, Yao DF, Qiu LW, Sai WL, Shen JJ, Yu HB, Wu XH, Li YM, Wang YL, Gu WJ. Characteristics of hepatic nuclear-transcription factor-kappa B expression and quantitative analysis in rat hepatocarcinogenesis. *Hepatobiliary Pancreat Dis Int* 2009; **8**: 504-509 [PMID: 19822494]

11 **Karin M**. Nuclear factor-kappaB in cancer development and progression. *Nature* 2006; **441**: 431-436 [PMID: 16724054]

12 **Hayden MS**, West AP, Ghosh S. SnapShot: NF-kappaB signaling pathways. *Cell* 2006; **127**: 1286-1287 [PMID: 17174900]

13 **O'Neil BH**, Bůzková P, Farrah H, Kashatus D, Sanoff H, Goldberg RM, Baldwin AS, Funkhouser WK. Expression of nuclear factor-kappaB family proteins in hepatocellular carcinomas. *Oncology* 2007; **72**: 97-104 [PMID: 18025803]

14 **Yan F**, Wang XM, Liu ZC, Pan C, Yuan SB, Ma QM. JNK1, JNK2, and JNK3 are involved in P-glycoprotein-mediated multidrug resistance of hepatocellular carcinoma cells. *Hepatobiliary Pancreat Dis Int* 2010; **9**: 287-295 [PMID: 20525557]

15 **Sun Z**, Zhao Z, Li G, Dong S, Huang Z, Ye L, Liang H, Qu J, Ai X, Zhang W, Chen X. Relevance of two genes in the multidrug resistance of hepatocellular carcinoma: in vivo and clinical studies. *Tumori* 2010; **96**: 90-96 [PMID: 20437864]

16 **Li G**, Dong S, Qu J, Sun Z, Huang Z, Ye L, Liang H, Ai X, Zhang W, Chen X. Synergism of hydroxyapatite nanoparticles and recombinant mutant human tumour necrosis factor-alpha in chemotherapy of multidrug-resistant hepatocellular carcinoma. *Liver Int* 2010; **30**: 585-592 [PMID: 19780956]

17 **Yan F**, Wang XM, Pan C, Ma QM. Down-regulation of extracellular signal-regulated kinase 1/2 activity in P-glycoprotein-mediated multidrug resistant hepatocellular carcinoma cells. *World J Gastroenterol* 2009; **15**: 1443-1451 [PMID: 19322916]

18 **Vainer GW**, Pikarsky E, Ben-Neriah Y. Contradictory functions of NF-kappaB in liver physiology and cancer. *Cancer Lett* 2008; **267**: 182-188 [PMID: 18479806]

19 **McCarty MF**, Block KI. Preadministration of high-dose salicylates, suppressors of NF-kappaB activation, may increase the chemosensitivity of many cancers: an example of proapoptotic signal modulation therapy. *Integr Cancer Ther* 2006; **5**: 252-268 [PMID: 16880431]

20 **Ho WC**, Dickson KM, Barker PA. Nuclear factor-kappaB induced by doxorubicin is deficient in phosphorylation and acetylation and represses nuclear factor-kappaB-dependent transcription in cancer cells. *Cancer Res* 2005; **65**: 4273-4281 [PMID: 15899819]

21 **Shareef MM**, Brown B, Shajahan S, Sathishkumar S, Arnold SM, Mohiuddin M, Ahmed MM, Spring PM. Lack of P-glycoprotein expression by low-dose fractionated radiation results from loss of nuclear factor-kappaB and NF-Y activation in oral carcinoma cells. *Mol Cancer Res* 2008; **6**: 89-98 [PMID: 18234965]

22 **Vander Borght S**, Komuta M, Libbrecht L, Katoonizadeh A, Aerts R, Dymarkowski S, Verslype C, Nevens F, Roskams T. Expression of multidrug resistance-associated protein 1 in hepatocellular carcinoma is associated with a more aggressive tumour phenotype and may reflect a progenitor cell origin. *Liver Int* 2008; **28**: 1370-1380 [PMID: 19055643]

23 **Wang J**, Tokoro T, Higa S, Kitajima I. Anti-inflammatory effect of pitavastatin on NF-kappaB activated by TNF-alpha in hepatocellular carcinoma cells. *Biol Pharm Bull* 2006; **29**: 634-639 [PMID: 16595893]

24 **Aravalli RN**, Steer CJ, Cressman EN. Molecular mechanisms of hepatocellular carcinoma. *Hepatology* 2008; **48**: 2047-2063 [PMID: 19003900]

25 **Butterfield LH**. Recent advances in immunotherapy for hepatocellular cancer. *Swiss Med Wkly* 2007; **137**: 83-90 [PMID: 17370144]

26 **Chang CS**, Huang SM, Lin HH, Wu CC, Wang CJ. Different expression of apoptotic proteins between HBV-infected and non-HBV-infected hepatocellular carcinoma. *Hepatogastroenterology* 2007; **54**: 2061-2068 [PMID: 18251160]

27 **Sato Y**, Kato J, Takimoto R, Takada K, Kawano Y, Miyanishi K, Kobune M, Sato Y, Takayama T, Matunaga T, Niitsu Y. Hepatitis C virus core protein promotes proliferation of human hepatoma cells through enhancement of transforming growth factor alpha expression via activation of nuclear factor-kappaB. *Gut* 2006; **55**: 1801-1808 [PMID: 16581947]

28 **Karin M**, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 2005; **5**: 749-759 [PMID: 16175180]

29 **Maeda S**, Omata M. Inflammation and cancer: role of nuclear factor-kappaB activation. *Cancer Sci* 2008; **99**: 836-842 [PMID: 18294278]

30 **Guo L**, Guo Y, Xiao S. Expression of tyrosine kinase Etk/Bmx and its relationship with AP-1- and NF-kappaB-associated proteins in hepatocellular carcinoma. *Oncology* 2007; **72**: 410-416 [PMID: 18196928]

31 **Assef Y**, Rubio F, Coló G, del Mónaco S, Costas MA, Kotsias BA. Imatinib resistance in multidrug-resistant K562 human leukemic cells. *Leuk Res* 2009; **33**: 710-716 [PMID: 18977528]

32 **Barraud L**, Merle P, Soma E, Lefrançois L, Guerret S, Chevallier M, Dubernet C, Couvreur P, Trépo C, Vitvitski L. Increase of doxorubicin sensitivity by doxorubicin-loading into nanoparticles for hepatocellular carcinoma cells in vitro and in vivo. *J Hepatol* 2005; **42**: 736-743 [PMID: 15826724]

33 **Xie Z**, Choong PF, Poon LF, Zhou J, Khng J, Jasinghe VJ, Palaniyandi S, Chen CS. Inhibition of CD44 expression in hepatocellular carcinoma cells enhances apoptosis, chemosensitivity, and reduces tumorigenesis and invasion. *Cancer Chemother Pharmacol* 2008; **62**: 949-957 [PMID: 18259754]

34 **Calvisi DF**, Pascale RM, Feo F. Dissection of signal transduction pathways as a tool for the development of targeted therapies of hepatocellular carcinoma. *Rev Recent Clin Trials* 2007; **2**: 217-236 [PMID: 18474008]

35 **Van Waes C**. Nuclear factor-kappaB in development, prevention, and therapy of cancer. *Clin Cancer Res* 2007; **13**: 1076-1082 [PMID: 17317814]

36 **Cho HA**, Park IS, Kim TW, Oh YK, Yang KS, Kim JS. Suppression of hepatitis B virus-derived human hepatocellular carcinoma by NF-kappaB-inducing kinase-specific siRNA using liver-targeting liposomes. *Arch Pharm Res* 2009; **32**: 1077-1086 [PMID: 19641890]


**P- Reviewer**: Guo ZJ, Hu YH **S- Editor**: Yu J
**L- Editor**: Filipodia **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12822

*World J Gastroenterol* 2015 December 7; 21(45): 12822-12834
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

Basic Study

# Heat shock pretreatment improves stem cell repair following ischemia-reperfusion injury *via* autophagy

Peng-Fei Qiao, Lei Yao, Xin-Chen Zhang, Guo-Dong Li, De-Quan Wu

Peng-Fei Qiao, Lei Yao, Xin-Chen Zhang, De-Quan Wu, Department of General Surgery, the Second Affiliated Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China

Guo-Dong Li, Department of General Surgery, the Fourth Affiliated Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China

Author contributions: Wu DQ and Qiao PF designed the research and drafted the manuscript; Qiao PF, Yao L, Zhang XC and Li GD carried out all the experiments; all the authors read and approved the final published version.

Supported by Grants from the Clinical Research Special Fund of Wu Jieping Medical Foundation, No. 320.6750.12263.

Institutional review board statement: All of the study procedures were reviewed and approved by the Medical Ethics Committee of Harbin Medical University, Harbin, China.

Institutional animal care and use committee statement: This study was conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Academy Press. All procedures involving animals were reviewed and approved by the Harbin Medical University Institutional Animal Care and Use Committee. All efforts were made to minimize suffering.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest in this study.

Data sharing statement: Technical appendices, statistical codes and datasets are available from the corresponding author at wudequanhum@163.com. Participants gave informed consent for data sharing. No additional data are available.



Correspondence to: De-Quan Wu, PhD, Professor, Department of General Surgery, the Second Affiliated Hospital of Harbin Medical University, No. 246, Xuefu Road, Nangang District, Harbin 150001, Heilongjiang Province, China. wudequanhmu@163.com
Telephone: +86-13796635887
Fax: +86-451-86605535

Received: April 16, 2015
Peer-review started: April 18, 2014
First decision: June 19, 2015
Revised: July 2, 2015
Accepted: September 2, 2015
Article in press: September 2, 2015
Published online: December 7, 2015

## Abstract

**AIM:** To investigate whether heat shock pretreatment (HSP) improves mesenchymal stem cell (MSC) repair *via* autophagy following hepatic ischemia-reperfusion injury (HIRI).

**METHODS:** Apoptosis of MSCs was induced by 250 mM hydrogen peroxide ($H_2O_2$) for 6 h. HSP was carried out using a 42 ℃ water bath for 1, 2 or 3 h. Apoptosis of MSCs was analyzed by flow cytometry, and Western blot was used to detect Bcl-2, Bax and cytochrome C expression. Autophagy of MSCs was analyzed by flow cytometry and transmission electron microscopy, and the expression of beclin Ⅰ and LC3-Ⅱ was detected by Western blot. MSCs were labeled *in vivo* with the fluorescent dye, CM-DiI, and subsequently transplanted into the portal veins of rats that had undergone HIRI. Liver levels of proliferating cell nuclear antigen (PCNA) were quantified by fluorescent microscopy. Serum aminotransferase activity and the extent of HIRI were also assessed at each time point.

**RESULTS:** HSP for 2 h reduced apoptosis of MSCs induced by $H_2O_2$ as seen by a decrease in apoptotic rate, a decrease in Bax and cytochrome C expression and an increase in Bcl-2 expression ($P < 0.001$). In addition, HSP for 2 h induced autophagy of MSCs exposed to $H_2O_2$ as shown by an increase in acidic vesicular organelle-positive cells, beclin 1 and LC3-Ⅱ expression, and autophagosome formation ($P < 0.05$). Treatment with 3-methyladenine attenuated HSP-induced autophagy and abolished the protective effects of HSP on the apoptosis of MSCs. Rapamycin failed to have additional effects on either autophagy or apoptosis compared with HSP alone. The phosphorylation of p38MAPK was significantly elevated and the phosphorylation of mTOR was downregulated in heat shock pretreated MSCs. Treatment with the p38MAPK inhibitor, SB203580, reduced HSP-induced autophagy in MSCs. *In vivo* studies showed that the transplantation of HSP-MSCs resulted in lower serum aminotransferase levels, lower Suzuki scores, improved histopathology and an increase in PCNA-positive cells ($P < 0.05$).

**CONCLUSION:** HSP effectively induces autophagy following exposure to $H_2O_2$ *via* the p38MAPK/mTOR pathway, which leads to enhanced MSC survival and improved MSC repair following HIRI in rats.

**Key words:** Hepatic ischemia-reperfusion injury; Heat shock pretreatment; Mesenchymal stem cells; Autophagy; Transplantation



**Core tip:** We investigated the interaction between autophagy and apoptosis in mesenchymal stem cells (MSCs) exposed to $H_2O_2$. We found that heat shock pretreatment (HSP)-induced autophagy served as a protective mechanism. HSP for 2 h improved the therapeutic potential of MSCs in the treatment of ischemia-reperfusion (I/R) injury in rats and enhanced autophagy *via* the p38MAPK/mTOR pathway, which is involved in the protective effects of HSP on $H_2O_2$-induced MSC apoptosis. Systemic administration led to an increase in HSP-MSCs homing to I/R liver cells compared with MSCs, resulting in a significant improvement in liver function, accelerated mitogenic response and alleviation of histopathological damage.

Qiao PF, Yao L, Zhang XC, Li GD, Wu DQ. Heat shock pretreatment improves stem cell repair following ischemia-reperfusion injury *via* autophagy. *World J Gastroenterol* 2015; 21(45): 12822-12834 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12822.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12822

## INTRODUCTION

During surgical trauma, particularly liver transplantation, hepatic ischemia-reperfusion injury (HIRI) may occur, which is associated with a significant reduction in liver function[1,2]. Effective treatment strategies aimed at reducing HIRI may therefore offer major benefits in hepatic surgery and liver transplantation. A previous study demonstrated the specific involvement of bone marrow mesenchymal stem cells (MSCs) in the repair of HIRI[3]. However, due to local hypoxia, inflammation, and especially oxidative stress in the targeted tissue, the transplanted MSCs did not withstand the difficult microenvironment caused by ischemia-reperfusion (I/R) injury. Thus, low cell survival reduced the therapeutic effect[4]. It was also reported that < 1% of transplanted MSCs survived to the fourth day in an immunodeficient mouse heart model[5]. The poor MSC survival rate was also observed after transplantation into lungs and kidneys with I/R injury[6,7]. Therefore, it is imperative to protect MSCs from oxidative stress and other pro-apoptotic factors to improve their therapeutic effect.

Heat shock pretreatment (HSP) is known to activate certain types of self-protective proteins and protects cells *in vitro* from various environmental insults[8-10]. Several reports have shown that HSP of transplanted cells enhanced their survival in a heart model both *in vivo* and *in vitro*[11,12]. Thus, we hypothesized that HSP of MSCs could enhance their survival following transplantation into the liver after I/R injury. Recently, the induction of autophagy was shown to be a novel method of protecting MSCs from apoptosis[13,14]. Several reports have shown that heat shock treatment can activate autophagy in multiple cell types[15,16]. However, it is unknown whether autophagy can be activated by heat shock treatment or how it affects MSCs.

Autophagy is an essential cellular mechanism that occurs in eukaryotic cells[17,18]. In recent years, it has been found that autophagy plays a vital role in cell apoptosis and its role depends on cell type and cellular conditions. Autophagy can lead to pro-survival pathways, while inappropriate autophagy can induce cell death[19]. Under ischemia or hypoxia/serum deprivation (H/SD), autophagy can protect MSCs from apoptosis by eliminating reactive oxygen species and damaged organelles to provide energy[13,20]. Moreover, H/SD-induced autophagy has also been demonstrated to induce apoptosis in some cell types. Autophagy can also directly promote type Ⅱ programmed cell death[21]. However, the functional role of autophagy in oxidative stress-induced apoptosis in MSCs has not been fully elucidated.

Mitogen-activated protein kinase (p38MAPK) is a positive regulator of autophagy and is regulated by heat shock treatment to improve cardiac cell survival[8]. p38MAPK can be activated in response to exogenous stress such as hypoxia, starvation and heat shock, which in turn activates mitogen-activated protein kinase kinases (MKK)-3/4/6 and their effector kinases to stimulate autophagy[22]. However, little is known about the function of the p38MAPK pathway

in regulating the activation of autophagy in MSCs following heat shock treatment.

The aim of this study was to examine the function of autophagy in MSC apoptosis induced by oxidative stress injury. Further, we investigated whether HSP activates autophagy *via* the p38MAPK/mTOR pathway to protect MSCs against apoptosis.

## MATERIALS AND METHODS

### Animals

Thirty-two male Sprague-Dawley rats (about 220 g; 10 wk old) from the Animal Center of the Second Affiliated Hospital, Harbin Medical University were used in this study. The rats were cared for in accordance with the guidelines published by the US National Institutes of Health. All study procedures were approved by the Harbin Medical University Institutional Animal Care and Use Committee. The study was conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Academy Press.

### Cell culture and treatment

MSCs were collected as previously described[3], and density centrifugation was performed to isolate MSCs[23]. The femurs and tibias from male Wistar rats aged 4 wk were flushed, and bone marrow cells were collected and then fractionated in Lymphoprep™ density solution. Following centrifugation at 800 × *g* for 20 min, the cells at the interface were collected and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, United States) containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were incubated at 37 ℃ with 95% humidity and 5% $CO_2$. After 48 h, the culture medium was changed to remove non-adherent cells. After the fourth passage, MSCs were washed with phosphate buffered saline (PBS), exposed to HSP for different time periods (1, 2 or 3 h) in a 42 ℃ water bath and then incubated at 37 ℃ in a humidified atmosphere containing 5% $CO_2$ for 24 h (HSP-MSC group). Control cells were cultured under normal conditions without HSP (MSC group). To simulate tissue I/R microenvironment *in vitro*, MSCs were treated with 250 mM $H_2O_2$ (Sigma-Aldrich, United States) for 6 h[24,25]. The autophagy inhibitor, 3-methyladenine (3-MA; 5 mM; Sigma-Aldrich, United States), the autophagy promoter, rapamycin (10 nM; Cell Signaling Technology, United States), or the p38MAPK inhibitor, SB203580 (5 mM; Beyotime, China), was added to further examine the role of autophagy on MSC apoptosis.

### Evaluation of autophagy and apoptosis by flow cytometry

Cell apoptosis was examined using the Annexin V-FITC/PI Kit (Becton-Dickinson, United States). Briefly, MSCs were collected in 200 mL medium. Following resuspension, approximately 10 mL of Annexin V solution were added and incubated for 15 min at room temperature in the dark. Then, 300 mL medium buffer and 5 mL propidium iodide (PI) were added and the cell suspension was incubated for 15 min at room temperature in the dark. The cell suspension was then immediately analyzed by flow cytometry (Becton-Dickinson, United States). Cell Quest software was used to analyze $10^4$ cells.

Cell autophagy was examined by detecting acidic vesicular organelles (AVO) using acridine orange (AO) stain (Solarbio, China) according to published protocols[26]. Briefly, cells were stained with 1 mg/mL AO for 15 min and collected in PBS. In AO-stained cells, the cytoplasm fluoresces bright green, whereas AVOs, including lysosomes and autolysosomes, fluoresce bright red. The green (510-530 nm) and red (650 nm) fluorescence emission from $10^4$ cells illuminated with blue (488 nm) excitation light was measured by flow cytometry using Cell Quest software.

### Transmission electron microscopy

MSCs were harvested and fixed with 2.5% glutaraldehyde at 4 ℃ for 2 h. Cells were then suspended in PBS containing 1% osmic acid at 4 ℃ for 2 h, Following dehydrating and embedding[13], ultrathin sections were prepared on uncoated copper grids using an Ultrotome (Leica, Reichert Ultracuts) and stained with uranyl acetate and lead citrate. Images were captured using a transmission electron microscope (JEM1230; JEOL).

### Western blot

Protein lysates were separated using SDS-PAGE and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, United States). Membranes were probed with the appropriate primary antibodies (Supplemental Table 1). Alexa Fluor® 680 donkey anti-mouse IgG (H + L) or Alexa Fluor® 680 donkey anti-rabbit IgG (H + L) were used as secondary antibodies (1:5000; Invitrogen, United States). Fluorophores were detected using the Odyssey™ Infrared Imaging System (Li-Cor, Lincoln, NE, United States).

### Labeling of MSCs

The transplanted MSCs were labeled with 10 μmol/L CM-Dil (Invitrogen, United States) according to published protocols[27].

### Model of HIRI and cell transplantation

HIRI in the rat model was performed as previously described[3]. Briefly, a midline laparotomy was performed following anesthesia administration with intraperitoneal sodium pentobarbital (60 mg/kg). The left lateral and medial lobes of the liver were then clamped at their bases using an atraumatic clip. Ischemia was induced in 70% of the segmental liver and prevented ischemia in the mesenteric veins[28]. Throughout the administration of anesthesia, body

temperature was monitored by a rectal probe and maintained at 37 ℃ by a heating lamp. The clamp was removed after 60 min, and 1 × $10^6$ CM-Dil-labeled MSCs or HSP-MSCs suspended in 200 μL PBS were immediately transplanted into the portal vein using a 30-gauge needle, in the MSC group and HSP-MSC group, respectively. The control group underwent laparotomy only and received 200 μL PBS. The 32 rats were randomly divided into 4 groups. At 24 h after transplantation, 2 mL blood was harvested from the inferior vena cava before the animals were sacrificed by cervical spine dislocation. Livers were harvested immediately.

### *Immunofluorescence microscopy*

The chest was opened following tracheal intubation and the rats were perfused with 4% paraformaldehyde (Sigma-Aldrich, United States) in 0.01 M PBS following an overdose of anesthesia (sodium pentobarbital; 100 mg/kg, intraperitoneal) for 2 min[29]. Harvested livers were cryopreserved in 30% sucrose at 4 ℃ overnight, embedded in optimal cutting temperature (OCT) compound, and cut into 4 μm-thick sections using a cryostat. The sections were rinsed twice with PBS, fixed in 4% paraformaldehyde for 20 min at room temperature, and washed three times with PBS. After permeabilization with 0.2% Triton X-100, the sections were blocked at 4 ℃ overnight in 1% BSA/0.05% Triton X-100. Sections were then incubated with an antibody against PCNA (1:200) at 37 ℃ for 2 h. After washing three times with PBS, the sections were incubated with Alexa Fluor® 488-conjugated Affinipure goat anti-rabbit IgG (H + L) secondary antibody (1:200; ZSGB-Bio, China) for 1 h at room temperature. After extensive washing, the sections were examined under a fluorescence microscope[30].

### *Measurement of liver function*

To evaluate the severity of HIRI, the serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by an automatic analyzer (Hitachi, Japan) as described previously[31].

### *Immunohistochemical staining*

Tissue sections of 1.5 cm × 1.5 cm × 2 mm were subjected to immunohistochemical staining to assess PCNA 24 h after cell transplantation. Immunohistochemical staining of sections for PCNA expression was performed by a standard streptavidin-biotin peroxidase complex method[32]. Tissue sections (4 mm) were deparaffinized and rehydrated by standard protocols, autoclaved at 95 ℃ for 20 min, and cooled to 30 ℃. Normal rabbit serum (10%) was used to block non-specific binding sites. Sections were then incubated with anti-PCNA primary antibody (1:100) in PBS containing 1% bovine serum albumin at 4 ℃ overnight. The sections were washed in PBS, incubated with biotinylated anti-rabbit IgG for 30 min at room temperature, and then a streptavidin-biotin peroxidase complex solution (Nichirei, Japan). The chromogen, 3, 3'-diaminobenzidine tetra-hydrochloride, was used as a 0.02% solution containing 0.005% $H_2O_2$ in 50 mmol/L ammonium acetate-citrate acid buffer (pH 6.0). Sections were counterstained with Mayer's hematoxylin and mounted. Negative controls were established by replacing the primary antibody with normal rabbit serum. No staining was detected in the negative controls.

### *Histological analysis*

The degree of HIRI was assessed by histological analysis as previously described[3].

### *Statistical analysis*

The data were expressed as the mean ± SD, and representative results were from at least three independent experiments. For quantitative continuous data, the differences between two groups were examined and the data were analyzed using *t*-tests. When multiple comparisons were possible, ANOVA coupled with Tukey's post-hoc test correction was used. $P < 0.05$ was considered statistically significant. Statistical analyses were carried out using SPSS version 21 (SPSS Inc., Chicago, IL, United States) or the GraphPad Prism 5.0 software package (GraphPad Software, Inc., La Jolla, CA, United States).

## RESULTS

### *Heat shock pretreatment protected MSCs exposed to $H_2O_2$ against apoptosis*

The apoptotic rate and levels of the pro-apoptotic proteins, Bax and cytochrome C, were all reduced. The anti-apoptotic protein, Bcl-2, was increased in the $HSP_{1h}$ and $HSP_{2h}$ groups compared to the control and $H_2O_2$ group (Figure 1; $P < 0.01$). However, in the $HSP_{3h}$ group, the apoptotic rate and expression of Bax and cytochrome C were increased, while Bcl-2 expression was reduced (Figure 1; $P < 0.01$). These results suggest that 2 h of HSP protected MSCs from $H_2O_2$-induced apoptosis.

### *HSP induced time-dependent autophagy in MSCs*

To examine whether HSP activated autophagy in MSCs, the cells were pretreated with heat shock for 1, 2 or 3 h, and then exposed to $H_2O_2$ for 6 h. The number of AVO-positive cells identified by flow cytometry was increased in the HSP group compared with the control group (Figure 2A; $P < 0.05$). Different durations of HSP led to a time-dependent increase in the action of autophagy in MSCs exposed to $H_2O_2$, which peaked in the $HSP_{3h}$ group ($P < 0.01$). HSP-MSCs showed a significant time-dependent increase in the expression of LC3B-Ⅱ and the autophagic marker, beclin 1, compared to the control group (Figure 2D). Autophagosomes observed in HSP-MSCs exposed to

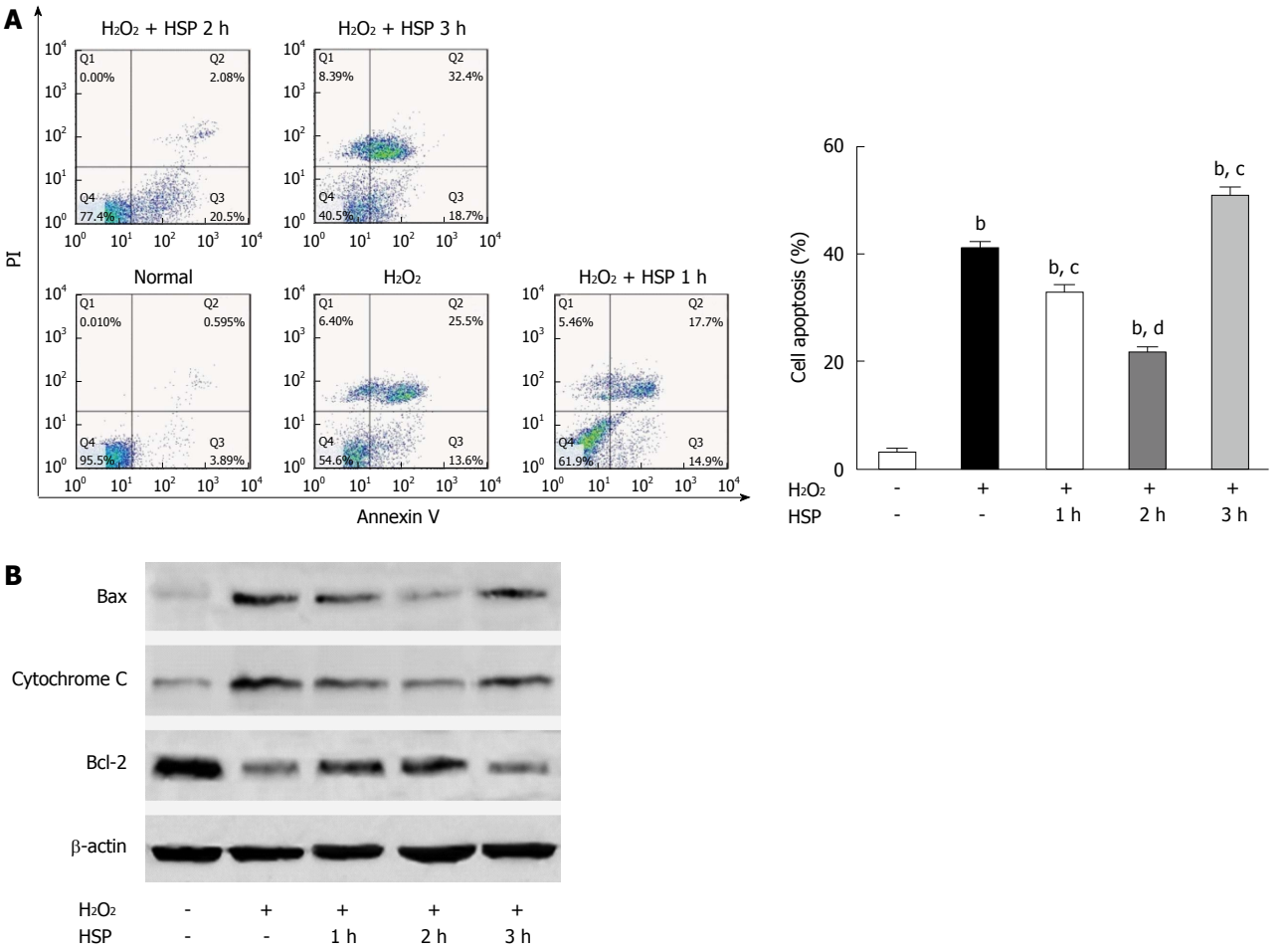



**Figure 1 Heat shock pretreatment protected mesenchymal stem cells from apoptosis induced by $H_2O_2$.** Apoptosis was analyzed using flow cytometry (A) and Western blot (B). The apoptotic rate and pro-apoptotic proteins Bax and cytochrome C were reduced, while the anti-apoptotic protein Bcl-2 was increased in the $HSP_{1h}$ and $HSP_{2h}$ group, particularly in the $HSP_{2h}$ group ($P < 0.01$). However, the apoptotic rate, and both Bax and cytochrome C were increased, while Bcl-2 was reduced in the $HSP_{3h}$ group. The data represent the results of three separate experiments. $^bP < 0.01$ compared with the normal control group; $^cP < 0.05$, $^dP < 0.01$ compared with the $H_2O_2$ group. HSP: Heat shock pretreatment.

$H_2O_2$ are shown in Figure 3. These results suggest that HSP promoted autophagic activity in MSCs exposed to $H_2O_2$ in a time-dependent manner.

### *HSP protects MSCs against $H_2O_2$-induced apoptosis by activating autophagy*

We found that $HSP_{2h}$ achieved the greatest protective effect against $H_2O_2$-induced apoptosis using flow cytometry and Western blot (Figure 1). To determine the role of autophagy in MSCs, we exposed cells to HSP for 2 h with 3-MA or rapamycin and $H_2O_2$ treatment for 6 h, and assessed autophagy and the apoptotic rate. Following 6 h of $H_2O_2$ treatment, 3-MA attenuated both the activation of autophagy and the anti-apoptotic capacity in MSCs treated with heat shock for 2 h, as shown by fewer AVO-positive MSCs (Figure 2C), lower expression of LC3-Ⅱ and beclin 1 (Figure 4B) and fewer autophagosomes in MSCs ($P < 0.01$; Figure 3). In addition, a higher apoptotic rate (Figure 4A), increased expression of Bax and cytochrome C, and decreased expression of Bcl-2 (Figure 4B) were found compared with the control group ($P < 0.01$) and the $HSP_{2h}$ group ($P < 0.05$). In addition, rapamycin failed to have any effect on autophagic activity and the apoptotic rate in MSCs pretreated with heat shock for 2 h. These results indicated that activation of autophagy by HSP for 2 h may serve as a protective mechanism against apoptosis in MSCs exposed to $H_2O_2$.

### *The p38MAPK/mTOR pathway is involved in HSP-induced autophagy*

To investigate whether HSP induced autophagy by activating the p38MAPK pathway, the p38MAPK inhibitor, SB203580, was used and the levels of autophagy were evaluated in HSP-MSCs exposed to $H_2O_2$. The results revealed that the expression of p38MAPK and mTOR did not significantly change in any of the groups. However, the phosphorylation of p38MAPK was upregulated and the phosphorylation of mTOR was downregulated in the $HSP_{2h}$ group compared with the control group (Figure 5). SB203580 reduced autophagy in the $HSP_{2h}$ group, as shown by a decrease in the number of AVO-positive MSCs ($P < 0.05$) (Figure 2C), expression of LC3-Ⅱ and beclin 1

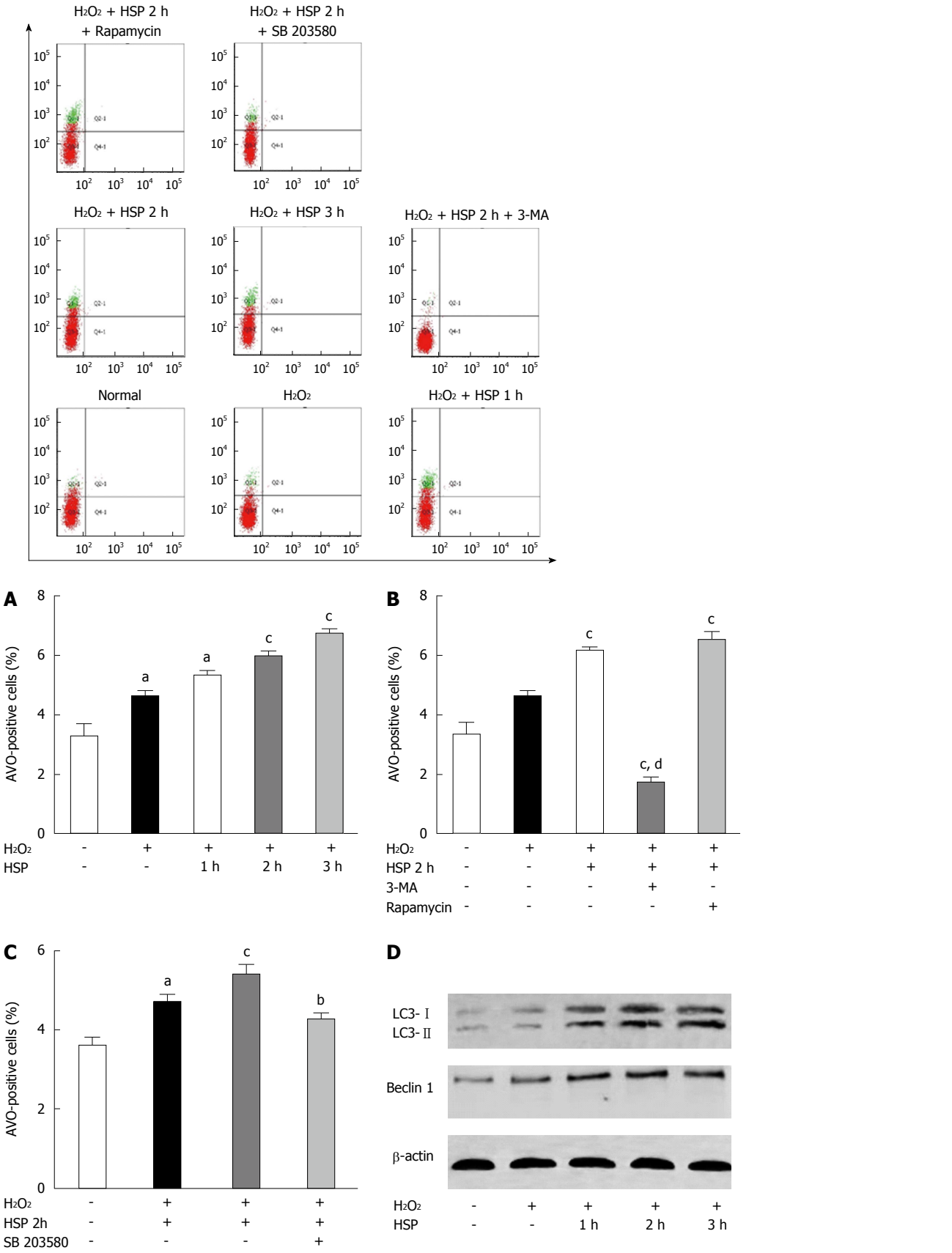



**Figure 2 Autophagy was determined by acidic vesicular organelle-positive mesenchymal stem cells (labeled in the circle) using a fluorescent dye (AO) and flow cytometry.** A: Different periods of heat shock pretreatment (HSP) ranging from 1 to 3 h led to a time-dependent increase in autophagy in mesenchymal stem cells (MSCs) exposed to $H_2O_2$, which peaked in the $HSP_{3h}$ group; B: 3-MA attenuated, whereas rapamycin failed to further increase HSP-induced autophagy; C: SB203580 significantly suppressed HSP-induced autophagy in MSCs exposed to $H_2O_2$; D: Western blot showed a significant time-dependent increase in expression of the autophagic marker LC3B-Ⅱ and beclin 1 in MSCs. The data represent the results of three separate experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ compared with the normal control group; $^{c}P < 0.05$, $^{d}P < 0.01$ compared with the $HSP_{2h}$ control group.

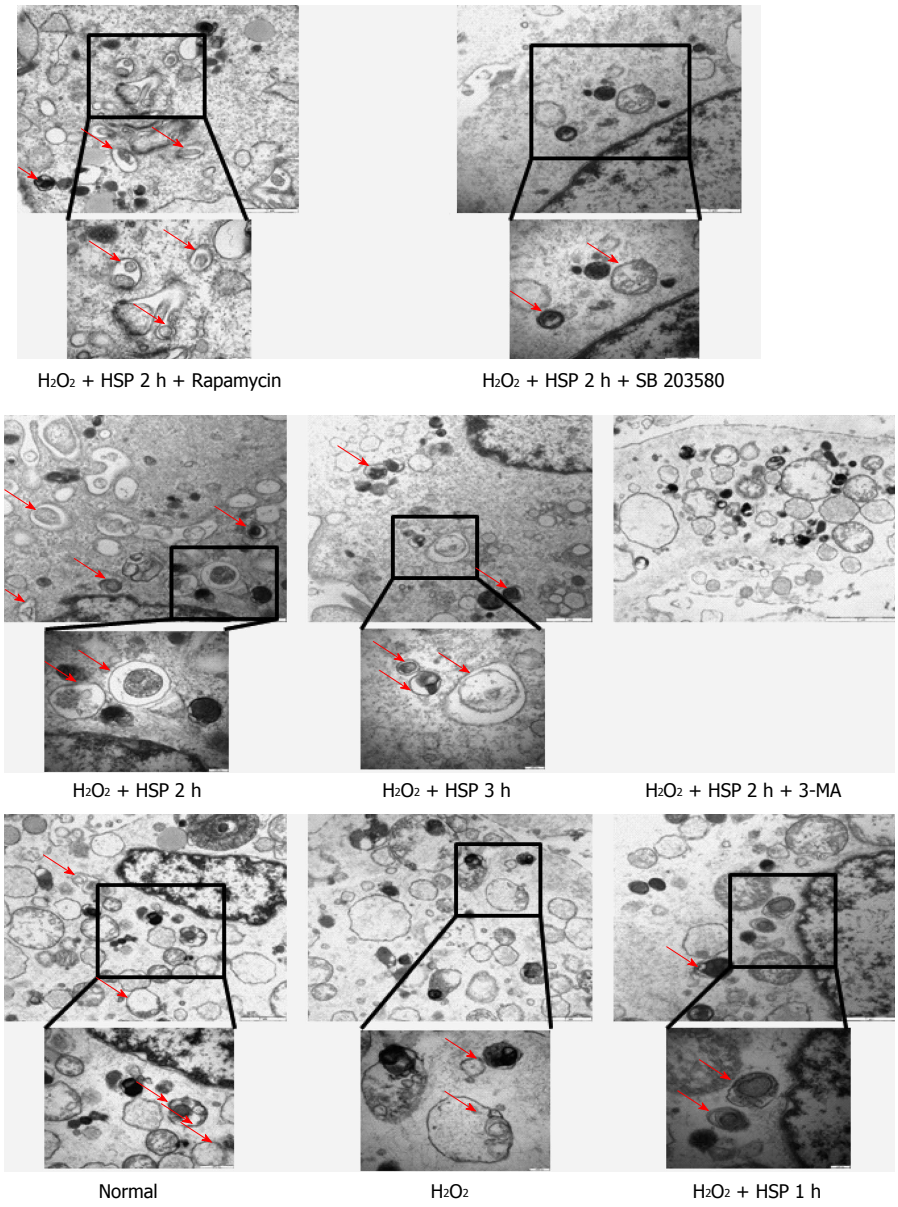



**Figure 3 Representative electron micrographs demonstrating autophagic vacuole formation in each group.** The arrows indicate the double-membrane vacuoles digesting organelles or cytosolic contents.

(Figure 5) and autophagosome formation (Figure 3). Furthermore, treatment with SB203580 abrogated the effects of p38MAPK phosphorylation, but failed to have any effect on the phosphorylation of mTOR. These data suggested that the p38MAPK/mTOR signaling pathway had a stimulatory role in the effects of HSP on MSC autophagy under $H_2O_2$ conditions.

### *HSP increased the homing and survival rate of transplanted MSCs to I/R livers in vivo*

We then investigated the survival rate and homing of transplanted MSCs to livers. Representative fluorescence microscopic images of MSCs after transplantation are shown in Figure 6. CM-Dil-labeled cells were detected only in sections that received transplanted MSCs. The total number of double-positive MSCs labeled by CM-Dil and PCNA in the HSP-MSC-treated group was higher than that in the MSC-treated group ($P < 0.05$). CM-Dil-labeled MSCs also showed PCNA reactivity.

### *HSP improved the therapeutic potential of MSCs in the treatment of HIRI in rats*

Twenty-four hours after MSC transplantation, liver function was assessed by serum AST and ALT levels. Compared with the control group, transplantation of MSCs improved liver function in rats. However, HSP-MSC-treated rats had lower AST and ALT levels compared with MSC-treated animals (Figure 7A; $P < 0.05$). A histological score was then assigned to the liver and the expression of PCNA was examined 24 h after transplantation. As expected, all I/R-induced livers showed sinusoidal congestion, cytoplasmic vacuolization and focal necrosis, which are indicative

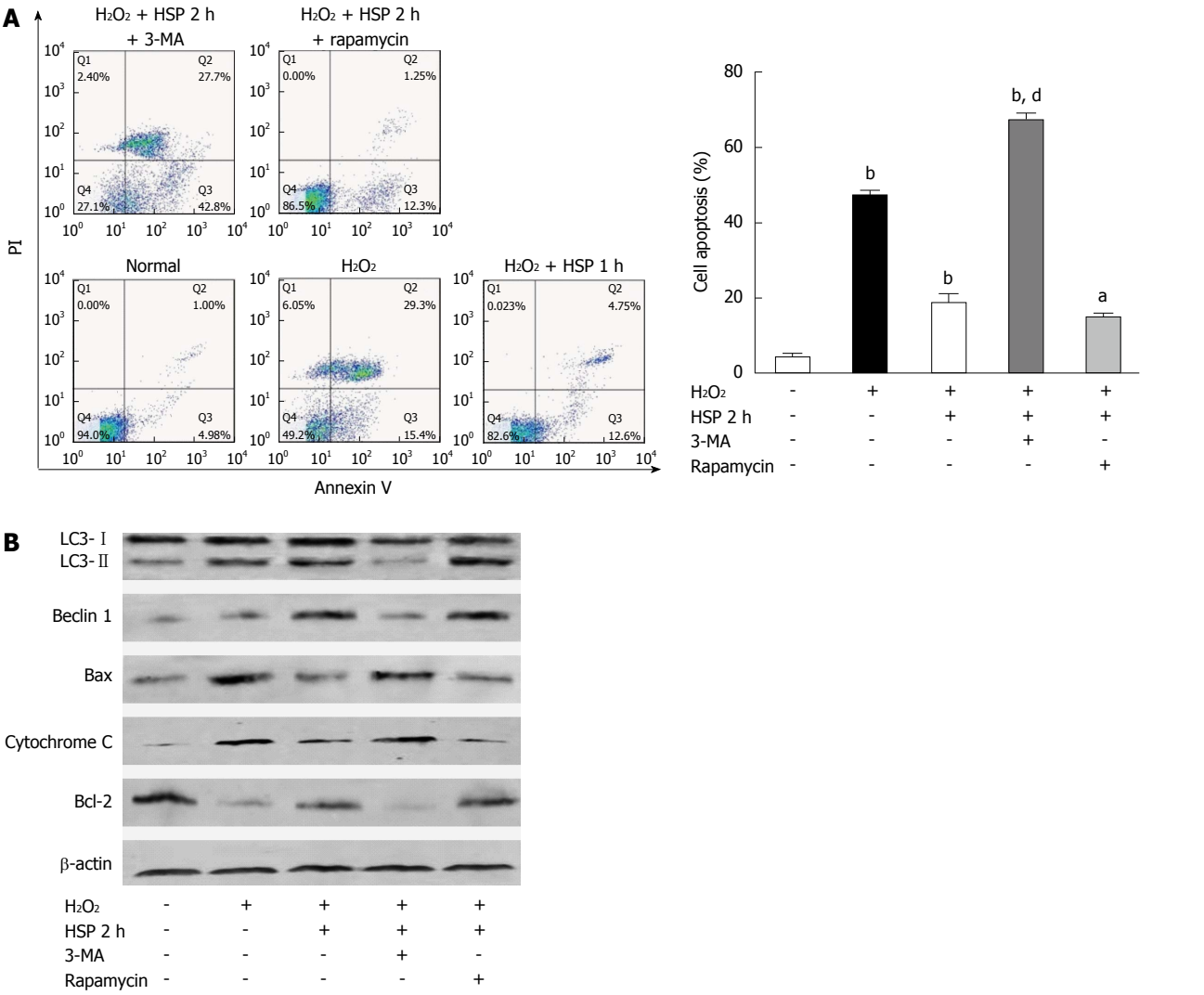



**Figure 4 Inhibition of autophagy abrogated the effects of heat shock pretreatment on apoptosis reduction in mesenchymal stem cells exposed to $H_2O_2$ treatment.** A: Flow cytometry indicated that 3-MA significantly increased the apoptotic rate in heat shock pretreatment (HSP)-treated mesenchymal stem cells; B: Western blot showed that 3-MA significantly increased Bax and cytochrome C expression, and decreased Bcl-2, LC3-Ⅱ and beclin 1 expression. The data represent the results of three separate experiments. $^aP < 0.05$, $^bP < 0.01$ compared with the control group; $^dP < 0.01$ compared with the $HSP_{2h}$ group.

of severe damage. When compared with the I/R control group and the MSC-treated group, the HSP-MSC-treated group showed significantly improved histopathology and lower Suzuki scores 24 h after transplantation (Figure 7B). Moreover, compared with the I/R control group and PBS-treated rats, the livers from HSP-MSC-treated and MSC-treated rats showed a significantly increased number of PCNA-positive cells. Interestingly, the number of PCNA-positive cells in livers from HSP-MSC-treated rats was significantly increased compared with MSC-treated rats (Figure 7C; $P < 0.05$).

## DISCUSSION

In the present study, we investigated the interaction between autophagy and apoptosis and the protective mechanism of autophagy activation by HSP in MSCs exposed to $H_2O_2$. Our results show that HSP for 2 h improves the therapeutic potential of MSCs in the treatment of HIRI in rats and enhances autophagy *via* the p38MAPK/mTOR pathway, which partly acted in the protective role of HSP on MSC apoptosis induced by $H_2O_2$. When administered systemically, more viable HSP-MSCs homed to the I/R liver compared with MSCs, which led to a significant improvement in liver function, an accelerated mitogenic response and alleviation of histopathological damage in the rat model.

In a previous study, we found that transplanted MSCs attenuated HIRI by suppressing oxidative stress and inhibiting apoptosis in rats[3]. However, the I/R microenvironment is detrimental to graft cells and induces cell death, thereby attenuating the therapeutic effect of stem cell transplantation[5-7]. Implanted MSCs must have a long life to ensure long-term MSC-based therapy in I/R tissues. It has been reported that short-term HSP can significantly improve

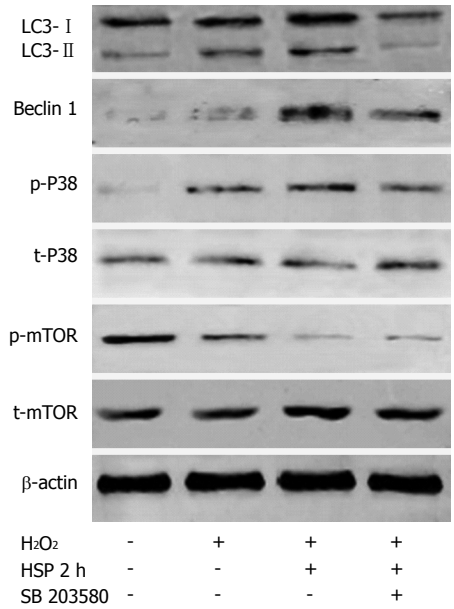



**Figure 5 The p38MAPK/mTOR pathway was involved in autophagy activation by heat shock pretreatment in mesenchymal stem cells.** Western blot showed that p-p38MAPK was upregulated and p-mTOR was downregulated by heat shock pretreatment (HSP) in mesenchymal stem cells. The p38MAPK inhibitor, SB203580, counteracted the effects of HSP on LC3-Ⅱ, beclin 1 and p-p38MAPK expression. The data represent the results of three separate experiments.

the viability of transplanted cells and thus enhance their tissue repairing capabilities in I/R tissue[10,11]. As $H_2O_2$ was previously shown to be a critical mediator of I/R-induced cell death[24,32], we induced a HIRI microenvironment by treating MSCs with $H_2O_2$ to investigate the function of HSP *in vitro*. We found that HSP for 2 h resulted in the most significant anti-apoptotic effects in MSCs exposed to $H_2O_2$ compared to the other groups. In addition, $H_2O_2$-induced apoptosis of MSCs was aggravated in the $HSP_{3h}$ group (Figure 1). More importantly, exposure to HSP for 2 h before transplantation enhanced the survival rate and therapeutic outcome of MSCs *in vivo*. These data suggest that HSP at 42 ℃ for 2 h was the optimal period for improving the effect of MSCs transplantation in the repair of HIRI in rats. The HSP procedure is a simple method to improve implanted cell survival with little risk and can be performed not only in the liver, but also other organs.

Autophagy has been implicated in many processes, including cell differentiation, growth, development and survival[33]. Autophagy can be activated by various stresses involved in mediating cell survival or death[25]. In the present study, we found that HSP ranging from 1 to 3 h leads to a time-dependent increase in the action of autophagy in MSCs exposed to $H_2O_2$ (Figure 2A and D; Figure 3). In addition to the anti-apoptotic effect of HSP in MSCs, these findings suggest that autophagy induced by HSP for 2 h results in the most significant anti-apoptotic effect in MSCs exposed to $H_2O_2$. We therefore performed HSP for 2 h to examine the effect of $H_2O_2$-induced apoptosis and the protective effect of autophagy against apoptosis in MSCs. The protective effect of autophagy against apoptosis has previously been reported in models of I/R injury[34], including a model using $H_2O_2$. One well-established view is that appropriate autophagy is essential for cell survival[35]. More recently, Herberg *et al*[20] reported that the SDF-1/CXCR4 axis plays a key role in mediating MSC survival exposed to $H_2O_2$ by activating autophagy. Consistent with these results, our data show that the autophagy inhibitor, 3-MA, abrogates the anti-apoptotic effect observed in the $HSP_{2h}$ group, and the autophagy inducer, rapamycin, does not reduce apoptosis of MSCs exposed to $H_2O_2$. These data suggest that moderate activation of autophagy mediated by HSP for 2 h may play a critical role in HSP to improve the survival of MSCs exposed to $H_2O_2$. It is known that autophagy is considered a double-edged sword in terms of cell survival. Moreover, we found that the activation of autophagy by HSP in MSCs is not paralleled by a corresponding increase in tolerance to $H_2O_2$-induced apoptosis. HSP for 1 and 2 h induced autophagy, which was an anti-apoptosis mechanism rather than a pro-apoptosis pathway in MSCs exposed to $H_2O_2$. Prolonged or excessive autophagy, which was mediated by HSP for 3 h, may digest essential components and lead to cell death. Thus, activation of autophagy may be a new mechanism in the process of HSP protecting MSCs from $H_2O_2$-induced apoptosis.

p38MAPK appears to have a dual role in that it has a positive or negative role in autophagy depending on conditions, cell type or type of cell stress[36-39]. In the present study, we assessed p38MAPK/mTOR pathway activation levels to determine the mechanisms underlying HSP-induced autophagy in MSCs exposed to $H_2O_2$. Interestingly, we found that HSP for 2 h increases p38MAPK activation and correspondingly alleviates mTOR activation. Moreover, p38MAPK inhibition abrogates autophagy induced by HSP for 2 h, but does not significantly impair mTOR suppression. In addition, our results indicate that treatment with rapamycin does not further induce autophagy of MSCs compared with HSP alone in the presence of $H_2O_2$, indicating that HSP may be involved in the same mechanism as rapamycin to activate autophagy in MSCs. These data suggest that the p38MAPK/mTOR signaling pathway may be involved in the mechanism of HSP-induced autophagy in MSCs exposed to $H_2O_2$.

To confirm the observations in the *in vitro* assay, we investigated the protective effect of HSP on MSCs *in vivo*. We determined the extent of MSCs localized in I/R livers of the recipient group by counting the number of CM-Dil fluorescent-labeled cells. It is well established that PCNA, which is synthesized in the cell nucleus, is a nuclear antigen related to the cell life cycle. PCNA is expressed in the G1 and S phases, and performs the essential function of providing replicative DNA polymerases in eukaryotic cells. The

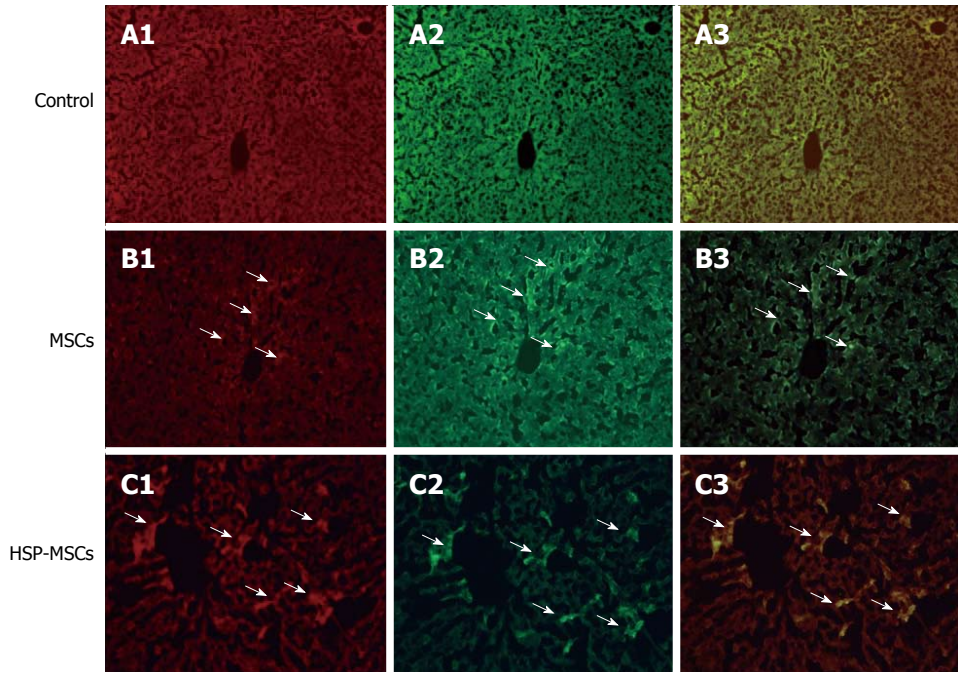
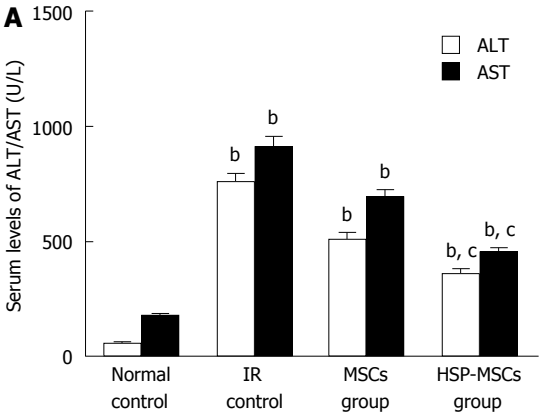
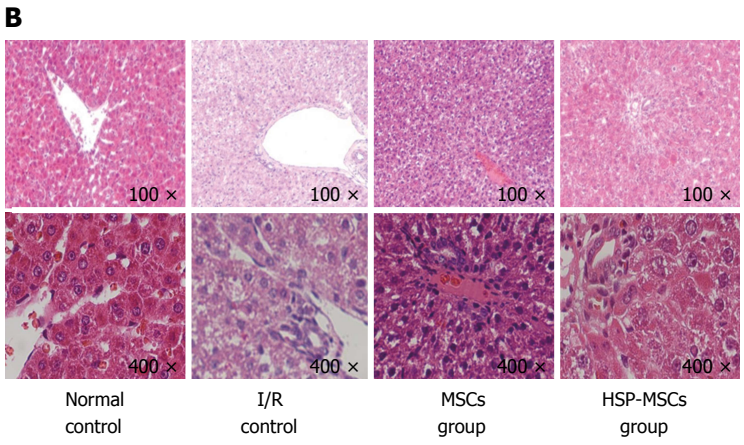
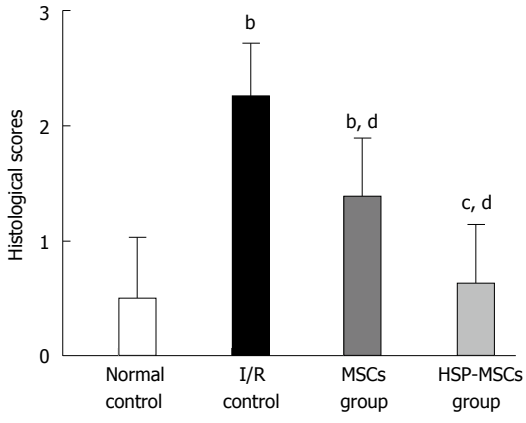



**Figure 6 Heat shock pretreatment increases the homing and survival rate of transplanted mesenchymal stem cells in I/R livers *in vivo*.** CM-Dil-labeled positive cells (red color, B1, C1), PCNA-conjugated with FITC (green color, B2, C2) and their co-localization (yellow color, B3, C3) were detected by immunofluorescence microscopy, respectively (magnification × 100). The total number of double-positive cells labeled by CM-Dil and PCNA in the heat shock pretreatment-mesenchymal stem cell (MSC)-treated group was higher than that in the MSC-treated group. The arrows indicate positive stained cells by CM-Dil, PCNA or their co-localization, respectively. The data represent the results of three separate experiments.



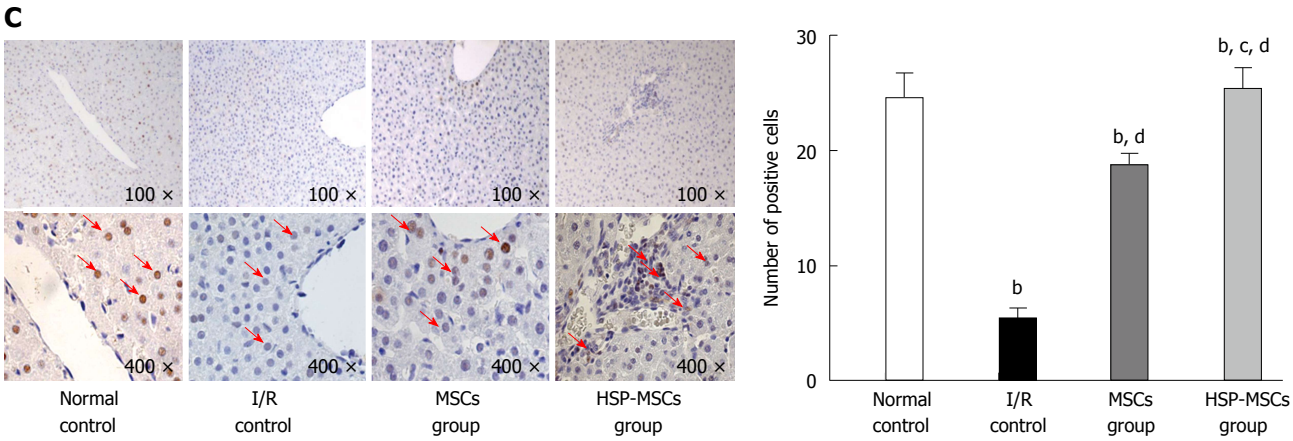



**Figure 7 Heat shock pretreatment improves the therapeutic potential of mesenchymal stem cells in the treatment of hepatic ischemia-reperfusion injury *in vivo*.** A: Serum aminotransferase levels were measured using an automatic analyzer following treatment; B: Histopathological analyses of livers from the normal control, I/R-control, mesenchymal stem cells (MSCs) and HSP-MSC groups. Liver tissue sections were stained with HE and scored according to the Suzuki Scoring System. Original magnification, × 100 and × 400, respectively, for each slide; C: Expression of PCNA by immunohistochemistry in liver tissues (magnification × 100 and × 400). The arrows indicate positive stained cells by PCNA. The data represent the results of three separate experiments. $^{b}P < 0.01$ compared with the normal control group; $^{d}P < 0.01$ compared with the I/R control group; $^{c}P < 0.05$ compared with the MSC group.

level of PCNA in resting cells is low, but is substantially increased in multiplying and transformed cells[40,41]. As shown in Figure 6, the HSP-MSCs group show more double-positive cells labeled by CM-Dil and PCNA than the MSCs group, which indicates that more HSP-MSCs subsequently underwent cell division and that HSP enhances the survival rate of transplanted MSCs in the liver. Furthermore, a marked decrease in serum aminotransferase levels, improved histopathology, lower Suzuki scores and an increased number of PCNA-positive cells in response to transplantation of HSP-MSCs were observed compared with the MSC group and the control group (Figure 7). These results indicate that HSP increases the homing and survival rate of transplanted MSCs, and thus improves the therapeutic potential of MSCs in the treatment of HIRI *in vivo*.

In summary, we found, for the first time, that HSP effectively enhances the homing and survival rate of MSCs, and thereby improves the therapeutic outcome of MSCs in the treatment of HIRI. The activation of autophagy *via* the p38MAPK/mTOR pathway may be a novel mechanism of HSP to improve the survival of MSCs exposed to $H_2O_2$. Activation of autophagy by HSP may be an attractive method of preventing apoptosis of MSCs and promoting their application in cellular therapies in regenerative medicine.

## COMMENTS

### Background

Mesenchymal stem cells (MSCs) exert a protective effect in hepatic ischemia-reperfusion injury (HIRI). However, due to local hypoxia, inflammation, and particularly oxidative stress in the targeted tissue, the transplanted MSCs do not withstand the difficult microenvironment due to ischemia-reperfusion (I/R) injury and low cell survival reduces the therapeutic effect. Autophagy is a complex "self-eating" process and can reduce apoptosis of MSCs exposed to $H_2O_2$. Heat shock pretreatment (HSP) is known to protect cells from various environmental insults and has been shown to induce autophagy in some cell lines. Previous studies show that HSP can regulate mitogen-activated protein kinase (p38MAPK), a positive modulator of autophagy in MSCs. Therefore, the authors designed this study to determine the role of HSP in autophagy activation *via* the p38MAPK/mTOR pathway to protect MSCs against apoptosis induced by oxidative stress injury.

### Research frontiers

Autophagy is an evolutionarily conserved process that occurs in all eukaryotic cells. Evidence suggests that under hypoxia/serum deprivation (H/SD) conditions, autophagy can protect MSCs by providing energy or eliminating reactive oxygen species and damaged organelles, and can reduce apoptosis. In addition, several reports show that HSP increases survival rate following cell transplantation in the heart. However, it is unknown whether autophagy can be activated by HSP or its effect and exact mechanism in MSCs.

### Innovations and breakthroughs

This study shows that activation of autophagy was a protective mechanism of HSP in MSCs. The results show that HSP for 2 h improves the therapeutic potential of MSCs in the treatment of HIRI in rats and enhances autophagy *via* the p38MAPK/mTOR pathway, which mediates, at least partly, the protective effects of HSP on MSC apoptosis exposed to $H_2O_2$. When administered systemically, more viable HSP-MSCs home to the I/R liver compared with MSCs, which leads to a significant improvement in liver function, an accelerated mitogenic response and the alleviation of histopathological damage in the rat model.

### Applications

This study indicates that HSP effectively enhances MSCs homing and survival rate, and thus improves the therapeutic outcome of MSCs in the treatment of HIRI in rats. The activation of autophagy *via* the p38MAPK/mTOR pathway may be a novel mechanism of HSP to enhance the survival of MSCs exposed to $H_2O_2$. The regulation of autophagy by HSP may be an attractive strategy in preventing apoptosis of MSCs, thus promoting their application in cellular therapies in regenerative medicine.

### Terminology

HIRI is an inevitable event and occurs in a number of clinical settings, including liver surgery, hemorrhagic shock with subsequent fluid resuscitation, sepsis, hepatic artery ligation, trauma, and some vascular lesions, and especially in

liver transplantation. Autophagy is an evolutionarily conserved process that occurs in all eukaryotic cells and is considered a double-edged sword in relation to cell survival. Heat shock pretreatment involves short-term exposure to mild hyperthermia that can significantly enhance cell tolerance and viability.

### Peer-review

The work presented here is an interesting contribution that demonstrates the interaction of autophagy with apoptosis on MSCs under $H_2O_2$ conditions, and the activation of autophagy as a protective mechanism of HSP on MSCs.

## REFERENCES


1 **Montalvo-Jave EE**, Escalante-Tattersfield T, Ortega-Salgado JA, Piña E, Geller DA. Factors in the pathophysiology of the liver ischemia-reperfusion injury. *J Surg Res* 2008; **147**: 153-159 [PMID: 17707862 DOI: 10.1016/j.jss.2007.06.015]

2 **Walsh KB**, Toledo AH, Rivera-Chavez FA, Lopez-Neblina F, Toledo-Pereyra LH. Inflammatory mediators of liver ischemia-reperfusion injury. *Exp Clin Transplant* 2009; **7**: 78-93 [PMID: 19715511]

3 **Jin G**, Qiu G, Wu D, Hu Y, Qiao P, Fan C, Gao F. Allogeneic bone marrow-derived mesenchymal stem cells attenuate hepatic ischemia-reperfusion injury by suppressing oxidative stress and inhibiting apoptosis in rats. *Int J Mol Med* 2013; **31**: 1395-1401 [PMID: 23589072 DOI: 10.3892/ijmm.2013.1340]

4 **Wang X**, Jameel MN, Li Q, Mansoor A, Qiang X, Swingen C, Panetta C, Zhang J. Stem cells for myocardial repair with use of a transarterial catheter. *Circulation* 2009; **120**: S238-S246 [PMID: 19752374 DOI: 10.1161/CIRCULATIONAHA.109.885236]

5 **Toma C**, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* 2002; **105**: 93-98 [PMID: 11772882 DOI: 10.1161/hc0102.101442]

6 **Atashi F**, Modarressi A, Pepper MS. The role of reactive oxygen species in mesenchymal stem cell adipogenic and osteogenic differentiation: a review. *Stem Cells Dev* 2015; **24**: 1150-1163 [PMID: 25603196 DOI: 10.1089/scd.2014.0484]

7 **Mias C**, Trouche E, Seguelas MH, Calcagno F, Dignat-George F, Sabatier F, Piercecchi-Marti MD, Daniel L, Bianchi P, Calise D, Bourin P, Parini A, Cussac D. Ex vivo pretreatment with melatonin improves survival, proangiogenic/mitogenic activity, and efficiency of mesenchymal stem cells injected into ischemic kidney. *Stem Cells* 2008; **26**: 1749-1757 [PMID: 18467662 DOI: 10.1634/stemcells.2007-1000]

8 **Yamashita N**, Hoshida S, Nishida M, Igarashi J, Taniguchi N, Tada M, Kuzuya T, Hori M. Heat shock-induced manganese superoxide dismutase enhances the tolerance of cardiac myocytes to hypoxia-reoxygenation injury. *J Mol Cell Cardiol* 1997; **29**: 1805-1813 [PMID: 9236135 DOI: 10.1006/jmcc.1997.0415]

9 **Venkatakrishnan CD**, Tewari AK, Moldovan L, Cardounel AJ, Zweier JL, Kuppusamy P, Ilangovan G. Heat shock protects cardiac cells from doxorubicin-induced toxicity by activating p38 MAPK and phosphorylation of small heat shock protein 27. *Am J Physiol Heart Circ Physiol* 2006; **291**: H2680-H2691 [PMID: 16782845 DOI: 10.1152/ajpheart.00395.2006]

10 **Jiang B**, Xiao W, Shi Y, Liu M, Xiao X. Heat shock pretreatment inhibited the release of Smac/DIABLO from mitochondria and apoptosis induced by hydrogen peroxide in cardiomyocytes and C2C12 myogenic cells. *Cell Stress Chaperones* 2005; **10**: 252-262 [PMID: 16184770 DOI: 10.1379/csc-124R.1]

11 **Suzuki K**, Smolenski RT, Jayakumar J, Murtuza B, Brand NJ, Yacoub MH. Heat shock treatment enhances graft cell survival in skeletal myoblast transplantation to the heart. *Circulation* 2000; **102**: III216-III221 [PMID: 11082390 DOI: 10.1161/01.CIR.102.suppl_3.III-216]

12 **Bhora FY**, Wise RM, Foster AH. Heat shock protects cardiomyocytes from hypoxia-mediated apoptosis by attenuation of nitric oxide. *In Vivo* 2000; **14**: 597-602 [PMID: 11125543]

13 **Zhang Q**, Yang YJ, Wang H, Dong QT, Wang TJ, Qian HY, Xu H. Autophagy activation: a novel mechanism of atorvastatin to protect mesenchymal stem cells from hypoxia and serum deprivation via AMP-activated protein kinase/mammalian target of rapamycin pathway. *Stem Cells Dev* 2012; **21**: 1321-1332 [PMID: 22356678 DOI: 10.1089/scd.2011.0684]

14 **Hou J**, Han ZP, Jing YY, Yang X, Zhang SS, Sun K, Hao C, Meng Y, Yu FH, Liu XQ, Shi YF, Wu MC, Zhang L, Wei LX. Autophagy prevents irradiation injury and maintains stemness through decreasing ROS generation in mesenchymal stem cells. *Cell Death Dis* 2013; **4**: e844 [PMID: 24113178 DOI: 10.1038/cddis.2013.338]

15 **Zhao Y**, Gong S, Shunmei E, Zou J. Induction of macroautophagy by heat. *Mol Biol Rep* 2009; **36**: 2323-2327 [PMID: 19152020 DOI: 10.1007/s110033-009-9451-4]

16 **Han J**, Xu X, Qin H, Liu A, Fan Z, Kang L, Fu J, Liu J, Ye Q. The molecular mechanism and potential role of heat shock-induced p53 protein accumulation. *Mol Cell Biochem* 2013; **378**: 161-169 [PMID: 23456460 DOI: 10.1007/s11010-013-1607-9]

17 **Virgin HW**, Levine B. Autophagy genes in immunity [J]. *Nature immunology* 2009; **10**: 461-470 [DOI: 10.1038/ni.1726]

18 **Chen W**, Zhang L, Zhang K, Zhou B, Kuo ML, Hu S, Chen L, Tang M, Chen YR, Yang L, Ann DK, Yen Y. Reciprocal regulation of autophagy and dNTP pools in human cancer cells. *Autophagy* 2014; **10**: 1272-1284 [PMID: 24905824 DOI: 10.4161/auto.28954]

19 **Ravikumar B**, Sarkar S, Davies JE, Futter M, Garcia-Arencibia M, Green-Thompson ZW, Jimenez-Sanchez M, Korolchuk VI, Lichtenberg M, Luo S, Massey DC, Menzies FM, Moreau K, Narayanan U, Renna M, Siddiqi FH, Underwood BR, Winslow AR, Rubinsztein DC. Regulation of mammalian autophagy in physiology and pathophysiology. *Physiol Rev* 2010; **90**: 1383-1435 [PMID: 20959619 DOI: 10.1152/physrev.00030.2009]

20 **Herberg S**, Shi X, Johnson MH, Hamrick MW, Isales CM, Hill WD. Stromal cell-derived factor-1β mediates cell survival through enhancing autophagy in bone marrow-derived mesenchymal stem cells. *PLoS One* 2013; **8**: e58207 [PMID: 23472159 DOI: 10.1371/journal.pone.0058207]

21 **Azad MB**, Chen Y, Henson ES, Cizeau J, McMillan-Ward E, Israels SJ, Gibson SB. Hypoxia induces autophagic cell death in apoptosis-competent cells through a mechanism involving BNIP3. *Autophagy* 2008; **4**: 195-204 [PMID: 18059169 DOI: 10.4161/auto.5278]

22 **Filippi-Chiela EC**, Bueno e Silva MM, Thomé MP, Lenz G. Single-cell analysis challenges the connection between autophagy and senescence induced by DNA damage. *Autophagy* 2015; **11**: 1099-1113 [PMID: 25701485 DOI: 10.0180/15548627.2015.1009795]

23 **Zhang XM**, Du F, Yang D, Yu CJ, Huang XN, Liu W, Fu J. Transplanted bone marrow stem cells relocate to infarct penumbra and co-express endogenous proliferative and immature neuronal markers in a mouse model of ischemic cerebral stroke. *BMC Neurosci* 2010; **11**: 138 [PMID: 20973978 DOI: 10.1186/1471-2202-11-138]

24 **Liu H**, Liu S, Li Y, Wang X, Xue W, Ge G, Luo X. The role of SDF-1-CXCR4/CXCR7 axis in the therapeutic effects of hypoxia-preconditioned mesenchymal stem cells for renal ischemia/reperfusion injury. *PLoS One* 2012; **7**: e34608 [PMID: 22511954 DOI: 10.1371/journal.pone.0034608]

25 **Shintani T**, Klionsky DJ. Autophagy in health and disease: a double-edged sword. *Science* 2004; **306**: 990-995 [PMID: 15528435 DOI: 10.1126/science.1099993]

26 **Paglin S**, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, Domingo D, Yahalom J. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res* 2001; **61**: 439-444 [PMID: 11212227]

27 **Tong J**, Ding J, Shen X, Chen L, Bian Y, Ma G, Yao Y, Yang F. Mesenchymal stem cell transplantation enhancement in myocardial infarction rat model under ultrasound combined with nitric oxide microbubbles. *PLoS One* 2013; **8**: e80186 [PMID: 24244646 DOI: 10.1371/journal.pone.0080186]

28 **Nauta RJ**, Tsimoyiannis E, Uribe M, Walsh DB, Miller D, Butterfield A. Oxygen-derived free radicals in hepatic ischemia and reperfusion injury in the rat. *Surg Gynecol Obstet* 1990; **171**: 120-125 [PMID: 2382188]

29 **Sheikh AY**, Lin SA, Cao F, Cao Y, van der Bogt KE, Chu P, Chang CP, Contag CH, Robbins RC, Wu JC. Molecular imaging of bone marrow mononuclear cell homing and engraftment in ischemic myocardium. *Stem Cells* 2007; **25**: 2677-2684 [PMID: 17628019 DOI: 10.1634/stemcells.2007-0041]

30 **Kim HJ**, Moradi H, Yuan J, Norris K, Vaziri ND. Renal mass reduction results in accumulation of lipids and dysregulation of lipid regulatory proteins in the remnant kidney. *Am J Physiol Renal Physiol* 2009; **296**: F1297-F1306 [PMID: 19357177 DOI: 10.1152/ajprenal.90761.2008]

31 **Katoh K**, Nakanishi Y, Akimoto S, Yoshimura K, Takagi M, Sakamoto M, Hirohashi S. Correlation between laminin-5 gamma2 chain expression and epidermal growth factor receptor expression and its clinicopathological significance in squamous cell carcinoma of the tongue. *Oncology* 2002; **62**: 318-326 [PMID: 12138239 DOI: 10.1159/000065063]

32 **Sachse A**, Wolf G. Angiotensin II-induced reactive oxygen species and the kidney. *J Am Soc Nephrol* 2007; **18**: 2439-2446 [PMID: 17687073 DOI: 10.1681/ASN.2007020149]

33 **Mathew R**, White E. Autophagy in tumorigenesis and energy metabolism: friend by day, foe by night. *Curr Opin Genet Dev* 2011; **21**: 113-119 [PMID: 21255998 DOI: 10.1016/j.gde.2010.12.008]

34 **Wang D**, Ma Y, Li Z, Kang K, Sun X, Pan S, Wang J, Pan H, Liu L, Liang D, Jiang H. The role of AKT1 and autophagy in the protective effect of hydrogen sulphide against hepatic ischemia/reperfusion injury in mice. *Autophagy* 2012; **8**: 954-962 [PMID: 22694815 DOI: 10.4161/auto.19927]

35 **Zhou J**, Wu S, Chen Y, Zhao J, Zhang K, Wang J, Chen S. microRNA-143 is associated with the survival of ALDH1+CD133+ osteosarcoma cells and the chemoresistance of osteosarcoma. *Exp Biol Med* (Maywood) 2015; **240**: 867-875 [PMID: 25576341 DOI: 10.1177/1535370214563893]

36 **Webber JL**, Tooze SA. Coordinated regulation of autophagy by p38alpha MAPK through mAtg9 and p38IP. *EMBO J* 2010; **29**: 27-40 [PMID: 19893488 DOI: 10.1038/emboj.2009.321]

37 **Comes F**, Matrone A, Lastella P, Nico B, Susca FC, Bagnulo R, Ingravallo G, Modica S, Lo Sasso G, Moschetta A, Guanti G, Simone C. A novel cell type-specific role of p38alpha in the control of autophagy and cell death in colorectal cancer cells. *Cell Death Differ* 2007; **14**: 693-702 [PMID: 17159917 DOI: 10.1038/sj.cdd.4402076]

38 **Doyle A**, Zhang G, Abdel Fattah EA, Eissa NT, Li YP. Toll-like receptor 4 mediates lipopolysaccharide-induced muscle catabolism via coordinate activation of ubiquitin-proteasome and autophagy-lysosome pathways. *FASEB J* 2011; **25**: 99-110 [PMID: 20826541 DOI: 10.1096/fj.10-164152]

39 **Matsuzawa T**, Kim BH, Shenoy AR, Kamitani S, Miyake M, Macmicking JD. IFN-γ elicits macrophage autophagy via the p38 MAPK signaling pathway. *J Immunol* 2012; **189**: 813-818 [PMID: 22675202 DOI: 10.4049/jimmunol.1102041]

40 **Maga G**, Hubscher U. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. *J Cell Sci* 2003; **116**: 3051-3060 [PMID: 12829735 DOI: 10.1242/jcs.00653]

41 **Shizhu J**, Xiangwei M, Xun S, Mingzi H, Bingrong L, Dexia K, Xinghong W, Fenghua P. Bone marrow mononuclear cell transplant therapy in mice with CCl4-induced acute liver failure. *Turk J Gastroenterol* 2012; **23**: 344-352 [PMID: 22965505 DOI: 10.4318/tjg.2012.0344]


**P- Reviewer**: Keyashian K, Kamiya T, Tsujikawa T **S- Editor**: Yu J
**L- Editor**: Filipodia **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12835

*World J Gastroenterol* 2015 December 7; 21(45): 12835-12842
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

Retrospective Cohort Study

# Nutritional care in hospitalized patients with chronic liver disease

Dep K Huynh, Shane P Selvanderan, Hugh AJ Harley, Richard H Holloway, Nam Q Nguyen

Dep K Huynh, Shane P Selvanderan, Hugh AJ Harley, Richard H Holloway, Nam Q Nguyen, Discipline of Medicine, University of Adelaide, North Terrace, Adelaide 5000, South Australia, Australia

Dep K Huynh, Hugh AJ Harley, Richard H Holloway, Nam Q Nguyen, Department of Gastroenterology and Hepatology, Royal Adelaide Hospital, North Terrace, Adelaide 5000, South Australia, Australia

Author contributions: Huynh DK, Nguyen NQ contributed to study concept and design, acquisition, analysis and interpretation of data; statistical analysis and drafting of the manuscript; Selvanderan SP contributed to data acquisition and revision of the manuscript; Harley HAJ contributed to study concept; analysis and interpretation of data and revision of the manuscript; Holloway RH contributed to study concept, analysis and interpretation of data and critical revision of manuscript for important intellectual content.

Institutional review board statement: This study was reviewed and approved by the Royal Adelaide Hospital Research Ethics Committee.

Informed consent statement: The need to obtain informed consent from subjects was waived by the Royal Adelaide Hospital Research Ethics Committee.

Conflict-of-interest statement: The authors do not have any conflict of interest to disclose.

Data sharing statement: No additional data are available.



Correspondence to: Dep K Huynh, MBBS, FRACP, Department of Gastroenterology and Hepatology, Royal Adelaide Hospital, North Terrace, Adelaide 5000, South Australia, Australia. dep.huynh@health.sa.gov.au
Telephone: +61-8-82225214
Fax: +61-8-82225885

Received: April 16, 2015
Peer-review started: April 18, 2015
First decision: May 18, 2015
Revised: June 11, 2015
Accepted: September 2, 2015
Article in press: September 2, 2015
Published online: December 7, 2015

## Abstract

**AIM:** To evaluate the practice of nutritional assessment and management of hospitalised patients with cirrhosis and the impact of malnutrition on their clinical outcome.

**METHODS:** This was a retrospective cohort study on patients with liver cirrhosis consecutively admitted to the Department of Gastroenterology and Hepatology at the Royal Adelaide Hospital over 24 mo. Details were gathered related to the patients' demographics, disease severity, nutritional status and assessment, biochemistry and clinical outcomes. Nutritional status was assessed by a dietician and determined by subjective global assessment. Estimated energy and protein requirements were calculated by Simple Ratio Method. Intake was estimated from dietary history and/or food charts, and represented as a percentage of estimated daily requirements. Median duration of follow up was 14.9 (0-41.4) mo.

**RESULTS:** Of the 231 cirrhotic patients (167 male, age: 56.3 ± 0.9 years, 9% Child-Pugh A, 42% Child-Pugh B and 49% Child-Pugh C), 131 (57%) had formal nutritional assessment during their admission and 74 (56%) were judged to have malnutrition. In-hospital

caloric (15.6 ± 1.2 kcal/kg *vs* 23.7 ± 2.3 kcal/kg, $P$ = 0.0003) and protein intake (0.65 ± 0.06 g/kg *vs* 1.01 ± 0.07 g/kg, $P$ = 0.0003) was significantly reduced in patients with malnutrition. Of the malnourished cohort, 12 (16%) received enteral nutrition during hospitalisation and only 6 (8%) received ongoing dietetic review and assessment following discharge from hospital. The overall mortality was 51%, and was higher in patients with malnutrition compared to those without (HR = 5.29, 95%CI: 2.31-12.1; $P$ < 0.001).

**CONCLUSION:** Malnutrition is common in hospitalised patients with cirrhosis and is associated with higher mortality. Formal nutritional assessment, however, is inadequate. This highlights the need for meticulous nutritional evaluation and management in these patients.

**Key words:** Liver cirrhosis; Nutrition assessment; Mortality; Malnutrition; Morbidity



**Core tip:** This is the first study to highlight the lack of nutritional assessment of hospitalised patients with cirrhosis. Despite the well-established prognostic value of nutrition, our study showed that almost half of hospitalized patients with cirrhosis did not have a formal nutritional assessment. The prevalence of malnutrition in this group of patients was high (56%) and in-hospital dietary intake was substantially reduced, even in patients with normal subjective global assessment. We also confirmed that malnutrition was an independent predictor of both short-term and long-term mortality.

Huynh DK, Selvanderan SP, Harley HAJ, Holloway RH, Nguyen NQ. Nutritional care in hospitalized patients with chronic liver disease. *World J Gastroenterol* 2015; 21(45): 12835-12842 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12835.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12835

## INTRODUCTION

Malnutrition is common in chronic liver disease, and occurs in 24%-66% of hospitalised patients with cirrhosis[1-4]. However, in contrast to the common occurrence of malnutrition reported in prospective studies, the largest retrospective population based study of hospitalised patients with cirrhosis reported a substantially lower prevalence of malnutrition at only 6.1%[5]. This suggests that malnutrition is likely under-recognised and inadvertently under-treated in hospitalised patients with liver cirrhosis. Currently, the extent to which nutritional assessment and support is implemented in routine clinical care is unknown.

Malnutrition in patients with liver cirrhosis is associated with increased morbidity including hepatic encephalopathy, variceal bleeding, refractory ascites, spontaneous bacterial peritonitis (SBP) and hepatorenal syndrome (HRS)[6,7]. While a small number of studies have shown that malnutrition is an independent predictor of mortality in patients with liver cirrhosis, the largest multi-centre prospective study demonstrated that reduction in muscle mass was associated with lower cumulative survival only in Childs A and B cirrhosis on univariate but not multivariate analysis[4,8] and survival in Childs C cirrhosis was not associated with nutritional status[8]. Evidence for malnutrition as a prognostic indicator in patients with decompensated cirrhosis has mostly been derived from the transplant population where severe but not mild or moderate malnutrition has been associated with pre-transplant mortality[9]. Pre-transplant reduced nutritional index have also been associated with increased and earlier post-transplant morbidity but not mortality[10,11]. The data relating to the impact of malnutrition on survival in patients with advanced liver disease who are not candidates for liver transplantation however, are limited.

The identification of malnutrition in patients with liver cirrhosis using traditional objective nutritional assessment parameters is often confounded by non-nutritional factors, including liver synthetic function and fluid status. While multi-compartmental body composition analyses have been used to define malnutrition in patients with cirrhosis more accurately, the expense and lack of availability of these techniques limits their use to the research setting. Subjective global assessment (SGA) is often applied to determine nutritional status in routine clinical practice[12]. While SGA may underestimate the frequency and severity of malnutrition[7,13,14], it has been reported to correlate better with disease severity than does body composition analysis[15]. Although nutritional assessment by SGA is a simple bedside tool, it is only one of a large number of undertakings expected of clinicians, and is often forgotten. Furthermore, appropriate nutritional intervention requires the establishment and frequent re-evaluation of nutrient requirements and intake levels which is more efficiently performed by dieticians. Early detection and management of nutritional deficiency by a dietician has been reported to be associated with an improvement in survival, as compared to that by a physician[16]. The aim of this study, therefore, was to evaluate the practice of nutritional assessment and management in hospitalised patients with cirrhosis in a non-liver transplant tertiary hospital, and to assess the impact of malnutrition on their clinical outcomes.

## MATERIALS AND METHODS

### *Study population*

A retrospective analysis was performed of all patients

with chronic liver disease and cirrhosis, who had been admitted to the Department of Gastroenterology and Hepatology in the Royal Adelaide Hospital, the largest tertiary referral hospital in South Australia, between January 2010 and December 2011. The patients were identified from a prospectively collected hospital database of all patients admitted with chronic liver disease. Cirrhosis was proven histologically or defined by evidence of chronic liver disease with the presence of portal hypertension or complications of hepatic decompensation on the basis of clinical, biochemical and radiological findings. The only exclusion criterion was elective admission for only day procedures (paracentesis or endoscopy). The project was approved by the Royal Adelaide Hospital Research Ethics Committee, and all patient data were de-identified to maintain confidentiality.

### Data collection

Detailed demographic and disease-specific data relating to the patients with liver cirrhosis were collected from careful review of case notes, electronic medical records, and prospectively compiled Gastroenterological departmental databases. The disease-specific variables recorded were: aetiology of liver disease, severity of liver disease as graded by Child-Pugh classification[17] and Model for End-Stage Liver Disease (MELD) score[18] and laboratory data including bilirubin, albumin, serum creatinine and international normalised ratio.

In addition to the hospital patient records, an independent record of nutritional assessment was prospectively maintained by the Dietetics Department. The evaluation of nutritional status was performed by a dedicated dietician specialized in gastrointestinal and liver disease, using the Subjective Global Assessment (SGA). The SGA entailed a medical history including weight change, dietary change, gastrointestinal symptoms, and functional impairment, and physical examination for subcutaneous fat store, muscle wasting, peripheral oedema and ascites and body mass index (BMI). BMI was calculated from dry weight following paracentesis or estimated by adjusting for ascites and peripheral oedema according to Mendenhall, 1992[19]. The recommended caloric and protein requirements were calculated using the simple ratio method. In accordance with the European Society of Clinical Nutrition and Metabolism guidelines[20], energy requirement was calculated as 35-40 kcal/kg per day and protein requirement as 1.2-1.5 g/kg/d[20]. In-hospital dietary intake was calculated from detailed 3 d dietary recall by the patient and daily food charts. Nutritional intervention and the route of nutritional supplementation (enteral, parenteral or oral) were recorded.

Clinical details relating to the hospital admission, patient progress and outcomes from the hospitalization, associated morbidities, and mortality were obtained from extensive review and cross check of hospital medical record, outpatient records, and electronic patient database (covering all major metropolitan hospitals and the 8 largest rural centres). If there was uncertainty, the patients as well as their primary care physician were contacted *via* telephone. Follow-up was from the time of the index admission until time of death, or censored at the date of last clinical encounter alive.

The primary outcomes of this study were: (1) the proportion of patients who had dietetic assessment and interventions; and (2) the impact of nutrition status on mortality. Secondary outcomes were the impact of nutritional status on hospital length of stay, infectious complications, liver-related morbidity and hospital readmission.

### Statistical analysis

Statistical analysis was carried out using SPSS version 22.0.0 for Windows. Parametric data are presented as mean ± SEM and non-parametric data as median (range). Categorical variables were compared using Chi-square and Fisher's exact test where appropriate. Quantitative variables were tested by *t*-test and, when the assumption for normality was not met, the Mann Whitney *U* test. Univariate analysis for survival was performed using Kaplan Meier method and differences in Kaplan Meier curves were tested for statistical significance using the log rank test. A multivariate Cox Proportional Hazards regression analysis of nutritional status on survival time was performed, controlling for age, disease severity (MELD score), the presence of hepatocellular carcinoma (HCC) and creatinine. As the proportional effect of malnutrition on the risk of death was not constant over time, the proportional hazards assumption of the standard Cox regression was not met for the malnutrition variable. Instead, the Cox regression model that allowed for a time-dependent effect of malnutrition on survival was utilized. $P < 0.05$ was considered statistically significant.

The statistical methods of this study was reviewed by Kylie Lange, Biostatistician, Centre of Research Excellence (CRE) in Translating Nutritional Science to Good Health, Discipline of Medicine, The University of Adelaide

## RESULTS

A total of 231 patients [167 male (72%); 56.3 ± 0.9 years] with cirrhosis were admitted over the 24 mo. The most common aetiologies of cirrhosis were alcoholic liver disease (56%), a combination of alcoholic liver disease and hepatitis C (12%) and hepatitis C (10%).The mean MELD score was 17.0 ± 0.5, with the majority of patients having Child-Pugh B (42%) and C (49%) disease. Only 9% ($n$ = 21) patients had Child-Pugh A cirrhosis. The median length of hospital stay was 7 d (range, 1-116 d). Other characteristics and details relating to the hospitalization

**Table 1 Baseline characteristics of hospitalised patients with liver cirrhosis *n* (%)**

| Variable | *n* = 231 |
|---|---|
| Gender | 167 M; 64 F |
| Age (yr) | 56.3 ± 0.9 |
| Aetiology | |
| Alcohol | 130 (56) |
| Alcohol and HCV | 28 (12) |
| HCV | 23 (10) |
| NAFLD | 19 (8) |
| Others | 31 (14) |
| Child-Pugh classification | |
| Childs A | 21 (9) |
| Childs B | 96 (42) |
| Childs C | 114 (49) |
| MELD Score | 17.0 ± 0.5 |
| Ascites | 135 (71) |
| Hepatic encephalopathy | 88 (38) |
| Albumin | 24.9 ± 0.3 |
| Bilirubin | 80.3 ± 7.1 |
| INR | 1.6 ± 0.04 |
| Median LOS (d) | 7 (1-116) |
| Admission with variceal bleeding | 30 (13) |
| 28-d mortality | 31 (13) |
| In-hospital mortality | 33 (14) |

HCV: Hepatitis C virus; NAFLD: Non-alcoholic liver disease; MELD: Model for End-Stage Liver Disease; INR: International normalised ratio; LOS: Length of stay.

are summarized in Table 1.

After a median follow-up period of 14.9 mo (range, 0.03-40.8 mo), 117 (51%) patients died, with a 1-year survival rate of 61% and a 3-year survival of 42%. The in-hospital mortality rate was 14% and the 28 d mortality rate was 13%. On multivariate Cox regression analysis, reduced survival was related to older age ($P < 0.001$), presence of HCC ($P < 0.001$) and greater disease severity as determined by MELD score ($P = 0.01$).

### *Prevalence of nutritional assessment, malnutrition and interventions*

Of the 231 hospitalised patients with cirrhosis, only 131 (57%) had dietetic assessment during their admission. On univariate analysis, Child-Pugh score (9.1 ± 0.9 *vs* 9.9 ± 0.2, $P = 0.01$), serum albumin (26.3 ± 0.7 *vs* 23.1 ± 0.4, $P = 0.004$) and the presence of ascites [OR 2.0 (95%CI: 1.1-3.5), $P = 0.02$] were factors that associated with referral for a formal dietetic evaluation. There were no differences in the length of hospital stay, in-hospital mortality and 28-d mortality between the patients assessed by dieticians and those who were not (Table 2). Of the 131 patients who had an initial nutritional assessment, 71 (54%) were subsequently reviewed by a dietician during their inpatient encounter. More importantly, only 6 (8%) of the malnourished patients attended outpatient dietetic clinic after discharge from hospital.

According to the SGA, 74/131 (56%) of the assessed patients were identified as "malnourished". Malnutrition was significantly associated with lower BMI (23.2 ± 0.8 kg/m$^2$ *vs* 26.3 ± 0.7 kg/m$^2$, $P = 0.005$) and poorer in-hospital oral caloric intake (1080 ± 91 kcal per day *vs* 1674 ± 114 kcal per day, $P = 0.0003$), and protein intake (99 ± 7 g *vs* 45 ± 4 g, $P < 0.0001$). When, dietary intake was measured as a percentage of daily requirements calculated from body weight, malnourished patients only met 45% (15.6 ± 1.2 kcal/kg) of their total caloric requirements compared to 68% (23.7 ± 2.3 kcal/kg) those that were not malnourished ($P = 0.0003$), and 54% (0.65 ± 0.06 g/kg) *vs* 84% (1.01 ± 0.07 g/kg) of their protein requirements in the malnourish and not malnourished cohort respectively ($P = 0.0003$). There was no association between malnutrition and age, gender, aetiology of liver disease, disease severity, the presence of ascites, and the presence of hepatic encephalopathy (Table 3).

**Table 2 Characteristics of hospitalised patients assessed by dietician *n* (%)**

| Variable | No nutritional assessment (*n* = 100) | Nutritional assessment (*n* = 131) | *P* value |
|---|---|---|---|
| Gender | 75M; 25F | 92M; 39F | 0.46 |
| Age | 57.6 ± 1.3 | 55.3 ± 1.2 | 0.23 |
| Aetiology-alcohol | 66 (66) | 99 (76) | 0.14 |
| Child-Pugh score | 9.1 ± 0.9 | 9.9 ± 0.2 | 0.01 |
| MELD score | 16.6 ± 0.8 | 17.4 ± 0.6 | 0.20 |
| Ascites | 63 (63) | 102 (78) | 0.02 |
| Hepatic encephalopathy | 35 (35) | 53 (40) | 0.40 |
| Albumin | 26.3 ± 0.7 | 23.1 ± 0.4 | 0.004 |
| Bilirubin | 71.4 ± 10.6 | 87.1 ± 9.6 | 0.15 |
| INR | 1.6 ± 0.05 | 1.7 ± 0.05 | 0.43 |
| Median LOS | 5 (1-116) | 9 (1-100) | 0.09 |
| Admission with variceal bleeding | 15 (15) | 15 (11) | 0.44 |
| 28-d mortality | 12 (12) | 19 (15) | 0.55 |
| In-hospital mortality | 12 (12) | 21 (16) | 0.45 |

MELD: Model for End-Stage Liver Disease; INR: International normalised ratio; LOS: Length of stay.

Of the patients assessed by a dietician and determined to be malnourished, only 28 (38%) patients received supplementation with oral nutritional supplements ($n = 14$), enteral nutrition ($n = 12$), parenteral nutrition ($n = 1$) and combined enteral-parenteral nutrition ($n = 1$). The remaining 46 (62%) patients received dietary advice alone. A low sodium diet was prescribed for 23 (31%) patients. Of the 12 patients who were enterally fed, four had a percutaneous endoscopic gastrostomy (PEG) inserted for long-term feeding. All four patients did not have ascites at the time of PEG insertion. For the remaining patients, the median duration of enteral feeding was 5 d (1-50 d). There were no complications relating to PEG or enteral feeding.

**Table 3 Characteristics of malnourished *vs* well-nourished cirrhotic patients**

| | Normal nutritional status ($n$ = 57) | Malnutrition ($n$ = 74) | $P$ value |
|---|---|---|---|
| Gender | 39M:18F | 53M:21F | 0.89 |
| Age | 54.9 ± 1.9 | 55.6 ± 1.6 | 0.89 |
| Aetiology-alcohol | 41 | 58 | 0.41 |
| Child-Pugh Classification | | | |
| Childs A | 3 | 4 | 1.00 |
| Childs B | 23 | 28 | 0.86 |
| Childs C | 31 | 42 | 0.70 |
| Child Pugh score | 9.72 ± 0.3 | 9.99 ± 0.3 | 0.48 |
| MELD score | 16.9 ± 0.8 | 17.8 ± 0.9 | 0.87 |
| Ascites | 44 | 58 | 1.00 |
| Hepatic encephalopathy | 23 | 30 | 1.00 |
| Albumin | 24.5 ± 0.8 | 23.3 ± 0.6 | 0.25 |
| Bilirubin | 85.3 ± 12.9 | 88.5 ± 13.9 | 0.90 |
| INR | 1.6 ± 0.1 | 1.7 ± 0.1 | 0.38 |
| Creatinine | 84.9 ± 8.6 | 91.6 ± 8.6 | 0.70 |
| BMI | 26.3 ± 0.7 | 23.2 ± 0.8 | 0.005 |
| 28-d mortality | 3 | 16 | 0.011 |
| In-hospital mortality | 3 | 18 | 0.0035 |

MELD: Model for End-Stage Liver Disease; INR: International normalised ratio; LOS: Length of stay; BMI: Body mass index.

### *Impact of malnutrition on clinical outcomes*

In-hospital mortality was significantly higher in cirrhotic patients who were malnourished compared to those who were not [24.3% *vs* 5.3%, $P$ = 0.0035; HR = 5.79 (95%CI: 1.61-20.77)]. On univariate analysis, malnutrition was associated with shortened survival ($P$ = 0.001; Figure 1). On multivariate Cox regression analysis, adjusting for age, presence of HCC, serum bilirubin, serum creatinine and disease severity, malnutrition remained an independent risk factor for reduced survival (HR = 5.29, 95%CI: 2.31-12.1; $P$ < 0.001). In-hospital caloric and protein intake of less than 25% of the calculated daily requirements was associated with shortened survival on univariate analysis (Figure 2), but was not an independent prognostic factor on multivariate analysis.

There was no relationship between malnutrition and the length of hospital stay (malnourished *vs* normal: 9 (1-100) d *vs* 9 (1-66) d, $P$ = 0.6), development of ascites (3.5% *vs* 2.7%, $P$ = 1.0), or encephalopathy (14% *vs* 12%, $P$ = 0.8). After discharge, there was a trend for more malnourished patients to be re-admitted for major liver related complications, such as spontaneous bacterial peritonitis, hepatorenal syndrome, and/or variceal bleeding than those without malnutrition (10.8% *vs* 5.6%, $P$ = 0.13).

## DISCUSSION

In addition to reinforcing the notion that malnutrition

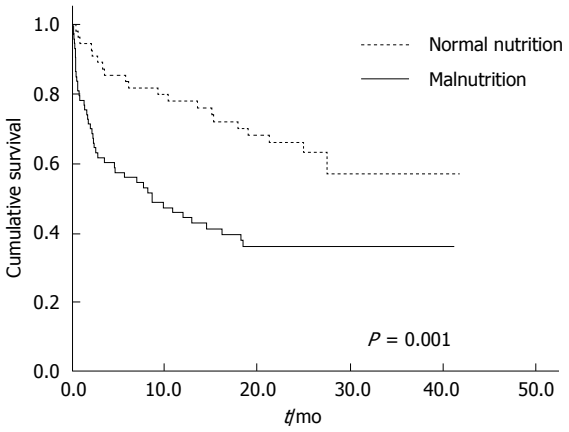



**Figure 1 Relationship between overall survival and the presence of malnutrition in patients with liver cirrhosis.**

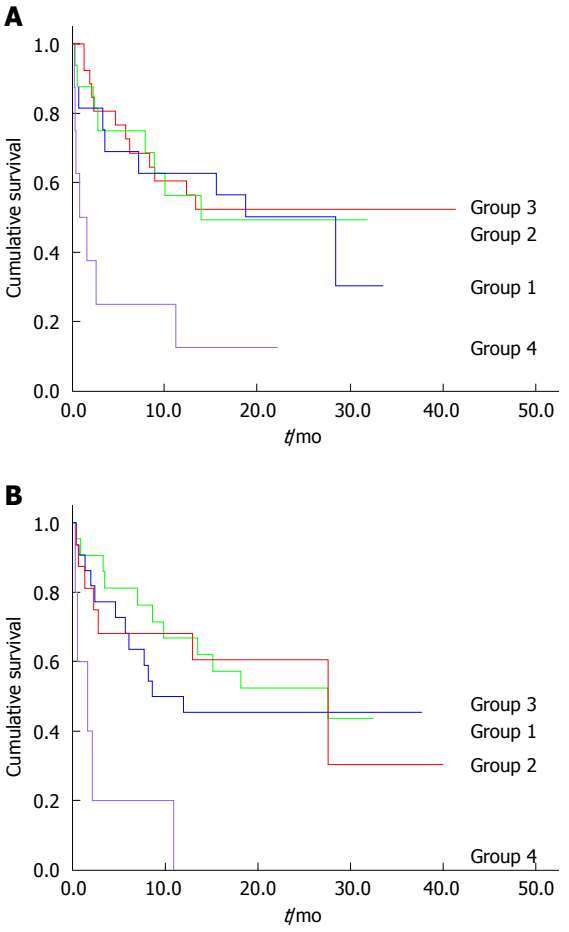



**Figure 2 Caloric and protein intake and survival.** A: Caloric intake and survival. Group 1: Caloric intake > 75% of recommended; Group 2: Caloric intake 50%-75% of recommended; Group 3: Caloric intake 25%-50% of recommended; Group 4: Caloric intake < 25% of recommended. Lower survival rates in group 4 *vs* 1 ($P$ = 0.014), group 4 *vs* 2 ($P$ = 0.017) and group 4 *vs* 3 ($P$ = 0.002). Pairwise comparisons between groups 1, 2 and 3 (not significant); B: Protein intake and survival. Group 1: Protein intake > 75% of recommended; Group 2: Protein intake 50%-75% of recommended; Group 3: Protein intake 25%-50% of recommended; Group 4: Protein intake < 25% of recommended. Lower survival rates in group 4 *vs* 1 ($P$ < 0.001), group 4 *vs* 2 ($P$ = 0.003) and group 4 *vs* 3 ($P$ = 0.005). Pairwise comparisons between groups 1, 2 and 3 (not significant).

is common in cirrhotic patients, the current study also highlights the following important issues: (1) formal dietetic assessment and intervention are not routine for hospitalized patients with liver cirrhosis and up to 43% of patients were not assessed; (2) daily caloric and protein intake in these patients was sub-optimal, even in patients who had normal SGA; (3) only one third of patients who were identified to have malnutrition and poor caloric intake were actively given nutritional supplement; (4) less than 10% of the identified malnourished patients had outpatient follow-up and, most importantly; and (5) the presence of "malnutrition" was associated with higher mortality. Together, our findings strongly support for a need of early dietetic evaluation in all hospitalized patients with chronic liver disease in order to identify subjects at-risk for "malnutrition", so that early nutritional intervention can be instituted during hospital admission and also after discharge.

To our knowledge, this is the first study to highlight the lack of dietetic assessment in hospitalised patients with liver cirrhosis. Despite the well-established prognostic value of nutrition, our study showed that almost half of hospitalized patients with cirrhosis did not have a formal nutritional assessment. While patients with hypoalbuminaemia and ascites were more likely to receive formal dietetic input, neither of these factors was predictive of nutritional status. More importantly, the prevalence of malnutrition in this group of patients is high (56%) and is in keeping with previously reports[3,4], which would support the notion that malnutrition is under-diagnosed in these patients in routine clinical care.

Poor nutrient intake is likely to be a major factor responsible for the development of malnutrition in these patients. Consistent with finding of a previous study[21], dietary intake was substantially reduced in our patients with liver cirrhosis, even in patients who had normal SGA (only 68% daily carbohydrate and 85% protein requirements), resulting in a negative energy balance, weight loss and the development of malnutrition. This reduction in nutrient intake in these patients is likely to be multi-factorial. Patients with cirrhosis frequently experience gastrointestinal symptoms[22,23] which may contribute to reduced nutrient intake[24]. Delayed gastric emptying has been reported in patients with cirrhosis and has been associated with post-prandial fullness and bloating[25]. Alteration in taste acuity has been associated with deficiencies in trace elements including zinc, magnesium and vitamin A[26,27], which are common in patients with cirrhosis. Appetite is also reduced owing to increased inflammatory cytokines and alterations in appetite regulating hormones in cirrhotic patient including leptin, ghrelin, PYY and CCK[25,28-32].

Paradoxically, the lack of nutrient intake also influences the function of the gastrointestinal tract. In healthy and critically ill subjects, enteral nutrient deprivation is associated with mucosal atrophy, increased intestinal permeability and infections[33,34]. The use of enteral nutrition in critically ill and surgical patients has been shown to prevent the development of these adverse changes to the gastrointestinal tract[34,35], and has been associated with reduced morbidity, particularly septic complications[36,37]. Given such poor oral intake in cirrhotic patients, it is very likely that similar gastrointestinal changes of nutritional deprivation will be observed. This may explain the increase in intestinal permeability seen in cirrhotic patients with malnutrition[38], which subjects these patients to a higher risk of infectious complications and hepatic encephalopathy[39]. Given our finding of increased mortality in malnourished patients and in patients with poor in-hospital caloric and protein intake, the role of routine oral nutritional supplement or even aggressive enteral nutrition on gut functions and defence needs further evaluation.

Another key finding in our study was that nutritional status as determined by subjective global assessment had a major impact on short-term and long-term mortality in hospitalised patients with liver cirrhosis. Studies examining the association between nutrition and survival have yielded different results. Compared to other studies, our patient population had more advanced liver disease with over ninety percent having decompensated cirrhosis, and a high mortality rate during hospital stay and the follow-up period.

Our findings are consistent with other studies that involved hospitalized patients with mostly decompensated cirrhosis[4,5,9,40], with similar hazard ratios of 2-5.3 for mortality[9,40]. Conversely, studies enrolling stable, and mostly compensated cirrhotic patients in the outpatient setting have failed to demonstrate an association between malnutrition and survival[7,8,21]. A potential explanation for the difference in the impact of nutritional status on outcomes between inpatients and outpatients is the lack of room for compensation in the already decompensated inpatients.

Despite the higher mortality in cirrhotic patients with malnutrition, we found no significant difference in liver related morbidity. This is contrary to previous studies which have found increased variceal bleeding, refractory ascites, spontaneous bacterial peritonitis (SBP) and hepatorenal syndrome (HRS) in malnourished patients[6,7]. The failure to find any difference in liver related morbidity in our study may be due to the patient population with advanced, decompensated cirrhosis, high prevalence of pre-existing morbidity, and the small number of subsequent new liver related complications.

We acknowledge that this is a retrospective review with the potential limitations associated with the study design. Nevertheless, the data were thoroughly collected and cross-checked from a number of prospective databases. Furthermore, all patients with liver cirrhosis who were admitted to the Department of Gastroenterology and Hepatology in the Royal Adelaide Hospital were included in the study and the sample size was sufficient to demonstrate differences

in the clinical outcomes. The strength of this study is that it provides information on nutritional care and the impact of malnutrition on clinical outcomes in patients with cirrhosis in the real-world setting. Although SGA may have a lower sensitivity for the detection of malnutrition compared to hand grip strength[7,41] and multi-compartmental body composition analysis[13], SGA is a simple, inexpensive and reliable bed-side tool to assess for malnutrition with an 80% inter-observer reproducibility rate[42].

In conclusion, although malnutrition is common in hospitalised patients with liver cirrhosis and is associated with higher short- and long-term mortality, nearly half of these patients have no dietetic assessment and intervention. These findings suggest that routine dietetic assessment should be performed on all patients who are admitted with chronic liver disease and related complication, so that at-risk subjects for "malnutrition" can be identified and early nutritional intervention can be instituted during hospitalisation but also after discharge.

## ACKNOWLEDGMENTS

The authors thank Dr Rahul Solanki for his help with the collection of data, and Kylie Lange for her expertise and assistance in performing the statistical analysis.

## COMMENTS

### Background

Malnutrition is common in patients with liver cirrhosis and is associated with increased morbidity including hepatic encephalopathy, variceal bleeding, refractory ascites, spontaneous bacterial peritonitis and hepatorenal syndrome. Malnutrition is also associated with increased mortality. Early detection and nutritional intervention by a dietician can improve survival.

### Research frontiers

Despite the prognostic importance of malnutrition in liver cirrhosis, little is known about the current practice of nutritional management in hospitalized patients with liver cirrhosis.

### Innovations and breakthroughs

This study shows that malnutrition is common in hospitalized patients with cirrhosis and is associated with increased mortality. However, this is the first study to highlight the lack of formal dietetic assessment and nutritional intervention in these patients.

### Applications

The study results suggest the need for more conscientious nutritional assessment and management in the routine clinical care of hospitalised patients with liver cirrhosis.

### Terminology

SGA is a simple bedside tool for nutritional assessment which entails a medical history including weight change, dietary change, gastrointestinal symptoms, and functional impairment, and physical examination for subcutaneous fat store, muscle wasting, peripheral oedema and ascites and body mass index.

### Peer-review

This study evaluated the practice of nutritional assessment and management of hospitalised patients with cirrhosis and the impact of malnutrition on their clinical outcome. The author found that malnutrition was common in hospitalised patients with cirrhosis and is associated with higher mortality. However, the formal nutritional assessment was inadequate currently. This highlights the need for meticulous nutritional evaluation and management in these patients. This is a well conducted and well written study. The experiments are described in detail, the results are shown nicely and the figures are impressive.

## REFERENCES

1 **Teiusanu A**, Andrei M, Arbanas T, Nicolaie T, Diculescu M. Nutritional status in cirrhotic patients. *Maedica* (Buchar) 2012; **7**: 284-289 [PMID: 23483873]

2 **Guglielmi FW**, Panella C, Buda A, Budillon G, Caregaro L, Clerici C, Conte D, Federico A, Gasbarrini G, Guglielmi A, Loguercio C, Losco A, Martines D, Mazzuoli S, Merli M, Mingrone G, Morelli A, Nardone G, Zoli G, Francavilla A. Nutritional state and energy balance in cirrhotic patients with or without hypermetabolism. Multicentre prospective study by the 'Nutritional Problems in Gastroenterology' Section of the Italian Society of Gastroenterology (SIGE). *Dig Liver Dis* 2005; **37**: 681-688 [PMID: 15978878 DOI: 10.1016/j.dld.2005.03.010]

3 **Campillo B**, Richardet JP, Scherman E, Bories PN. Evaluation of nutritional practice in hospitalized cirrhotic patients: results of a prospective study. *Nutrition* 2003; **19**: 515-521 [PMID: 12781851]

4 **Alberino F**, Gatta A, Amodio P, Merkel C, Di Pascoli L, Boffo G, Caregaro L. Nutrition and survival in patients with liver cirrhosis. *Nutrition* 2001; **17**: 445-450 [PMID: 11399401]

5 **Sam J**, Nguyen GC. Protein-calorie malnutrition as a prognostic indicator of mortality among patients hospitalized with cirrhosis and portal hypertension. *Liver Int* 2009; **29**: 1396-1402 [PMID: 19602136 DOI: 10.1111/j.1478-3231.2009.02077.x]

6 **Møller S**, Bendtsen F, Christensen E, Henriksen JH. Prognostic variables in patients with cirrhosis and oesophageal varices without prior bleeding. *J Hepatol* 1994; **21**: 940-946 [PMID: 7699257]

7 **Alvares-da-Silva MR**, Reverbel da Silveira T. Comparison between handgrip strength, subjective global assessment, and prognostic nutritional index in assessing malnutrition and predicting clinical outcome in cirrhotic outpatients. *Nutrition* 2005; **21**: 113-117 [PMID: 15723736 DOI: 10.1016/j.nut.2004.02.002]

8 **Merli M**, Riggio O, Dally L. Does malnutrition affect survival in cirrhosis? PINC (Policentrica Italiana Nutrizione Cirrosi). *Hepatology* 1996; **23**: 1041-1046 [PMID: 8621131 DOI: 10.1002/hep.510230516]

9 **Gunsar F**, Raimondo ML, Jones S, Terreni N, Wong C, Patch D, Sabin C, Burroughs AK. Nutritional status and prognosis in cirrhotic patients. *Aliment Pharmacol Ther* 2006; **24**: 563-572 [PMID: 16827812 DOI: 10.1111/j.1365-2036.2006.03003.x]

10 **Figueiredo F**, Dickson ER, Pasha T, Kasparova P, Therneau T, Malinchoc M, DiCecco S, Francisco-Ziller N, Charlton M. Impact of nutritional status on outcomes after liver transplantation. *Transplantation* 2000; **70**: 1347-1352 [PMID: 11087151]

11 **Kakiyama G**, Pandak WM, Gillevet PM, Hylemon PB, Heuman DM, Daita K, Takei H, Muto A, Nittono H, Ridlon JM, White MB, Noble NA, Monteith P, Fuchs M, Thacker LR, Sikaroodi M, Bajaj JS. Modulation of the fecal bile acid profile by gut microbiota in cirrhosis. *J Hepatol* 2013; **58**: 949-955 [PMID: 23333527 DOI: 10.1016/j.jhep.2013.01.003]

12 **Detsky AS**, McLaughlin JR, Baker JP, Johnston N, Whittaker S, Mendelson RA, Jeejeebhoy KN. What is subjective global assessment of nutritional status? *JPEN J Parenter Enteral Nutr* 1987; **11**: 8-13 [PMID: 3820522]

13 **Figueiredo FA**, Perez RM, Freitas MM, Kondo M. Comparison of three methods of nutritional assessment in liver cirrhosis: subjective global assessment, traditional nutritional parameters, and body composition analysis. *J Gastroenterol* 2006; **41**: 476-482 [PMID: 16799890]

14 **Naveau S**, Belda E, Borotto E, Genuist F, Chaput JC. Comparison of clinical judgment and anthropometric parameters for evaluating nutritional status in patients with alcoholic liver disease. *J Hepatol* 1995; **23**: 234-235 [PMID: 7499801]

15 **Vulcano DS**, Carvalhaes MA, Bakonyi Neto A. Evaluation of nutritional indicators and body composition in patients with advanced liver disease enrolled for liver transplantation. *Acta Cir Bras* 2013; **28**: 733-739 [PMID: 24114303]

16 **Iwasa M**, Iwata K, Hara N, Hattori A, Ishidome M, Sekoguchi-Fujikawa N, Mifuji-Moroka R, Sugimoto R, Fujita N, Kobayashi Y, Takei Y. Nutrition therapy using a multidisciplinary team improves survival rates in patients with liver cirrhosis. *Nutrition* 2006; **29**: 1418-1421 [PMID: 24103520 DOI: 10.1016/j.nut.2013.05.016]

17 **Pugh RN**, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; **60**: 646-649 [PMID: 4541913]

18 **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]

19 **Mendenhall CL**. Protein-calorie malnutrition in alcholic liver disease. In: Wartson RR, Watz IB, editors. Nutrition and Alcohol. Boca Raton: CRC Press, 1992: 363-384

20 **Plauth M**, Cabré E, Riggio O, Assis-Camilo M, Pirlich M, Kondrup J, Ferenci P, Holm E, Vom Dahl S, Müller MJ, Nolte W. ESPEN Guidelines on Enteral Nutrition: Liver disease. *Clin Nutr* 2006; **25**: 285-294 [PMID: 16707194 DOI: 10.1016/j.clnu.2006.01.018]

21 **Huisman EJ**, Trip EJ, Siersema PD, van Hoek B, van Erpecum KJ. Protein energy malnutrition predicts complications in liver cirrhosis. *Eur J Gastroenterol Hepatol* 2011; **23**: 982-989 [PMID: 21971339 DOI: 10.1097/MEG.0b013e32834aa4bb]

22 **Galati JS**, Monsour HP, Dyer CH, Seagren S, Quigley EMM. A survey or the frequency of gastrointestinal complaints in patients with chronic liver disease. *Gastroenterology* 1995; **108**: A1068

23 **Kalaitzakis E**, Simrén M, Olsson R, Henfridsson P, Hugosson I, Bengtsson M, Björnsson E. Gastrointestinal symptoms in patients with liver cirrhosis: associations with nutritional status and health-related quality of life. *Scand J Gastroenterol* 2006; **41**: 1464-1472 [PMID: 17101578 DOI: 10.1080/00365520600825117]

24 **Kalaitzakis E**, Josefsson A, Castedal M, Henfridsson P, Bengtsson M, Andersson B, Björnsson E. Gastrointestinal symptoms in patients with cirrhosis: a longitudinal study before and after liver transplantation. *Scand J Gastroenterol* 2013; **48**: 1308-1316 [PMID: 24063547 DOI: 10.3109/00365521.2013.836755]

25 **Kalaitzakis E**, Sadik R, Holst JJ, Ohman L, Björnsson E. Gut transit is associated with gastrointestinal symptoms and gut hormone profile in patients with cirrhosis. *Clin Gastroenterol Hepatol* 2009; **7**: 346-352 [PMID: 19200458 DOI: 10.1016/j.cgh.2008.11.022]

26 **Madden AM**, Bradbury W, Morgan MY. Taste perception in cirrhosis: its relationship to circulating micronutrients and food preferences. *Hepatology* 1997; **26**: 40-48 [PMID: 9214450 DOI: 10.1002/hep.510260106]

27 **Russell RM**, Cox ME, Solomons N. Zinc and the special senses. *Ann Intern Med* 1983; **99**: 227-239 [PMID: 6349457]

28 **Kalaitzakis E**, Bosaeus I, Ohman L, Björnsson E. Altered post-prandial glucose, insulin, leptin, and ghrelin in liver cirrhosis: correlations with energy intake and resting energy expenditure. *Am J Clin Nutr* 2007; **85**: 808-815 [PMID: 17344504]

29 **Campillo B**, Sherman E, Richardet JP, Bories PN. Serum leptin levels in alcoholic liver cirrhosis: relationship with gender, nutritional status, liver function and energy metabolism. *Eur J Clin Nutr* 2001; **55**: 980-988 [PMID: 11641747 DOI: 10.1038/sj.ejcn.1601255]

30 **Valentini L**, Schuetz T, Omar A, Gläser S, Kasim E, Nowotny P, Kroencke T, Ockenga J. Abnormal plasma peptide YY(3-36) levels in patients with liver cirrhosis. *Nutrition* 2011; **27**: 880-884 [PMID: 21819934]

31 **Paloheimo LI**, Clemmesen O, Dalhoff K, Rehfeld JF. Plasma cholecystokinin and its precursors in hepatic cirrhosis. *J Hepatol* 1997; **27**: 299-305 [PMID: 9288604]

32 **Kanayama S**, Himeno S, Higashimoto Y, Yamasaki Y, Kitani T, Tarui S. Plasma cholecystokinin-octapeptide like immunoreactivity in patients with hepatic cirrhosis. *Life Sci* 1987; **41**: 1915-1920 [PMID: 3657391]

33 **Hernandez G**, Velasco N, Wainstein C, Castillo L, Bugedo G, Maiz A, Lopez F, Guzman S, Vargas C. Gut mucosal atrophy after a short enteral fasting period in critically ill patients. *J Crit Care* 1999; **14**: 73-77 [PMID: 10382787]

34 **Kudsk KA**. Effect of route and type of nutrition on intestine-derived inflammatory responses. *Am J Surg* 2003; **185**: 16-21 [PMID: 12531438]

35 **McClave SA**, Heyland DK. The physiologic response and associated clinical benefits from provision of early enteral nutrition. *Nutr Clin Pract* 2009; **24**: 305-315 [PMID: 19483060 DOI: 10.1177/0884533609335176]

36 **Adams S**, Dellinger EP, Wertz MJ, Oreskovich MR, Simonowitz D, Johansen K, Oreskovich MR, Simonowitz D, Johansen K. Enteral versus parenteral nutritional support following laparotomy for trauma: a randomized prospective trial. *J Trauma* 1986; **26**: 882-891 [PMID: 3095558]

37 **Moore FA**, Moore EE, Jones TN, McCroskey BL, Peterson VM. TEN versus TPN following major abdominal trauma--reduced septic morbidity. *J Trauma* 1989; **29**: 916-922; discussion 922-923 [PMID: 2501509]

38 **Norman K**, Pirlich M, Schulzke JD, Smoliner C, Lochs H, Valentini L, Bühner S. Increased intestinal permeability in malnourished patients with liver cirrhosis. *Eur J Clin Nutr* 2012; **66**: 1116-1119 [PMID: 22872031 DOI: 10.1038/ejcn.2012.104]

39 **Kalaitzakis E**, Olsson R, Henfridsson P, Hugosson I, Bengtsson M, Jalan R, Björnsson E. Malnutrition and diabetes mellitus are related to hepatic encephalopathy in patients with liver cirrhosis. *Liver Int* 2007; **27**: 1194-1201 [PMID: 17919230 DOI: 10.1111/j.1478-3231.2007.01562.x]

40 **Abad-Lacruz A**, Cabré E, González-Huix F, Fernández-Bañares F, Esteve M, Planas R, Llovet JM, Quer JC, Gassull MA. Routine tests of renal function, alcoholism, and nutrition improve the prognostic accuracy of Child-Pugh score in nonbleeding advanced cirrhotics. *Am J Gastroenterol* 1993; **88**: 382-387 [PMID: 8438845]

41 **Ferreira LG**, Anastácio LR, Lima AS, Correia MI. Assessment of nutritional status of patients waiting for liver transplantation. *Clin Transplant* 2011; **25**: 248-254 [PMID: 20236138 DOI: 10.1111/j.1399-0012.2010.01228.x]

42 **Hasse J**, Strong S, Gorman MA, Liepa G. Subjective global assessment: alternative nutrition-assessment technique for liver-transplant candidates. *Nutrition* 1993; **9**: 339-343 [PMID: 8400590]


**P- Reviewer**: Gong ZG, Liang XS **S- Editor**: Yu J
**L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12843

*World J Gastroenterol* 2015 December 7; 21(45): 12843-12850
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

Retrospective Cohort Study

# Laparoscopic *vs* open partial colectomy in elderly patients: Insights from the American College of Surgeons - National Surgical Quality Improvement Program database

Umashankkar Kannan, Vemuru Sunil K Reddy, Amar N Mukerji, Vellore S Parithivel, Ajay K Shah, Brian F Gilchrist, Daniel T Farkas

Umashankkar Kannan, Vemuru Sunil K Reddy, Amar N Mukerji, Vellore S Parithivel, Ajay K Shah, Brian F Gilchrist, Daniel T Farkas, Department of Surgery, Bronx-Lebanon Hospital Center, Bronx, NY 10457, United States

Author contributions: Kannan U conceived of the idea; Kannan U, Reddy VSK, Mukerji AN, Parithivel VS, Shah AK, Gilchrist BF and Farkas DT designed the research; Kannan U collected the data; Kannan U and Farkas DT interpreted and analyzed the data; Kannan U and Farkas DT wrote the paper; Kannan U, Reddy VSK, Mukerji AN, Parithivel VS, Shah AK and Gilchrist BF edited the paper; Farkas DT supervised the project and performed the final revisions.

Institutional review board statement: This paper used public data with no protected health information (all the data is de-identified before going in the database) and is exempt from the Institutional Review Board.

Informed consent statement: No identifiable patients are involved in this study, and informed consent is not applicable.

Conflict-of-interest statement: None of the authors have any conflicts of interest related to the manuscript.

Data sharing statement: The original anonymous dataset is available on request from the corresponding author at dfarkas@bronxleb.org.



Correspondence to: Daniel T Farkas, Department of Surgery, Bronx-Lebanon Hospital Center, 1650 Selwyn Avenue, Suite 4E, Bronx, NY 10457, United States. dfarkas@bronxleb.org
Telephone: +1-718-5901800

Received: June 20, 2015
Peer-review started: June 25, 2015
First decision: July 19, 2015
Revised: August 15, 2015
Accepted: October 13, 2015
Article in press: October 13, 2015
Published online: December 7, 2015

## Abstract

**AIM:** To compare the outcomes between the laparoscopic and open approaches for partial colectomy in elderly patients aged 65 years and over using the American College of Surgeons - National Surgical Quality Improvement Program (ACS NSQIP) database.

**METHODS:** The ACS NSQIP database for the years 2005-2011 was queried for all patients 65 years and above who underwent partial colectomy. 1:1 propensity score matching using the nearest- neighbor method was performed to ensure both groups had similar pre-operative comorbidities. Outcomes including post-operative complications, length of stay and mortality were compared between the laparoscopic and open groups. $\chi^2$ and Fisher's exact test were used for discrete variables and Student's *t*-test for continuous variables. $P < 0.05$ was considered significant and odds ratios with 95%CI were reported when applicable.

**RESULTS:** The total number of patients in the ACS NSQIP database of the years 2005-2011 was 1777035. We identified 27604 elderly patients who underwent partial colectomy with complete data sets. 12009 (43%) of the cases were done laparoscopically and

15595 (57%) were done with open. After propensity score matching, there were 11008 patients each in the laparoscopic (LC) and open colectomy (OC) cohorts. The laparoscopic approach had lower post-operative complications (LC 15.2%, OC 23.8%, $P < 0.001$), shorter length of stay (LC 6.61 d, OC 9.62 d, $P < 0.001$) and lower mortality (LC 1.6%, OC 2.9%, $P < 0.001$).

**CONCLUSION:** Even after propensity score matching, elderly patients in the ACS NSQIP database having a laparoscopic partial colectomy had better outcomes than those having open colectomies. In the absence of specific contraindications, elderly patients requiring a partial colectomy should be offered the laparoscopic approach.

**Key words:** Colectomy; Laparoscopic; Outcomes; Elderly; National Surgical Quality Improvement Program



**Core tip:** Elderly patients having partial colectomies are at greater risk for complications due to a higher incidence of comorbidities. This study looked at patients aged 65 and above in a nationally validated database from the American College of Surgeons National Surgical Quality Improvement Program. Patients having a laparoscopic partial colectomy, when compared to an open partial colectomy, had fewer complications, shorter lengths of stay and decreased mortality.

Kannan U, Reddy VSK, Mukerji AN, Parithivel VS, Shah AK, Gilchrist BF, Farkas DT. Laparoscopic *vs* open partial colectomy in elderly patients: Insights from the American College of Surgeons - National Surgical Quality Improvement Program database. *World J Gastroenterol* 2015; 21(45): 12843-12850 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12843.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12843

## INTRODUCTION

The role of laparoscopy has been well documented in the field of colorectal surgery[1,2]. The laparoscopic approach has been associated with reduced post-operative pain, less morbidity, shorter lengths of stay, lower costs, fewer adhesions and a lower incidence of hernias[3,4]. Despite these well-established advantages, the minimally invasive approach has been underutilized with a recent study showing adoption rates of up to 40%[5,6]. The elderly population, aged 65 years and above, is one of the higher risk groups due to the presence of comorbidities and as a result is at increased risk for post-operative events[7]. Consequently, caring for such patients can present unique challenges. Laparoscopic cholecystectomy has been shown to be safe in this age group[8,9]. A laparoscopic colectomy requires a longer operating time than a laparoscopic cholecystectomy[10] and operative duration has been shown to be correlated with postoperative complications as well as length of stay[11,12]. On the other hand, open colorectal surgery in the elderly is itself associated with increased morbidity and mortality[13]. Hence it becomes important to look specifically in this elderly cohort and analyze if the laparoscopic approach is advantageous when compared with the conventional open approach.

The aim of this study is to compare the outcomes between elderly patients undergoing laparoscopic partial colectomy (LC) and open partial colectomy (OC). There are various studies showing improved outcomes with the laparoscopic approach but there are very few reports on its impact specifically in the elderly population[14,15]. The studies that are available are generally single institution studies based on small sample sizes[16-18]. The present work is based on a very large sample size from the American College of Surgeons National Surgical Quality Improvement Program (ACS NSQIP) database. ACS NSQIP is the first nationally validated, risk adjusted, outcome based program to measure and improve the quality of surgical care. The number of variables, cases and centers participating in the ACS NSQIP database have been progressively increasing over the years. In 2012, ACS NSQIP included over 150 variables involving about 543885 cases from 315 academic and community-based hospitals in United States. The variables collected include data on preoperative risk factors, intraoperative variables, 30-d postoperative mortality and morbidity outcomes for all patients aged 18 years and older undergoing major surgical procedures in the inpatient and outpatient settings. A trained surgical clinical reviewer captures this data prospectively by a variety of methods including medical chart abstraction using an 8 d cycle. To date, the ACS NSQIP has had a 95% success rate in capturing the 30 d outcomes for all cases in the database. The accuracy and reproducibility of its data has been well documented[19].

In studies that use nonrandomized databases such as the ACS NSQIP there is a potential for selection bias. In order to minimize these problems, propensity score matching was employed in this study. This allowed the two groups of patients to be more closely matched, which provides a more accurate comparison of the outcomes between the two groups.

## MATERIALS AND METHODS

### *Patient selection*

Participant user files (PUF) from the years 2005 to 2011 of the ACS NSQIP database were combined into a single database. Patients undergoing a partial colectomy were included. Current Procedural Terminology (CPT) codes 44205, 44204 and 44207 were chosen as representing laparoscopic right, left and sigmoid colectomies respectively. CPT codes

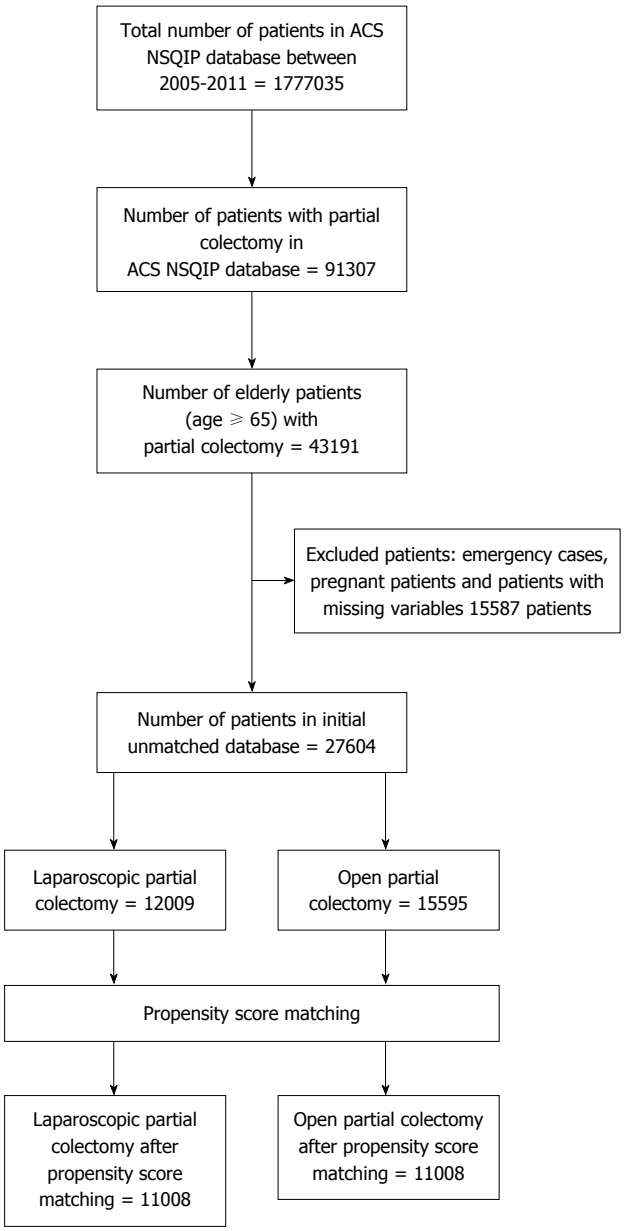



**Figure 1 Flowchart outlining patient selection.**

44160, 44140 and 44145 were chosen as representing open right, left and sigmoid colectomies. Rectal procedures were not included, and neither were cases with colostomies or ileostomies. We excluded emergency cases, pregnant patients and database entries with missing data.

### *Propensity score matching*

A propensity 1:1 matched analysis was then performed to identify similar patients groups in the LC and OC groups. The first step of the propensity score matching consisted of fitting a logistic regression to model the probability of receiving a LC. The covariates included in the regression were age, body mass index (BMI), sex, site of surgery, American Society of Anesthesiology (ASA) class 3 and above, cardiac, pulmonary, neurological, renal and bleeding disorders, diabetes mellitus and steroid usage. In the second step, LC patients were matched with OC patients based on the predicted probability (propensity score). The result was more balanced groups in the selected covariates.

### *Statistical analysis*

The two groups were then compared using a bivariate analysis approach. Patient demographics, comorbidities, intraoperative complications and postoperative complications were then compared between the two groups using bivariate analysis. $\chi^2$ test was used for categorical variables and student's *t* test was used for continuous variables. Fisher's exact test was used for categorical variables with very small expected frequencies. Results were reported as mean (± SD) for continuous variables and frequency for nominal and ordinal variables. *P* values < 0.05 were considered significant. OR with 95%CI were reported when applicable. Analyses were performed using SPSS 20 (IBM SPSS Inc., Chicago, IL).

## RESULTS

The NSQIP database between 2005 and 2011 had a total of 1777035 patients. Of these patients, there were 91307 who had a partial colectomy, with 43191 aged 65 and above (Figure 1). 15587 patients were excluded on the basis of being emergent cases, pregnant patients, or the dataset not being complete in the ACS NSQIP database. This left an index unmatched database of 27604 elderly partial colectomy patients. This original group had 12009 (43%) laparoscopic patients and 15595 (57%) open patients. The preoperative characteristics of these groups are outlined in Table 1. The groups are different in almost all demographic and comorbidity categories.

After propensity matching, we were left with a total of 22016 patients - 11008 patients in each of the laparoscopic and open cohorts. These are the groups that were used for comparison in our study, and their pre-operative characteristics are compared in Table 2. As a result of the propensity matching the two groups are essentially similar in terms of their demographics and rate of comorbidities. There were no significant differences between the groups in terms of cardiac, pulmonary, neurological, renal, hematological or endocrine comorbidities.

Intraoperative characteristics of the matched cohorts are shown in Table 3. The operative duration in the LC cohort is slightly higher than in the OC cohort (LC 152 min, OC 144 min, $P < 0.001$). The LC cohort had slightly lower need for transfusions (LC 2.3%, OC 2.8%, $P < 0.001$). There was no statistically significant difference in intraoperative events, such as cardiac arrest, myocardial infarction and unplanned intubation between the groups, although these numbers were quite small.

**Table 1 Preoperative characteristics of elderly patients undergoing partial colectomy before propensity score matching *n* (%)**

| Characteristics | Laparoscopic colectomy (*n* = 12009) | Open colectomy (*n* = 15595) | *P* value |
|---|---|---|---|
| Age, mean ± SD | 75.94 ± 7.15 | 74.87 ± 6.92 | < 0.001 |
| BMI, mean ± SD | 27.30 ± 6.38 | 27.17 ± 7.09 | < 0.001 |
| Gender | | | 0.416 |
| Female | 7401 (61.6) | 9536 (61.1) | |
| Male | 4608 (38.4) | 6059 (38.9) | |
| Procedure | | | < 0.001 |
| Right partial colectomy | 3122 (25.9) | 3773 (24.2) | |
| Left partial colectomy | 6614 (55.1) | 8492 (54.5) | |
| Sigmoidectomy | 2273 (19.0) | 3330 (21.3) | |
| Indication | | | < 0.001 |
| Benign | 4769 (39.7) | 5447 (34.9) | |
| Malignant | 4544 (37.8) | 7454 (47.8) | |
| Unknown | 2696 (22.4) | 2694 (17.3) | |
| ASA class | | | |
| Ⅰ | 155 (1.2) | 115 (0.7) | |
| Ⅱ | 5418 (45.2) | 4960 (31.8) | |
| Ⅲ | 5956 (49.6) | 9225 (59.2) | |
| Ⅳ | 477 (4.0) | 1278 (8.2) | |
| Ⅴ | 3 (0.02) | 17 (0.1) | |
| ASA Ⅲ, Ⅳ or Ⅴ | 6436 (53.6) | 10520 (67.5) | < 0.001 |
| Alcohol | 359 (2.9) | 408 (2.6) | 0.061 |
| Current smoker | 1062 (8.8) | 1689 (10.8) | < 0.001 |
| Cardiac diseases | 8489 (70.7) | 11350 (72.8) | < 0.001 |
| Hypertension | 8232 (68.5) | 10936 (70.1) | 0.005 |
| History of CHF | 119 (1.0) | 297 (1.9) | < 0.001 |
| History of MI | 56 (0.5) | 169 (1.1) | < 0.001 |
| History of angina | 80 (0.7) | 200 (1.3) | < 0.001 |
| History of PCI | 957 (8.0) | 1431 (9.2) | < 0.001 |
| History of PCS | 959 (8.0) | 1495 (9.6) | < 0.001 |
| Pulmonary diseases | 836 (7.0) | 1519 (9.7) | < 0.001 |
| Dyspnea | 1600 (13.3) | 2723 (17.4) | < 0.001 |
| History of COPD | 821 (6.8) | 1428 (9.2) | < 0.001 |
| Ventilator dependent | 4 (0.02) | 58 (0.4) | < 0.001 |
| Pneumonia | 20 (0.2) | 71 (0.5) | < 0.001 |
| Neurological disease | 994 (8.3) | 1689 (10.8) | < 0.001 |
| History of CVA | 347 (2.9) | 613 (3.9) | < 0.001 |
| History of CVANO | 355 (3.0) | 591 (3.8) | < 0.001 |
| History of TIA | 516 (4.3) | 801 (5.1) | 0.002 |
| History of tumor CNS | 11 (0.1) | 18 (0.1) | 0.545 |
| Renal diseases | 62 (0.5) | 173 (1.1) | < 0.001 |
| History of renal failure | 15 (0.1) | 56 (0.4) | < 0.001 |
| Dialysis dependent | 52 (0.4) | 137 (0.9) | < 0.001 |
| Bleeding disorders | 487 (4.1) | 1009 (6.5) | < 0.001 |
| Steroid use[1] | 365 (3.0) | 580 (3.7) | 0.002 |
| Diabetes | 3166 (26.4) | 3193 (20.5) | < 0.001 |

[1]Use of steroid in the 30 d prior to surgery for a chronic medical condition. BMI: Body mass index; ASA: American Society of Anesthesiologists; CHF: Congestive heart failure; MI: Myocardial infarction; PCI: Percutaneous coronary intervention; PCS: Previous cardiac surgery; PVD: Peripheral vascular disease; COPD: Chronic pulmonary obstructive disease; CVA: Cerebrovascular accident with neurological deficit; CVANO: Cerebrovascular accident without neurological deficit; TIA: Transient ischemic attack; Tumor CNS: Tumor involving central nervous system.

**Table 2 Preoperative characteristics of elderly patients undergoing partial colectomy after propensity score matching *n* (%)**

| Characteristics | Laparoscopic colectomy (*n* = 11008) | Open colectomy (*n* = 11008) | *P* value |
|---|---|---|---|
| Age | | | |
| mean ± SD | 75.12 ± 6.97 | 75.21 ± 6.98 | 0.857 |
| median ± IQR | 74 ± 11 | 74 ± 12 | 0.332 |
| BMI (mean ± SD) | 27.31 ± 6.42 | 27.23 ± 7.05 | 0.368 |
| Gender | | | 0.021 |
| Female | 6685 (60.7) | 6852 (62.2) | |
| Male | 4323 (39.3) | 4156 (37.8) | |
| Procedure | | | 0.013 |
| Right partial colectomy | 2549 (23.2) | 2736 (24.8) | 0.003 |
| Left partial colectomy | 6237 (56.7) | 6099 (55.4) | 0.061 |
| Sigmoidectomy | 2222 (20.1) | 2173 (19.8) | 0.409 |
| Indication | | | < 0.001 |
| Benign | 4361 (39.6) | 3966 (36.0) | |
| Malignant | 4193 (38.1) | 5431 (49.3) | |
| Unknown | 2454 (22.3) | 1611 (14.6) | |
| ASA class | | | |
| Ⅰ | 127 (1.1) | 110 (1.0) | |
| Ⅱ | 4563 (41.5) | 4563 (41.5) | |
| Ⅲ | 5852 (53.2) | 5673 (51.5) | |
| Ⅳ | 463 (4.2) | 658 (6.0) | |
| Ⅴ | 3 (0.02) | 4 (0.03) | |
| ASA Ⅲ, Ⅳ or Ⅴ | 6318 (57.4) | 6335 (57.5) | 0.817 |
| Alcohol | 341 (3.0) | 289 (2.6) | 0.036 |
| Current smoker | 1051 (9.5) | 993 (9.0) | 0.178 |
| Cardiac diseases | 7905 (71.8) | 7845 (71.3) | 0.370 |
| Hypertension | 7654 (69.5) | 7614 (69.2) | 0.559 |
| History of CHF | 116 (1.1) | 134 (1.2) | 0.252 |
| History of MI | 51 (0.5) | 57 (0.5) | 0.563 |
| History of angina | 75 (0.6) | 86 (0.7) | 0.384 |
| History of PCI | 902 (8.2) | 807 (7.3) | 0.017 |
| History of PCS | 924 (8.4) | 894 (8.1) | 0.463 |
| Pulmonary diseases | 815 (7.4) | 778 (7.1) | 0.336 |
| Dyspnea | 1526 (13.7) | 1513 (13.7) | 0.432 |
| History of COPD | 801 (7.3) | 758 (6.9) | 0.259 |
| Ventilator dependent | 3 (0.02) | 3 (0.02) | 1.000 |
| Pneumonia | 18 (0.2) | 24 (0.2) | 0.354 |
| Neurological disease | 921 (8.4) | 896 (8.1) | 0.540 |
| History of CVA | 333 (3.0) | 305 (2.8) | 0.261 |
| History of CVANO | 323 (2.9) | 307 (2.8) | 0.518 |
| History of TIA | 347 (3.2) | 353 (3.2) | 0.818 |
| History of tumor CNS | 10 (0.1) | 6 (0.1) | 0.317 |
| Renal diseases | 57 (0.5) | 55 (0.5) | 0.850 |
| History of renal failure | 12 (0.1) | 13 (0.1) | 0.841 |
| Dialysis dependent | 49 (0.4) | 48 (0.4) | 0.919 |
| Bleeding disorders | 466 (4.2) | 425 (3.9) | 0.161 |
| Steroid use[1] | 353 (3.2) | 318 (2.9) | 0.170 |
| Diabetes | 2077 (18.9) | 2040 (18.5) | 0.522 |

[1]Use of steroid in the 30 d prior to surgery for a chronic medical condition. BMI: Body mass index; ASA: American Society of Anesthesiologists; CHF: Congestive heart failure; MI: Myocardial infarction; PCI: Percutaneous coronary intervention; PCS: Previous cardiac surgery; PVD: Peripheral vascular disease; COPD: Chronic pulmonary obstructive disease; CVA: Cerebrovascular accident with neurological deficit; CVANO: Cerebrovascular accident without neurological deficit; TIA: Transient ischemic attack; Tumor CNS: Tumor involving central nervous system.

Postoperative 30-d outcomes of both the cohorts are shown in Table 4. There were significantly fewer complications in the LC group (LC 15.2%, OC 23.8%, $P < 0.001$). These were lower in every single subcategory of cardiac, pulmonary, renal and infectious complications. Patients in the LC cohort had lower rates of unplanned return to the operating room (LC 4.1%, OC 5.3%, $P < 0.001$). Length of stay was shorter in the laparoscopic group (LC 6.61 d, OC 9.62 d, $P < 0.001$). Finally, the laparoscopic cohort had lower

**Table 3 Intraoperative characteristics of elderly patients undergoing partial colectomy after propensity score matching**

| Characteristics | Laparoscopic colectomy ($n$ = 11008) | Open colectomy ($n$ = 11008) | $P$ value |
|---|---|---|---|
| Operative time (min), mean ± SD | 151.75 ± 66.15 | 143.56 ± 78.60 | < 0.001 |
| Anesthesia time (min), mean ± SD | 209.47 ± 78.54 | 200.72 ± 91.29 | < 0.001 |
| Blood transfusions, $n$ (%) | 250 (2.3) | 307 (2.8) | < 0.001 |
| Intraoperative occurrence | | | 0.143 |
| Cardiac arrest | 1 | 5 | |
| Myocardial infarction | 3 | 5 | |
| Unplanned intubation | 5 | 11 | |

mortality than the open colectomy group (LC 1.6%, OC 2.9%, $P$ < 0.001).

## DISCUSSION

This study demonstrates that laparoscopic partial colectomy has better outcomes than open partial colectomy in the elderly patient. There are fewer complications, shorter lengths of stay, and lower mortality. A recent meta-analysis of colorectal surgery by Antoniou *et al*[20] involving 66592 patients supports our conclusion of lower mortality (2.2% in laparoscopic *vs* 5.4% in open approach) and overall morbidity (19.3% *vs* 26.7% in open approach). Similar results are reported in the meta-analysis by Seishima *et al*[21] showing LC to have lower risk of perioperative mortality (OR = 0.55, $P$ < 0.01) and postoperative complications (OR = 0.55, $P$ < 0.01) when compared with open surgery. In a randomized control study involving 535 patients by Frasson *et al*[22], the laparoscopic approach was associated with an overall complication rate of 20% in comparison to 42% in the open group. Senagore *et al*[23] also report similar results in the laparoscopic group with shorter length of stay and lower direct hospital costs. These positive trends are also seen in octogenarians[16]. In a pooled analysis involving 11 studies, the laparoscopic approach was associated with lower incidence of postoperative cardiac complications, wound complications, earlier return of bowel function and shorter lengths of stay[17]. All of these small sample size studies and meta-analyses concluded that LC is safer and has better short term outcomes. Our analysis based on a large well validated ACS NSQIP database replicates these findings in a very large database of patients and confirms the benefits of the minimally invasive approach. More importantly, our study employed propensity matching to make sure the groups of patients were similar pre-operatively, and still found the same results.

The most common complication after colorectal surgery is surgical site infection (SSI)[24]. SSI in colorectal surgery is associated with significant economic burden and prolonged recovery, and it affects the quality of life significantly[25]. Our analysis showed an infection rate of 10.6% in the LC group in comparison with 17.7% in the OC group. A similar ACS NSQIP based analysis of colorectal procedures involving all age groups show an infection rate of 9.5% with the laparoscopic approach in comparison to 16% with the open approach[26]. The reasons for lower SSI with the laparoscopic approach might include reduced blood transfusions and reduced wound contact with the colon[27]. Elderly age is known to be a risk factor for respiratory and cardiac complications. This is probably due to higher incidence

**Table 4 Post-operative outcomes of elderly patients undergoing partial colectomy after propensity score matching $n$ (%)**

| Characteristics | Laparoscopic colectomy ($n$ = 11008) | Open colectomy ($n$ = 11008) | $P$ value | OR/CI |
|---|---|---|---|---|
| Overall complications[1] | 1676 (15.2) | 2622 (23.8) | < 0.001 | 1.56 (1.48-1.65) |
| Cardiac complications | 109 (1.0) | 160 (1.5) | 0.002 | 1.47 (1.15-1.87) |
| Cardiac arrest | 50 (0.4) | 80 (0.7) | 0.008 | 1.60 (1.13-2.28) |
| Myocardial infarction | 71 (0.6) | 88 (0.8) | 0.176 | 1.24 (0.91-1.69) |
| Pulmonary complications | 489 (4.4) | 729 (6.6) | < 0.001 | 1.49 (1.33-1.67) |
| Pneumonia | 239 (2.2) | 375 (3.4) | < 0.001 | 1.57 (1.34-1.84) |
| Unplanned intubation | 217 (2.0) | 326 (3.0) | < 0.001 | 1.50 (1.27-1.78) |
| Ventilated for more than 48 h | 165 (1.5) | 294 (2.7) | < 0.001 | 1.78 (1.48-2.15) |
| Pulmonary emboli | 69 (0.6 ) | 87 (0.8) | 0.148 | 1.26 (0.92-1.73) |
| Deep venous thrombosis | 97 (0.9) | 179 (1.6) | < 0.001 | 1.85 (1.44-2.36) |
| Renal complications | 421 (3.8) | 656 (6.0) | < 0.001 | 1.56 (1.38-1.76) |
| Progressive renal insufficiency | 54 (0.4) | 98 (0.9) | < 0.001 | 1.81 (1.30-2.53) |
| Acute renal failure | 50 (0.4) | 72 (0.7) | 0.046 | 1.44 (1.01-2.06) |
| Urinary tract infection | 339 (3.1) | 524 (4.8) | < 0.001 | 1.55 (1.35-1.77) |
| Infections | 1170 (10.6) | 1951 (17.7) | < 0.001 | 1.67 (1.56-1.78) |
| Sepsis | 274 (2.5) | 468 (4.3) | < 0.001 | 1.71 (1.46-1.98) |
| Septic shock | 156 (1.4) | 284 (2.6) | < 0.001 | 1.82 (1.50-2.21) |
| Superficial incisional infection | 582 (5.3) | 968 (8.8) | < 0.001 | 1.66 (1.51-1.84) |
| Deep incisional infection | 96 (0.9) | 150 (1.4) | 0.001 | 1.56 (1.21-2.02) |
| Organ space infection | 206 (1.9) | 347 (3.2) | < 0.001 | 1.68 (1.42-1.99) |
| Wound disruption | 84 (0.8) | 144 (1.3) | < 0.001 | 1.71 (1.31-2.24) |
| Return to operating room | 451 (4.1) | 588 (5.3) | < 0.001 | 1.30 (1.16-1.47) |
| Length of stay (d), mean ± SD | 6.61 ± 6.73 | 9.62 ± 8.33 | < 0.001 | - |
| 30-d mortality | 173 (1.6) | 316 (2.9) | < 0.001 | 1.83 (1.52-2.19) |

[1]Any one of cardiac, pulmonary, renal or infectious complications.

of comorbidities in this cohort and preoperative comorbidities have shown to be an important predictor of postoperative adverse outcomes[28,29]. After matching for pre-operative cardiac, respiratory, neurological and renal comorbidities, our study showed a lower incidence of pneumonia and need for mechanical ventilation, as well as lower incidence of cardiac and renal complications with the laparoscopic approach. These lower rates of complications seen with the laparoscopic approach are probably related to there being less pain related splinting of diaphragm causing less postoperative atelectasis and pneumonia[30,31]. While our study does not explicitly look at cost, length of stay impacts the costs, and LC was associated with a significantly shorter recovery period[32].

The main strength of this study is its large sample size from a robust database. The ACS NSQIP database is one of the largest, well validated, risk adjusted current database designed to track surgical outcomes based on relevant set of perioperative variables. ACS NSQIP database has been demonstrated to improve outcomes and decrease expenses[33-35]. The hallmark of this data is that it is collected prospectively and strictly audited. This prevents the pitfalls of accruing data from small institutional databases. Another strength of our study is the use of propensity matching which further minimizes the bias associated with patient selection.

We recognize that there are limitations to our study. Although the ACS NSQIP database is one of the largest available, it does not represent every hospital as it includes only participating hospitals. In addition, we are only able to analyze the data that has been recorded. For example we can only group the patients into colectomies based on which part of the colon was removed, but any differences in surgical technique have not been recorded. And in terms of complications we have access to the number of complications in each category, but the severity of each complication (*e.g.,* Clavien-Dindo classification) is not available. Nonetheless, the extremely large size of the database allows for many of these deficiencies to not impact on the results. It is unlikely that the complications in one group were all severe while the complications in the other group were all minor.

Another limitation is the non-randomized nature associated with any large database. These are essentially cohort studies, and there is always a chance for selection bias. However, by incorporating propensity score matching into this study, we have significantly minimized the risk of this. And finally, other factors such as the surgeon's experience and procedure volume are known to impact the outcomes but were not explored in this study[36].

Of course, each patient and each situation needs to be evaluated individually. In some cases there might be specific reasons why an open approach might be preferable, such as extensive prior surgery, a large mass or phlegmon, or surgeon comfort level. However, in the absence of specific contraindications our study showed better results with a laparoscopic approach.

In conclusion, this ACS NSQIP based study shows that even in elderly patients laparoscopic partial colectomy has better outcomes than those seen with open partial colectomy. There were decreases in every category of complications, they had shorter lengths of stay and lower mortality. This improvement in outcomes was seen even after matching the two groups by propensity scoring, ensuring that the two groups had similar rates of pre-operative comorbidities. Although a randomized control trial could be done to further the strength of the evidence, we feel the current data indicates that elderly patients requiring a partial colectomy should be offered a laparoscopic approach unless otherwise contraindicated.

## COMMENTS

### Background

Traditionally colon surgeries were done with an open approach. The introduction of laparoscopic techniques and improvement in the technology and instruments enabled the surgeons to adopt the laparoscopic approach for colectomies. Patients aged 65 years and above have increased comorbidities and are at risk for more complications, especially with longer operations. The question is whether the benefits of laparoscopic colectomy extend to the elderly population as well.

### Research frontiers

The major areas to evaluate are mortality, morbidity and lengths of stay. Mortality and morbidity are generally considered within the first 30 d, and morbidity can be further subdivided into different types of complications, such as cardiac, pulmonary, renal and infectious complications.

### Innovations and breakthroughs

The current study used a very large national database that has been well validated, the American College of Surgeons National Surgical Quality Improvement Program (ACS NSQIP) database. In addition, the current study employed propensity matching in order to minimize any pre-operative differences between the open and laparoscopic groups.

### Applications

This study shows that the laparoscopic approach was associated with lower mortality, lower complications of every type and shorter lengths of stay. This is important when surgeons are faced with elderly patients requiring a partial colectomy. This study points to the laparoscopic approach being the preferred method in the absence of any contraindications. Future research areas would include a large scale randomized control trial in this patient population.

### Terminology

Partial colectomy refers to removal of a part of the colon. In this particular study this included an anastomosis between the remaining parts, as colostomy cases were excluded. Elderly in this study referred to patients aged 65 and above. Propensity matching refers to the statistical methods employed in order to ensure that two different groups are matched to become similar with respect to the chosen variables.

### Peer-review

This is a large database study of colorectal surgery in the elderly, which is overall well written and nicely structured. The conception of the study is simple but the results are robust and well backed by the rather straightforward method.

## REFERENCES


1 **Clinical Outcomes of Surgical Therapy Study Group**. A comparison of laparoscopically assisted and open colectomy for colon cancer. *N Engl J Med* 2004; **350**: 2050-2059 [PMID: 15141043 DOI: 10.1056/NEJMoa032651]

2 **Vaid S**, Tucker J, Bell T, Grim R, Ahuja V. Cost analysis of laparoscopic versus open colectomy in patients with colon cancer: results from a large nationwide population database. *Am Surg* 2012; **78**: 635-641 [PMID: 22643256]

3 **Bartels SA**, Vlug MS, Hollmann MW, Dijkgraaf MG, Ubbink DT, Cense HA, van Wagensveld BA, Engel AF, Gerhards MF, Bemelman WA. Small bowel obstruction, incisional hernia and survival after laparoscopic and open colonic resection (LAFA study). *Br J Surg* 2014; **101**: 1153-1159 [PMID: 24977342 DOI: 10.1002/bjs.9585]

4 **Wilson MZ**, Dillon PW, Hollenbeak CS, Stewart DB. How do risk factors for mortality and overall complication rates following laparoscopic and open colectomy differ between inpatient and post-discharge phases of care? A retrospective cohort study from NSQIP. *Surg Endosc* 2014; **28**: 3392-3400 [PMID: 24928234 DOI: 10.1007/s00464-014-3609-4]

5 **Robinson CN**, Chen GJ, Balentine CJ, Sansgiry S, Marshall CL, Anaya DA, Artinyan A, Albo D, Berger DH. Minimally invasive surgery is underutilized for colon cancer. *Ann Surg Oncol* 2011; **18**: 1412-1418 [PMID: 21213059 DOI: 10.1245/s10434-010-1479-0]

6 **Simorov A**, Shaligram A, Shostrom V, Boilesen E, Thompson J, Oleynikov D. Laparoscopic colon resection trends in utilization and rate of conversion to open procedure: a national database review of academic medical centers. *Ann Surg* 2012; **256**: 462-468 [PMID: 22868361 DOI: 10.1097/SLA.0b013e3182657ec5]

7 **Finlayson E**, Fan Z, Birkmeyer JD. Outcomes in octogenarians undergoing high-risk cancer operation: a national study. *J Am Coll Surg* 2007; **205**: 729-734 [PMID: 18035254 DOI: 10.1016/j.jamcollsurg.2007.06.307]

8 **Dubecz A**, Langer M, Stadlhuber RJ, Schweigert M, Solymosi N, Feith M, Stein HJ. Cholecystectomy in the very elderly--is 90 the new 70? *J Gastrointest Surg* 2012; **16**: 282-285 [PMID: 22143419 DOI: 10.1007/s11605-011-1708-2]

9 **Nielsen LB**, Harboe KM, Bardram L. Cholecystectomy for the elderly: no hesitation for otherwise healthy patients. *Surg Endosc* 2014; **28**: 171-177 [PMID: 23996332 DOI: 10.1007/s00464-013-3144-8]

10 **Daley BJ**, Cecil W, Clarke PC, Cofer JB, Guillamondegui OD. How slow is too slow? Correlation of operative time to complications: an analysis from the Tennessee Surgical Quality Collaborative. *J Am Coll Surg* 2015; **220**: 550-558 [PMID: 25728140 DOI: 10.1016/j.jamcollsurg.2014.12.040]

11 **Evans C**, Lim J, Gatzen C, Huang A. Factors influencing laparoscopic colorectal operative duration and its effect on clinical outcome. *Surg Laparosc Endosc Percutan Tech* 2012; **22**: 437-442 [PMID: 23047389 DOI: 10.1097/SLE.0b013e31826020a3]

12 **Turrentine FE**, Wang H, Simpson VB, Jones RS. Surgical risk factors, morbidity, and mortality in elderly patients. *J Am Coll Surg* 2006; **203**: 865-877 [PMID: 17116555 DOI: 10.1016/j.jamcollsurg.2006.08.026]

13 **Alves A**, Panis Y, Mathieu P, Mantion G, Kwiatkowski F, Slim K. Postoperative mortality and morbidity in French patients undergoing colorectal surgery: results of a prospective multicenter study. *Arch Surg* 2005; **140**: 278-283, discussion 284 [PMID: 15781793 DOI: 10.1001/archsurg.140.3.278]

14 **Senagore AJ**, Stulberg JJ, Byrnes J, Delaney CP. A national comparison of laparoscopic vs. open colectomy using the National Surgical Quality Improvement Project data. *Dis Colon Rectum* 2009; **52**: 183-186 [PMID: 19279409 DOI: 10.1007/DCR.0b013e31819ad4a4]

15 **Webb S**, Rubinfeld I, Velanovich V, Horst HM, Reickert C. Using National Surgical Quality Improvement Program (NSQIP) data for risk adjustment to compare Clavien 4 and 5 complications in open and laparoscopic colectomy. *Surg Endosc* 2012; **26**: 732-737 [PMID: 22038161 DOI: 10.1007/s00464-011-1944-2]

16 **Lian L**, Kalady M, Geisler D, Kiran RP. Laparoscopic colectomy is safe and leads to a significantly shorter hospital stay for octogenarians. *Surg Endosc* 2010; **24**: 2039-2043 [PMID: 20174947 DOI: 10.1007/s00464-010-0900-x]

17 **Grailey K**, Markar SR, Karthikesalingam A, Aboud R, Ziprin P, Faiz O. Laparoscopic versus open colorectal resection in the elderly population. *Surg Endosc* 2013; **27**: 19-30 [PMID: 22752280 DOI: 10.1007/s00464-012-2414-1]

18 **Chautard J**, Alves A, Zalinski S, Bretagnol F, Valleur P, Panis Y. Laparoscopic colorectal surgery in elderly patients: a matched case-control study in 178 patients. *J Am Coll Surg* 2008; **206**: 255-260 [PMID: 18222377 DOI: 10.1016/j.jamcollsurg.2007.06.316]

19 American College of Surgeons National Surgical Quality Improvement Program (ACS NSQIP). available from: URL: https://www.facs.org/quality-programs/acs-nsqip

20 **Antoniou SA**, Antoniou GA, Koch OO, Pointner R, Granderath FA. Laparoscopic colorectal surgery confers lower mortality in the elderly: a systematic review and meta-analysis of 66,483 patients. *Surg Endosc* 2015; **29**: 322-333 [PMID: 24986017 DOI: 10.1007/s00464-014-3672-x]

21 **Seishima R**, Okabayashi K, Hasegawa H, Tsuruta M, Shigeta K, Matsui S, Yamada T, Kitagawa Y. Is laparoscopic colorectal surgery beneficial for elderly patients? A systematic review and meta-analysis. *J Gastrointest Surg* 2015; **19**: 756-765 [PMID: 25617077 DOI: 10.1007/s11605-015-2748-9]

22 **Frasson M**, Braga M, Vignali A, Zuliani W, Di Carlo V. Benefits of laparoscopic colorectal resection are more pronounced in elderly patients. *Dis Colon Rectum* 2008; **51**: 296-300 [PMID: 18197453 DOI: 10.1007/s10350-007-9124-0]

23 **Senagore AJ**, Madbouly KM, Fazio VW, Duepree HJ, Brady KM, Delaney CP. Advantages of laparoscopic colectomy in older patients. *Arch Surg* 2003; **138**: 252-256 [PMID: 12611568]

24 **Wick EC**, Vogel JD, Church JM, Remzi F, Fazio VW. Surgical site infections in a "high outlier" institution: are colorectal surgeons to blame? *Dis Colon Rectum* 2009; **52**: 374-379 [PMID: 19333034 DOI: 10.1007/DCR.0b013e31819a5e45]

25 **Mahmoud NN**, Turpin RS, Yang G, Saunders WB. Impact of surgical site infections on length of stay and costs in selected colorectal procedures. *Surg Infect* (Larchmt) 2009; **10**: 539-544 [PMID: 19708769 DOI: 10.1089/sur.2009.006]

26 **Kiran RP**, El-Gazzaz GH, Vogel JD, Remzi FH. Laparoscopic approach significantly reduces surgical site infections after colorectal surgery: data from national surgical quality improvement program. *J Am Coll Surg* 2010; **211**: 232-238 [PMID: 20670861 DOI: 10.1016/j.jamcollsurg.2010.03.028]

27 **Poon JT**, Law WL, Wong IW, Ching PT, Wong LM, Fan JK, Lo OS. Impact of laparoscopic colorectal resection on surgical site infection. *Ann Surg* 2009; **249**: 77-81 [PMID: 19106679 DOI: 10.1097/SLA.0b013e31819279e3]

28 **Liu LL**, Leung JM. Predicting adverse postoperative outcomes in patients aged 80 years or older. *J Am Geriatr Soc* 2000; **48**: 405-412 [PMID: 10798467]

29 **Masoomi H**, Kang CY, Chen A, Mills S, Dolich MO, Carmichael JC, Stamos MJ. Predictive factors of in-hospital mortality in colon and rectal surgery. *J Am Coll Surg* 2012; **215**: 255-261 [PMID: 22640532 DOI: 10.1016/j.jamcollsurg.2012.04.019]

30 **Frazee RC**, Roberts JW, Okeson GC, Symmonds RE, Snyder SK, Hendricks JC, Smith RW. Open versus laparoscopic cholecystectomy. A comparison of postoperative pulmonary function. *Ann Surg* 1991; **213**: 651-653; discussion 653-654 [PMID: 1828139]

31 **Schwenk W**, Böhm B, Witt C, Junghans T, Gründel K, Müller JM. Pulmonary function following laparoscopic or conventional colorectal resection: a randomized controlled evaluation. *Arch Surg* 1999; **134**: 6-12; discussion 13 [PMID: 9927122]

32 **Noblett SE**, Horgan AF. A prospective case-matched comparison of clinical and financial outcomes of open versus laparoscopic colorectal resection. *Surg Endosc* 2007; **21**: 404-408 [PMID: 17180293 DOI: 10.1007/s00464-006-9016-8]

33 **McNelis J**, Castaldi M. "The National Surgery Quality Improvement Project" (NSQIP): a new tool to increase patient safety and cost efficiency in a surgical intensive care unit. *Patient Saf Surg* 2014; **8**: 19 [PMID: 24817910 DOI: 10.1186/1754-9493-8-19]
34 **Ingraham AM**, Richards KE, Hall BL, Ko CY. Quality improvement in surgery: the American College of Surgeons National Surgical Quality Improvement Program approach. *Adv Surg* 2010; **44**: 251-267 [PMID: 20919525]
35 **Hall BL**, Hamilton BH, Richards K, Bilimoria KY, Cohen ME, Ko CY. Does surgical quality improve in the American College of Surgeons National Surgical Quality Improvement Program: an evaluation of all participating hospitals. *Ann Surg* 2009; **250**: 363-376 [PMID: 19644350 DOI: 10.1097/SLA.0b013e3181b4148f]
36 **Liu CJ**, Chou YJ, Teng CJ, Lin CC, Lee YT, Hu YW, Yeh CM, Chen TJ, Huang N. Association of surgeon volume and hospital volume with the outcome of patients receiving definitive surgery for colorectal cancer: A nationwide population-based study. *Cancer* 2015; **121**: 2782-2790 [PMID: 25892632 DOI: 10.1002/cncr.29356]

**P- Reviewer:** Diener MK, Galvao FHF, Li XX, Symeonidis NG
**S- Editor**: Yu J **L- Editor**: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12851

*World J Gastroenterol* 2015 December 7; 21(45): 12851-12856
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

Retrospective Study

# Clinicopathological characteristics of clinical early gastric cancer in the upper-third stomach

Daisuke Ichikawa, Shuhei Komatsu, Toshiyuki Kosuga, Hirotaka Konishi, Kazuma Okamoto, Atsushi Shiozaki, Hitoshi Fujiwara, Eigo Otsuji

Daisuke Ichikawa, Shuhei Komatsu, Toshiyuki Kosuga, Hirotaka Konishi, Kazuma Okamoto, Atsushi Shiozaki, Hitoshi Fujiwara, Eigo Otsuji, Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, Kyoto 6028566, Japan

Author contributions: Ichikawa D designed this research; Ichikawa D, Komatsu S, Kosuga T, Konishi H, Okamoto K, Shiozaki A, Fujiwara H and Otsuji E performed this research; Ichikawa D wrote the manuscript.

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

Conflict-of-interest statement: The authors have no conflicts of interest to disclose.

Data sharing statement: No additional data are available.



Correspondence to: Daisuke Ichikawa, MD, PhD, Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 6028566, Japan. ichikawa@koto.kpu-m.ac.jp
Telephone: +81-75-2515527
Fax: +81-75-2515522

Received: March 18, 2015
Peer-review started: March 19, 2015
First decision: April 23, 2015
Revised: May 16, 2015
Accepted: August 31, 2015
Article in press: August 31, 2015
Published online: December 7, 2015

## Abstract

**AIM:** To elucidate the clinicopathological characteristics of clinically early gastric cancer in the upper-third stomach and to clarify treatment precautions.

**METHODS:** A total of 683 patients with clinical early gastric cancer were enrolled in this retrospective study, 128 of whom had gastric cancer in the upper-third stomach (U group). All patients underwent a double contrast barium examination, endoscopy, and computed tomography (CT), and were diagnosed preoperatively based on the findings obtained. The clinicopathological features of these patients were compared with those of patients with gastric cancer in the middle- and lower-third stomach (ML group). We also compared clinicopathological factors between accurate-diagnosis and under-diagnosis groups in order to identify factors affecting the accuracy of a preoperative diagnosis of tumor depth.

**RESULTS:** Patients in the U group were older ($P$ = 0.029), had a higher ratio of males to females ($P$ = 0.015), and had more histologically differentiated tumors ($P$ = 0.007) than patients in the ML group. A clinical under-diagnosis occurred in 57 out of 683 patients (8.3%), and was more frequent in the U group than in the ML group (16.4% *vs* 6.3%, $P$ < 0.0001). Therefore, the rates of lymph node metastasis and lymphatic invasion were slightly higher in the U group than in the ML group ($P$ = 0.071 and 0.082, respectively). An under-diagnosis was more frequent in histologically undifferentiated tumors ($P$ = 0.094) and in those larger than 4 cm ($P$ = 0.024). The median

follow-up period after surgery was 56 mo (range, 1-186 mo). Overall, survival and disease-specific survival rates were significantly lower in the U group than in the ML group ($P$ = 0.016 and 0.020, respectively). However, limited operation-related cancer recurrence was not detected in the U group in the present study.

**CONCLUSION:** Clinical early gastric cancer in the upper-third stomach has distinguishable characteristics that increase the risk of a clinical under-diagnosis, especially in patients with larger or undifferentiated tumors.

**Key words:** Upper-third stomach; Diagnosis; Gastric cancer



**Core tip:** The clinicopathological features of patients with gastric cancer in the upper-third stomach (U group) were compared with those of patients with gastric cancer in the middle- and lower-third stomach (ML group). The rate of clinical under-diagnoses was significantly higher in the U group than in the ML group and more frequent in histologically undifferentiated tumors and in those larger than 4 cm.

Ichikawa D, Komatsu S, Kosuga T, Konishi H, Okamoto K, Shiozaki A, Fujiwara H, Otsuji E. Clinicopathological characteristics of clinical early gastric cancer in the upper-third stomach. *World J Gastroenterol* 2015; 21(45): 12851-12856 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12851.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12851

## INTRODUCTION

Although the incidence of gastric cancer (GC) has recently plateaued, the frequency of GC in the upper-third stomach has increased[1-4]. In Asian countries, the detection of early GC in the upper-third stomach has also been increasing[2,3]. Less invasive treatment options, such as endoscopic submucosal dissection (ESD) and laparoscopic proximal gastrectomy, have recently been performed on patients with early GC in the upper-third stomach in an attempt to preserve postoperative functions and improve the quality of life of these patients[5-9].

These recent findings prompted us to investigate the clinicopathological characteristics of early GC in the upper-third of the stomach. Treatment strategies are generally selected based on the preoperative findings of several examinations; therefore, we herein focused on patients with clinical early GC (T1) diagnosed preoperatively. In the present study, we retrospectively examined the clinicopathological characteristics of clinical early GC in the upper-third stomach and compared them with those in other regions. We also determined treatment precautions for patients with clinical early GC in the upper-third stomach.

## MATERIALS AND METHODS

### Patients

A total of 1856 patients with GC were admitted to Kyoto Prefectural University of Medicine between 1997 and 2013. Of these, 814 patients were diagnosed preoperatively with early GC (clinical T1) and underwent gastrectomy at our University Hospital. Patients with GC in the remnant stomach and with multiple GC detected previously were excluded from this study. A total of 683 patients with clinical T1 GC were enrolled in this retrospective study, 128 of whom had GC in the upper-third stomach. Of these, 59 patients underwent proximal gastrectomy. Lymph node dissection was performed based on the Guidelines of the Japanese Gastric Cancer Association[10].

### Evaluations

All patients underwent a double contrast barium examination, endoscopy, and computed tomography (CT) and were diagnosed preoperatively based on the findings obtained. Tumor depth was judged according to previously described criteria[11,12]. Briefly, the endoscopic criteria for mucosal cancer were a smooth surface protrusion, shallow and even depression, erosion with slight marginal elevation, or a flat or superficial spreading lesion. The criteria for submucosal cancer were an irregular or nodular surface with or without abnormal converging folds, such as clubbing and abrupt cutting, an irregular-based ulcer with marginal mucosal elevation, or marked depression with interrupted enlarged folds. The criteria for T2 or higher tumors were irregular based ulceration surrounded by a tumorous bank or marked depression when the tips of converging folds were elevated and merged. In CT examinations, non-visualized lesions and tumors confined to the inner or middle layers of the gastric wall were diagnosed as clinical T1 tumors, and full-thickness wall thickening with/without an irregular surface on the outer layer surrounding the tumors were diagnosed as clinical T2 or higher tumors[13-15]. Endoscopic ultrasonography was also performed in some patients, and the depth of tumor invasion was assessed based on the generally accepted 5-layer sonographic structure of the gastric wall, as recommended by the Union Internationale Contre le Cancer (UICC)/American Joint Cancer Committee (AJCC). The clinicopathological features of these patients were reviewed retrospectively from hospital records and compared with those of patients with GC in the middle- and lower-third stomach. *Helicobacter pylori* infection was not necessarily examined in all cases in this study, therefore, we

**Table 1 Clinicopathological characteristics of cT1 gastric cancer in the upper-third stomach**

| | | Upper | Middle or Lower | *P* value |
|---|---|---|---|---|
| Age (yr) | | 66.1 | 62.8 | 0.029 |
| Sex | Male | 94 | 344 | |
| | Female | 34 | 211 | 0.015 |
| Macroscopic[1] | Localized | 37 | 148 | |
| | Diffuse | 91 | 405 | 0.620 |
| | Unknown | 0 | 2 | |
| Histology | Diff.[2] | 87 | 301 | |
| | Undiff.[3] | 41 | 247 | 0.0072 |
| | Unknown | 0 | 7 | |
| Size (mm) | | 30.9 | 28.9 | 0.340 |
| pT[4] | T1 | 107 | 519 | |
| | T2 | 21 | 35 | < 0.0001 |
| | Unknown | 0 | 1 | |
| pN[5] | Negative | 115 | 523 | |
| | Positive | 13 | 32 | 0.071 |
| ly[6] | Negative | 98 | 457 | |
| | Positive | 27 | 82 | 0.082 |
| | Unknown | 3 | 16 | |
| v[7] | Negative | 110 | 497 | |
| | Positive | 15 | 42 | 0.130 |
| | Unknown | 3 | 16 | |

[1]Macroscopic: Macroscopic findings; [2]Diff.: Differentiated adenocarcinoma; [3]Undiff.: Undifferentiated adenocarcinoma; [4]pT: Pathological T-category; [5]pN: Pathological lymph node metastasis; [6]ly: Lymphatic invasion; [7]v: Venous invasion.

could not compare infection rates between the two groups. We also compared clinicopathological factors between accurate-diagnosis and under-diagnosis groups in order to identify the factors affecting the accuracy of a preoperative diagnosis of tumor depth. The macroscopic and microscopic classifications of GC were based on the Japanese Classification of Gastric Carcinoma[10].

### *Statistical analysis*

Continuous data were compared using the *t*-test or Mann-Whitney *U* test. The $\chi^2$ test was used to evaluate differences in the proportion of clinicopathological variables. Overall survival (OS) and disease-specific survival (DSS) rates were calculated by the Kaplan-Meier method, with the date of gastrectomy as the starting point. Only deaths from postoperative complications and GC recurrence were considered in the analysis of DSS. Differences in survival were examined by the log-rank test. All statistical analyses were performed using Stat View 5.0 software (SAS Institute, Cary, NC, United States). The significance of differences was accepted at $P < 0.05$.

**Table 2 Comparison of clinicopathological factors between accurate- and under-diagnosis groups**

| | | Accurate diagnosis | Under diagnosis | *P* value |
|---|---|---|---|---|
| Age (yr) | < 65 | 44 | 9 | |
| | ⩾ 65 | 63 | 12 | 0.088 |
| Sex | Male | 77 | 17 | |
| | Female | 30 | 4 | 0.390 |
| Macroscopic[1] | Localized | 33 | 4 | |
| | Diffuse | 74 | 17 | 0.280 |
| Histology | Diff.[2] | 76 | 11 | |
| | Undiff.[3] | 31 | 10 | 0.094 |
| Size (mm) | < 40 | 81 | 11 | |
| | ⩾ 40 | 26 | 10 | 0.024 |
| | Unknown | 3 | 1 | |

[1]Macroscopic: Macroscopic findings; [2]Diff.: Differentiated adenocarcinoma; [3]Undiff.: Undifferentiated adenocarcinoma.

## RESULTS

### *Clinicopathological features of clinical T1 GC in the upper-third stomach*

The mean patient age was 63.4 years (range, 28-89 years), and the male: female ratio was 1.79:1. The median tumor size was 29.3 mm (range, 5-145 mm). The clinicopathological characteristics of patients and tumors in the upper-third stomach (U group) and middle- and lower-third of the stomach (ML group) are shown in Table 1. Patients in the U group were older, had a higher ratio of males to females, and had more histologically differentiated tumors than patients in the ML group. The number of pathological T2 or deeper tumors that had been clinically under-diagnosed was significantly higher in the U group than in the ML group. Therefore, the rates of lymph node metastasis and lymphatic invasion were slightly higher in the U group than in the ML group.

### *Factors affecting the accuracy of a preoperative diagnosis of tumor depth*

A clinical under-diagnosis occurred in 57 out of 683 patients (8.3%) and was more frequent in the U group than in the ML group (16.4% *vs* 6.3%). The clinicopathological features of patients in the U group with an accurate-diagnosis and under-diagnosis are listed in Table 2. Although an under-diagnosis was more frequent in large and histologically undifferentiated tumors, the histological difference was not significant.

### *Long-term prognosis of clinical T1 GC in the upper-third stomach*

The median follow-up period after surgery was 56 mo (range, 1-186 mo). Thirty-four deaths, including 10

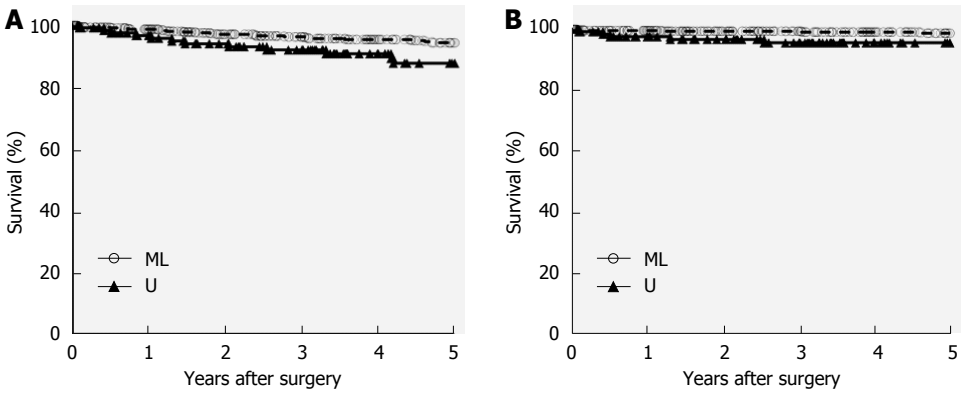



**Figure 1 Comparison of survival curves in patients with clinically early gastric cancer in the upper-third stomach (U group) and in the more distal stomach (ML group).** A: Overall survival (OS); B: Disease-specific survival (DSS). OS and DSS rates were significantly lower in the U group than in the ML group ($P$ = 0.016 and 0.020, respectively).

disease-related deaths, occurred during the follow-up period. Recurrence was noted in six patients (two and four patients in the U and ML groups, respectively), while four patients (three and one patients in U and ML groups, respectively) died of postoperative complications. Recurrence patterns were peritoneal dissemination in two patients, para-aortic lymph node metastasis in two, and hematogenous metastasis in two. OS and DSS rates were significantly lower in the U group than in the ML group (Figure 1). However, limited operation-related cancer recurrence was not detected in the U group in the present study.

## DISCUSSION

The present study clearly showed that clinical T1 GC in the upper-third stomach has features that distinguish it from GC in other regions of the stomach, including older patients, a higher ratio of males to females, and more histologically differentiated tumors. Regarding the histological type, Kunisaki *et al*[16] also reported that patients with tumors in the upper-third stomach more frequently had differentiated tumors. However, the frequency of tumor differentiation may vary markedly between different countries, as previously reported[17].

The results of the present study revealed that clinical T1 GC in the U group was associated with a higher incidence of under-diagnosis of advanced GC (T2 or higher) in pathological examinations compared to the ML group. Since the extent of gastric resection and lymph node dissection is slightly narrower in such limited treatment options, accurate preoperative diagnoses are crucial for determining individualized treatment strategies. Early GC, which is confined to the mucosa and/or submucosa, has been diagnosed preoperatively based on the findings of upper barium contrast examinations and gastroscopy[11,12]. Endoscopic ultrasonography and multi-detector computed tomography have recently been utilized for more accurate diagnoses; however, preoperative under-diagnoses represent a frequent problem in the clinical staging of early GC[18-20]. The major drawback of this study was that endoscopic ultrasonography was not performed on all of the study patients. However, several recent studies indicated that endoscopic ultrasonography did not impact pretreatment staging of tumor depth, especially in patients with early GC[21-24]. The diagnostic accuracy of the depth of tumor invasion is considered to be affected by several factors[25,26]. Kim *et al*[26] reported that histologically undifferentiated-type tumors were associated with lower diagnostic accuracy of endoscopic assessments in preoperatively predicted tumor invasion, and the probability of a clinical under-diagnosis was significantly high. However, the number of histologically differentiated-type tumors was significantly higher in the U group than in the ML group in this study; therefore, the histological type was not involved in the under-diagnosis of clinical T1 in the U group. Other possible explanations for the predisposition toward an under-diagnosis are anatomy-related factors. Muscle bundles of the lamina muscularis mucosae are separated by wide spaces, are relatively sparse, and have a reticular arrangement in the cardia. In the more distal stomach, the spaces between the muscle bundles are narrower with a more dense reticular arrangement and a linear arrangement[27]. Therefore, superficial cancer may be vulnerable to infiltration to the muscle layer of the gastric wall. Another explanation is that fixation of the gastric wall to the diaphragm and retroperitoneum *via* a bare area of the stomach may reduce changes in the luminal face, which may play a role in the discrepancy observed between clinical and pathological diagnoses of tumor infiltration. Further investigations are needed in order to elucidate the exact reasons why tumors in the upper-third stomach are predisposed to clinical under-diagnosis.

Functional preservation operations, such as proximal gastrectomy and/or limited lymph node dissection, are now more likely to be performed on patients with clinically early GC in the upper-third stomach[6-8]. Previous studies demonstrated that

proximal gastrectomy with regional lymphadenectomy was satisfactory for early GC in the upper-third stomach[6,8,28,29]; however, populations were collected based on pathological examinations in most of these studies. In these conservative operations, clinical under-diagnoses carry the potential risk of incomplete treatments. This study clearly demonstrated that a clinical under-diagnosis correlated with the presence of large and undifferentiated tumors; therefore, the potential risk of clinical underestimations needs to be considered in patients with these tumors.

The present study also investigated the long-term outcomes of clinical T1 GC in the upper-third stomach and compared them with those of patients who had GC in the more distal stomach. Patients with clinical T1 GC in the ML group had significantly better OS and DSS rates than those in the U group; however, the older mean age and higher rates of fatal complications in the U group appeared to be associated with decreased survival rates.

In conclusion, clinically early GC in the upper-third stomach has distinguishable characteristics from the more distal stomach, and the risk of a clinical under-diagnosis is greater in GC in the upper-third stomach, especially in patients with undifferentiated tumors or those larger than 4 cm. Particular attention is needed for the indication of limited operations in patients with those tumors.

## COMMENTS

### Background

Although the incidence of gastric cancer has recently plateaued, the frequency of gastric cancer in the upper-third stomach has increased. In Asian countries, the detection of early gastric cancer in the upper-third stomach has also been increasing.

### Research frontiers

The authors herein investigated the clinicopathological characteristics of early gastric cancer in the upper-third of the stomach and also determined treatment precautions for patients with clinical early gastric cancer in the upper-third stomach.

### Innovations and breakthroughs

Treatment strategies are generally selected based on the preoperative findings of several examinations; therefore, the authors focused on patients with clinical early gastric cancer diagnosed preoperatively in this retrospective study.

### Applications

Clinically early gastric cancer in the upper-third stomach has distinguishable characteristics from the more distal stomach, and the risk of a clinical under-diagnosis is greater in upper-third stomach cancer, especially in patients with undifferentiated tumors or those larger than 4 cm.

### Terminology

Clinically early gastric cancer was diagnosed preoperatively based on the findings of a double contrast barium examination, endoscopy, and computed tomography.

### Peer-review

The authors clearly demonstrated that clinically early gastric cancer in the upper-third stomach has distinguishable characteristics from the distal stomach that increase the risk for clinical under-diagnosis. Therefore, particular attention is needed for the indication of limited operations in patients with these tumors.

## REFERENCES


1 **Salvon-Harman JC**, Cady B, Nikulasson S, Khettry U, Stone MD, Lavin P. Shifting proportions of gastric adenocarcinomas. *Arch Surg* 1994; **129**: 381-388; discussion 388-389 [PMID: 7512326 DOI: 10.1001/archsurg.1994.01420280053007]

2 **Okabayashi T**, Gotoda T, Kondo H, Inui T, Ono H, Saito D, Yoshida S, Sasako M, Shimoda T. Early carcinoma of the gastric cardia in Japan: is it different from that in the West? *Cancer* 2000; **89**: 2555-2559 [PMID: 11135215 DOI: 10.1002/1097-0142(20001215)89:12]

3 **Ahn HS**, Lee HJ, Yoo MW, Jeong SH, Park DJ, Kim HH, Kim WH, Lee KU, Yang HK. Changes in clinicopathological features and survival after gastrectomy for gastric cancer over a 20-year period. *Br J Surg* 2011; **98**: 255-260 [PMID: 21082693 DOI: 10.1002/bjs.7310]

4 **Takeno S**, Hashimoto T, Maki K, Shibata R, Shiwaku H, Yamana I, Yamashita R, Yamashita Y. Gastric cancer arising from the remnant stomach after distal gastrectomy: a review. *World J Gastroenterol* 2014; **20**: 13734-13740 [PMID: 25320511 DOI: 10.3748/wjg.v20.i38.13734]

5 **Numata N**, Oka S, Tanaka S, Kagemoto K, Sanomura Y, Yoshida S, Arihiro K, Shimamoto F, Chayama K. Risk factors and management of positive horizontal margin in early gastric cancer resected by en bloc endoscopic submucosal dissection. *Gastric Cancer* 2015; **18**: 332-338 [PMID: 24737447 DOI: 10.1007/s10120-014-0368-9]

6 **Katai H**, Morita S, Saka M, Taniguchi H, Fukagawa T. Long-term outcome after proximal gastrectomy with jejunal interposition for suspected early cancer in the upper third of the stomach. *Br J Surg* 2010; **97**: 558-562 [PMID: 20169569 DOI: 10.1002/bjs.6944]

7 **Hiki N**, Nunobe S, Kubota T, Jiang X. Function-preserving gastrectomy for early gastric cancer. *Ann Surg Oncol* 2013; **20**: 2683-2692 [PMID: 23504120 DOI: 10.1245/s10434-013-2931-8]

8 **Ichikawa D**, Komatsu S, Kubota T, Okamoto K, Shiozaki A, Fujiwara H, Otsuji E. Long-term outcomes of patients who underwent limited proximal gastrectomy. *Gastric Cancer* 2014; **17**: 141-145 [PMID: 23558459]

9 **Yoon JY**, Shim CN, Chung SH, Park W, Chung H, Lee H, Shin SK, Lee SK, Lee YC, Park JC. Impact of tumor location on clinical outcomes of gastric endoscopic submucosal dissection. *World J Gastroenterol* 2014; **20**: 8631-8637 [PMID: 25024619 DOI: 10.3748/wjg.v20.i26.8631]

10 **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]

11 **Sano T**, Okuyama Y, Kobori O, Shimizu T, Morioka Y. Early gastric cancer. Endoscopic diagnosis of depth of invasion. *Dig Dis Sci* 1990; **35**: 1340-1344 [PMID: 2226095 DOI: 10.1007/BF01536738]

12 **Choi J**, Kim SG, Im JP, Kim JS, Jung HC, Song IS. Endoscopic prediction of tumor invasion depth in early gastric cancer. *Gastrointest Endosc* 2011; **73**: 917-927 [PMID: 21316050 DOI: 10.1016/j.gie.2010.11.053]

13 **Furukawa K**, Miyahara R, Itoh A, Ohmiya N, Hirooka Y, Mori K, Goto H. Diagnosis of the invasion depth of gastric cancer using MDCT with virtual gastroscopy: comparison with staging with endoscopic ultrasound. *AJR Am J Roentgenol* 2011; **197**: 867-875 [PMID: 21940574 DOI: 10.2214/AJR.10.5872]

14 **Hasegawa S**, Yoshikawa T, Shirai J, Fujikawa H, Cho H, Doiuchi T, Yoshida T, Sato T, Oshima T, Yukawa N, Rino Y, Masuda M, Tsuburaya A. A prospective validation study to diagnose serosal invasion and nodal metastases of gastric cancer by multidetector-row CT. *Ann Surg Oncol* 2013; **20**: 2016-2022 [PMID: 23266583 DOI: 10.1245/s10434-012-2817-1]

15 **Mehmedović A**, Mesihović R, Saray A, Vanis N. Gastric cancer staging: EUS and CT. *Med Arch* 2014; **68**: 34-36 [PMID: 24783909

DOI: 10.5455/medarh.2014.68.34-36]
16 **Kunisaki C**, Shimada H, Nomura M, Matsuda G, Otsuka Y, Ono H, Akiyama H. Surgical outcome in patients with gastric adenocarcinoma in the upper third of the stomach. *Surgery* 2005; **137**: 165-171 [PMID: 15674196 DOI: 10.1016/j.surg.2004.06.005]
17 **Shim JH**, Song KY, Jeon HM, Park CH, Jacks LM, Gonen M, Shah MA, Brennan MF, Coit DG, Strong VE. Is gastric cancer different in Korea and the United States? Impact of tumor location on prognosis. *Ann Surg Oncol* 2014; **21**: 2332-2339 [PMID: 24599411 DOI: 10.1245/s10434-014-3608-7]
18 **Makino T**, Fujiwara Y, Takiguchi S, Tsuboyama T, Kim T, Nushijima Y, Yamasaki M, Miyata H, Nakajima K, Mori M, Doki Y. Preoperative T staging of gastric cancer by multi-detector row computed tomography. *Surgery* 2011; **149**: 672-679 [PMID: 21310453 DOI: 10.1016/j.surg.2010.12.003]
19 **Ganpathi IS**, So JB, Ho KY. Endoscopic ultrasonography for gastric cancer: does it influence treatment? *Surg Endosc* 2006; **20**: 559-562 [PMID: 16446988 DOI: 10.1007/s00464-005-0309-0]
20 **Shirahige A**, Suzuki H, Oda I, Sekiguchi M, Mori G, Abe S, Nonaka S, Yoshinaga S, Sekine S, Kushima R, Saito Y, Fukagawa T, Katai H. Fatal submucosal invasive gastric adenosquamous carcinoma detected at surveillance after gastric endoscopic submucosal dissection. *World J Gastroenterol* 2015; **21**: 4385-4390 [PMID: 25892891 DOI: 10.3748/wjg.v21.i14.4385]
21 **Choi J**, Kim SG, Im JP, Kim JS, Jung HC, Song IS. Comparison of endoscopic ultrasonography and conventional endoscopy for prediction of depth of tumor invasion in early gastric cancer. *Endoscopy* 2010; **42**: 705-713 [PMID: 20652857 DOI: 10.1055/s-0030-1255617]
22 **Choi J**, Kim SG, Im JP, Kim JS, Jung HC, Song IS. Is endoscopic ultrasonography indispensable in patients with early gastric cancer prior to endoscopic resection? *Surg Endosc* 2010; **24**: 3177-3185 [PMID: 20490559 DOI: 10.1007/s00464-010-1112-0]
23 **Lee HH**, Lim CH, Park JM, Cho YK, Song KY, Jeon HM, Park CH. Low accuracy of endoscopic ultrasonography for detailed T staging in gastric cancer. *World J Surg Oncol* 2012; **10**: 190 [PMID: 22978534 DOI: 10.1186/1477-7819-10-190]
24 **Hwang SW**, Lee DH. Is endoscopic ultrasonography still the modality of choice in preoperative staging of gastric cancer? *World J Gastroenterol* 2014; **20**: 13775-13782 [PMID: 25320515 DOI: 10.3748/wjg.v20.i38.13775]
25 **Akashi K**, Yanai H, Nishikawa J, Satake M, Fukagawa Y, Okamoto T, Sakaida I. Ulcerous change decreases the accuracy of endoscopic ultrasonography diagnosis for the invasive depth of early gastric cancer. *Int J Gastrointest Cancer* 2006; **37**: 133-138 [PMID: 18080789 DOI: 10.1007/s12029-007-9004-9]
26 **Kim JH**, Song KS, Youn YH, Lee YC, Cheon JH, Song SY, Chung JB. Clinicopathologic factors influence accurate endosonographic assessment for early gastric cancer. *Gastrointest Endosc* 2007; **66**: 901-908 [PMID: 17963876 DOI: 10.1016/j.gie.2007.06.012]
27 **Akashi Y**, Noguchi T, Nagai K, Kawahara K, Shimada T. Cytoarchitecture of the lamina muscularis mucosae and distribution of the lymphatic vessels in the human stomach. *Med Mol Morphol* 2011; **44**: 39-45 [PMID: 21424936 DOI: 10.1007/s00795-010-0503-6]
28 **Ohdaira H**, Nimura H, Takahashi N, Mitsumori N, Kashiwagi H, Narimiya N, Yanaga K. The possibility of performing a limited resection and a lymphadenectomy for proximal gastric carcinoma based on sentinel node navigation. *Surg Today* 2009; **39**: 1026-1031 [PMID: 19997796 DOI: 10.1007/s00595-009-3993-x]
29 **Zhao P**, Xiao SM, Tang LC, Ding Z, Zhou X, Chen XD. Proximal gastrectomy with jejunal interposition and TGRY anastomosis for proximal gastric cancer. *World J Gastroenterol* 2014; **20**: 8268-8273 [PMID: 25009402 DOI: 10.3748/wjg.v20.i25.8268]

**P- Reviewer**: Osawa S, Reshetnyak VI **S- Editor**: Yu J
**L- Editor**: Filipodia **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12857

*World J Gastroenterol* 2015 December 7; 21(45): 12857-12864
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

Retrospective Study

# Innovative technique of needlescopic grasper-assisted single-incision laparoscopic common bile duct exploration: A comparative study

Say-June Kim, Kee-Hwan Kim, Chang-Hyeok An, Jeong-Soo Kim

Say-June Kim, Department of Surgery, Daejeon St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Daejeon 301-723, South Korea

Kee-Hwan Kim, Chang-Hyeok An, Jeong-Soo Kim, Department of Surgery, Uijeongbu St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Uijeongbu-shi 480-717, Gyeonggi-do, South Korea.

Author contributions: Kim KH designed the study and approved the final version for publication; Kim SJ, An CH and Kim JS contributed equally to acquiring and analyzing the data; Kim SJ and Kim KH interpreted the data; Kim SJ wrote the manuscript.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee at Uijeongbu St. Mary's Hospital (IRB code: UC15RISI0027).

Informed consent statement: The IRB has approved the informed consent procedures would appropriately be waived for all subject due to the nature of this study (retrospective analysis).

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

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at keehwan@catholic.ac.kr.



Correspondence to: Kee-Hwan Kim, MD, PhD, Department of Surgery, Uijeongbu St. Mary's Hospital, College of Medicine, The Catholic University of Korea, #271, Cheonbo-ro, Uijeongbu-shi 480-717, Gyeonggi-do, South Korea. keehwan@catholic.ac.kr
Telephone: +82-31-8203562
Fax: +82-31-8472717

Received: March 26, 2015
Peer-review started: March 30, 2015
First decision: May 18, 2015
Revised: May 22, 2015
Accepted: September 2, 2015
Article in press: September 2, 2015
Published online: December 7, 2015

## Abstract

**AIM:** To investigate the safety and feasibility of needlescopic grasper-assisted single-incision laparoscopic common bile duct exploration (nSIL-CBDE) by comparing the surgical outcomes of this technique with those of conventional laparoscopic CBDE (CL-CBDE).

**METHODS:** We retrospectively analyzed the clinical data of patients who underwent CL-CBDE or nSIL-CBDE for the treatment of common bile duct (CBD) stones between January 2000 and December 2014. For performing nSIL-CBDE, a needlescopic grasper was also inserted through a direct puncture below the right subcostal line after introducing a single-port through the umbilicus. The needlescopic grasper helped obtain the critical view of safety by retracting the gallbladder laterally and by preventing crossing or conflict between laparoscopic instruments. The gallbladder was then partially dissected from the liver bed and used for retraction. CBD stones were usually extracted through a longitudinal supraduodenal choledochotomy, mostly using flushing a copious amount of normal saline through

a ureteral catheter. Afterward, for the certification of CBD clearance, CBDE was performed mostly using a flexible choledochoscope. The choledochotomy site was primarily closed without using a T-tube, and simultaneous cholecystectomies were performed.

**RESULTS:** During the study period, 40 patients underwent laparoscopic CBDE. Of these patients, 20 underwent CL-CBDE and 20 underwent nSIL-CBDE. The operative time for nSIL-CBDE was significantly longer than that for CL-CBDE (238 ± 76 min *vs* 192 ± 39 min, $P = 0.007$). The stone clearance rate was 100% (40/40) in both groups. Postoperatively, the nSIL-CBDE group required less intravenous analgesic (pethidine) (46.5 ± 63.5 mg/kg *vs* 92.5 ± 120.1 mg/kg, $P = 0.010$) and had a shorter hospital stay than the CL-CBDE group (3.8 ± 2.0 d *vs* 5.1 ± 1.7 d, $P = 0.010$). There was no significant difference in the incidence of postoperative complications between the two groups.

**CONCLUSION:** The results of this study suggest that nSIL-CBDE could be safe and feasible while improving cosmetic outcomes when performed by surgeons trained in conventional laparoscopic techniques.

**Key words:** Choledocholithiasis; Choledochotomy; Common bile duct exploration;Laparoscopy; Single-incision laparoscopic surgery



**Core tip:** Though single-incision laparoscopic surgery has been applied in various fields worldwide, the reports on the single-incision laparoscopic common bile duct exploration (SIL-CBDE) are very limited, possibly due to technical difficulties. In this study, we were intended to overcome these difficulties by using additional 2-mm needlscopic grasper. This grasper is separately entered into the right abdomen by a puncture, and helps to form a stable trangulation with the transumbilically placed instruments. Our SIL-CBDE resulted in comparable surgical outcomes as conventional laparoscopic CBDE, indicating that the nSIL-CBDE is not only cosmetically acceptable but also provides both operative safety and feasibility.

Kim SJ, Kim KH, An CH, Kim JS. Innovative technique of needlescopic grasper-assisted single-incision laparoscopic common bile duct exploration: A comparative study. *World J Gastroenterol* 2015; 21(45): 12857-12864 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12857.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12857

## INTRODUCTION

The introduction of single-incision laparoscopic surgery (SILS) has offered significant advantages to patients, including nearly scarless wounds and reduced wound morbidity[1-11]. In the same time, it has also increased the burden on surgeons because the entire operation is performed solely through one small incision. Accordingly, SILS is usually limited to operations in which the procedure is uncomplicated or less technically demanding[12-14]. Common bile duct exploration (CBDE) is a complicated surgery that includes a series of steps such as choledochoscopic lithotripsy and common bile duct (CBD) repair. Hence, reports on single-incision laparoscopic (SIL) CBDE are limited[15-17].

Recent advances in techniques and instruments have led to a progression of laparoscopic CBDE procedure. Moreover, current trends of no T-tube implementation during CBDE have further simplified the procedure[18-23]. Thus, these changes have opened wide the way for SIL-CBDE. Here, we describe needlescopic grasper-assisted single-incision CBDE (nSIL-CBDE). This technique is a modification of SIL-CBDE in order to reproduce the environment of laparoscopic CBDE by the addition of a very fine (2-mm) needlescopic grasper apart from the umbilical port. Simple addition of the needlescopic grasper to SIL-CBDE was expected to simultaneously enhance operative proficiency and minimize wound trauma. In this study, we aimed to demonstrate the safety and operative feasibility of nSIL-CBDE by comparing the surgical outcomes of nSIL-CBDE with those of conventional laparoscopic CBDE (CL-CBDE).

## MATERIALS AND METHODS

### *Study design and data collection*

We performed a retrospective analysis of prospectively collected data from patients who underwent laparoscopic CBDE - either CL-CBDE or nSIL-CBDE - for stone(s) in the Department of Surgery, Uijeongbu St. Mary's Hospital, College of Medicine, the Catholic University of Korea, between January 2000 and December 2014. The Ethics Committee at Uijeongbu St. Mary's Hospital (IRB code: UC15RISI0027) approved the study. A total 40 laparoscopic CBDE procedures were performed during the study period.

Preoperatively, all the patients underwent ultrasonography, computed tomography, or magnetic resonance cholangiography. The inclusion criteria for CL-CBDE and nSIL-CBDE were identical and included all types of CBD stones that were estimated from preoperative evaluations, regardless of the severity of the inflammation or a history of abdominal laparotomy. In March 2012, we first performed nSIL-CBDE, and since then nSIL-CBDE was considered first for all the patients with CBD stone(s). The exclusion criteria for both CL-CBDE and nSIL-CBDE included CBD stone(s) combined with hepatolithiasis, lesion(s) suspected to be malignant, an American Society of Anesthesiologists (ASA) physical status classification of IV or V, severe medical conditions such as a recent history of myo-

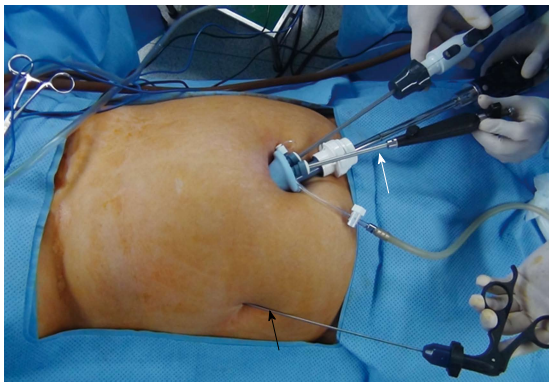


**Figure 1 External view of needlescopic grasper-assisted single-incision laparoscopic common bile duct exploration.** The needlescopic grasper (black arrow) was used for traction through a direct puncture on the right abdomen along the right anterior axillary line. The snake liver retractor (white arrow) was used for cephalad traction of the liver to obtain better visualization.

cardial infarction, and combined operation(s) outside the biliary tract. The total follow-up duration was 61.1 (6-171) mo.

The operative time was defined as the interval between the initial skin incision and the completion of wound closure as documented by the anesthesiologist. Open conversion was defined as completion of the operation with an incision for open surgery. In terms of postoperative complications, a minor bile leak was defined as bile drainage from the drain site prior to the seventh postoperative day that did not require radiological or operative interventions.

### *Needlescopic grasper-assisted single-incision laparoscopic common bile duct exploration technique*

Under general anesthesia, patients were placed in the supine position, and the monitor was placed on the right-hand side of the patient, opposite the operating surgeon. In performing CL-CBDE, standard 4-port approach was utilized: one 10-mm infra-umbilical port for laparoscope, one 5-mm subxipholdal, one 5-mm right flank, and one 5-mm ports along the midclavicular line below the right subcostal region. After meticulous dissecting the Calot's triangle, the critical view of safety was obtained. The cystic artery was clipped and divided, and then cystic duct was clipped. After making 5- to 10-mm vertical choledochotomy, CBD stone retrieval was attempted using a Stonebasket forcep (Olympus), Fogarty catheter, or triflange forceps (through a rigid nephroscope). To confirm the clearance of CBD, intraoperative cholangiography or flexible choledochoscopic exploration was performed. After CBD repairing, the gallbladder was completely removed from the liver, and trocar sites were repaired.

In performing nSIL-CBDE, a single, 25-mm vertical incision was made on the umbilicus and a dissection performed down to the peritoneum. Thereafter, a commercial single-port (SILSTM port; Covidien, Mansfield, MA) was then introduced through the umbilicus. The instruments used through the single port were a 5-mm grasper (Echicon Endosurgery, Cincinnati, OH), liver retractor (Snake retractor; Snowden Pencer, Tucker, GA), and laparoscope (Endoeye Flex 10 mm videoscope; Olympus, Tokyo, Japan). The grasper was replaced intermittently with a hook cautery and suction probe (Endopath Electrosurgery Probe Plus II; Echicon Endo-Surgery, Cincinnati, OH) for meticulous dissection. The snake retractor played a substantial role in achieving operative vision by pushing the hepatic hilum upwards in the direction of the cephalad. EndograbTM internal retractor (Virtual Ports, Misgav, Israel) was intermittently used to assist in obtaining optimal retraction of the liver.

After abdominal insufflation, a needlescopic grasper (Minilap Grasper, Stryker, San Jose, CA or EndoRelief, Hirata Precision Co., Japan) was also inserted through a direct puncture below the right subcostal line along the right anterior axillary line (Figure 1). The needlescopic grasper helped obtain the critical view of safety (CVS)[24] by retracting the gallbladder laterally and by preventing crossing or conflict between laparoscopic instruments.

After dissection and identification of cystic artery and duct, they were ligated with 5-mm Hem-o-lok® clips (Weck Closure Systems, Research Triangle Park, NC), and then transected using laparoscopic scissors. The gallbladder was partially dissected from the liver bed and used for retraction. For CBD stone(s) retrieval, a longitudinal supraduodenal choledochotomy was made using an endoknife (Karl-Storz, Tuttlingen, Germany). Stones and debris from the CBD were extracted either by flushing a copious amount of normal saline through a ureteral catheter (3 - 5 French) or by using a Fogarty balloon catheter.

Afterwards, for the detection of residual CBD stone(s), CBDE was performed using a flexible choledochoscope (11-Fr, 30°; Karl-Storz). During choledochoscope manipulation (Figure 2), the needlescopic grasper effectively assisted the insertion of the choledochoscope into the CBD and changes in direction. It gently pushed the choledochoscope toward upper and lower part of the CBD to properly visualize both directions, enabling thorough visualization of both the upper (up to the right and left hepatic ducts) and lower (down to the papilla) portion of the CBD. Any residual stones were removed using a Stonebasket forcep or Fogarty catheter. In extracting residual stones, we selectively used a rigid nephroscope (17-Fr, 6°; Karl-Storz). It accommodates wide graspers, thereby making it possible to extract large stones under the direct vision. Therefore, it was commonly indicated at the time of failing to extract stones using stonebasket forcep or Fogarty catheter.

After CBD clearance, the choledochotomy site was primarily closed without using a T-tube. Initially, it was interruptedly repaired using a laparoscopic suturing device (Lap-suture®; Sejong Medical, Seoul, Korea).

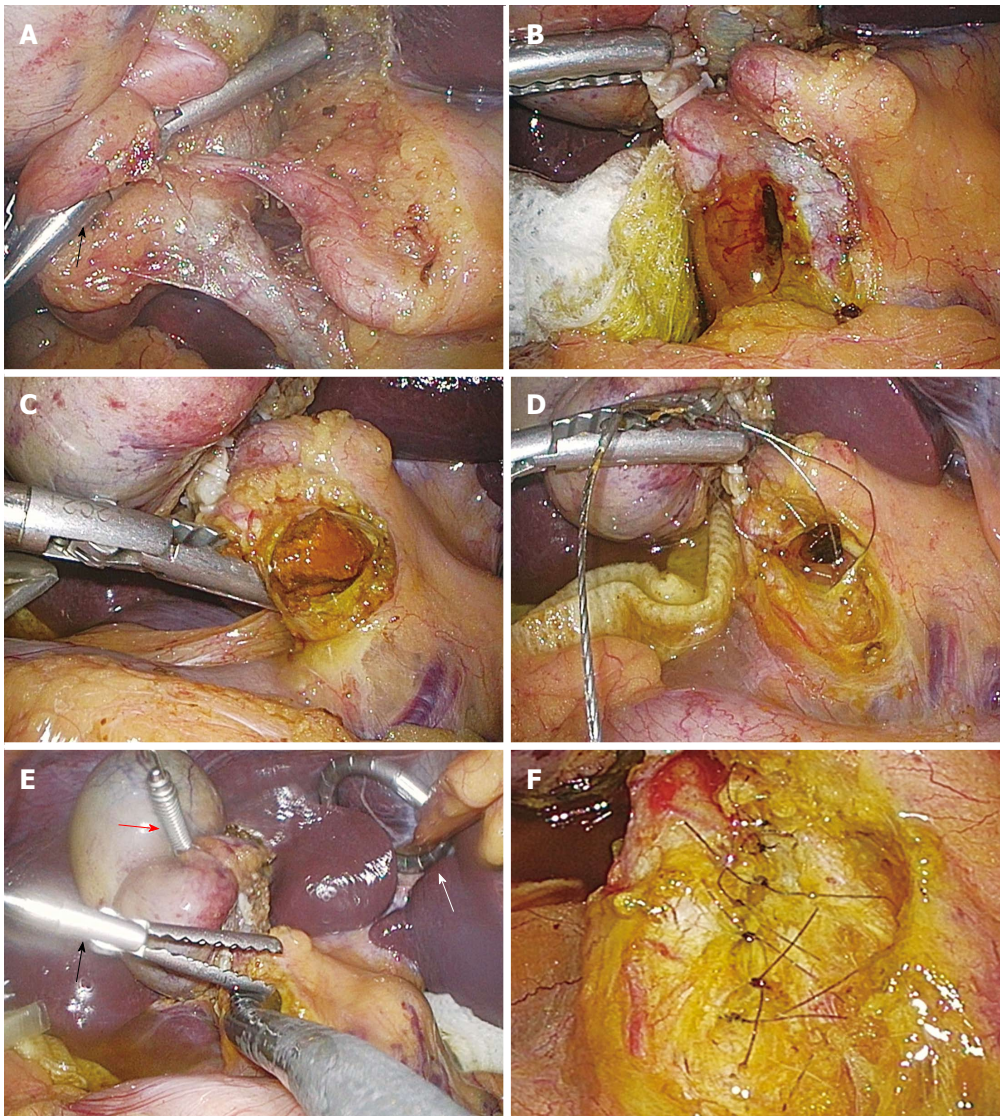



**Figure 2 Operative illustrations of needlescopic grasper-assisted single-incision laparoscopic common bile duct exploration.** The needlescopic grasper helped with obtaining a critical view of safety by retracting the gallbladder laterally (A). After ligation and transection of the cystic artery and duct, the common bile duct (CBD) was opened longitudinally using an endoknife (B) The CBD stones were extracted through various methods including direct extraction (C) or the use of a stone basket (D). After extraction of the CBD stones, completion choledochoscopy was performed to check for the presence of remnant stone(s) (E). After CBD clearance, the choledochotomy was primarily closed using either interrupted (F) or running sutures. Black arrows indicates lateral retraction of the gallbladder by the needlescopic grasper. A white and a red arrows indicate the snake retractor and Endograb, respectively.

Lap-suture is a pre-knotted suturing material which enables convenient suturing just by suturing, passing the needle through the pre-knotted hole, and then tightening it by pushing using a bar. Thereafter, it was replaced with continuous suturing using an absorbable monofilament suture (PDS®6/0 Ethicon; Johnson and Johnson, Somerville, NJ). During intracorporeal suturing, the needlescopic grasper assisted a 5-mm straight needle holder (ENDOPATH Needle holder; Ethicon Endo-surgery) which had been entered *via* the transumbilical port.

We next dissected the gallbladder completely away from the liver. The resected gallbladder was placed in a specimen bag and was subsequently extracted from the abdomen through the umbilical wound. A Jackson-Pratt drain was placed in the subhepatic space, if necessary. After abdominal deflation, intraumbilical fascial defects and transumbilical skin incisions were closed with interrupted sutures.

### *Statistical analysis*

Numeric data were presented as the mean and standard deviation or as the median and range. Continuous variables were analyzed using independent *t*-tests or Wilcoxon rank-sum tests. Categorical variables or proportions were compared using Pearson $\chi^2$ tests or Fisher exact tests when appropriate. Statistical analyses were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL). All *P* values were two-tailed. Statistical significance was accepted for *P*

**Table 1 Demographic and preoperative patient characteristics *n* (%)**

| | CL-LCBDE (*n* = 20) | nSIL-CBDE (*n* = 20) | *P* value |
|---|---|---|---|
| Age (yr) | 51.0 ± 19.3 | 60.5 ± 17.2 | 0.068 |
| Male:female | 9:11 | 9:11 | |
| Body mass index (kg/m²) | | | 0.064 |
| mean ± SD | 25.00 ± 3.97 | 22.55 ± 2.69 | |
| median (range) | 25.10 (19.60-34.80) | 22.45 (17.16-27.69) | |
| ASA classification | | | 0.010 |
| 1 | 3 (15) | 10 (50) | |
| 2 | 15 (75) | 10 (50) | |
| 3 | 2 (10) | 0 (0) | |
| Comorbidity | | | 0.231 |
| CCI = 0 | 17 (85) | 20 (100) | |
| CCI ⩾ 1 | 3 (15) | 0 (0) | |
| Previous abdominal surgery) | 4 (20) | 4 (20) | 1.000 |
| Laboratory parameters | | | |
| Total leukocyte count (cells/mm³) | 9117 ± 3756 | 9123 ± 2569 | 0.274 |
| ALT (IL/dL) | 319 ± 267 | 215 ± 240 | 0.068 |
| Total bilirubin (mg/dL) | 3.71 ± 2.24 | 2.18 ± 2.02 | 0.079 |
| Alkaline phosphatase (IL/dL) | 610 ± 321 | 453 ± 342 | 0.069 |
| Radiological parameters | | | |
| Gallbladder wall thickening (⩾ 4 mm) | 4 (20) | 7 (35) | 0.480 |
| Acute cholangitis | 17 (85) | 14 (70) | 0.294 |
| CBD stone(s) | | | 1.000 |
| Definite | 18 (90) | 19 (95) | |
| Suspicious | 2 (10) | 1 (5) | |
| Indication of CBDE | | | 0.025 |
| Primary intervention | 7 (35) | 15 (75) | |
| Secondary intervention | 13 (65) | 5 (25) | |
| After ERCP failure | 7 (35) | 5 (25) | |
| Remnant CBD stone(s) after ERCP | 6 (30) | 0 (0) | |
| Admission route | | | 1.000 |
| ER | 12 (60) | 13 (65) | |
| OPD | 8 (40) | 7 (35) | |

ALT: Alanine transaminase; CBD: Common bile duct; CCI: Charlson comorbidity index; CL-CBDE: Conventional laparoscopic common bile duct exploration; ER: Emergency room; ERCP: Endoscopic retrograde cholangio pancreatography; nSIL-CBDE: Needlescopic grasper-assisted single-incision laparoscopic common bile duct exploration; OPD: Outpatient clinic.

values < 0.05.

## RESULTS

### *Comparison of the baseline characteristics between patients who underwent conventional laparoscopic or needlescopic grasper-assisted single-incision laparoscopic common bile duct exploration*

A total of 40 patients who underwent laparoscopic CBDE during the study period were included in this study. Of these patients, 20 (50%) underwent CL-CBDE and 20 (50%) underwent nSIL-CBDE. The mean patient age was 55.8 ± 18.7 (25-81) years. Male patients accounted for 45% (18/40) of the total patients in the study. The mean body mass index (BMI) was 23.8 ± 3.8 (17.2-34.8) kg/m$^2$. Table 1 shows a summary of comparisons in patient demographics and preoperative clinical parameters. The two operative groups were similar in age, sex, and BMI. Laboratory and radiological variables were also comparable between the two groups. However, a comparison of the ASA classifications in the two groups revealed that patients with more severe conditions were included in the CL-CBDE group ($P$ = 0.010).

We classified the operative indications for laparoscopic CBDE into two categories: primary intervention and secondary intervention. The secondary intervention category was further subdivided into the inability or failure of therapeutic endoscopic retrograde cholangiopancreatography (ERCP) and remnant CBD stone(s) after ERCP attempt(s). We compared the operative indications between the two groups. CL-CBDE group included 37% (7/21) of patients with primary intervention, and nSIL-CBDE group included 75% (15/21) of patients with primary intervention ($P$ = 0.025).

### *Comparison of operative and postoperative outcomes*

We next compared the operative results following CL-CBDE and nSIL-CBDE (Table 2). The operative time in the nSIL-CBDE group was significantly longer than that of the CL-CBDE group (238 ± 76 min *vs* 192 ± 39 min, $P$ = 0.007). There was no significant difference in the estimated blood loss. The mean size and number of CBD stones that were retrieved were comparable. Successful retrievals of CBD stone(s) were reported in both groups, and there was no open conversion or addition of another port(s) during the procedure. Two methods were used to detect remnant stone(s) after CBD stone retrieval: completion cholangiography and completion choledochoscopy. All CL-CBDE patients, but not all nSIL-CBDE patients, underwent completion cholangiography ($P$ = 0.018). Instead, all the nSIL-CBDE patients underwent completion choledochoscopy. In addition, Jackson-Pratt drains were less frequently placed in the nSIL-CBDE patients than in the CL-CBDE patients (15% *vs* 95.0%, $P$ < 0.001).

Postoperatively, the nSIL-CBDE group had lesser intravenous analgesic (pethidine) administration (46.5 ± 63.5 mg/kg *vs* 92.5 ± 120.1 mg/kg, $P$ = 0.010) and shorter duration of hospitalization (3.8 ± 2.0 d *vs* 5.1 ± 1.7 d, $P$ = 0.010). Postoperative complications were comparable between the two groups. Specifically, CL-CBDE group included one patient with infected subhepatic fluid collection, and nSIL-CBDE group included one patient with minor bile leak and the other patient with subhepatic hematoma.

## DISCUSSION

To our best knowledge, this study was the first to compare CL-CBDE with nSIL-CBDE. Although nSIL-

**Table 2 Operative results of patients who underwent laparoscopic common bile duct exploration *n* (%)**

| | CL-LCBDE (*n* = 20) | nSIL-CBDE (*n* = 20) | *P* value |
|---|---|---|---|
| Operative time (skin to skin, min) | | | 0.007 |
| mean ± SD | 192 ± 39 | 238 ± 76 | |
| median (range) | 197.5 (115-245) | 215.0 (125-365) | |
| Estimated blood loss (mL) | 132 ± 309 | 75 ± 161 | 0.101 |
| Completion cholangiogram | 5 (25) | 0 (0) | 0.018 |
| Completion choledochoscopy | 16 (80) | 20 (100) | 0.037 |
| Stone clearance | 20 (100) | 20 (100) | 1.000 |
| CBD stone | | | 0.348 |
| Single CBD stone | 12 (60) | 9 (45) | |
| Multiple CBD stones | 8 (40) | 11 (55) | |
| Mean number of CBD stone extracted | 1.9 ± 1.7 | 3.0 ± 3.2 | 0.316 |
| The largest diameter of CBD stone (mm) | | | 0.624 |
| mean ± SD | 12.3 ± 3.8 | 14.1 ± 4.6 | |
| median (range) | 14.0 (5.0-15.8) | 13.6 (7.0-20.0) | |
| Open conversion | 0 | 0 | 1.000 |
| Addition of another port | 0 | 0 | 1.000 |
| Gallbladder pathology | | | 0.234 |
| Gangrene/empyema | 1 (5) | 4 (20) | |
| Acute inflammation | 3 (15) | 3 (15) | |
| Chronic inflammation | 16 (80) | 13 (65) | |
| Drain placed | 19 (95) | 3 (15) | < 0.001 |
| Pethidine dose (mg/kg) | 92.5 ± 120.1 | 46.5 ± 63.5 | 0.010 |
| Postoperative hospital stay (d) | | | 0.010 |
| mean ± SD | 5.1 ± 1.7 | 3.8 ± 2.0 | |
| median (range) | 5.0 (2.0-8.0) | 3.0 (2.0-9.0) | |
| Postoperative complication | | | 0.275 |
| Infected subhepatic collection | 1 | 0 | |
| Minor bile leak | 0 | 1 | |
| Subhepatic seroma | 0 | 1 | |

CBD: Common bile duct; CL-CBDE: Conventional laparoscopic common bile duct exploration; nSIL-CBDE: Needlescopic grasper-assisted single-incision laparoscopic common bile duct exploration.

CBD increased the total operative time, it significantly reduced both intravenous analgesic administration and the length of hospital stay. The two groups were similar in other variables, including the CBD clearance rate, open conversion rate, and incidence of postoperative complications. Besides, nSIL-CBDE has intuitive advantages of cosmetic superiority and lessened wound morbidity. Therefore, we conclude that nSIL-CBDE is a reasonable alternative to CL-CBDE when performed by a surgeon with sufficient laparoscopic experience.

Patients prefer cosmesis and quality of life, as long as their safety is guaranteed[25]. nSIL-CBDE provides cosmetic superiority because it reproduces laparoscopic CBDE environment using the nearly scarless incisions. nSIL-CBDE exclusively leaves two trivial wounds; one is usually concealed in the umbilicus and the other is often negligible because it is made by a puncture of a 2-mm instrument. Moreover, though we could not detect possibly due to relatively small patient population, nSIL-CBDE is expected to reduce wound morbidity, as the number of port(s) is correlated with the number of the port-related complications, including trocar site hernia, and injury to the vessels, bowel, and other intra-abdominal organs[26-28]. Therefore, we believe that reducing the possibility of wound morbidity would be a major advantage of nSIL-CBDE.

Although SILS has been applied in various fields during the past two decades[11,12,29,30], technical difficulties has prevented widespread use of SIL-CBDE to the patients with CBD stone(s). However, as SILS experiences have been accumulated, the more refined surgical techniques and instruments have been overcoming the significant proportion of the technical difficulties. For instance, it has been considered challenging to appropriately manipulate the choledochoscope during SIL-CBDE. In nSIL-CBDE, however, this difficulty can be circumvented by the addition of a convenient guiding grasper which is aligned with the laparoscope in a triangular shape. Likewise, nSIL-CBDE provides a similar environment as CL-CBDE in terms of the handling of the choledochoscope.

CBD repair is one of the most challenging step during laparoscopic CBDE and has been performed with various techniques. The degree of difficulty is particularly increased when the T-tube is inserted into the CBD. However, data from a recent meta-analysis have provided evidence that primary closure instead of T-tube drainage is superior in terms of operative time, overall postoperative complications, and postoperative hospital stay associated with laparoscopic CBDE[31,32]. In our study, we performed primary closure in all patients who underwent laparoscopic CBDE. After retrieval of CBD stones, open CBD can be primarily closured by either interrupted or continuous sutures. We initially performed interrupted suturing using laparoscopic suture loops, but later changed to continuous sutures using 6.0 PDS. Recently, an increasing number of reports have described the use of continuous suturing, which is also supported by favorable long-term outcomes[18,19,22,33]. We think that although continuous suturing may initially result in increased operative time, with practice, it may actually shorten the total operative time.

We believe that nSIL-CBDE is particularly advantageous in CBD repair. During CL-CBDE, the needle holder, which is usually inserted through an epigastric port, reaches the CBD at an oblique angle. This alignment often hinders the stable performance of CBD repair. However, during nSIL-CBDE, the needle holder is inserted through the umbilicus and reaches the CBD nearly vertically, which allows comfortable suturing. We used two kinds of needlescopic graspers for assistance: the Minilap™ and Endorelief™ graspers. While the Minilap™ has a 2-mm forceps, Endorelief™ has a 5-mm forceps, enabling a grasping power equivalent to that of a 5-mm grasper. After a period of testing, we preferred to use the Endorelief™, because it provided an operative efficiency that was equivalent

to that of the 5-mm grasper.

The nSIL-CBDE group had significantly longer operative times than the CL-CBDE group (237.8 ± 76.4 min *vs* 192.1 ± 39.0 min, $P = 0.007$). The most of nSIL-CBDE were performed during a learning period. Therefore, we expected that the operative times of nSIL-CBDE would be shorter once the learning curve was completed. In addition, although the series of nSIL-CBDE are our early experience, nSIL-CBDE significantly reduced the requirement for intravenous analgesic administration ($P = 0.010$) and the duration of hospitalization ($P = 0.010$). However, drain placement is one of the factors influencing postoperative pain[34], and nSIL-CBDE group lesser placed the drain (15% *vs* 95%, $P < 0.001$). Therefore, further investigation is required to determine whether or not nSIL-CBDE has the potential to reduce postoperative pain.

This study had several limitations. Because it was a retrospective study with a small sample size, future prospective randomized trials are required to definitively determine the efficacy and safety of nSIL-CBDE. In addition, the patient distribution was not completely balanced between the two groups. CL-CBDE group included more patients who had high ASA score ($P = 0.010$). However, it seems not to be substantial because no differences observed in the other variables reflecting degree of inflammation, such as the serum leukocyte count, total bilirubin level, and radiologic findings, between the two groups. Finally, there was a chronological difference between the two groups. A total series of nSIL-CBDE was followed by a series of CL-CBDE. Because both CL-CBDE and nSIL-CBDE are same laparoscopic surgery by nature, the nSIL-CBDE technique could be refined with accumulated experience. However, because most of the nSIL-CBDE were performed during its learning period, we think the comparable surgical outcomes of nSIL-CBDE demonstrate the operative feasibility of this technique.

In conclusion, although nSIL-CBDE increased the total operative time, it could maintain the critical view of safety throughout the procedure. nSIL-CBDE showed similar surgical outcomes as CL-CBDE, especially in the CBD clearance rate and the incidences of open conversion and postoperative complications. Moreover, nSIL-CBDE significantly reduced requirement of postoperative analgesics and length of the postoperative hospital stay. Therefore, we think nSIL-CBDE could provide both operative safety and feasibility while improving cosmetic outcomes.

## COMMENTS

### Background

Common bile duct exploration (CBDE) is a complicated surgery that includes a strenuous series of steps, including choledochoscopic lithotripsy and common bile duct (CBD) repair. Hence, reports on single-incision laparoscopic (SIL) CBDE are limited. The authors were intended to overcome these limitations by the addition of a very fine (2-mm) needlescopic grasper apart from the umbilical port.

### Research frontiers

In contrast to conventional laparoscopic CBDE which usually requires four incisions for trocar insertion, needlescopic grasper-assisted single-incision laparoscopic (nSIL) CBDE only requires one umbilical incision and a negligible 2-mm incision.

### Innovations and breakthroughs

SIL-CBDE is very strenuous operation due to the difficulties provoked by the processes of choledochoscopic lithotripsy and common bile duct (CBD) repair. The results shows that simple addition of the needlescopic grasper to SIL-CBDE enhances operative proficiency while minimizing wound trauma. It was more facilitated by recent trends of primary CBD closure instead of T-tube drainage after CBDE.

### Applications

Although CL-CBDE is a difficult operation, SIL-CBDE is even more difficult. nSIL-CBDE does not require the operator's surgical skill enough to perform SIL-CBDE; only the surgical skill to perform CL-CBDE is sufficient. In addition, nSIL-CBDE seems to be accustomed within a relatively short learning curve. Therefore, nSIL-CBDE could be safe and feasible while improving cosmetic outcomes when performed by surgeons trained in conventional laparoscopic techniques.

### Peer-review

This manuscript has novelty. This is the first study comparing needlescopic grasper-assisted single incision with conventional laparoscopic common bile duct exploration.

## REFERENCES


1 **Kim SJ**, Ryu GO, Choi BJ, Kim JG, Lee KJ, Lee SC, Oh ST. The short-term outcomes of conventional and single-port laparoscopic surgery for colorectal cancer. *Ann Surg* 2011; **254**: 933-940 [PMID: 22107740 DOI: 10.1097/SLA.0b013e318237826b]

2 **Weiss HG**, Brunner W, Biebl MO, Schirnhofer J, Pimpl K, Mittermair C, Obrist C, Brunner E, Hell T. Wound complications in 1145 consecutive transumbilical single-incision laparoscopic procedures. *Ann Surg* 2014; **259**: 89-95 [PMID: 23426333 DOI: 10.1097/SLA.0b013e31827b7818]

3 **Ahmed K**, Wang TT, Patel VM, Nagpal K, Clark J, Ali M, Deeba S, Ashrafian H, Darzi A, Athanasiou T, Paraskeva P. The role of single-incision laparoscopic surgery in abdominal and pelvic surgery: a systematic review. *Surg Endosc* 2011; **25**: 378-396 [PMID: 20623239 DOI: 10.1007/s00464-010-1208-6]

4 **Asakuma M**, Hayashi M, Komeda K, Shimizu T, Hirokawa F, Miyamoto Y, Okuda J, Tanigawa N. Impact of single-port cholecystectomy on postoperative pain. *Br J Surg* 2011; **98**: 991-995 [PMID: 21538340 DOI: 10.1002/bjs.7486]

5 **Chow A**, Purkayastha S, Nehme J, Darzi LA, Paraskeva P. Single incision laparoscopic surgery for appendicectomy: a retrospective comparative analysis. *Surg Endosc* 2010; **24**: 2567-2574 [PMID: 20336322 DOI: 10.1007/s00464-010-1004-3]

6 **Hodgett SE**, Hernandez JM, Morton CA, Ross SB, Albrink M, Rosemurgy AS. Laparoendoscopic single site (LESS) cholecystectomy. *J Gastrointest Surg* 2009; **13**: 188-192 [PMID: 19031097 DOI: 10.1007/s11605-008-0735-0]

7 **Lee PC**, Lo C, Lai PS, Chang JJ, Huang SJ, Lin MT, Lee PH. Randomized clinical trial of single-incision laparoscopic cholecystectomy versus minilaparoscopic cholecystectomy. *Br J Surg* 2010; **97**: 1007-1012 [PMID: 20632264 DOI: 10.1002/bjs.7087]

8 **Marks J**, Tacchino R, Roberts K, Onders R, Denoto G, Paraskeva P, Rivas H, Soper N, Rosemurgy A, Shah S. Prospective randomized controlled trial of traditional laparoscopic cholecystectomy versus single-incision laparoscopic cholecystectomy: report of preliminary data. *Am J Surg* 2011; **201**: 369-72; discussion 372-3 [PMID: 21367381 DOI: 10.1016/j.amjsurg.2010.09.012]

9 **Raman JD**, Bagrodia A, Cadeddu JA. Single-incision, umbilical laparoscopic versus conventional laparoscopic nephrectomy: a

comparison of perioperative outcomes and short-term measures of convalescence. *Eur Urol* 2009; **55**: 1198-1204 [PMID: 18715694 DOI: 10.1016/j.eururo.2008.08.019]
10 **Teixeira J**, McGill K, Koshy N, McGinty J, Todd G. Laparoscopic single-site surgery for placement of adjustable gastric band--a series of 22 cases. *Surg Obes Relat Dis* 2010; **6**: 41-45 [PMID: 19560980 DOI: 10.1016/j.soard.2009.03.220]
11 **Vestweber B**, Alfes A, Paul C, Haaf F, Vestweber KH. Single-incision laparoscopic surgery: a promising approach to sigmoidectomy for diverticular disease. *Surg Endosc* 2010; **24**: 3225-3228 [PMID: 20464419 DOI: 10.1007/s00464-010-1090-2]
12 **Carus T**. Current advances in single-port laparoscopic surgery. *Langenbecks Arch Surg* 2013; **398**: 925-929 [PMID: 24037311 DOI: 10.1007/s00423-013-1113-2]
13 **Goel R**, Lomanto D. Controversies in single-port laparoscopic surgery. *Surg Laparosc Endosc Percutan Tech* 2012; **22**: 380-382 [PMID: 23047376 DOI: 10.1097/SLE.0b013e3182615776]
14 **Kim SJ**, Choi BJ, Lee SC. Overview of single-port laparoscopic surgery for colorectal cancers: past, present, and the future. *World J Gastroenterol* 2014; **20**: 997-1004 [PMID: 24574772 DOI: 10.3748/wjg.v20.i4.997]
15 **Chuang SH**, Chen PH, Chang CM, Tsai YF, Lin CS. Single-incision laparoscopic common bile duct exploration with conventional instruments: an innovative technique and a comparative study. *J Gastrointest Surg* 2014; **18**: 737-743 [PMID: 24347312 DOI: 10.1007/s11605-013-2420-1]
16 **Shibao K**, Higure A, Yamaguchi K. Laparoendoscopic single-site common bile duct exploration using the manual manipulator. *Surg Endosc* 2013; **27**: 3009-3015 [PMID: 23436088 DOI: 10.1007/s00464-013-2837-3]
17 **Yeo D**, Mackay S, Martin D. Single-incision laparoscopic cholecystectomy with routine intraoperative cholangiography and common bile duct exploration via the umbilical port. *Surg Endosc* 2012; **26**: 1122-1127 [PMID: 22170316 DOI: 10.1007/s00464-011-2009-2]
18 **Abellán Morcillo I**, Qurashi K, Abrisqueta Carrión J, Martinez Isla A. Laparoscopic common bile duct exploration. Lessons learned after 200 cases. *Cir Esp* 2014; **92**: 341-347 [PMID: 24559592 DOI: 10.1016/j.ciresp.2013.02.010]
19 **Decker G**, Borie F, Millat B, Berthou JC, Deleuze A, Drouard F, Guillon F, Rodier JG, Fingerhut A. One hundred laparoscopic choledochotomies with primary closure of the common bile duct. *Surg Endosc* 2003; **17**: 12-18 [PMID: 12364990 DOI: 10.1007/s00464-002-9012-6]
20 **Dong ZT**, Wu GZ, Luo KL, Li JM. Primary closure after laparoscopic common bile duct exploration versus T-tube. *J Surg Res* 2014; **189**: 249-254 [PMID: 24746254 DOI: 10.1016/j.jss.2014.03.055]
21 **Lee HM**, Min SK, Lee HK. Long-term results of laparoscopic common bile duct exploration by choledochotomy for choledocholithiasis: 15-year experience from a single center. *Ann Surg Treat Res* 2014; **86**: 1-6 [PMID: 24761400 DOI: 10.4174/astr.2014.86.1.1]
22 **Zhang HW**, Chen YJ, Wu CH, Li WD. Laparoscopic common bile duct exploration with primary closure for management of choledocholithiasis: a retrospective analysis and comparison with conventional T-tube drainage. *Am Surg* 2014; **80**: 178-181 [PMID: 24480219]
23 **Zhou Y**, Wu XD, Fan RG, Zhou GJ, Mu XM, Zha WZ, Jia J. Laparoscopic common bile duct exploration and primary closure of choledochotomy after failed endoscopic sphincterotomy. *Int J Surg* 2014; **12**: 645-648 [PMID: 24879343 DOI: 10.1016/j.ijsu.2014.05.059]
24 **Strasberg SM**, Hertl M, Soper NJ. An analysis of the problem of biliary injury during laparoscopic cholecystectomy. *J Am Coll Surg* 1995; **180**: 101-125 [PMID: 8000648]
25 **Varadarajulu S**, Tamhane A, Drelichman ER. Patient perception of natural orifice transluminal endoscopic surgery as a technique for cholecystectomy. *Gastrointest Endosc* 2008; **67**: 854-860 [PMID: 18355816 DOI: 10.1016/j.gie.2007.09.053]
26 **Rao PP**, Rao PP, Bhagwat S. Single-incision laparoscopic surgery - current status and controversies. *J Minim Access Surg* 2011; **7**: 6-16 [PMID: 21197236 DOI: 10.4103/0972-9941.72360]
27 **Lowry PS**, Moon TD, D'Alessandro A, Nakada SY. Symptomatic port-site hernia associated with a non-bladed trocar after laparoscopic live-donor nephrectomy. *J Endourol* 2003; **17**: 493-494 [PMID: 14565880 DOI: 10.1089/089277903769013649]
28 **Marcovici I**. Significant abdominal wall hematoma from an umbilical port insertion. *JSLS* 2001; **5**: 293-295 [PMID: 11548838]
29 **Froghi F**, Sodergren MH, Darzi A, Paraskeva P. Single-incision Laparoscopic Surgery (SILS) in general surgery: a review of current practice. *Surg Laparosc Endosc Percutan Tech* 2010; **20**: 191-204 [PMID: 20729685 DOI: 10.1097/SLE.0b013e3181ed86c6]
30 **Greaves N**, Nicholson J. Single incision laparoscopic surgery in general surgery: a review. *Ann R Coll Surg Engl* 2011; **93**: 437-440 [PMID: 21929912 DOI: 10.1308/003588411X590358]
31 **Wu X**, Yang Y, Dong P, Gu J, Lu J, Li M, Mu J, Wu W, Yang J, Zhang L, Ding Q, Liu Y. Primary closure versus T-tube drainage in laparoscopic common bile duct exploration: a meta-analysis of randomized clinical trials. *Langenbecks Arch Surg* 2012; **397**: 909-916 [PMID: 22644601 DOI: 10.1007/s00423-012-0962-4]
32 **Yin Z**, Xu K, Sun J, Zhang J, Xiao Z, Wang J, Niu H, Zhao Q, Lin S, Li Y. Is the end of the T-tube drainage era in laparoscopic choledochotomy for common bile duct stones is coming? A systematic review and meta-analysis. *Ann Surg* 2013; **257**: 54-66 [PMID: 23059495 DOI: 10.1097/SLA.0b013e318268314b]
33 **Hua J**, Lin S, Qian D, He Z, Zhang T, Song Z. Primary closure and rate of bile leak following laparoscopic common bile duct exploration via choledochotomy. *Dig Surg* 2015; **32**: 1-8 [PMID: 25613528 DOI: 10.1159/000368326]
34 **Eidy M**, Jesmi F, Raygan F, Pishgahroudsari M, Pazouki A. Evaluating the Effect of Drain Site on Abdominal Pain after Laparoscopic Gastric Bypass Surgery for Morbid Obesity: A Randomized Controlled Trial. *Bariatr Surg Pract Patient Care* 2015; **10**: 38-41 [PMID: 25830079 DOI: 10.1089/bari.2014.0032]

**P- Reviewer**: Kassir R, Wada H **S- Editor**: Yu J
**L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12865

*World J Gastroenterol* 2015 December 7; 21(45): 12865-12872
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

Retrospective Study

# Oddi sphincter preserved cholangioplasty with hepatico-subcutaneous stoma for hepatolithiasis

Yu-Gui Lian, Wei-Tao Zhang, Zhi Xu, Xiao-Feng Ling, Li-Xin Wang, Chun-Sheng Hou, Gang Wang, Long Cui, Xiao-Si Zhou

Yu-Gui Lian, Wei-Tao Zhang, Zhi Xu, Xiao-Feng Ling, Li-Xin Wang, Chun-Sheng Hou, Gang Wang, Long Cui, Xiao-Si Zhou, Department of General Surgery, Peking University Third Hospital, Beijing 100191, China

Author contributions: Lian YG, Xu Z and Zhou XS designed the research; Lian YG, Zhang WT, Xu Z, Ling XF, Wang LX, Hou CS, Wang G and Cui L performed the research; Lian YG and Zhang WT analyzed the data; and Lian YG wrote this paper.

Institutional review board statement: The study was reviewed and approved by the Peking University Third Hospital Medical Science Research Ethics Committee.

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

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

Data sharing statement: No additional data are available.



Correspondence to: Zhi Xu, MD, Department of General Surgery, Peking University Third Hospital, No. 49 Huayuan Beilu, Beijing 100191, China. xuzhi123456@sohu.com
Telephone: +86-10-82267331
Fax: +86-10-62010334

Received: June 2, 2015
Peer-review started: June 3, 2015
First decision: June 19, 2015
Revised: July 9, 2015
Accepted: August 31, 2015
Article in press: August 31, 2015
Published online: December 7, 2015

## Abstract

**AIM:** To evaluate the long-term outcomes of Oddi sphincter preserved cholangioplasty with hepatico-subcutaneous stoma (OSPCHS) and risk factors for recurrence in hepatolithiasis.

**METHODS:** From March 1993 to December 2012, 202 consecutive patients with hepatolithiasis underwent OSPCHS at our department. The Oddi sphincter preserved procedure consisted of common hepatic duct exploration, stone extraction, hilar bile duct plasty, establishment of subcutaneous stoma to the bile duct. Patients with recurrent stones can undergo stone extraction and/or biliary drainage *via* the subcutaneous stoma which can be incised under local anesthesia. The long-term results were reviewed. Cox regression model was employed to analyze the risk factors for stone recurrence.

**RESULTS:** Ninety-seven (48.0%) OSPCHS patients underwent hepatic resection concomitantly. The rate of surgical complications was 10.4%. There was no perioperative death. The immediate stone clearance rate was 72.8%. Postoperative cholangioscopic lithotomy raised the clearance rate to 97.0%. With a median follow-up period of 78.5 mo (range: 2-233 mo), 24.8% of patients had recurrent stones, 2.5% had late development of cholangiocarcinoma, and the mortality rate was 5.4%. Removal of recurrent stones and/or drainage of inflammatory bile *via* subcutaneous stoma were conducted in 44 (21.8%) patients. The clearance rate of recurrent stones was 84.0% after subsequent choledochoscopic lithotripsy *via* subcutaneous stoma.

Cox regression analysis showed that residual stone was an independent prognostic factor for stone recurrence.

**CONCLUSION:** In selected patients with hepatolithiasis, OSPCHS achieves excellent long-term outcomes, and residual stone is an independent prognostic factor for stone recurrence.

**Key words:** Hepatolithiasis; Stone recurrence; Sphincter of Oddi; Hepaticoplasty; Hepatectomy



**Core tip:** The treatment of hepatolithiasis remains a great challenge among various biliary operations. Residual and recurrent stones are the most troublesome problem after surgery. The present study introduces an optional technique (OSPCHS) for hepatolithiasis. This procedure keeps the Oddi sphincter intact and reduces the postoperative reflux cholangitis, and OSPCHS also stresses the clearance of hepatobiliary lesions. Moreover, OSPCHS provides the recurrent patients with the minimal invasive treatment to avoid major surgery. OSPCHS generates a satisfactory long-term outcome for hepatolithiasis.

Lian YG, Zhang WT, Xu Z, Ling XF, Wang LX, Hou CS, Wang G, Cui L, Zhou XS. Oddi sphincter preserved cholangioplasty with hepatico-subcutaneous stoma for hepatolithiasis. *World J Gastroenterol* 2015; 21(45): 12865-12872 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12865.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12865

## INTRODUCTION

Hepatolithiasis is a common disease in Southeast Asia and is particularly prevalent in China[1]. It is characterized by repeated attacks of acute bacterial cholangitis with subsequent formation of pigment stones and strictures in the biliary system. Hepatolithiasis, at present, is still difficult to treat because of the high rate of residual or recurrent stones[1-3].

The definitive management of primary hepatolithiasis is to use a multidisciplinary approach, aiming to remove all biliary stones, establish adequate drainage to the biliary system, resect nonfunctioning liver segments that harbor bacteria and serve as foci of infection[3-5]. There are various treatment modalities for hepatolithiasis, including hepatectomy, intrahepatic duct exploration *via* choledochotomy, percutaneous transhepatic cholangioscopic lithotripsy (PTCSL), and Oddi sphincter preserved cholangioplasty with hepatico-subcutaneous stoma (OSPCHS)[6-9]. However, postoperative residual and recurrent stones occur in 20% of patients treated with these therapies[10]. Biliary-enteric anastomosis, which mostly includes choledochoduodenostomy and hepaticojejunostomy, is one of the most common procedures used for hepatolithiasis. However, our previous reports supported that choledochoduodenostomy was not an ideal approach to reduce cholangitis in hepatolithiasis and was not the best choice in the management of hepatolithiasis due to the loss of the anti-reflux function of the sphincter of Oddi[8,11]. Herman *et al*[12] found that patients who underwent liver resection associated with hepaticojejunostomy had a significantly higher recurrence rate of symptoms than patients submitted to liver resection alone (41.2% *vs* 0%, respectively, $P = 0.0006$). In the management of hepatolithiasis, we emphasize both complete eradication of hepatobiliary lesions and keeping the Oddi sphincter intact. In 1993, OSPCHS for hepatolithiasis was developed at our hospital and quickly spread to other hospitals in China[8].

Until now, the long-term outcomes of OSPCHS and risk factors for recurrence in hepatolithiasis have not been reported. In the current study, we reviewed the cases with hepatolithiasis treated surgically at our center in the past 20 years retrospectively and evaluated the long-term outcomes of OSPCHS and risk factors for recurrence in hepatolithiasis.

## MATERIALS AND METHODS

### *Patient data*

From March 1993 to December 2012, 202 hepatolithiasis cases who underwent OSPCHS were analyzed retrospectively. The patients ranged in age from 18 to 83 years with a median age of 55.0 years. The male to female ratio was 75:127. Of the 202 patients, 136 (67.6%) had at least one attack of acute cholangitis, 62 (30.7%) had previous biliary surgery, and 154 (76.2%) had extrahepatic bile duct stones. The demographic data of these patients are shown in Table 1. Preoperative evaluations included ultrasonography, computed tomography (CT), magnetic resonance cholangiopancreatography (MRCP), endoscopic retrograde cholangiopancreatography (ERCP), and/or percutaneous transhepatic cholangiography (PTC). The primary long-term outcome measure was stone recurrence. Secondary long-term outcome measures were the development of cholangiocarcinoma and mortality. Stone recurrence was defined as new bile duct stone formation after complete initial clearance.

### *Operative indications*

The indications for OSPCHS in patients with hepatolithiasis were as follows: (1) no evidence of sphincter of Oddi dysfunction; (2) no indication of common bile duct (CBD) resection; (3) hilar bile duct stenosis; (4) biliary strictures were not completely corrected with hepatectomy alone; and (5) bilateral or diffuse hepatolithiasis. The indications for simultaneous hepatectomy included: (1) hepatolithiasis associated

**Table 1 Patient characteristics *n* (%)**

| | Patients ($n$ = 202) |
|---|---|
| Age | 55 (18-83) |
| Male:female | 75:127 |
| Presentation | |
| Acute cholangitis | 36 (67.3) |
| Jaundice | 8 (4.0) |
| Liver abscess | 0 |
| Abdominal pain | 54 (26.7) |
| Acute pancreatitis | 4 (2.0) |
| Previous biliary operation | 62 |
| Cholecystectomy | 25 (12.4) |
| Cholecystectomy plus CBD exploration | 23 (11.4) |
| CBD exploration | 6 (3.0) |
| Hepatectomy | 8 (4.0) |
| Concomitant extrahepatic stones | 154 |
| GB stones | 14 (6.9) |
| CBD stones | 94 (46.5) |
| GB + CBD stones | 46 (22.8) |
| Stone location | |
| Left | 58 (28.7) |
| Right | 34 (16.8) |
| Both | 110 (54.4) |
| Intrahepatic stricture | 118 (58.4) |
| Liver atrophy | 52 (25.7) |
| Biliary cirrhosis | 27 (3.5) |

CBD: Common bile duct.

with biliary stenosis; (2) atrophy or fibrosis of the affected liver segment(s) or lobe; (3) the presence of liver abscess; (4) Child-Pugh A liver function without biliary cirrhosis; and (5) the patient's general condition was good and could tolerate the operation.

### *Surgical procedure*

A right subcostal incision was made. Exploration of the CBD and hepatic duct was conducted through the incision. First, the primary or secondary bile duct stricture rings were incised. The choledochofiberscope was employed to remove the stone. Large and impacted stones were fragmented with plasma shock wave lithotripsy (PSWL)[13]. Subsequently, the opened bile ducts were stitched together to form a "hepatobiliary basin" (Figure 1A). Second, a segment of the jejunum 12-15 cm long with a vascular pedicle was intercepted 15-20 cm away from the Treitz ligament, and was stretched upward to the hilar *via* the colon, during which care was taken to avoid twisting the mesentery (Figure 1B). The proximal end of the free jejunum was closed. The jejunum segment was then flushed using 0.9% saline *via* the distal end. End-to-end anastomosis was performed between the proximal and distal ends of the jejunum. The third step consisted of reconstructing a subcutaneous stoma. Specifically, side-to-end anastomosis was performed between the distal end of the free jejunum segment and the "hepatobiliary basin" with an anastomotic diameter of approximately 3-5 cm (Figure 1C). The proximal end of the free jejunum segment was settled subcutaneously in the upper

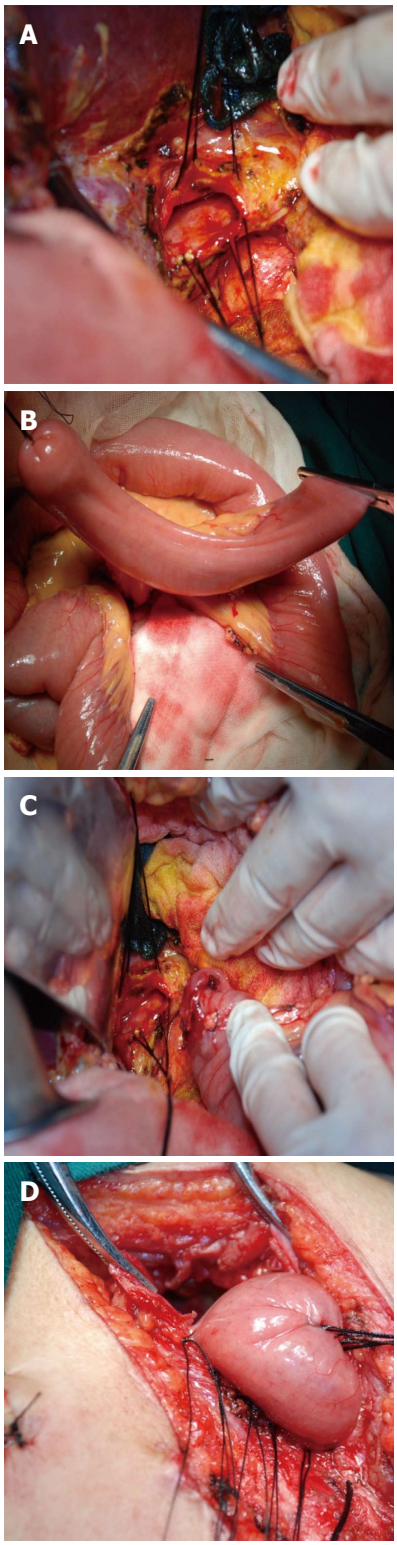



**Figure 1 Connecting the opened neighboring bile ducts to form a "hepatobiliary basin" (A); Obtaining a 12-15 cm free jejunum segment with a vascular pedicle (B); Performing a side-to-end anastomosis between the distal end of the free jejunum segment and the "hepatobiliary basin" (C); and Embedment in the skin after the closure of the free jejunum segment at its proximal end (D).**

1/3 segment of the incision, and was marked on the skin (Figure 1D). During the operations, all patients received cholangiography, CBD exploration, and T tube

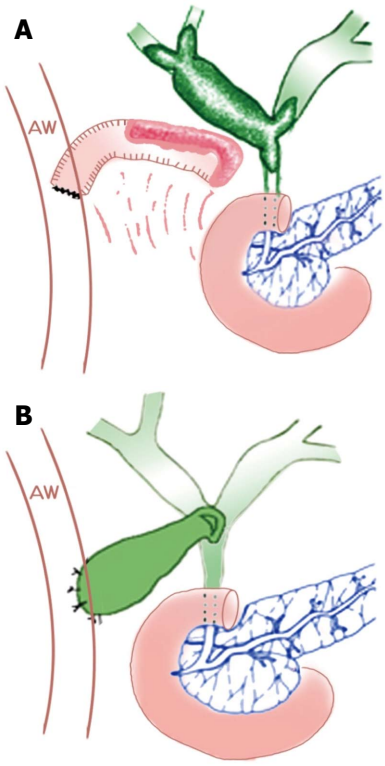



**Figure 2 Oddi sphincter preserved cholangioplasty with hepatico-subcutaneous stoma in which the free jejunum segment served as the subcutaneous stoma (A) or in which the gallbladder served as the subcutaneous stoma (B).** AW: Abdominal wall.

drainage. Drains were placed routinely in the Winslow foramen and/or the subphrenic space in all patients. There are two types of subcutaneous stoma: free jejunum as a subcutaneous stoma (Figure 2A) and gallbladder as a subcutaneous stoma (Figure 2B). The procedure using the gallbladder as a subcutaneous stoma has been previously described by our group[14]. If there were indications for liver resection, the stone-affected liver was also resected.

### Follow-up

All patients had regular follow-up every 3 mo for the first year, and twice a year thereafter. The symptoms, physical examination, liver function tests and abdominal ultrasound, CT or MRCP were recorded. The median follow-up period was 78.5 mo (range: 2-233 mo).

### Statistical analysis

Demographic and follow-up data of all patients were collected. Data were analyzed retrospectively. Overall survival was measured from the date of surgery to the time of death. Grouped data are expressed as median (range). Univariate analysis was conducted using $\chi^2$ test. Cumulative rates of stone recurrence were analyzed by the Kaplan-Meier and log-rank test. The Cox regression model was used to analyze potential risk factors associated with stone recurrence. $P < 0.05$ was considered statistically significant. Data analysis was performed using the SPSS version 22 (IBM, Armonk, New York, United States).

**Table 2 Types of operation**

| | Type of operation | *n* (%) |
|---|---|---|
| Subcutaneous stoma (*n* = 202) | Gallbladder as a subcutaneous stoma | 99 (49.0) |
| | Free jejunum as a subcutaneous stoma | 103 (51.0) |
| Hepatectomy (*n* = 97) | Left lateral sectionectomy | 65 (67.0) |
| | Left hepatectomy | 19 (19.6) |
| | Segmentectomy of segment 6 | 5 (5.2) |
| | Segmentectomy of segment 5 | 1 (1.0) |
| | Left hepatectomy + right anterior segmentectomy | 1 (1.0) |
| | Segmentectomy of segment 3 | 1 (1.0) |
| | Segmentectomy of segment 5 + segment 6 | 1 (1.0) |
| | Segmentectomy of segment 6 + segment 7 | 3 (3.1) |
| | Segmentectomy of segment 7 + segment 8 | 1 (1.0) |

## RESULTS

### Intraoperative results

The operative procedures are listed in Table 2. Among the 202 patients, 105 underwent OSPCHS alone, and 97 underwent OSPCHS combined with hepatectomy. Left lateral sectionectomy was the major procedure for hepatolithiasis, followed by left hepatectomy.

### Short-term outcomes

The short-term outcomes are shown in Table 3. The overall operative morbidity and hospital mortality rates were 10.4% and 0%, respectively. The most common complication was biliary leakage, followed by wound infection. Three patients (bile leakage = 1, hemobilia = 1, intra-abdominal bleeding = 1) received reoperations, and the remaining 18 cases underwent conservative management and recovered. The immediate stone clearance rate was 72.8%, and after additional choledochoscopic lithotripsy, the final stone clearance rate was 97.0%. Residual stones could not be completely removed in 6 patients, because of the uncorrected bile duct stenosis ($n$ = 1) and stone location at peripheral bile ducts ($n$ = 5) unreachable by cholangioscopy.

### Long-term outcomes

The long-term outcomes are shown in Table 4. With a median follow-up period of 78.5 mo, 50 (24.8%) patients developed recurrent stones. Among these, 28 (13.9%) appeared as acute cholangitis, which was cured using subcutaneous-stoma drainage plus antibiotics. Forty-four of fifty patients underwent stone extraction through subcutaneous stoma, while 2 patients refused. Recurrent stones were completely removed in 42 of 50 patients after 1 to 7 sessions

**Table 3 Operative short-term outcomes**

| Short-term outcome | Patients ($n$ = 202) | Management |
|---|---|---|
| Complications | 21 (10.4) | |
| Biliary leakage | 9 (4.5) | Observation for 8 patients, and reoperation for 1 patient |
| Wound infection | 5 (2.5) | Dressing |
| Intra-abdominal bleeding | 2 (1.0) | Observation for 1 patient, and reoperation for 1 patient |
| Hemobilia | 2 (1.0) | Observation for 1 patient, and reoperation for 1 patient |
| Low limb thrombosis | 1 (0.5) | Observation |
| Pleural effusion | 1 (0.5) | Observation |
| Abdominal fungal infection | 1 (0.5) | Antibiotic |
| Perioperative mortality | 0 | |
| Immediate stone clearance | 147 (72.8) | |
| Final stone clearance | 196 (97.0) | |

**Table 4 Long-term outcomes of surgery**

| Long-term outcome | $n$ (%) |
|---|---|
| Stone recurrence | 50 (24.8) |
| Recurrent attack of acute cholangitis | 28 (13.9) |
| Utilization of subcutaneous stoma | 42 (20.8) |
| Clearance of recurrent stones | 42 (84.0) |
| Development of cholangiocarcinoma | 5 (2.5) |
| Death | 11 (5.4) |

**Table 5 Risk factors associated with stone recurrence identified with Cox regression model**

| Variable | Univariate analysis, $P$ value | Multivariate analysis | | |
|---|---|---|---|---|
| | | HR | 95%CI | $P$ value |
| Age | 0.572 | | | |
| Gender | 0.883 | | | |
| Previous biliary surgery | 0.237 | | | |
| Biliary stenosis | 0.689 | | | |
| Extrahepatic stones | 0.417 | | | |
| Stone distribution | 0.217 | | | |
| Hepatectomy | 0.191 | | | |
| Types of subcutaneous stoma | 0.414 | | | |
| Residual stones | 0.008 | 2.587 | 1.396-4.795 | 0.003 |

of cholangioscopic lithotomy. After the complete eradication of recurrent stones, the subcutaneous stoma was then closed. The final clearance rate of recurrent stones was 84.0%.

Eleven patients died during the follow-up, and 6 deaths were not related to the hepatolithiasis. Five (2.5% of the total) cases died of hepatolithiasis associated cholangiocarcinoma, 4 of whom developed stone recurrence and unresectable intrahepatic cholangiocarcinoma, and the rest 1 patient underwent radical operation. The total mortality rate was 5.4% at the end of follow-up.

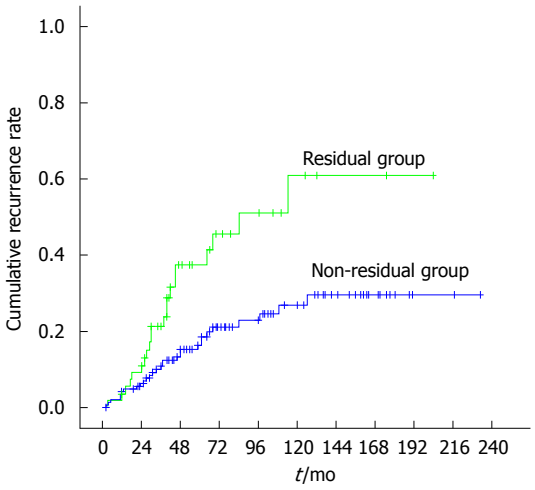



**Figure 3 Cumulative stone recurrence curves of the residual group ($n$ = 55) and non-residual group ($n$ = 147) (log-rank test, $P$ = 0.001).**

### *Risk factors for stone recurrence*

A total of nine factors were selected to identify the potential risk factors possibly associated with postoperative stone recurrence, including six patient factors [age (≥ 50 years *vs* < 50 years), gender (male *vs* female), biliary stenosis (yes *vs* no), previous biliary surgery (yes *vs* no), extrahepatic stones (yes *vs* no), and stone distribution (unilateral *vs* bilateral)], two operation factors [hepatectomy (yes *vs* no) and subcutaneous stoma type (gallbladder *vs* free jejunum)], and one postoperative factor [residual stones (yes *vs* no)]. All factors were analyzed with the Cox regression model, which indicated that only residual stone was an independent prognostic factor for stone recurrence (Table 5). The stone recurrence rate was significantly higher in patients with residual stones, and there was a significant difference in the cumulative stone recurrence rate between the two groups as revealed by the Kaplan-Meier log-rank test ($P$ = 0.001) (Figure 3).

## DISCUSSION

Hepatolithiasis is usually complicated by recurrent cholangitis, pancreatitis and liver abscess, and it even leads to secondary biliary cirrhosis and cholangiocarcinoma[15]. In China, many investigators and surgeons have been exploring this issue for more than half a century. The famous basic principle of hepatolithiasis management, "clearance of stones, correction of strictures, removal of hepatobiliary lesions, and restoration of bile drainage", has been widely acknowledged in China and around the world[2,16,17]. Following these principles, the definitive surgical approach for each patient should be designed individually according to the divergent hepatobiliary lesions.

Theoretically, partial hepatectomy is the most definitive treatment for hepatolithiasis because it removes all of the hepatic stones and strictured bile ducts within the resected liver segment(s), thus reducing the subsequent risk of recurrent stones and cholangiocarcinoma[3,7,18,19]. However, approximately 50% of intrahepatic lesions of stones and ductal strictures are distributed bilaterally, even diffusely. Under these complicated conditions, thorough resection of the affected liver is impossible. Uncorrected strictures inevitably result in recurrent cholangitis. We found that keeping the function of the sphincter of Oddi intact is useful to reduce cholangitis in patients with uncorrected strictures or remnant stones due to the anti-reflux function of the sphincter of Oddi[11]. Therefore, it is suggested that the sphincter of Oddi should be preserved if there is no laxity or restriction.

According to the review of the previous literature, the present study is so far the only one that evaluated the long-term results of OSPCHS and factors for recurrence in hepatolithiasis. The pivot of OSPCHS is to ensure that the sphincter of Oddi has normal function. However, it is difficult to make a definitive evaluation preoperatively, although ultrasonography, CT, ERCP, MRCP, PTC, and intraoperative finding can provide useful data. Tan *et al*[2] proposed that the functional status of the sphincter of Oddi was accurately assessed by choledochoscopic manometry *via* T-tube or by peroral endoscopic manometry postoperatively. We have established a relatively simple method for intraoperative judgement: if the intraoperative exploration shows that a 16-Fr catheter can pass through the orifice of the duodenum ampulla, the sphincter of Oddi can be considered to have normal function.

Residual and recurrent stones are the most troublesome problem after treatment for hepatolithiasis[7]. Therefore, absent residual or recurrent stones and cholangitis were regarded as an important way to evaluate the efficacy of all modalities for hepatolithiasis treatment. The incidence of residual stones has been markedly reduced from 62.3% to 19.8%[10,16]. In our study, the residual stone rate after OSPCHS for hepatolithiasis was 27.2% and was comparable with the two above-mentioned reports. This study indicated that residual stones was an independent risk factor for stone recurrence. According to our clinical practice, the intraoperative key in treating complicated hepatolithiasis is resection of the affected liver, correction of strictures and removal of impacted stones. It is suggested that the appropriate time frame for the operation is within 6 h, and additional postoperative choledochoscopic lithotripsy can significantly improve the stone clearance rate. In this study, the final stone clearance rate was much higher (97%) with the use of choledochoscopy and PSWL compared with the immediate stone clearance rate (72.8%).

Intraoperative ultrasonic scanning can detect stones as small as 2 mm with an accuracy rate of 98.7%[20]. Application of the intraoperative ultrasound-guided fiberoptic choledochoscope can significantly reduce the residual stone rate of intrahepatic biliary calculi (5.4% *vs* 19%, respectively, $P = 0.025$) and significantly improve the efficacy of hepatolithiasis[21]. With the rapid development of computer technology, digital medicine has become a new direction in surgery. Compared with traditional hepatectomy, hepatectomy for hepatolithiasis based on 3-dimensional reconstruction technique had a significantly lower stone residual rate (0% *vs* 9.5%) and stone recurrence rate (3.6% *vs* 23.8%)[16].

With the advent of endoscopic and image-directed percutaneous approaches, it is increasingly uncommon to require a surgical approach for recurrent stones[22,23]. Various noninvasive procedures, such as PTCSL and peroral cholangioscopy, have been reported[24,25]. Although PTCSL has been widely applied in Western countries with the advantage of avoiding operation, there still exist complications, including low stone clearance, hemobilia, and percutaneous tract dilation, which may incur pain to the patients[2,26]. Similarly, peroral cholangioscopic lithotomy also attains a lower stone clearance rate (64%) as reported in the previous report[27]. For the convenience in the eradication of recurrent stones, Beckingham *et al*[28] designed a subparietal hepaticojejunal access loop and indicated that the access loop permits long-term access to the intrahepatic ducts, allowing removal of stones with minimal patient discomfort and low morbidity. However, this approach permanently undermines the physiological anti-reflux function of the sphincter of Oddi, possibly increasing the recurrence rate of stones.

In 1993, we developed a new operative procedure in which hepaticoplasty was performed using a free segment of the jejunum or gallbladder for a subcutaneous stoma and preservation of the sphincter of Oddi[14]. Its aim was to set up a tunnel between the biliary duct and tela subcutanea to remove recurrent stones and drain the bile in recurrent cholangitis in a minimally invasive way. In our study, patients with recurrent stones underwent stone clearance and biliary drainage *via* the subcutaneous stoma which can be incised under local anesthesia whenever the patients present with symptoms suggestive of cholangitis. The follow-up data showed that subcutaneous stoma offers the advantage of permanent percutaneous access to intrahepatic ducts and is readily available for reusage subsequently without the need for further surgery if stones recur at a later stage.

Hepatolithiasis is a well-known risk factor for the occurrence of intrahepatic cholangiocarcinoma[1,29]. The occurrence rate of intrahepatic cholangiocarcinoma in patients with hepatolithiasis ranges from 2% to 12%[30]. In our study, 2.5% of patients developed cholangiocarcinoma during follow-up. The development of cholangiocarcinoma is the main factor com-

promising the long-term survival in patients with hepatolithiasis[1,16]. Unfortunately, regardless of advances in diagnosis modalities, diagnosing cholangiocarcinoma in patients with hepatolithiasis remains challenging, and the diagnostic rate has been reported to span from 0% to 37.5%[16]. Hence, a suspicion of malignancy is necessary when managing patients with hepatolithiasis, and careful follow-up for cholangiocarcinoma is needed.

However, this study has its limitations. As it is not a prospective and randomized controlled trial and was conducted in a single center, it may somewhat produce some bias. Clinically, it is very difficult to design a control group in which the patients' Oddi sphincter will be damaged iatrogenically. In the near future we will carry out a survey in multiple centers to further investigate the advantages and disadvantages of OSPCHS.

In summary, this study shows that OSPCHS can be a safe and effective treatment option for well-selected patients with hepatolithiasis, and it achieves excellent long-term outcomes. A combination of different treatment modalities is conducive to decreasing the residual stone rate and improving the results of patients with hepatolithiasis.

## COMMENTS

### Background

Hepatolithiasis is a prevalent disease in China, and surgical treatment is needed because of the serious complications such as acute cholangitis and biliary cirrhosis. However, the long-term outcomes of Oddi sphincter preserved cholangioplasty with hepatico-subcutaneous stoma (OSPCHS) and risk factors for recurrence in hepatolithiaisis remain unclear.

### Research frontiers

Many reports have showed that partial hepatectomy is the most definitive treatment for hepatolithiasis. However, the high incidence of residual and recurrent stones is still a major challenge. The high incidence of reflux cholangitis caused by traditional hepaticojejunostomy is the major complication during follow-up. In this study, the authors tried to introduce an optional approach for effective and minimally invasive therapy of recurrent stones.

### Innovations and breakthroughs

The authors created an optional technique (OSPCHS) for hepatolithiasis. The hepatobiliary lesions could be resected, with simultaneous construction of a subcutaneous stoma. The subcutaneous stoma allowed to remove recurrent stones or drain the inflammatory bile and avoid major reoperation. The high incidence of reflux cholangitis caused by hepaticojejunostomy could be avoided by preserving the sphincter of Oddi.

### Applications

Lian YG *et al* performed OSPCHS for treatment of hepatolithiasis. OSPCHS achieves good long-term outcomes, and residual stone is an independent prognostic factor for stone recurrence. This study may present a strategy for treatment of complicated hepatolithiasis and patients with a high risk of recurrent stones.

### Terminology

OSPCHS in well-selected patients is safe and effective for treatment of hepatolithiasis.

### Peer-review

This manuscript addresses the outcome of OSPCHS for hepaticolithiasis. This paper is interesting.

## REFERENCES


1 **Li SQ**, Liang LJ, Peng BG, Hua YP, Lv MD, Fu SJ, Chen D. Outcomes of liver resection for intrahepatic stones: a comparative study of unilateral versus bilateral disease. *Ann Surg* 2012; **255**: 946-953 [PMID: 22504194 DOI: 10.1097/SLA.0b013e31824dedc2]

2 **Tan J**, Tan Y, Chen F, Zhu Y, Leng J, Dong J. Endoscopic or laparoscopic approach for hepatolithiasis in the era of endoscopy in China. *Surg Endosc* 2015; **29**: 154-162 [PMID: 25027471 DOI: 10.1007/s00464-014-3669-5]

3 **Yang T**, Lau WY, Lai EC, Yang LQ, Zhang J, Yang GS, Lu JH, Wu MC. Hepatectomy for bilateral primary hepatolithiasis: a cohort study. *Ann Surg* 2010; **251**: 84-90 [PMID: 20032719 DOI: 10.1097/SLA.0b013e3181b2f374]

4 **Vetrone G**, Ercolani G, Grazi GL, Ramacciato G, Ravaioli M, Cescon M, Varotti G, Del Gaudio M, Quintini C, Pinna AD. Surgical therapy for hepatolithiasis: a Western experience. *J Am Coll Surg* 2006; **202**: 306-312 [PMID: 16427557 DOI: 10.1016/j.jamcollsurg.2005.09.022]

5 **Lai EC**, Ngai TC, Yang GP, Li MK. Laparoscopic approach of surgical treatment for primary hepatolithiasis: a cohort study. *Am J Surg* 2010; **199**: 716-721 [PMID: 19959158 DOI: 10.1016/j.amjsurg.2009.02.007]

6 **Yoon YS**, Han HS, Shin SH, Cho JY, Min SK, Lee HK. Laparoscopic treatment for intrahepatic duct stones in the era of laparoscopy: laparoscopic intrahepatic duct exploration and laparoscopic hepatectomy. *Ann Surg* 2009; **249**: 286-291 [PMID: 19212183 DOI: 10.1097/SLA.0b013e31818eea56]

7 **Cheon YK**, Cho YD, Moon JH, Lee JS, Shim CS. Evaluation of long-term results and recurrent factors after operative and nonoperative treatment for hepatolithiasis. *Surgery* 2009; **146**: 843-853 [PMID: 19744434 DOI: 10.1016/j.surg.2009.04.009]

8 **Ling XF**, Xu Z, Wang LX, Hou CS, Xiu DR, Zhang TL, Zhou XS. Long-term outcomes of choledochoduodenostomy for hepatolithiasis. *Chin Med J* (Engl) 2010; **123**: 137-141 [PMID: 20137359 DOI: 10.3760/cma.j.issn.0366-6999.2010.02.002]

9 **Nuzzo G**, Clemente G, Giovannini I, De Rose AM, Vellone M, Sarno G, Marchi D, Giuliante F. Liver resection for primary intrahepatic stones: a single-center experience. *Arch Surg* 2008; **143**: 570-573; discussion 574 [PMID: 18559750 DOI: 10.1001/archsurg.143.6.570]

10 **Uchiyama K**, Kawai M, Ueno M, Ozawa S, Tani M, Yamaue H. Reducing residual and recurrent stones by hepatectomy for hepatolithiasis. *J Gastrointest Surg* 2007; **11**: 626-630 [PMID: 17468921 DOI: 10.1007/s11605-006-0024-8]

11 **Ling X**, Xu Z, Wang L, Hou C, Xiu D, Zhang T, Zhou X. Is Oddi sphincterotomy an indication for hepatolithiasis? *Surg Endosc* 2009; **23**: 2268-2272 [PMID: 19184214 DOI: 10.1007/s00464-008-0304-3]

12 **Herman P**, Perini MV, Pugliese V, Pereira JC, Machado MA, Saad WA, D'Albuquerque LA, Cecconello I. Does bilioenteric anastomosis impair results of liver resection in primary intrahepatic lithiasis? *World J Gastroenterol* 2010; **16**: 3423-3426 [PMID: 20632446 DOI: 10.3748/wjg.v16.i27.3423]

13 **Xu Z**, Wang L, Zhang N, Deng S, Xu Y, Zhou X. Clinical applications of plasma shock wave lithotripsy in treating postoperative remnant stones impacted in the extra- and intrahepatic bile ducts. *Surg Endosc* 2002; **16**: 646-649 [PMID: 11972206 DOI: 10.1007/s00464-001-8164-2]

14 **Cui L**, Xu Z, Ling XF, Wang LX, Hou CS, Wang G, Zhou XS. Laparoscopic hepaticoplasty using gallbladder as a subcutaneous tunnel for hepatolithiasis. *World J Gastroenterol* 2014; **20**: 3350-3355 [PMID: 24695884 DOI: 10.3748/wjg.v20.i12.3350]

15 **Cheung MT**, Wai SH, Kwok PC. Percutaneous transhepatic

choledochoscopic removal of intrahepatic stones. *Br J Surg* 2003; **90**: 1409-1415 [PMID: 14598423 DOI: 10.1002/bjs.4327]
16 **Fang CH**, Liu J, Fan YF, Yang J, Xiang N, Zeng N. Outcomes of hepatectomy for hepatolithiasis based on 3-dimensional reconstruction technique. *J Am Coll Surg* 2013; **217**: 280-288 [PMID: 23870220 DOI: 10.1016/j.jamcollsurg.2013.03.017]
17 **Dong J**, Lau WY, Lu W, Zhang W, Wang J, Ji W. Caudate lobe-sparing subtotal hepatectomy for primary hepatolithiasis. *Br J Surg* 2012; **99**: 1423-1428 [PMID: 22961524 DOI: 10.1002/bjs.8888]
18 **Cho JY**, Han HS, Yoon YS, Shin SH. Outcomes of laparoscopic liver resection for lesions located in the right side of the liver. *Arch Surg* 2009; **144**: 25-29 [PMID: 19153321 DOI: 10.1001/archsurg.2008.510]
19 **Lee TY**, Chen YL, Chang HC, Chan CP, Kuo SJ. Outcomes of hepatectomy for hepatolithiasis. *World J Surg* 2007; **31**: 479-482 [PMID: 17334864 DOI: 10.1007/s00268-006-0441-6]
20 **Li F**, Cheng J, He S, Li N, Zhang M, Dong J, Jiang L, Cheng N, Xiong X. The practical value of applying chemical biliary duct embolization to chemical hepatectomy for treatment of hepatolithiasis. *J Surg Res* 2005; **127**: 131-138 [PMID: 15936771 DOI: 10.1016/j.jss.2005.02.029]
21 **Pan W**, Xu E, Fang H, Deng M, Xu R. Surgical treatment of complicated hepatolithiasis using the ultrasound-guided fiberoptic choledochoscope. *Surg Endosc* 2011; **25**: 497-502 [PMID: 20614140 DOI: 10.1007/s00464-010-1200-1]
22 **Matsushima K**, Soybel DI. Operative management of recurrent choledocholithiasis. *J Gastrointest Surg* 2012; **16**: 2312-2317 [PMID: 22907776 DOI: 10.1007/s11605-012-1968-5]
23 **Tsuyuguchi T**, Miyakawa K, Sugiyama H, Sakai Y, Nishikawa T, Sakamoto D, Nakamura M, Yasui S, Mikata R, Yokosuka O. Ten-year long-term results after non-surgical management of hepatolithiasis, including cases with choledochoenterostomy. *J Hepatobiliary Pancreat Sci* 2014; **21**: 795-800 [PMID: 25070702 DOI: 10.1002/jhbp.134]
24 **Uenishi T**, Hamba H, Takemura S, Oba K, Ogawa M, Yamamoto T, Tanaka S, Kubo S. Outcomes of hepatic resection for hepatolithiasis. *Am J Surg* 2009; **198**: 199-202 [PMID: 19249730 DOI: 10.1016/j.amjsurg.2008.08.020]
25 **Lee SE**, Jang JY, Lee JM, Kim SW. Selection of appropriate liver resection in left hepatolithiasis based on anatomic and clinical study. *World J Surg* 2008; **32**: 413-418 [PMID: 18196323 DOI: 10.1007/s00268-007-9355-1]
26 **Chen C**, Huang M, Yang J, Yang C, Yeh Y, Wu H, Chou D, Yueh S, Nien C. Reappraisal of percutaneous transhepatic cholangioscopic lithotomy for primary hepatolithiasis. *Surg Endosc* 2005; **19**: 505-509 [PMID: 15959714 DOI: 10.1007/s00464-004-8125-5]
27 **Okugawa T**, Tsuyuguchi T, K C S, Ando T, Ishihara T, Yamaguchi T, Yugi H, Saisho H. Peroral cholangioscopic treatment of hepatolithiasis: Long-term results. *Gastrointest Endosc* 2002; **56**: 366-371 [PMID: 12196774 DOI: 10.1016/s0016-5107(02)70040-0]
28 **Beckingham IJ**, Krige JE, Beningfield SJ, Bornman PC, Terblanche J. Subparietal hepaticojejunal access loop for the long-term management of intrahepatic stones. *Br J Surg* 1998; **85**: 1360-1363 [PMID: 9782013 DOI: 10.1046/j.1365-2168.1998.00827.x]
29 **Kim HJ**, Kim JS, Suh SJ, Lee BJ, Park JJ, Lee HS, Kim CD, Bak YT. Cholangiocarcinoma Risk as Long-term Outcome After Hepatic Resection in the Hepatolithiasis Patients. *World J Surg* 2015; **39**: 1537-1542 [PMID: 25648078 DOI: 10.1007/s00268-015-2965-0]
30 **Tabrizian P**, Jibara G, Shrager B, Schwartz ME, Roayaie S. Hepatic resection for primary hepatolithiasis: a single-center Western experience. *J Am Coll Surg* 2012; **215**: 622-626 [PMID: 22921329 DOI: 10.1016/j.jamcollsurg.2012.07.005]

**P- Reviewer:** Shrestha A **S- Editor**: Ma YJ **L- Editor**: Wang TQ
**E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12873

*World J Gastroenterol* 2015 December 7; 21(45): 12873-12881
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

Retrospective Study

# Updated experiences with minimally invasive McKeown esophagectomy for esophageal cancer

Ju-Wei Mu, Shu-Geng Gao, Qi Xue, You-Sheng Mao, Da-Li Wang, Jun Zhao, Yu-Shun Gao, Jin-Feng Huang, Jie He

Ju-Wei Mu, Shu-Geng Gao, Qi Xue, You-Sheng Mao, Da-Li Wang, Jun Zhao, Yu-Shun Gao, Jin-Feng Huang, Jie He, Department of Thoracic Surgical Oncology, Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

**Author contributions:** Mu JW and He J designed and performed the research, analyzed the data and wrote the manuscript; Gao SG, Xue Q, Mao YS, Wang DL, Zhao J, Gao YS and Huang JF performed the research.

**Supported by** The Fund of Capital Health Technology Development Priorities Research Project, No. 2014-1-4021.

**Institutional review board statement:** Institutional Review Board of Cancer Hospital, Chinese Academy of Medical Science reviewed and approved this study.

**Informed consent statement:** Informed consent was given by patients preoperatively. All included patients accepted the possibility to collect their patient data.

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

**Data sharing statement:** No additional data are available.



**Correspondence to:** Jie He, MD, PhD, Professor, Department of Thoracic Surgical Oncology, Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China. hejie@cicams.ac.cn
**Telephone:** +86-10-87788207
**Fax:** +86-10-67787079

**Received:** September 12, 2015
**Peer-review started:** September 15, 2015
**First decision:** October 22, 2015
**Revised:** October 27, 2015
**Accepted:** November 9, 2015
**Article in press:** November 9, 2015
**Published online:** December 7, 2015

## Abstract

**AIM:** To update our experiences with minimally invasive McKeown esophagectomy for esophageal cancer.

**METHODS:** We retrospectively reviewed the medical records of 445 consecutive patients who underwent minimally invasive McKeown esophagectomy between January 2009 and July 2015 at the Cancer Hospital of Chinese Academy of Medical Sciences and used 103 patients who underwent open McKeown esophagectomy in the same period as controls. Among 375 patients who underwent total minimally invasive McKeown esophagectomy, 180 in the early period were chosen for the study of learning curve of total minimally invasive McKeown esophagectomy. These 180 minimally invasive McKeown esophagectomies performed by five surgeons were divided into three groups according to time sequence as group 1 ($n$ = 60), group 2 ($n$ = 60) and group 3 ($n$ = 60).

**RESULTS:** Patients who underwent total minimally invasive McKeown esophagectomy had significantly less intraoperative blood loss than patients who underwent hybrid minimally invasive McKeown esophagectomy or open McKeown esophagectomy (100 mL *vs* 300 mL *vs* 200 mL, $P$ = 0.001). However, there were no significant differences in operation time, number of harvested lymph nodes, or postoperative morbidity including

incidence of pulmonary complication and anastomotic leak between total minimally invasive McKeown esophagectomy, hybrid minimally invasive McKeown esophagectomy and open McKeown esophagectomy groups. There were no significant differences in 5-year survival between these three groups (60.5% *vs* 47.9% *vs* 35.6%, $P$ = 0.735). Patients in group 1 had significantly longer duration of operation than those in groups 2 and 3. There were no significant differences in intraoperative blood loss, number of harvested lymph nodes, or postoperative morbidity including incidence of pulmonary complication and anastomotic leak between groups 1, 2 and 3.

**CONCLUSION:** Total minimally invasive McKeown esophagectomy was associated with reduced intraoperative blood loss and comparable short term and long term survival compared with hybrid minimally invasive McKeown esophagectomy or open Mckeown esophagectomy. At least 12 cases are needed to master total minimally invasive McKeown esophagectomy in a high volume center.

**Key words:** Surgical procedures; Minimally invasive; Esophagectomy; Outcome; Learning curve



**Core tip:** Total minimally invasive McKeown esophagectomy had reduced intraoperative blood loss and comparable short term and long term survival compared with hybrid minimally invasive McKeown esophagectomy or open Mckeown esophagectomy. At least 12 cases are needed to master total minimally invasive McKeown esophagectomy in a high volume cancer center.

Mu JW, Gao SG, Xue Q, Mao YS, Wang DL, Zhao J, Gao YS, Huang JF, He J. Updated experiences with minimally invasive McKeown esophagectomy for esophageal cancer. *World J Gastroenterol* 2015; 21(45): 12873-12881 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12873.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12873

## INTRODUCTION

Esophageal cancer is a growing concern and is the eighth most common cancer worldwide[1]. According to statistics of esophageal cancer in China, the incidence and death rates were 22.14 per 100000 person-years and 16.77 per 100000 person-years in 2009, respectively, being the top one in the world[2]. For resectable carcinoma of the esophagus, surgery remains the gold standard of treatment. Minimally invasive esophagectomy (MIE) was introduced into clinical practice in 1992 in order to minimize the surgical injury reaction and reduce the morbidity and mortality rates of esophagectomy[3]. However, concerns existed for whether MIE may reduce systematic inflammatory response syndrome and provide comparable oncologic clearance with open esophagectomy even 5 years ago[4].

In the past 5 years, several studies including one randomized controlled trial reported reduced postoperative pulmonary complication rates, comparable oncologic clearance and similar long term survival between MIE and open esophagectomy[5-14]. Our previous study demonstrated reduced morbidity rate and comparable oncologic clearance in minimally invasive McKeown esophagectomy group compared with open McKeown esophagectomy[9]. We started minimally invasive McKeown esophagectomy in 2009. Here, we will review these minimally invasive McKeown esophagectomies and focus on short term outcome, long term survival and learning curve of minimally invasive McKeown esophagectomy.

## MATERIALS AND METHODS

### *General information*

This study was approved by the Institutional Review Board of Cancer Hospital, Chinese Academy of Medical Science. The medical records of 445 consecutive patients who underwent minimally invasive McKeown esophagectomy between January 2009 and July 2015 at the Cancer Hospital of Chinese Academy of Medical Sciences were retrospectively reviewed. In the same period, 103 patients received open McKeown esophagectomy. The clinical variables of the paired groups were compared, including age, sex, body mass index (BMI), neoadjuvant therapy, tumor location, duration of operation, intraoperative blood loss, number of harvested lymph nodes, differentiation, American Joint Committee on Cancer (AJCC) stage, morbidity rate, rate of anatomic leakage, pulmonary morbidity rate, mortality rate and length of hospital stay. Esophageal cancer staging was carried out according to the AJCC 2009 cancer staging system[15]. All involved patients gave their informed consent prior to study inclusion. A randomized, controlled trial of neoadjuvant treatment has shown a survival benefit in locally advanced esophageal carcinoma as compared with esophagectomy alone in 2012[16]. Since then, we adopted chemotherapy or chemoradiotherapy as an alternative for locally advanced esophageal cancer.

### *Surgical technique*

MIE includes total minimally invasive McKeown esophagectomy and hybrid minimally invasive McKeown esophagectomy[8]. The former consists of thoracoscopic esophagectomy, laparoscopic gastric preparation and gastroesophageal cervical anastomosis, while there are thoracoscopic esophagectomy plus open gastric preparation or laparoscopic gastric preparation plus open esophagectomy in the hybrid minimally invasive

McKeown esophagectomy group. Since 2009, total minimally invasive McKeown esophagectomy has been introduced and in use. The selection criteria for patients to either total MIE, hybrid MIE or open esophagectomy were mainly based on the clinical stage and the experiences of surgeons. Patients with early stage esophageal cancer received more minimally invasive esophagectomies than open esophagectomies, and surgeons who received training in minimally invasive thoracic surgery more performed minimally invasive esophagectomies than open esophagectomies.

### *Minimally invasive McKeown esophagectomy*

**Thoracoscopic phase:** The patient was placed in the left lateral decubitus position. The position of the double-lumen tube was verified, and single-lung ventilation was used. Four thoracoscopic ports were established. A 10 mm port was placed at the seventh intercostal space, just along the anterior axillary line, for the camera. Another 10 mm port was placed at the eighth or ninth intercostal space, posterior to the axillary line, for the dissection instrument (ultrasonic coagulating shears) and passage of the end-to-end circular stapler (EEA; Covidien or Johnson) or Hem-lock. A 5 mm port was placed in the anterior axillary line, at the third or fourth intercostal space, and this was used to pass a fan-shaped retractor to retract the lung anteriorly and allow exposure of the esophagus. A 5 mm port was placed just below the subscapular tip to place the instruments for retraction and counter traction. The inferior pulmonary ligament was divided. The mediastinal pleura overlying the esophagus was divided and opened to the level of the azygous vein to expose the thoracic esophagus. The azygous vein was then dissected and divided with an endoscopic vascular stapler or Hem-lock. The thoracic esophagus, alone with the periesophageal tissue and mediastinal lymph nodes, was circumferentially mobilized from the diaphragm to the level of inlet of the thorax. Mediastinal lymphadenectomy was done for every patient, and the resected lymph nodes included left recurrent and right subclavian,paratracheal, subcarinal, left and right bronchial, lower posterior mediastinum, para-aortic, and para-oesophageal lymph nodes. The chest was inspected closely, and hemostasis was verified. Chest tube was routinely placed.

**Laparoscopic phase:** The patient was placed in a supine position. A pneumoperitoneum (12-14 cm $H_2O$) was established by $CO_2$ injection through an umbilical port. A total of five abdominal ports (three 5 mm and two 10 mm) were used. After placement of the ports, the first step of the laparoscopic phase was an exploration of the abdomen to rule out advanced disease. The mobilization of the stomach was started with the division of the greater curvature using a Harmonic scalpel (Ethicon Endo-Surgery, OH, United States). The short gastric vessels were then divided. The gastrocolic omentum was then divided, with care taken to preserve the right gastroepiploic artery. The posterior attachments of the stomach were then divided after retraction of the stomach anteriorly. The left gastric vessel was divided at its origin from the celiac trunk with an endoscopic gastrointestinal anastomosis (GIA) stapler or Hem-lock. Lymphatic tissues around vessels were included in the resection. Subsequently, the right crus was visualized and dissected, followed by dissecting and defining the left crura of the diaphragm. The abdominal/distal esophagus was dissected as far as possible toward the distal end. The gastric conduit was made extracorporeally. Pyloroplasty or gastric drainage procedure was not routinely performed in our study. We inserted duodenal nutrition tube before anastomosis in the operation. The abdomen was inspected to make sure that hemostasis was adequate and the incisions were closed.

**Cervical anastomosis:** After laparoscopic phase and thoracoscopic phase, a 4 to 6 cm horizontal neck incision was made to expose the cervical esophagus. Careful dissection was performed down until the thoracic dissection plane was encountered, generally quite easily since the VATS dissection was continued well into the thoracic inlet. The esophagogastric specimen was pulled out of the neck incision and the cervical esophagus divided high. The specimen was removed from the field. An anastomosis was performed between the cervical esophagus and gastric tube using standard techniques (mechanical stapled or handsewn anastomosis in an end-to-side fashion).

**Open Mckeown esophagectomy:** The first stage was started with a right posterolateral thoracotomy. The mediastinal pleura overlying the esophagus were divided with electrotome. The thoracic esophagus, alone with the periesophageal tissue and mediastinal lymph nodes, was circumferentially mobilized from the diaphragm to the level of the inlet of the thorax.

The second stage is the mobilization of the stomach which was started with the division of the greater curvature using ultrasonic coagulating shears. The short gastric vessels were divided with ultrasonic coagulating shears as well. The gastrocolic omentum was then divided, with care taken to preserve the right gastroepiploic artery. The posterior attachments of the stomach were then divided after retraction of the stomach anteriorly. The left gastric vessel was divided at its origin from the celiac trunk with sutures. Lymphatic tissues around vessels were included in the resection. Subsequently, the abdominal esophagus was dissected as far as possible toward the distal end. Pyloroplasty was not routinely performed. The abdomen is inspected to make sure that hemostasis was adequate and the incisions were closed. For the last stage, the cervical incision was made and then anastomosis was performed like minimally invasive esophagectomy.

**Table 1 Clinical characteristics of patients receiving minimally invasive McKeown esophagectomy *n* (%)**

| Clinical variable | Value |
|---|---|
| Age (yr) | 60 (36-79) |
| Male gender | 341 (76.6) |
| Neoadjuvant radiotherapy | 21 (4.7) |
| Neoadjuvant chemotherapy | 30 (6.7) |
| Location | |
| Upper | 96 (21.6) |
| Middle | 292 (65.6) |
| Lower | 57 (12.8) |
| Type of surgery | |
| Total MIME | 375 (84.3) |
| Hybrid MIME | 70 (15.7) |

MIME: Minimally invasive McKeown esophagectomy.

**Postoperative care:** The patients were placed in an intensive care unit or discharged to ward directly from operation room according to the judgement of anesthetist. Assessment of recurrent laryngeal nerve injury was done on the 1st d postoperatively. Postoperative respiratory tract management included chest physiotherapy and early ambulation. Patient-controlled analgesia was given to every patient to control postoperative pain.

### *Learning curve of total minimally invasive McKeown esophagectomy*

In order to study the learning curve of total minimally invasive McKeown esophagectomy, we selected data of 180 patients who underwent total minimally invasive McKeown esophagectomy in the early period which was performed by five senior thoracic oncologic surgeons who majored in thoracic surgical oncology over 20 years. All 180 patients were divided into three groups according to time sequence from January 2009 to August 2013 as group 1 ($n$ = 60), from September 2013 to November 2013 as group 2 ($n$ = 60) and from December 2013 to group 3 ($n$ = 60).

### *Statistical analysis*

The SPSS software package 16.0 for Windows was used for statistical analyses. Data are presented as median value (interquartile range) for continuous variables, and percentages for dichotomous variables. Continuous variables were analyzed using ANOVA test or nonparametric test, and categorical variables were analyzed using Fisher exact test. Survival was estimated using Kaplan-Meier method and log-rank tests were used to analyze differences between curves. The significant level was set as a $P$ value less than 0.05.

## RESULTS

### *Clinical characteristics*

From January 2009 to June 2015, 445 cases of minimally invasive McKeown esophagectomy were conducted at our hospital. In this cohort, the median age was 60 years (range, 36-79 years) and there were 341 males and 104 females. Twenty-one patients underwent neoadjuvant radiotherapy, and 30 patients underwent neoadjuvant chemotherapy. Other clinical variables are displayed in Table 1. Five patients were converted into open thoracotomy and laparotomy and the reasons for conversion are displayed in Table 2.

**Table 2 Reasons for conversion of patients receiving minimally invasive McKeown esophagectomy**

| Reason | Number |
|---|---|
| Rupture of trachea | 1 |
| Pleural adhesion | 2 |
| Adhesion of abdominal cavity | 2 |

The cohort was divided into three groups based on operative technique used. Of 548 McKeown esophagectomies, there were 375 total minimally invasive McKeown esophagectomies, 70 hybrid minimally invasive McKeown esophagectomies and 103 open McKeown esophagectomies. The selection of which approach was based on the opinion of surgeons. Patients who underwent minimally invasive McKeown esophagectomy were older than patients who underwent open Mckeown esophagectomy. Patients who underwent open McKeown esophagectomy were more in the proximal third of the esophagus and more received neoadjuvant chemotherapy and radiotherapy (Table 3) .

### *Perioperative outcomes of patients undergoing three types of Mckeown esophagectomy*

As shown in Table 4, patients who underwent total minimally invasive McKeown esophagectomy had significantly less intraoperative blood loss than patients who underwent hybrid minimally invasive McKeown esophagectomy and open McKeown esophagectomy. However, there were no significant differences in duration of operation, number of harvested lymph nodes, or postoperative morbidity including incidence of pulmonary complication and anastomotic leak between total minimally invasive McKeown esophagectomy, hybrid minimally invasive McKeown esophagectomy and open McKeown esophagectomy groups.

### *Survival*

Kaplan-Meier plots depict the long term survival of patients who underwent three types of operation: total minimally invasive McKeown esophagectomy, hybrid minimally invasive McKeown esophagectomy and open McKeown esophagectomy (Figure 1). There were no significant differences in 5-year survival between these three types (60.3% *vs* 47.9% *vs* 35.3%, $P$ = 0.579).

### *Learning curve of total minimally invasive McKeown esophagectomy*

Patients in group 1 ($n$ = 60) had significantly longer duration of operation than those in groups 2 ($n$

**Table 3 Clinical characteristics of patients receiving McKeown esophagectomy *n* (%)**

| Clinical variable | Total MIME (*n* = 375) | Hybrid MIME (*n* = 70) | Open McKeown esophagectomy (*n* = 103) | *P* value |
|---|---|---|---|---|
| Age (yr) | 59 (54-65) | 62 (55-67) | 56 (52-63) | 0.024 |
| Sex (Male) | 289 (77.1) | 52 (74.3) | 84 (81.6) | 0.490 |
| BMI (kg/m$^2$) | 23 (21-25) | 22 (20-25) | 23 (20-24) | 0.100 |
| Tumor location | | | | < 0.001 |
| Upper | 78 (20.8) | 18 (25.7) | 58 (56.3) | |
| Middle | 248 (66.1) | 44 (62.9) | 39 (37.9) | |
| Lower | 49 (13.1) | 8 (11.4) | 6 (5.8) | |
| Neoadjuvant chemotherapy | 21 (5.6) | 9 (12.9) | 11 (10.7) | 0.042 |
| Neoadjuvant radiotherapy | 16 (4.3) | 5 (7.1) | 11 (10.7) | 0.043 |

MIME: Minimally invasive McKeown esophagectomy; BMI: Body mass index; AJCC: American Joint Committee on Cancer.

**Table 4 Perioperative outcomes of patients receiving Mckeown esphagectomy *n* (%)**

| Clinical variable | Total MIME (*n* = 375) | Hybrid MIME (*n* = 70) | Open McKeown esophagectomy (*n* = 103) | *P* value |
|---|---|---|---|---|
| Duration of operation (min) | 330 (270-420) | 370 (305-435) | 340 (320-400) | 0.323 |
| Intraoperative blood loss (mL) | 100 (100-200) | 300 (100-300) | 200 (100-300) | 0.001 |
| Number of harvested lymph nodes | 22 (16-31) | 19 (14-29) | 25 (19-32) | 0.293 |
| AJCC staging | | | | 0.085 |
| 0 | 1 (0.3) | 1 (1.4) | 0 (0) | |
| I | 108 (28.8) | 19 (27.1) | 17 (16.5) | |
| II | 172 (45.9) | 33 (47.1) | 49 (47.6) | |
| III | 94 (25.1) | 17 (24.3) | 37 (35.9) | |
| Differentiation | | | | 0.685 |
| High | 107 (28.6) | 18 (25.7) | 35 (34.0) | |
| Middle | 209 (55.9) | 40 (57.1) | 50 (48.5) | |
| Low | 58 (15.5) | 12 (17.1) | 18 (17.5) | |
| Complete resection | 374 (99.7) | 70 (100) | 103 (100) | 0.794 |
| Overall Morbidity | 73 (19.5) | 13 (18.6) | 22 (21.6) | 0.864 |
| Pulmonary complications | 11 (2.9) | 2 (2.9) | 6 (5.8) | 0.347 |
| Leakage | 46 (12.3) | 10 (14.3) | 9 (8.7) | 0.493 |
| In-hospital mortality | 2 (0.5) | 0 (0) | 1.0 (0) | 0.696 |
| Length of hospital stay (d) | 16 (14-24) | 18 (16-27) | 21 (16-28) | 0.078 |

MIME: Minimally invasive McKeown esophagectomy; AJCC: American Joint Committee on Cancer.

**Table 5 Comparison of perioperative outcomes of patients who underwent total minimally invasive McKeown esophagectomy in the early period *n* (%)**

| Clinical variable | Group 1 (*n* = 60) | Group 2 (*n* = 60) | Group 3 (*n* = 60) | *P* value |
|---|---|---|---|---|
| Duration of operation (min) | 350 (285-450) | 303 (270-373) | 300 (240-370) | 0.004 |
| Intraoperative blood loss (mL) | 300 (125-375) | 200 (100-300) | 100 (100-300) | 0.081 |
| Number of harvested lymph nodes | 21 (17-30) | 22 (16-31) | 21 (16-26) | 0.866 |
| Overall morbidity | 10 (16.7) | 13 (21.7) | 14 (23.3) | 0.643 |
| Pulmonary morbidity | 2 (3.3) | 3 (5.0) | 0 (0) | 0.237 |
| Leakage | 5 (8.3) | 7 (11.7) | 11 (18.3) | 0.248 |
| In-hospital mortality | 1 (1.7) | 0 (0) | 0 (0) | 0.366 |
| Length of hospital stay (d) | 17 (14-22) | 20 (14-31) | 15 (12-21) | 0.335 |

= 60) and 3 (*n* = 60). There were no significant differences in intraoperative blood loss, number of harvested lymph nodes, or postoperative morbidity including incidence of pulmonary complication and anastomotic leak between groups 1, 2 and 3 (Table 5). Five surgeons performed 180 total minimally invasive McKeown esophagectomies. There were no significant differences in the short term outcomes or oncologic clearance between these three groups. The duration of operation got steady after the first 60 cases for 5 surgeons, suggesting that 12 cases were needed for a senior surgeon to master total minimally invasive McKeown esophagectomy at our hospital, a high volume cancer center. Then we analyzed the learning curve for each of 5 surgeons and found that all surgeons had a trend of reduction of duration of operation. Of 5 surgeons, there were significant differences in duration of operation between surgeons

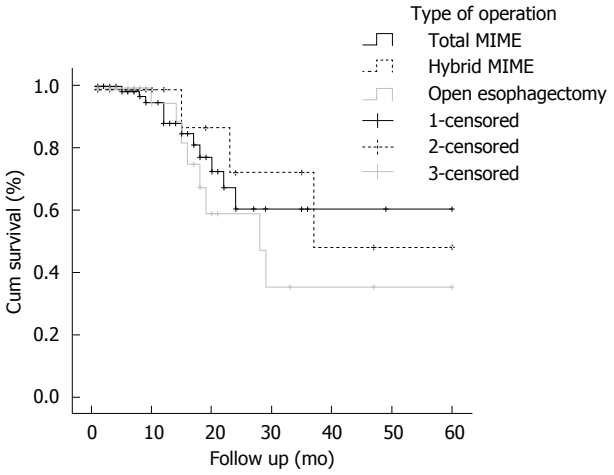



**Figure 1 Kaplan-Meier analysis of three types of operation.** There were no significant differences in 5-yr survival between total minimally invasive McKeown esophagectomy, hybrid minimally invasive McKeown esophagectomy and open Mckeown esophagectomy (60.3% *vs* 47.9% *vs* 35.3%, $P = 0.579$). MIME: Minimally invasive McKeown esophagectomy.

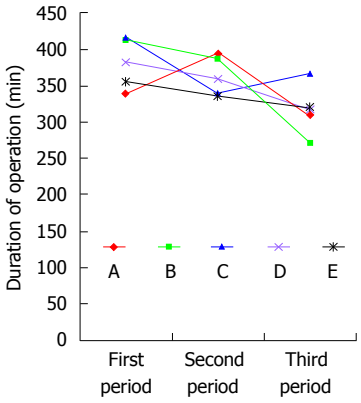



**Figure 2 Learning curve of total minimally invasive McKeown esophagectomy of five surgeons.** Durations of operation for five surgeons in three periods are as follows: surgeon A (340 ± 57 min *vs* 395 ± 105 min *vs* 310 ± 61 min, $P = 0.037$); surgeon B (413 ± 109 min *vs* 387 ± 110 min *vs* 272 ± 58 min, $P = 0.002$); surgeon C (418 ± 65 min *vs* 339 ± 116 min *vs* 367 ± 74 min, $P = 0.098$); surgeon D (383 ± 105 min *vs* 359 ± 82 min *vs* 317 ± 116 min, $P = 0.287$); and surgeon E (355 ± 123 min *vs* 337 ± 77 min *vs* 320 ± 159 min, $P = 0.789$).

A and B, while there were no significant differences in duration of operation between surgeons C, D and E (Figure 2).

## DISCUSSION

In this study, we found that patients who underwent total minimally invasive McKeown esophagectomy had similar short term outcome and long term survival compared with patients who underwent hybrid minimally invasive McKeown esophagectomy or open Mckeown esophagectomy.

The feasibility of MIE has been well established in our center as previously reported[9]. Recently, a meta-analysis involving 13267 patients demonstrated reduced in-hospital mortality in patients who underwent MIE compared with patients who underwent open esophagectomy[14]. In that study, the mortality rates were 3.0% and 4.6% in MIE and open esophagectomy group, respectively[14]. Also, a significant effect of MIE was observed in that study in reducing the risk of pulmonary complications compared with open esophagectomy (17.8% *vs* 20.4%)[14]. We did not observe any reduction of incidence of morbidity or mortality in MIE group compared with open esophagectomy group. However, there was a trend in our study that the rate of pulmonary complication decreased in total minimally invasive group and hybrid minimally invasive group compared with open group, with the pulmonary complication rates of 2.9%, 2.9% and 5.8%, respectively. Relatively small number of samples in our study may account for the reason. There was no significant difference in the rate of anastomotic leak after esophagectomy between MIE and open esophagectomy group in our study, which is consistent with the result of the meta-analysis[14].

The results of a large randomized, controlled trial of neoadjuvant treatment demonstrated a survival benefit in locally advanced esophageal carcinoma as compared with esophagectomy alone, with a five-year survival of 47% in neoadjuvant treatment group compared with 34% in the surgery group[16]. And since then, some surgeons at our hospital adopted neoadjuvant treatment as an alternative for locally advanced esophageal carcinoma to surgery alone. The rate of neoadjuvant treatment for locally advanced esophageal carcinoma was only 20% at our hospital. A low fraction of patients with esophageal squamous cell carcinoma in the study of van Hagen *et al*[16] (around 20%) may preclude the application of neoadjuvant treatment at our hospital. Several meta-analyses demonstrated consistent results of survival advantage of neoadjuvant treatment plus surgery over surgery alone for resectable esophageal adenocarcinoma[17-21]. However, there were limited data regarding the survival advantage of neoadjuvant treatment plus surgery over surgery alone for resectable esophageal squamous cell carcinoma. More studies of neoadjuvant treatment on esophageal squamous cell carcinoma are needed to define the role of neoadjuvant treatment in locally advanced esophageal squamous cell carcinoma.

We found a similar oncologic clearance rate as demonstrated by no difference in number of dissected lymph nodes between MIE and open esophagectomy group. And we found similar 5-year survival between MIE and open esophagectomy group. Recently, a propensity score-matched comparison study showed similar lymph node harvest and equal oncologic survival in MIE and open esophagectomy group which are similar to our results[11]. Two recent studies showed better long term survival in MIE group compared with open esophagectomy group, and select bias may lead to the results[12,13]. In these two studies, more early tumors were selected in MIE group and more advanced cancers in open esophagectomy group[12,13]. More

studies are needed to clarify the survival advantage of MIE over open esophagectomy.

Many surgeons reported less intraoperative blood loss in MIE group than in open esophagectomy group[12,13,22]. In this study, we observed a similar result. It is reported that perioperative blood transfusion was a negative prognostic factor for long-term survival in esophageal cancer after esophagectomy. Therefore, less intraoperative bleeding may lessen the need for perioperative transfusion, which may increase long term survival of patients who received MIE[23].

Apart from perioperative morbidity and long term survival, other measures including quality of life questionnaires such as European Organization for Research on Treatment of Cancer quality of life questionnaire-C30 (EORTC QLQ-C30) and QLQ-0ES18 and cost analysis were used to assess the difference between minimally invasive and open esophagectomies[24,25]. More importantly, quality of life measures could be a tool to provide clinical information from patients' perspective suggesting cancer recurrence[26]. Indeed, an ongoing multicenter prospective study organized and led by our hospital are being performed to compare the effects between minimally invasive McKeown esophagectomy and open McKeown esophagectomy in China[27]. The measures included perioperative morbidity, mortality and long term survival. Also, quality of life questionnaires (EORTC QLQ-C30 and QLQ-0ES18) are included in this ongoing study. Owing to the retrospective nature of this study, we did not include the quality of life questionnaires in the analysis. Reduced cost of minimally invasive esophagectomy compared with open esophagectomy has been demonstrated in our early study[9]. Therefore, minimally invasive esophagectomy had the advantages of decreased intraoperative blood and reduced cost compared with open esophagectomy, with comparable perioperative morbidity and mortality, and long term survival. Although minimally invasive esophagectomy is technically changing, it is a valuable procedure for the surgical treatment of esophageal cancer patients in specialized centers[28].

Learning curve of a new technique is an important issue in clinical practice, which may influence the outcome of patients and training of the surgeons. The risk of increased technical problems when applying a new procedure is not uncommon[29]. As Tao reported that minimally invasive approaches were demonstrated to decrease the risk of functional complications including arrhythmia, pulmonary infection, acute lung injury (ALI), ileus, acute renal failure or acute hepatic failure but not technical problems including perioperative bleeding, chylothorax, recurrent laryngeal nerve palsy (RLNP), and anastomotic leakage. In their study, functional complications between open esophagectomy and MIE group were 32.0% and 1.79%, respectively, while technical complications were 12.0% and 23.9%, respectively[29]. In our study, there was no significant difference in technical problems including anastomotic leak between patients who underwent total minimally invasive McKeown esophagectomy, hybrid minimally invasive McKeown esophagectomy and open McKeown esophagectomy. However, the duration of operation decreased significantly in groups 2 and 3 than in group 1, suggesting that increment of number of procedures would improve the surgeon's performance. Also, there was a trend that intraoperative blood loss decreased as the surgeon's experiences increased. However, there were no significant differences in the number of harvested lymph nodes or postoperative morbidity including incidence of pulmonary complication and anastomotic leak between groups 1, 2 and 3. Therefore, a new MIE program can be implemented safely with comparable oncologic clearance rate and postoperative morbidity rate after approximately 12 cases for a surgeon at a high volume cancer center. Lin *et al*[30] reported that surgery skill can be reached after 40 cases. In their study, an attending doctor who performed 40 cases may reach the plateau of learning curve. However, in our study, senior doctors with over 20 years of experiences with thoracic surgery who performed only 12 cases can overcome the skill obstacle.

The limitation of this study mainly comes from its retrospective nature, which carries a risk of selection bias. For example, there were more patients who had the tumor in the upper third of the esophagus in open esophagectomy group. Second, the patients were from one hospital, which may not be generalized in other medical centers. Last, rates of local and distant recurrences, and long term survival analysis are needed to determine the oncologic clearance apart from the comparison of number of harvested lymph nodes.

In conclusion, total minimally invasive McKeown esophagectomy had reduced intraoperative blood loss and comparable short term and long term survival compared with hybrid minimally invasive McKeown esophagectomy or open Mckeown esophagectomy. At least 12 cases are needed to master the technique in a high volume cancer center.

## COMMENTS

### Background

Open McKeown esophagectomy is a complex surgery for upper third esophageal cancer with higher morbidity rate than open Ivor Lewis and Sweet esophagectomy. Minimally invasive esophagectomy is a new technique which aims to reduce systematic inflammatory response syndrome and perioperative morbidity rate.

### Research frontiers

In the past 5 years, several studies including one randomized controlled trial reported reduced postoperative pulmonary complication rates, comparable oncologic clearance and similar long term survival. However, few studies focused on the comparison of open McKeown esophagectomy and minimally invasive McKeown esophagectomy.

### *Innovations and breakthroughs*

This study again reinforced the feasibility of minimally invasive McKeown esophagectomy and extended previous study of learning curve of minimally invasive McKeown esophagectomy that at least 12 cased are needed to reach the plateau of this technique.

### *Applications*

The results of this study may provide new data for thoracic surgeons who majored in esophageal surgery that minimally invasive McKeown esophagectomy is feasible and is associated with less intraoperative blood loss. Most importantly, performing 12 cases of minimally invasive McKeown esophagectomies may reach the plateau of this technique.

### *Peer-review*

This manuscript compared totally minimally invasive esophagectomy (MIE), hybrid MIE and open three stage (McKeowm) oesophagectomy. The authors found the procedure ontologically safe in terms of lymph node yield and long term survival and technically safe in terms of blood loss, operating time, morbidity and mortality. This is an interesting manuscript and clearly written and organized.

## REFERENCES


1 **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]

2 **Chen W**, Zheng R, Zhang S, Zhao P, Li G, Wu L, He J. Report of incidence and mortality in China cancer registries, 2009. *Chin J Cancer Res* 2013; **25**: 10-21 [PMID: 23372337 DOI: 10.3978/j.issn.1000-9604.2012.12.04]

3 **Cuschieri A**, Shimi S, Banting S. Endoscopic oesophagectomy through a right thoracoscopic approach. *J R Coll Surg Edinb* 1992; **37**: 7-11 [PMID: 1573620]

4 **Herbella FA**, Patti MG. Minimally invasive esophagectomy. *World J Gastroenterol* 2010; **16**: 3811-3815 [PMID: 20698044 DOI: 10.3748/wjg.v16.i30.3811]

5 **Nafteux P**, Moons J, Coosemans W, Decaluwé H, Decker G, De Leyn P, Van Raemdonck D, Lerut T. Minimally invasive oesophagectomy: a valuable alternative to open oesophagectomy for the treatment of early oesophageal and gastro-oesophageal junction carcinoma. *Eur J Cardiothorac Surg* 2011; **40**: 1455-1463; discussion 1463-1464 [PMID: 21514837 DOI: 10.1016/j.ejcts.2011.01.086]

6 **Tsujimoto H**, Takahata R, Nomura S, Yaguchi Y, Kumano I, Matsumoto Y, Yoshida K, Horiguchi H, Hiraki S, Ono S, Yamamoto J, Hase K. Video-assisted thoracoscopic surgery for esophageal cancer attenuates postoperative systemic responses and pulmonary complications. *Surgery* 2012; **151**: 667-673 [PMID: 22244180 DOI: 10.1016/j.surg.2011.12.006]

7 **Biere SS**, van Berge Henegouwen MI, Maas KW, Bonavina L, Rosman C, Garcia JR, Gisbertz SS, Klinkenbijl JH, Hollmann MW, de Lange ES, Bonjer HJ, van der Peet DL, Cuesta MA. Minimally invasive versus open oesophagectomy for patients with oesophageal cancer: a multicentre, open-label, randomised controlled trial. *Lancet* 2012; **379**: 1887-1892 [PMID: 22552194 DOI: 10.1016/S0140-6736(12)60516-9]

8 **Mu JW**, Chen GY, Sun KL, Wang DW, Zhang BH, Li N, Lv F, Mao YS, Xue Q, Gao SG, Zhao J, Wang DL, Li ZS, Lei WD, Gao YS, Zhang L, Huang JF, Shao K, Su K, Yang K, Zhao L, Feng FY, Wang YG, Li J, He J. Application of video-assisted thoracic surgery in the standard operation for thoracic tumors. *Cancer Biol Med* 2013; **10**: 28-35 [PMID: 23691442 DOI: 10.7497/j.issn.2095-3941.2013.01.005]

9 **Mu J**, Yuan Z, Zhang B, Li N, Lyu F, Mao Y, Xue Q, Gao S, Zhao J, Wang D, Li Z, Gao Y, Zhang L, Huang J, Shao K, Feng F, Zhao L, Li J, Cheng G, Sun K, He J. Comparative study of minimally invasive versus open esophagectomy for esophageal cancer in a single cancer center. *Chin Med J* (Engl) 2014; **127**: 747-752 [PMID: 24534234]

10 **Yang J**, Lyu B, Zhu W, Chen J, He J, Tang S. A retrospective cohort comparison of esophageal carcinoma between thoracoscopic and laparoscopic esophagectomy and open esophagectomy. *Zhonghua Wai Ke Za Zhi* 2015; **53**: 378-381 [PMID: 26082255]

11 **Wang H**, Shen Y, Feng M, Zhang Y, Jiang W, Xu S, Tan L, Wang Q. Outcomes, quality of life, and survival after esophagectomy for squamous cell carcinoma: A propensity score-matched comparison of operative approaches. *J Thorac Cardiovasc Surg* 2015; **149**: 1006-1014; discussion 1014-1015.e4 [PMID: 25752374 DOI: 10.1016/j.jtcvs.2014.12.063]

12 **Palazzo F**, Rosato EL, Chaudhary A, Evans NR, Sendecki JA, Keith S, Chojnacki KA, Yeo CJ, Berger AC. Minimally invasive esophagectomy provides significant survival advantage compared with open or hybrid esophagectomy for patients with cancers of the esophagus and gastroesophageal junction. *J Am Coll Surg* 2015; **220**: 672-679 [PMID: 25667145 DOI: 10.1016/j.jamcollsurg.2014.12.023]

13 **Burdall OC**, Boddy AP, Fullick J, Blazeby J, Krysztopik R, Streets C, Hollowood A, Barham CP, Titcomb D. A comparative study of survival after minimally invasive and open oesophagectomy. *Surg Endosc* 2015; **29**: 431-437 [PMID: 25125095 DOI: 10.1007/s00464-014-3694-4]

14 **Zhou C**, Zhang L, Wang H, Ma X, Shi B, Chen W, He J, Wang K, Liu P, Ren Y. Superiority of Minimally Invasive Oesophagectomy in Reducing In-Hospital Mortality of Patients with Resectable Oesophageal Cancer: A Meta-Analysis. *PLoS One* 2015; **10**: e0132889 [PMID: 26196135 DOI: 10.1371/journal.pone.0132889]

15 **Rice TW**, Blackstone EH, Rusch VW. 7th edition of the AJCC Cancer Staging Manual: esophagus and esophagogastric junction. *Ann Surg Oncol* 2010; **17**: 1721-1724 [PMID: 20369299 DOI: 10.1245/s10434-010-1024-1]

16 **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]

17 **Wang F**, Wang YM, He W, Li XK, Peng FH, Yang XL, Fan QX. Chemoradiotherapy followed by surgery could improve the efficacy of treatments in patients with resectable esophageal carcinoma. *Chin Med J* (Engl) 2013; **126**: 3138-3145 [PMID: 23981626]

18 **Ronellenfitsch U**, Schwarzbach M, Hofheinz R, Kienle P, Kieser M, Slanger TE, Burmeister B, Kelsen D, Niedzwiecki D, Schuhmacher C, Urba S, van de Velde C, Walsh TN, Ychou M, Jensen K. Preoperative chemo(radio)therapy versus primary surgery for gastroesophageal adenocarcinoma: systematic review with meta-analysis combining individual patient and aggregate data. *Eur J Cancer* 2013; **49**: 3149-3158 [PMID: 23800671 DOI: 10.1016/j.ejca.2013.05.029]

19 **Kumagai K**, Rouvelas I, Tsai JA, Mariosa D, Klevebro F, Lindblad M, Ye W, Lundell L, Nilsson M. Meta-analysis of postoperative morbidity and perioperative mortality in patients receiving neoadjuvant chemotherapy or chemoradiotherapy for resectable oesophageal and gastro-oesophageal junctional cancers. *Br J Surg* 2014; **101**: 321-338 [PMID: 24493117 DOI: 10.1002/bjs.9418]

20 **Deng J**, Wang C, Xiang M, Liu F, Liu Y, Zhao K. Meta-analysis of postoperative efficacy in patients receiving chemoradiotherapy followed by surgery for resectable esophageal carcinoma. *Diagn Pathol* 2014; **9**: 151 [PMID: 25030066 DOI: 10.1186/1746-1596-9-151]

21 **Kumagai K**, Rouvelas I, Tsai JA, Mariosa D, Lind PA, Lindblad M, Ye W, Lundell L, Schuhmacher C, Mauer M, Burmeister BH, Thomas JM, Stahl M, Nilsson M. Survival benefit and additional value of preoperative chemoradiotherapy in resectable gastric and gastro-oesophageal junction cancer: a direct and adjusted indirect comparison meta-analysis. *Eur J Surg Oncol* 2015; **41**: 282-294 [PMID: 25491892 DOI: 10.1016/j.ejso.2014.11.039]

22 **Kubo N**, Ohira M, Yamashita Y, Sakurai K, Toyokawa T, Tanaka

H, Muguruma K, Shibutani M, Yamazoe S, Kimura K, Nagahara H, Amano R, Ohtani H, Yashiro M, Maeda K, Hirakawa K. The impact of combined thoracoscopic and laparoscopic surgery on pulmonary complications after radical esophagectomy in patients with resectable esophageal cancer. *Anticancer Res* 2014; **34**: 2399-2404 [PMID: 24778050]

23 **Kakuta T**, Kosugi S, Kanda T, Ishikawa T, Hanyu T, Suzuki T, Wakai T. Prognostic factors and causes of death in patients cured of esophageal cancer. *Ann Surg Oncol* 2014; **21**: 1749-1755 [PMID: 24510184 DOI: 10.1245/s10434-014-3499-7]

24 **Scarpa M**, Valente S, Alfieri R, Cagol M, Diamantis G, Ancona E, Castoro C. Systematic review of health-related quality of life after esophagectomy for esophageal cancer. *World J Gastroenterol* 2011; **17**: 4660-4674 [PMID: 22180708 DOI: 10.3748/wjg.v17.i42.4660]

25 **Ecker BL**, Savulionyte GE, Datta J, Dumon KR, Kucharczuk J, Williams NN, Dempsey DT. Laparoscopic transhiatal esophagectomy improves hospital outcomes and reduces cost: a single-institution analysis of laparoscopic-assisted and open techniques. *Surg Endosc* 2015; Epub ahead of print [PMID: 26416370]

26 **Chang YL**, Tsai YF, Chao YK, Wu MY. Quality-of-life measures as predictors of post-esophagectomy survival of patients with esophageal cancer. *Qual Life Res* 2015; Epub ahead of print [PMID: 26238649]

27 ClinicalTrials.gov Web site. Traditional three incisions vs minimally invasive thoracol-laparoscopic esophagectomy for esophageal cancer. Accessed February 3, 2015. Available from: URL: https://www.clinicaltrials.gov/ct2/show/record/NCT02355249

28 **Rustgi AK**, El-Serag HB. Esophageal carcinoma. *N Engl J Med* 2014; **371**: 2499-2509 [PMID: 25539106 DOI: 10.1056/NEJMra1314530]

29 **Mao T**, Fang W, Gu Z, Guo X, Ji C, Chen W. Comparison of perioperative outcomes between open and minimally invasive esophagectomy for esophageal cancer. *Thorac Cancer* 2015; **6**: 303-306 [PMID: 26273376 DOI: 10.1111/1759-7714.12184]

30 **Lin J**, Kang M, Chen C, Lin R, Zheng W, Zhug Y, Deng F, Chen S. Thoracolaparoscopy oesophagectomy and extensive two-field lymphadenectomy for oesophageal cancer: introduction and teaching of a new technique in a high-volume centre. *Eur J Cardiothorac Surg* 2013; **43**: 115-121 [PMID: 22518035 DOI: 10.1093/ejcts/ezs151]

**P- Reviewer**: Merrett ND, Shimi SM **S- Editor**: Gong ZM
**L- Editor**: Wang TQ **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12882

*World J Gastroenterol* 2015 December 7; 21(45): 12882-12887
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

Prospective Study

# Comparison of Stretta procedure and toupet fundoplication for gastroesophageal reflux disease-related extra-esophageal symptoms

Chao Yan, Wei-Tao Liang, Zhong-Gao Wang, Zhi-Wei Hu, Ji-Min Wu, Chao Zhang, Mei-Ping Chen

Chao Yan, Wei-Tao Liang, Zhong-Gao Wang, Zhi-Wei Hu, Ji-Min Wu, Mei-Ping Chen, Department of Gastroesophageal Reflux Disease, Second Artillery General Hospital of Chinese People's Liberation Army, Beijing 100088, China

Chao Yan, Wei-Tao Liang, Zhong-Gao Wang, Chao Zhang, Department of Vascular Surgery, Xuan Wu Hospital, Capital Medical University, Beijing 100053, China

Author contributions: Yan C, Liang WT, Wang ZG, Hu ZW, Wu JM, Zhang C and Chen MP designed and performed the research; Yan C and Liang WT contributed the analytical tools; Yan C analyzed the data and wrote the manuscript; all authors read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the institutional review boards of the Department of Vascular Surgery, Xuan Wu Hospital, and the Department of Gastroesophageal Reflux Disease, Second Artillery General Hospital of Chinese People's Liberation Army (Beijing, China).

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

Conflict-of-interest statement: The authors of this manuscript have no conflicts of interest to disclose.

Data sharing statement: There are no additional data available.



Correspondence to: Zhong-Gao Wang, MD, Department of Vascular Surgery, Xuan Wu Hospital, Capital Medical University, No. 45 Changchun Street, Beijing 100053, China. zhonggaowang@hotmail.com
Telephone: +86-10-62015718

Received: May 18, 2015
Peer-review started: May 22, 2015
First decision: July 10, 2015
Revised: August 9, 2015
Accepted: September 30, 2015
Article in press: September 30, 2015
Published online: December 7, 2015

## Abstract

**AIM:** To compare the outcomes between the Stretta procedure and laparoscopic toupet fundoplication (LTF) in patients with gastroesophageal reflux disease (GERD)-related extra-esophageal symptoms.

**METHODS:** From January 2011 to February 2012, a total of 98 patients diagnosed with GERD-related extra-esophageal symptoms who met the inclusion criteria were enrolled in this study. All patients who either underwent the Stretta procedure or LTF treatment have now completed the 3-year follow-up. Primary outcome measures, including frequency and severity of extra-esophageal symptoms, proton pump inhibitor (PPI) use, satisfaction, and postoperative complications, were assessed. The results of the Stretta procedure and LTF therapy were analyzed and compared.

**RESULTS:** There were 47 patients in the Stretta group and 51 patients in the LTF group. Ninety patients were available at the 3-year follow-up. The total of the frequency and severity scores for every symptom improved in both groups ($P < 0.05$). Improvement

in symptom scores of cough, sputum, and wheezing did not achieve statistical significance between the two groups ($P > 0.05$). However, the score for globus hysterics was different between the Stretta group and the LTF group (4.9 ± 2.24 *vs* 3.2 ± 2.63, $P < 0.05$). After the Stretta procedure and LTF treatment, 29 and 33 patients in each group achieved PPI therapy independence (61.7% *vs* 64.7%, $P = 0.835$). The patients in the LTF group were more satisfied with their quality of life than those in the Stretta procedure group ($P < 0.05$). Most complications resolved without intervention within two weeks; however, two patients in the LTF group still suffered from severe dysphagia 2 wk after the operation, and it improved after bougie dilation treatment in both patients.

**CONCLUSION:** The Stretta procedure and LTF were both safe and effective for the control of GERD-related extra-esophageal symptoms and the reduction of PPI use.

**Key words:** Gastroesophageal reflux disease; Extra-esophageal symptoms; Laparoscopic Toupet fundoplication; Stretta procedure; Proton pump inhibitor use



**Core tip:** Laparoscopic toupet fundoplication offers equivalent symptom relief and a significantly lower risk of postoperative dysphagia compared with laparoscopic Nissen fundoplication. The Stretta procedure and laparoscopic Toupet fundoplication have been demonstrated to be effective and safe in controlling gastroesophageal reflux disease-related symptoms as minimally invasive procedures. Few studies have been conducted to compare the outcome between laparoscopic Toupet fundoplication and the Stretta procedure. In this prospective study, we compared the outcomes of patients who underwent the Stretta procedure and laparoscopic Toupet fundoplication and evaluated the efficacy of the techniques in controlling gastroesophageal reflux disease-related extra-esophageal symptoms.

Yan C, Liang WT, Wang ZG, Hu ZW, Wu JM, Zhang C, Chen MP. Comparison of Stretta procedure and toupet fundoplication for gastroesophageal reflux disease-related extra-esophageal symptoms. *World J Gastroenterol* 2015; 21(45): 12882-12887 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12882.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12882

## INTRODUCTION

Gastroesophageal reflux disease (GERD) is a condition that develops when the reflux of stomach contents causes troublesome symptoms and/or complications[1]. The typical symptoms of GERD are heartburn and regurgitation. The prevalences of extra-esophageal symptoms, including pneumonia, asthma, bronchitis, laryngitis, pharyngitis, chronic cough, wheezing, oral ulcers, and snoring, are significantly increased in symptomatic gastroesophageal reflux patients[2]. GERD has been confirmed to be one of the three main causes of chronic cough, accounting for approximately 20% of all cases[3].

Several randomized controlled trials strongly support antireflux surgery as an effective alternative for patients with disease that is not controlled well by medical therapy[4,5]. Laparoscopic Toupet fundoplication (LTF) offers equivalent symptom relief and has a significantly lower risk of postoperative complications compared with laparoscopic Nissen fundoplication (LNF)[6,7]. The earliest endoluminal technique for GERD, radiofrequency energy delivery to the gastroesophageal junction (Stretta), was introduced in 2000. Some studies on the Stretta procedure have confirmed not only an improvement in GERD symptoms and quality of life but also greater efficacy and safety compared with other surgical techniques[8].

LTF and the Stretta procedure have been used to treat selected patients with GERD-related extra-esophageal symptoms. This study compared the efficacy and safety of these two procedures, which were performed to control extra-esophageal symptoms in GERD patients.

## MATERIALS AND METHODS

### Subjects

From January 2011 to February 2012, we clinically treated a total of 98 patients with the Stretta procedure and LTF who suffered from extra-esophageal symptoms and completed 3 years of follow-up. All patients failed to respond to medical treatment or opted for surgery despite effective medical management.

The inclusion criteria were as follows: (1) GERD diagnosed by endoscopic evidence of esophagitis or abnormal esophageal pH, a DeMeester score ⩾ 14.7 with symptom correlation of ⩾ 50%, and/or > 73 reflux episodes during 24-h ambulatory impedance monitoring; (2) lower than normal lower esophageal sphincter (LES) pressure detected by esophageal manometry; (3) endoscopically confirmed Los Angeles grade A or B esophagitis; (4) non-hiatal hernia or small (< 2 cm) hiatal hernia; and (5) age ⩾ 18 years. Patients with central nervous system diseases, connective tissue diseases, previous esophageal or gastric surgery, esophageal stricture, shortened esophagus, impaired distal esophageal peristalsis, Barrett's esophagus, autoimmune diseases, collagen vascular diseases, and/or coagulation disorders were excluded.

### Treatment

LTF was performed with five ports under general

anesthesia. After dissecting the gastro-hepatic ligament with a harmonic scalpel, a window was created behind the lower esophagus. Then, the diaphragmatic crura were carefully dissected, and approximately 5 cm of the distal esophagus was mobilized, while the mediastinal structures, including the pleura, pericardium, vagus nerves, and aorta, were identified and preserved. In all cases, the gastric fundus was dissected by dividing the short gastric vessels. The diaphragmatic crura were sewed behind the esophagus with 1-2 non-absorbable sutures, and a posterior 270° and 2-cm-long fundoplication was constructed with 5-6 interrupted non-absorbable sutures.

For the Stretta procedure, after the patient was sedated with a combination of intravenously administered fentanyl and midazolam, the distance to the gastro-esophageal junction was measured under gastroscopy. Then, the endoscope was withdrawn, and a radiofrequency-delivering catheter, consisting of a flexible balloon-basket assembly with four electrode needle sheaths, was introduced orally using a guide wire. The balloon was inflated 2 cm proximal to the squamo-columnar junction, the electrode needles were deployed, and RF energy was delivered for 1 min. The needles were then withdrawn, the balloon was deflated, and the catheter was rotated 45°. This process was serially repeated every 0.5 cm inward to cover an area 2 cm above and 0.5 cm below the squamo-columnar junction.

### *Outcome assessment*

The primary outcome measures of this study were frequency and severity of the extra-esophageal GERD symptoms, including cough, sputum, wheezing, and globus hysterics. Data on these outcome measures were collected through a standardized questionnaire using the 6-point Likert scale system. More specifically, the frequency was graded as 0 (none), 1 (less than once per week), 2 (once or twice per week), 3 (three or four times per week), 4 (five or six times per week), and 5 (more than six times per week); the severity was graded as 0 (none), 1 (slight), 2 (mild), 3 (moderate), 4 (severe), and 5 (extremely severe). The total of the frequency and severity scores for each outcome measure was designated as the symptom score. Other outcome measures included medication independence, satisfaction, and reoperation complications. The questionnaires were prepared in simplified Chinese and administered to the subjects before LTF or the Stretta procedure and at 6 months and 1 and 3 years post-treatment.

### *Statistical analysis*

Data were analyzed with SPSS 17.0 software (SPSS Inc., Chicago, IL, United States) and are presented as the mean ± SD for continuous variables and as frequencies and proportions for categorical variables.

**Table 1 Baseline characteristics of the study population**

| Characteristic/ parameter | Stretta ($n$ = 47) | LTF ($n$ = 51) | $P$ value |
|---|---|---|---|
| Age (yr) | 45.9 ± 10.7 | 49.7 ± 11.5 | 0.095 |
| Male ratio, $n$ (%) | 19 (40.4) | 28 (54.9) | 0.163 |
| Symptom score[1] | | | |
| Cough | 7.7 ± 0.57 (20/47) | 7.4 ± 0.82 (25/51) | 0.110 |
| Sputum | 7.5 ± 0.70 (19/47) | 7.4 ± 0.90 (19/51) | 0.560 |
| Wheezing | 7.7 ± 0.81 (29/47) | 7.9 ± 0.93 (33/51) | 0.181 |
| Globus hysterics | 7.4 ± 0.64 (27/47) | 7.2 ± 0.73 (28/51) | 0.171 |

[1]The symptom score was designated as the total of the frequency score and the severity score for each symptom. Values are presented as mean ± SD or $n$ (%). LTF: Laparoscopic toupet fundoplication.

For the statistical analyses, normality was assessed by the Kolmogorov-Smirnov test. For continuous outcomes measured by the questionnaire, we performed comparisons between the 0- and 6-mo values and the 1- and 3-year values using the paired $t$-test or the Wilcoxon matched pairs signed rank sum test, as appropriate. To assess differences in outcomes between the Stretta and LTF procedures, we performed comparisons by the independent-samples $t$-test or Wilcoxon Mann-Whitney test, as appropriate. Analyses of dichotomous data (*e.g.*, medication independence) were performed using the chi-square statistic. A $P$ value less than 0.05 was considered statistically significant.

## RESULTS

A total of 47 patients were included in the Stretta group, and 51 patients were included in the LTF group. Ninety-six patients were available at the 1-year follow-up, and 90 patients were available at the 3-year follow-up. In the Stretta group, one patient underwent re-operation during the first postoperative year, and six patients underwent re-operation within 3 postoperative years. The mean duration of the Stretta procedure was 50 min, and the mean duration of the Toupet procedure was 100 min. The average hospitalization period of patients who underwent the Stretta procedure was 4.4 d, and that of patients who underwent the Toupet procedure was 5.3 d. The baseline symptom scores were similar between the two groups (Table 1).

### *Efficacy*

There were no differences in the pre-treatment symptom scores between patients in the Stretta and LTF groups. The extra-esophageal symptom scores for cough, sputum, wheezing, and globus hysterics improved in both groups. Evaluation at the 3-year follow-up demonstrated a statistically significant improvement in all extra-esophageal symptoms of GERD (Figure 1). Differences in the improvement of symptom scores for cough, sputum, and wheezing

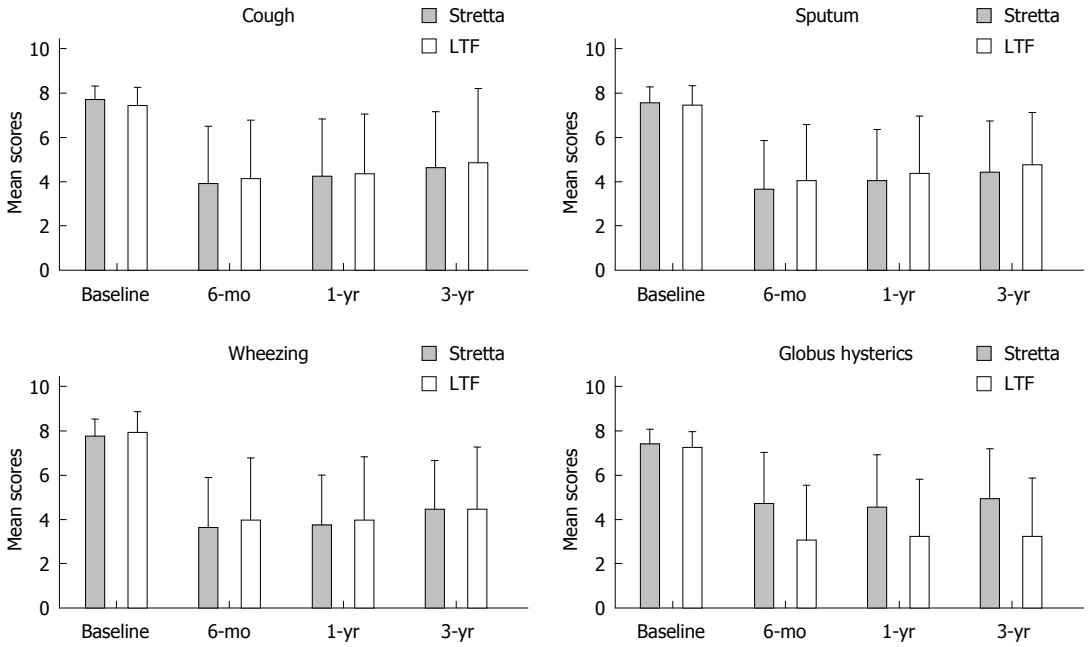



**Figure 1 Preoperative and postoperative symptom scores in the Stretta and laparoscopic Toupet fundoplication groups.**

**Table 2 Post-treatment complications in patients after laparoscopic toupet fundoplication and the Stretta procedure *n* (%)**

| | Stretta (*n* = 47) | LTF (*n* = 51) | *P* value |
|---|---|---|---|
| Fever | 5 (10.6) | 1 (2.0) | 0.073 |
| Pharyngeal pain | 9 (19.1) | 0 (0) | 0.001[1] |
| Retrosternal discomfort | 14 (29.8) | 8 (15.7) | 0.095 |
| Diarrhea | 4 (8.51) | 9 (17.6) | 0.183 |
| Abdominal distention | 0 (0) | 5 (9.8) | 0.028[1] |
| Dysphagia | 0 (0) | 5 (9.8) | 0.028[1] |

[1]*P* value less than 0.05.

between the two groups were not statistically significant. However, the score for globus hysterics in the Stretta group decreased to 4.9 ± 2.24, while that in the LTF group decreased to 3.2 ± 2.63 ($P < 0.05$) (Table 2).

### *Satisfaction*

From baseline to the 3-year follow-up, the mean patient satisfaction score improved from 28.19 to 51.49 in the Stretta group and from 31.76 to 69.80 in the Toupet group. Patients were more satisfied with their quality of life after undergoing LTF than after undergoing the Stretta procedure ($P < 0.05$) (Table 3).

### *Medication requirements*

At baseline, 100% of the patients were on daily proton pump inhibitor (PPI) therapy. The rate of medication use decreased significantly from baseline following surgical intervention. PPI independence was defined as the complete elimination of PPI use or PPI use less than once weekly. There were no significant differences in any parameters measured between the different follow-up time points. After the Stretta and LTF procedures, 29 (61.7%) and 33 (64.7%) patients in each group achieved PPI therapy independence (61.7% *vs* 64.7%, $P = 0.835$).

### *Complications*

No patients experienced perforation, infection, or death. The recorded complications after therapy included fever, pharyngeal pain, retrosternal discomfort, diarrhea, abdominal distention, and dysphagia. Most complications resolved without intervention within two weeks (Table 3). However, two patients still suffered from severe dysphagia at 2 wk post-operation in the LTF group, and it improved after bougie dilation treatment in both patients.

## DISCUSSION

GERD is considered a significant public health problem. According to a population-based survey, the prevalence of symptom-defined GERD in China is 3.1%[9]. PPI therapy is the main option for the treatment of GERD-related extra-esophageal symptoms. A follow-up study showed that most GERD patients with chronic cough showed an improvement in that symptom after 2 years of medical treatment[10]. However, clinical experience shows that 20%-30% of patients with GERD continue to have persistent reflux symptoms even while taking a PPI daily[11]. Therefore, surgical and Stretta therapy should be considered in patients whose disease has failed to respond to medical management and in patients who are unwilling to consent to lifelong medicine intake or have extra-esophageal manifestations.

This prospective study investigated the efficacy and safety of the Stretta procedure and LTF surgical

**Table 3 Post-treatment complications in patients after laparoscopic Toupet fundoplication and the Stretta procedure**

| Characteristic/ parameter value | 6-mo follow-up | | | 1-yr follow-up | | | 3-yr follow-up | | |
|---|---|---|---|---|---|---|---|---|---|
| | Stretta ($n$ = 47) | LTF ($n$ = 51) | $P$ value | Stretta ($n$ = 47) | LTF ($n$ = 51) | $P$ value | Stretta ($n$ = 47) | LTF ($n$ = 51) | $P$ value |
| Symptom score | | | | | | | | | |
| cough | 3.9 ± 2.55 | 4.1 ± 2.65 | 0.780 | 4.2 ± 2.59 | 4.3 ± 2.72 | 0.921 | 4.6 ± 2.52 | 4.8 ± 2.36 | 0.785 |
| Sputum | 3.6 ± 2.24 | 4.0 ± 2.54 | 0.638 | 4.0 ± 2.32 | 4.3 ± 2.60 | 0.695 | 4.4 ± 2.29 | 4.7 ± 2.35 | 0.628 |
| Wheezing | 3.6 ± 2.26 | 3.9 ± 2.83 | 0.635 | 3.7 ± 2.28 | 3.9 ± 2.86 | 0.784 | 4.4 ± 2.23 | 4.4 ± 2.83 | 0.982 |
| Globus hysterics | 4.7 ± 2.30 | 3.0 ± 2.53 | 0.015 | 4.5 ± 2.38 | 3.2 ± 2.58 | 0.040 | 4.9 ± 2.24 | 3.2 ± 2.63 | 0.013 |
| PPI independence, $n$ (%) | 32 (68.1) | 38 (74.5) | 0.510 | 31 (66.0) | 36 (70.6) | 0.668 | 29 (61.7) | 33 (64.7) | 0.835 |
| Satisfaction | 59.26 ± 34.80 | 72.55 ± 28.50 | 0.041 | 56.49 ± 34.78 | 72.16 ± 28.68 | 0.016 | 51.49 ± 32.77 | 69.80 ± 28.44 | 0.004 |

The symptom score was designated as the total of the frequency score and the severity score for each symptom. Values are presented as the mean ± SD or $n$ (%). PPI: Proton pump inhibitor; LTF: Laparoscopic toupet fundoplication.

therapy in patients with extra-esophageal symptoms and compared the outcomes following these two therapeutic methods. Oelschlager *et al*[12] demonstrated that laparoscopic anti-reflux surgery improved atypical symptoms, such as cough and hoarseness. Our previous study showed that the Stretta procedure improves GERD-related respiratory symptoms[13]. In this study, both procedures effectively decreased the frequency and severity of GERD-associated extra-esophageal symptoms. We compared the clinical efficacy of the two therapeutic methods. No significant differences in the improvement of extra-esophageal symptoms, such as cough, sputum, or wheezing, and no difference in the frequency of PPI independence were observed between the two groups. However, LTF surgical therapy was more effective in improving globus hysterics.

Dr. Zhonggao Wang hypothesized four phases through which GERD insults the airway: (1) gastro-esophageal phase, the generator; (2) pharyngeal phase, the reactor; (3) naso-oral phase, the effector; and (4) laryngotracheal phase, the asthmatic or laryngotracheal irritation/spasm stimulator[14]. The Stretta procedure is thought to improve the reflux barrier of the LES and to reduce transient LES relaxations that occur due to ablation or demodulation of vagal afferent fibers in the vicinity of the sphincter[15], while fundoplication is thought to significantly decrease acid exposure and significantly increase LES pressure[16]. In our previous study, laparoscopic LNF and Stretta RF were shown to be capable of effectively and safely controlling GERD symptoms in selected patients, but LNF showed a greater improvement of symptoms than Stretta RF[17]. In the current study, we compared LTF with Stretta RF to evaluate whether these different methods have similar abilities to control the different phases of GERD to provide a reference for clinical treatment.

This was an uncontrolled, nonrandomized study that did not include pH or motility outcomes. Regarding the medication requirement of GERD patients, the rate of PPI medication usage decreased significantly from baseline following surgical intervention. However, changes in respiratory drug use were not examined. In future research, we will include a larger study population and will evaluate changes in respiratory drug use.

In summary, through this 3-year prospective observational study, we demonstrated that LTF and the Stretta procedure were both effective in controlling GERD extra-esophageal symptoms and reducing PPI use. However, LTF achieved a greater symptom improvement and resulted in greater patient satisfaction.

## COMMENTS

### Background

Laparoscopic Toupet fundoplication (LTF) is a major anti-reflux surgical procedure for gastroesophageal reflux disease (GERD). The endoluminal technique for GERD (Stretta) was introduced in 2000. These two techniques are both minimally invasive treatments.

### Research frontiers

Although LTF and the Stretta procedure have been used to treat selected patients with GERD-related extra-esophageal symptoms as minimally invasive procedures, few studies have been conducted to compare the efficacy and safety of these two procedures.

### Innovations and breakthroughs

To the authors' knowledge, the study was the first to compare the efficacy and safety of these two procedures to determine which procedure is optimal for the treatment of GERD-related extra-esophageal symptoms.

### Applications

This research showed that LTF and the Stretta procedure were both effective in controlling GERD-related extra-esophageal symptoms and reducing PPI use. LTF yielded a greater improvement of symptoms and resulted in greater patient satisfaction.

### Peer-review

The results are worth to be published. Authors have to apply sample size analysis in order to justify their results in each of the primary outcomes (two means $t$-test for independent samples).

## REFERENCES

1 **Vakil N**, van Zanten SV, Kahrilas P, Dent J, Jones R; Global Consensus Group. The Montreal definition and classification

of gastroesophageal reflux disease: a global evidence-based consensus. *Am J Gastroenterol* 2006; **101**: 1900-1920; quiz 1943 [PMID: 16928254 DOI: 10.1111/j.15720241.2006.00630.X]
2 **Wang JH**, Luo JY, Dong L, Gong J, Tong M. Epidemiology of gastroesophageal reflux disease: a general population-based study in Xi'an of Northwest China. *World J Gastroenterol* 2004; **10**: 1647-1651 [PMID: 15162542]
3 **Tsoukali E**, Sifrim D. Investigation of extraesophageal gastro-esophageal reflux disease. *Ann Gastroenterol* 2013; **26**: 290-295 [PMID: 24714277]
4 **Ciovica R**, Gadenstätter M, Klingler A, Lechner W, Riedl O, Schwab GP. Quality of life in GERD patients: medical treatment versus antireflux surgery. *J Gastrointest Surg* 2006; **10**: 934-939 [PMID: 16843863 DOI: 10.1016/j.gassur.2006.04.001]
5 **Lundell L**, Miettinen P, Myrvold HE, Hatlebakk JG, Wallin L, Malm A, Sutherland I, Walan A; Nordic GORD Study Group. Seven-year follow-up of a randomized clinical trial comparing proton-pump inhibition with surgical therapy for reflux oesophagitis. *Br J Surg* 2007; **94**: 198-203 [PMID: 17256807 DOI: 10.1016/s0090-3671(08)79237-1]
6 **Velanovich V**. Commentary on the SAGES Guidelines for Surgical Treatment of Gastroesophageal Reflux Disease. *Surg Endosc* 2010; **24**: 2645-2646 [PMID: 20665049 DOI: 10.1007/s00464-010-1266-9]
7 **Shan CX**, Zhang W, Zheng XM, Jiang DZ, Liu S, Qiu M. Evidence-based appraisal in laparoscopic Nissen and Toupet fundoplications for gastroesophageal reflux disease. *World J Gastroenterol* 2010; **16**: 3063-3071 [PMID: 20572311 DOI: 10.3748/wjg.v16.i24.3063]
8 **Noar M**, Squires P, Noar E, Lee M. Long-term maintenance effect of radiofrequency energy delivery for refractory GERD: a decade later. *Surg Endosc* 2014; **28**: 2323-2333 [PMID: 24562599 DOI: 10.1007/s00464-014-3461-6]
9 **He J**, Ma X, Zhao Y, Wang R, Yan X, Yan H, Yin P, Kang X, Fang J, Hao Y, Li Q, Dent J, Sung JJ, Zou D, Wallander MA, Johansson S, Liu W, Li Z. A population-based survey of the epidemiology of symptom-defined gastroesophageal reflux disease: the Systematic Investigation of Gastrointestinal Diseases in China. *BMC Gastroenterol* 2010; **10**: 94 [PMID: 20707933 DOI: 10.1186/1471-230X-10-94]
10 **Jaspersen D**, Labenz J, Willich SN, Kulig M, Nocon M, Leodolter A, Lind T, Meyer-Sabellek W, Vieth M, Stolte M, Malfertheiner P. Long-term clinical course of extra-oesophageal manifestations in patients with gastro-oesophageal reflux disease. A prospective follow-up analysis based on the ProGERD study. *Dig Liver Dis* 2006; **38**: 233-238 [PMID: 16413233 DOI: 10.1016/j.dld.2005.10.026]
11 **Fass R**. Proton-pump inhibitor therapy in patients with gastro-oesophageal reflux disease: putative mechanisms of failure. *Drugs* 2007; **67**: 1521-1530 [PMID: 17661525 DOI: 10.2165/00003495-200767110-00001]
12 **Oelschlager BK**, Quiroga E, Parra JD, Cahill M, Polissar N, Pellegrini CA. Long-term outcomes after laparoscopic antireflux surgery. *Am J Gastroenterol* 2008; **103**: 280-287; quiz 288 [PMID: 17970835 DOI: 10.1111/j.1572-0241.2007.01606.x]
13 **Liang WT**, Wu JM, Wang F, Hu ZW, Wang ZG. Stretta radiofrequency for gastroesophageal reflux disease-related respiratory symptoms: a prospective 5-year study. *Minerva Chir* 2014; **69**: 293-299 [PMID: 25267020]
14 **Wang ZG**, Wu JM, Liu JJ, Wang LY, Lai YG, Ibrahim IM, Wang XJ, Dardik H. Respiratory distress resulting from gastroesophageal reflux is not asthma, but laryngotracheal irritation, spasm, even suffocation. *Chin Med Sci J* 2009; **24**: 130-132 [PMID: 19618613 DOI: 10.1016/S1001-9294(09)60076-6]
15 **Dughera L**, Navino M, Cassolino P, De Cento M, Cacciotella L, Cisarò F, Chiaverina M. Long-Term Results of Radiofrequency Energy Delivery for the Treatment of GERD: Results of a Prospective 48-Month Study. *Diagn Ther Endosc* 2011; **2011**: 507157 [PMID: 22110288 DOI: 10.1155/2011/507157]
16 **Stefanidis D**, Hope WW, Kohn GP, Reardon PR, Richardson WS, Fanelli RD; SAGES Guidelines Committee. Guidelines for surgical treatment of gastroesophageal reflux disease. *Surg Endosc* 2010; **24**: 2647-2669 [PMID: 20725747 DOI: 10.1007/s00464-010-1267-8]
17 **Liang WT**, Wu JN, Wang F, Hu ZW, Wang ZG, Ji T, Zhan XL, Zhang C. Five-year follow-up of a prospective study comparing laparoscopic Nissen fundoplication with Stretta radiofrequency for gastroesophageal reflux disease. *Minerva Chir* 2014; **69**: 217-223 [PMID: 24987969]

**P- Reviewer**: Sgourakis G **S- Editor**: Ma YJ **L- Editor**: Wang TQ
**E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12888

*World J Gastroenterol* 2015 December 7; 21(45): 12888-12895
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

Randomized Controlled Trial

# Effect of posture on $^{13}$C-urea breath test in partial gastrectomy patients

Shu-Ming Yin, Fan Zhang, Dong-Mei Shi, Ping Xiang, Li Xiao, Yi-Qin Huang, Gan-Sheng Zhang, Zhi-Jun Bao

Shu-Ming Yin, Fan Zhang, Dong-Mei Shi, Ping Xiang, Li Xiao, Yi-Qin Huang, Gan-Sheng Zhang, Zhi-Jun Bao, Division of Gastroenterology, Huadong Hospital, Shanghai Medical College of Fudan University, Shanghai 200040, China

Author contributions: Yin SM and Zhang F contributed equally to this work; Yin SM and Zhang F designed the research, analyzed the data and wrote the manuscript; Shi DM, Xiang P, Xiao L, Huang YQ and Zhang GS performed the research; Bao ZJ designed the research and integrated the sections.

Supported by The Guidance Project of Science and Technology Commission of Shanghai Municipality, No. 134119a1700; the Appropriate Project of Shanghai Municipal Health Bureau, No. 2013SY049; Shanghai Key Laboratory of Clinical Geriatric Medicine, No. 13DZ2260700; and the Scientific Research Projects of Shanghai Municipal Health Bureau, No. 20134377.

Institutional review board statement: The study was reviewed and approved by the Medical Ethics Committee of Huadong Hospital Affiliated to Fudan University.

Clinical trial registration statement: This study is registered at http://www.chictr.org.cn/listbycreater.aspx. The registration identification number is ChiCTR-ICQ-15006798.

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

Conflict-of-interest statement: We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Data sharing statement: No additional data are available.



Correspondence to: Dr. Zhi-Jun Bao, Division of Gastroenterology, Huadong Hospital, Shanghai Medical College of Fudan University, No. 221 West Yan'an Road, Shanghai 200040, China. xinyi8681@sina.com
Telephone: +86-21-62487221
Fax: +86-21-62490486

Received: April 19, 2015
Peer-review started: April 21, 2015
First decision: July 10, 2015
Revised: August 3, 2015
Accepted: September 30, 2015
Article in press: September 30, 2015
Published online: December 7, 2015

## Abstract

**AIM:** To investigate whether posture affects the accuracy of $^{13}$C-urea breath test ($^{13}$C-UBT) for *Helicobacter pylori* (*H. pylori*) detection in partial gastrectomy patients.

**METHODS:** We studied 156 consecutive residual stomach patients, including 76 with *H. pylori* infection (infection group) and 80 without *H. pylori* infection (control group). *H. pylori* infection was confirmed if both the rapid urease test and histology were positive during gastroscopy. The two groups were divided into four subgroups according to patients' posture during the $^{13}$C-UBT: subgroup A, sitting position; subgroup B, supine position; subgroup C, right lateral recumbent position; and subgroup D, left lateral recumbent position. Each subject underwent the following modified $^{13}$C-UBT: 75 mg of $^{13}$C-urea (powder) in 100 mL of citric acid solution was administered, and a mouth wash was performed immediately; breath samples were then collected at baseline and at 5-min intervals up to 30 min while the position was maintained. Seven breath

samples were collected for each subject. The cutoff value was 2.0‰.

**RESULTS:** The mean delta over baseline (DOB) values in the subgroups of the infection group were similar at 5 min ($P$ > 0.05) and significantly higher than those in the corresponding control subgroups at all time points ($P$ < 0.01). In the infection group, the mean DOB values in subgroup A were higher than those in other subgroups within 10 min and peaked at the 10-min point (12.4‰ ± 2.4‰). The values in subgroups B and C both reached their peaks at 15 min (B, 13.9‰ ± 1.5‰; C, 12.2‰ ± 1.7‰) and then decreased gradually until the 30-min point. In subgroup D, the value peaked at 20 min (14.7‰ ± 1.7‰). Significant differences were found between the values in subgroups D and B at both 25 min ($t$ = 2.093, $P$ = 0.043) and 30 min ($t$ = 2.141, $P$ = 0.039). At 30 min, the value in subgroup D was also significantly different from those in subgroups A and C (D *vs* C: $t$ = 6.325, $P$ = 0.000; D *vs* A: $t$ = 5.912, $P$ = 0.000). The mean DOB values of subjects with Billroth Ⅰ anastomosis were higher than those of subjects with Billroth Ⅱ anastomosis irrespectively of the detection time and posture ($P$ > 0.05).

**CONCLUSION:** Utilization of the left lateral recumbent position during the procedure and when collecting the last breath sample may improve the diagnostic accuracy of the $^{13}$C-UBT in partial gastrectomy patients.

**Key words:** *Helicobacter pylori*; $^{13}$C-urea breath test; Gastrectomy; Position



**Core tip:** The efficiency of the $^{13}$C-urea breath test in the diagnosis of *Helicobacter pylori* (*H. pylori*) infection in patients after gastrectomy is still controversial. Many factors may affect the diagnostic accuracy, and posture is especially important. We suggest that residual stomach patients should be kept in the horizontal position on the left side during the procedure and when collecting the last breath sample in order to improve the accuracy of detection of *H. pylori* infection.

Yin SM, Zhang F, Shi DM, Xiang P, Xiao L, Huang YQ, Zhang GS, Bao ZJ. Effect of posture on $^{13}$C-urea breath test in partial gastrectomy patients. *World J Gastroenterol* 2015; 21(45): 12888-12895 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12888.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12888

## INTRODUCTION

*Helicobacter pylori* (*H. pylori*), a spiral gram-negative bacterium, can colonize epithelial cells of the gastric mucosa under micro-aerobic growth condition. *H. pylori* infection leads to multiple gastric disorders, including chronic active gastritis, ulcer, adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma[1-3]. It has also been considered one of the factors inducing residual gastric mucosa carcinogenesis in postoperative patients with early-stage gastric carcinoma[4]. Therefore, it is crucial to accurately detect whether *H. pylori* is present in patients who underwent partial gastrectomy.

Due to the lack of specific clinical manifestations, *H. pylori* detection in residual stomach relies on additional examinations. Although the $^{13}$C-urea breath test ($^{13}$C-UBT), a noninvasive diagnostic method for *H. pylori* infection, is inferior to bacterial culture and histological examinations[5], it represents a fast, safe, and reliable technology which is able to accurately determine *H. pylori* infection in an intact stomach. Accordingly, it has been widely used in the general population[6,7]. However, a standardized international protocol defining specific steps, detection methods, and the cutoff value for the $^{13}$C-UBT is currently lacking. In addition, $^{13}$C-UBT diagnosis is limited by some factors, such as fasting and mouth washing, dose and dosage form of $^{13}$C-urea (tablet, capsule, or powder), presence of test meal, time of breath sample collection and storage, and cutoff values[5,8-10]. Since the bacterial load is lower and emptying of the stomach is faster in residual stomach subjects, there are some disputes on the efficiency of the $^{13}$C-UBT in gastrectomy patients[5,11-15]. In particular, the influence of posture on the results of the $^{13}$C-UBT in detecting *H. pylori* infection in partial gastrectomy patients has been a focus of attention. In the existing reports on the $^{13}$C-UBT diagnosis and treatment for *H. pylori* infection in such patients, researchers from different countries utilized the conventional $^{13}$C-UBT protocol for the general population[7,16,17]. Gastric remnant subjects were kept in the sitting position when breath samples were collected, and they usually maintained this position between the collections[14,18]. Only a few studies included the horizontal supine position[15] or the horizontal position on the left side[11,12]. Although Togashi *et al*[9] have performed a preliminary study of different positions (left-lateral horizontal, sitting, or supine position), which position is more suitable for gastrectomy subjects is not yet fully addressed.

The purpose of the present study was to assess whether the position of partial gastrectomy patients during the test affects the diagnostic accuracy of $^{13}$C-UBT for *H. pylori* infection. We attempted to develop a convenient as well as reliable means for *H. pylori* detection and follow-up after eradication therapy in patients who underwent partial gastrectomy.

## MATERIALS AND METHODS

### Subjects

The infection group consisted of 76 patients with partial gastrectomy who visited Huadong Hospital Affiliated

**Table 1 Characteristics of the patients in the infection and control groups**

| | Infection group | | | | Statistic | *P* value | Control group | | | | Statistic | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A (*n* = 19) | B (*n* = 19) | C (*n* 19) | D (*n* = 19) | | | A (*n* = 20) | B (*n* = 20) | C (*n* = 20) | D (*n* = 20) | | |
| Age (yr) | 59.9 ± 11.2 | 63.1 ± 9.2 | 66.1 ± 10.9 | 66.0 ± 12.5 | $F$ = 1.341 | 0.268 | 58.7 ± 12.1 | 62.0 ± 8.5 | 65.2 ± 10.7 | 61.7 ± 14.1 | $F$ = 1.081 | 0.362 |
| Sex (M:F) | 12:7 | 14:5 | 14:5 | 13:6 | $\chi^2$ = 0.686 | 0.877 | 15:5 | 14:6 | 15:5 | 13:7 | $\chi^2$ = 0.671 | 0.880 |
| Indication for gastrectomy | | | | | $\chi^2$ = 0.452 | 0.929 | | | | | $\chi^2$ = 0.251 | 0.969 |
| Peptic ulcer | 7 | 8 | 6 | 7 | - | - | 5 | 6 | 5 | 6 | - | - |
| Early-stage gastric cancer | 12 | 11 | 13 | 12 | - | - | 15 | 14 | 15 | 14 | - | - |
| Reconstructive procedure | | | | | - | - | | | | | - | - |
| B-Ⅰ | 11 | 11 | 11 | 11 | - | - | 10 | 10 | 10 | 10 | - | - |
| B-Ⅱ | 8 | 8 | 8 | 8 | - | - | 10 | 10 | 10 | 10 | - | - |
| Interval (yr) | 8.9 ± 5.9 | 7.4 ± 5.1 | 7.3 ± 3.9 | 7.9 ± 4.9 | $F$ = 0.429 | 0.733 | 8.6 ± 6.0 | 7.6 ± 5.8 | 7.8 ± 5.0 | 8.4 ± 4.6 | $F$ = 0.150 | 0.929 |

to Fudan University from November 2012 to March 2015. *H. pylori* infection was confirmed by histopathological examination. The following inclusion criteria were used: the time interval after the subtotal gastrectomy was at least 1 year; the surgical procedure was distal gastrectomy with Billroth Ⅰ or Ⅱ (B-Ⅰ or B-Ⅱ) anastomosis; the indication for surgery included benign peptic ulcer or early gastric cancer; endoscopy, histological examination, and rapid urease test (RUT) were performed before and after the operation.

The control group contained 80 patients who underwent partial gastrectomy during the same period, met the inclusion criteria, and were *H. pylori*-negative based on histological examination. Patient characteristics such as age, sex, disease etiology, reconstruction method, and postoperative course in the control group were matched to those in the infected group.

In both groups, the subjects were divided into four subgroups according to their posture after $^{13}$C-urea administration: subgroup A, sitting position; subgroup B, supine position on a bed; subgroup C, horizontal position on the right side; as well as subgroup D, horizontal position on the left side. The patients were assigned randomly to A, B, C, or D subgroup according to their reconstruction method. The following exclusion criteria were used: *H. pylori* eradication therapy prior to the present study; treatment with antibiotics, proton pump inhibitors, $H_2$-receptor antagonists, or bismuth salts within 1 mo before the study; absence of endoscopic examination, RUT, pre- and post-operative histological detection for *H. pylori*; presence of test contraindications; distal gastrectomy without B-Ⅰ or B-Ⅱ anastomosis; and previous gastrointestinal surgery history. The subjects were excluded if they fulfilled any of the above criteria.

The research protocol was approved by the Ethical Committee of Huadong Hospital Affiliated to Fudan University. All individuals provided written informed consent. The characteristics of the patients in the infection and control groups are shown in Table 1.

### $^{13}$C-UBT procedure

Modified $^{13}$C-UBT test was conducted in each participant within a week after the endoscopy. Overnight fasting was required before the test. In the following morning, breath samples were taken at baseline ($T_0$) and at 5-min intervals up to 30 min ($T_5$, $T_{10}$, $T_{15}$, $T_{20}$, $T_{25}$, and $T_{30}$) after an oral administration of $^{13}$C-labelled urea powder (75 mg/100 mL citric acid solution; AltaChem Pharma Ltd., Canada) and an immediate mouth wash to remove the residual compound. The first breath sample was taken in the sitting position. The patients were then placed in the positions according to their subgroups and maintained them for 30 min while the rest of the breath samples were collected. These gas samples were collected separately for analysis of the $^{13}CO_2/^{12}CO_2$ ratio ($\Delta^{13}CO_2$, ‰) with an isotope mass spectrometer (IRIS 3, Frankfurt, Germany), which was normalized using a standard gas sample. The analysis was performed by Wagner Analysen Technik GmbH (Bremen, Germany). Differences between the values at $T_5$, $T_{10}$, $T_{15}$, $T_{20}$, $T_{25}$, and $T_{30}$ and those at $T_0$ were presented as delta over baseline (DOB, $\Delta\delta$, ‰). Based on the related reports[5,11,12] and our previous study of 194 samples[19], the cutoff value for this diagnostic test was defined as 2.0‰. Subjects with a DOB > 2.0‰ were considered *H. pylori*-positive,

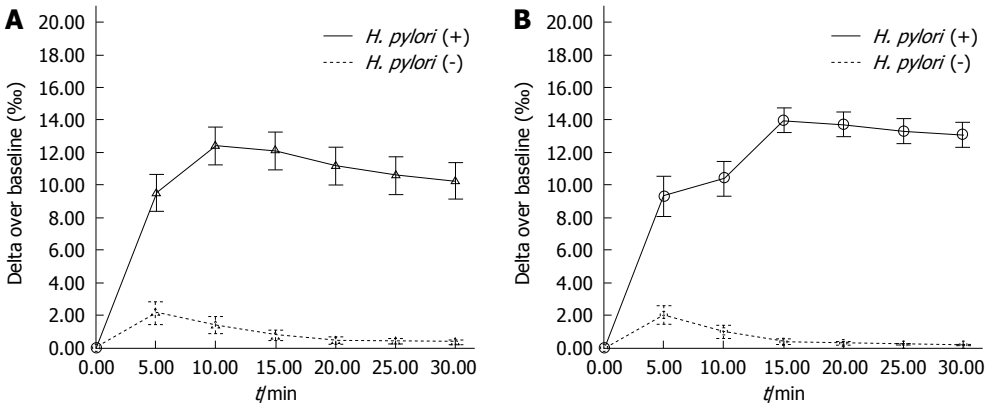



**Figure 1 Mean delta over baseline values for the *Helicobacter pylori*-positive or -negative subjects in the sitting position at each time point.** A: Subgroup A; B: Subgroup B.

whereas those with a DOB < 2.0‰ were considered *H. pylori*-negative.

### *Reference standard for H. pylori infection diagnosis*

Four gastric mucosa biopsy samples were collected separately from the greater curvature of the mid-to-high body as well as the gastric side of the anastomotic stoma, in that order (2 samples from each position), during endoscopy for RUT and histological examination. A positive RUT result was defined as a color alteration from yellow to red during a 24-h period. In most patients, this color change occurred within 120 min. For histological examinations such as hematoxylin and eosin (HE) or Giemsa staining, curved rods were used to identify *H. pylori* in a sectioned specimen. The result of the histological examination was considered positive if *H. pylori* was detected at any site. Only patients with positive RUT and positive histological test were defined as the ones with *H. pylori* infection[1]. Conversely, a patient was considered uninfected when both tests were negative. If inconsistent results were obtained between the RUT and histological test, the corresponding patients were excluded. All biopsy specimens were assessed by a single pathologist who was blinded to the results of endoscopic examinations and UBT for *H. pylori*.

### *Cancer staging system*

We used the cancer staging system from the American Joint Committee on Cancer (AJCC) Cancer Staging Manual (version 7$^{th}$)[20].

### *Statistical analysis*

Statistical Product and Service Solutions (SPSS) software (version 16.0) was used in this study for all statistical analyses. Continuous variables are expressed as mean ± SD and analyzed by Student's *t* test, one-way analysis of variance or Wilcoxon's rank-sum test. Classified variables were analyzed by $\chi^2$ test or Fisher's exact test. *P* values < 0.05 were defined as statistical significance.

## RESULTS

The patients in the infection group (76 subjects) and control group (80 subjects) were divided into four subgroups: A, B, C, and D. As a result, there were 19 infected patients (B-Ⅰ, 11 subjects; B-Ⅱ, 8 subjects) and 20 uninfected patients (B-Ⅰ, 10 subjects; B-Ⅱ, 10 subjects) in each subgroup. No statistically significant differences were found in age, sex, indications for gastrectomy, or postoperative course between the subgroups within the infection group and the control group ($P > 0.05$). The subjects in the infection and control groups were placed in the sitting position, supine position, and right or left lateral recumbent position according to their subgroups. Significantly higher DOB values for each subgroup in the infection group were detected compared with the control group at $T_5$ and thereafter regardless of the patients' posture ($P < 0.01$). No borderline or false-negative results were found in any position and at any time point in the infection group. In the control group, no borderline or false-positive results were found in any position at $T_{10}$, $T_{15}$, $T_{20}$, $T_{25}$, or $T_{30}$. The mean DOB values for the four subgroups of each group are plotted in Figures 1 and 2.

According to the DOB value curves in the infection group (Figure 3), the mean DOB values in the subgroups were similar at $T_5$ ($F = 0.421$, $P = 0.738$). The mean values in subgroup A were higher than in other subgroups within the first 10 min. At $T_{10}$, the mean DOB value was 12.4‰ ± 2.4‰, exceeding the mean DOB value in subgroup D (11.2‰ ± 2.1‰, $t = 1.617$, $P = 0.115$) and being significantly higher than those in subgroups B (10.4‰ ± 2.4‰, $t = 2.634$, $P = 0.012$) and C (9.9‰ ± 1.6‰, $t = 3.811$, $P = 0.001$). The values in subgroups B and C both reached their peaks (B: 13.9‰ ± 1.5‰, C: 12.2‰ ± 1.7‰) at $T_{15}$ and then both decreased gradually until 30 min. In subgroup D, the value peaked (14.7‰ ± 1.7‰) at $T_{20}$ and was significantly higher than those at $T_5$, $T_{10}$, and $T_{15}$ ($F = 30.628$, $P = 0.000$) but did not differ

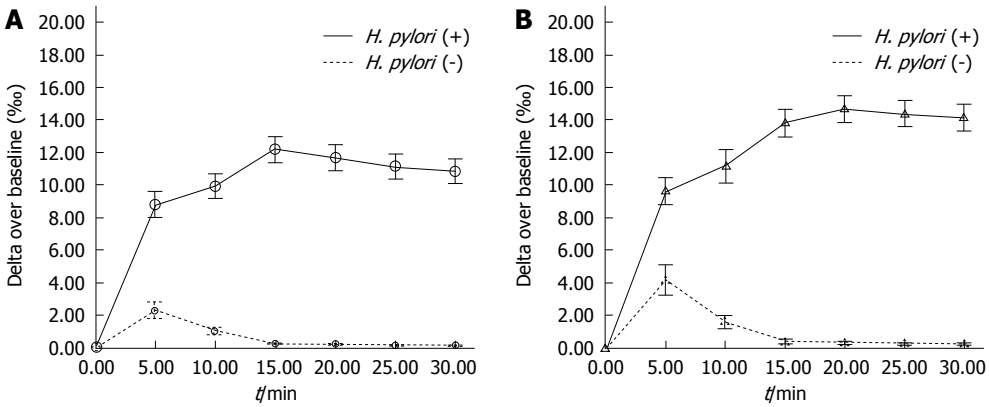



**Figure 2 Mean delta over baseline values for the *Helicobacter pylori*-positive or -negative subjects in the right (A)/left (B) lateral recumbent position at each time point.** A: Subgroup C; B: Subgroup D.

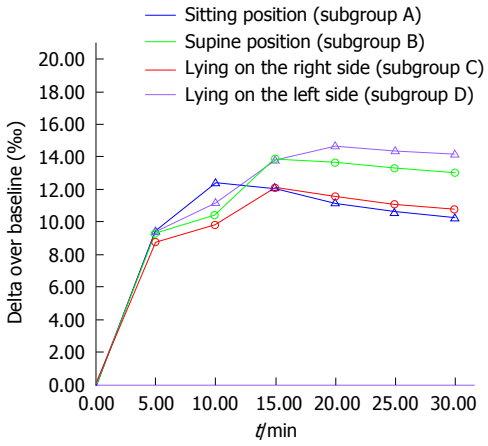



**Figure 3 Mean delta over baseline value curves in the four subgroups of the infection group.**

significantly from those at $T_{25}$ and $T_{30}$ ($F$ = 0.396, $P$ = 0.675). At $T_{20}$, no statistically significant values were determined ($t$ = 1.812, $P$ = 0.078) between the subgroups D and B (13.7‰ ± 1.60‰), but significant differences were observed between the values in these two subgroups at both $T_{25}$ (D: 14.4‰ ± 1.69‰, B: 13.3‰ ± 1.60‰, $t$ = 2.093, $P$ = 0.043) and $T_{30}$ (D: 14.2‰ ± 1.7‰, B: 13.1‰ ± 1.6‰, $t$ = 2.141, $P$ = 0.039). At $T_{30}$, the value in subgroup D was also significantly different from those in subgroups A (10.3‰ ± 2.3‰, $t$ = 5.912, $P$ = 0.000) and C (10.8‰ ± 1.6‰, $t$ = 6.325, $P$ = 0.000).

In the infection group, the mean DOB values of the subjects with B-Ⅰ anastomosis were higher than those of the subjects with B-Ⅱ anastomosis irrespectively of the time point and position. However, these differences (except at $T_5$, $T_{10}$, and $T_{15}$ in subgroup D) were not statistically significant ($P$ > 0.05). Table 2 shows the reconstructive procedure and mean DOB values for the subjects in the infection group.

## DISCUSSION

The $^{13}$C-UBT is an internationally recognized gold standard for *H. pylori* infection detection and anti-*H. pylori* drug efficacy monitoring[2]. It has been widely recommended, even for children, gravidas, and the elderly[7]. However, the application of $^{13}$C-UBT in diagnosing *H. pylori* infection in partial gastrectomy patients remains controversial. In spite of the fact that the gastric remnant is not suitable for colonization by *H. pylori* and its survival, the bacteria can still be transmitted *via* fecal-oral, gastric-oral, oral-oral, and other ways. Gisbert *et al*[5] concluded that the $^{13}$C-UBT was not suitable for gastric remnant patients who underwent Billroth gastrectomy as the ingested $^{13}$C-urea passed through the stomach faster and entered the duodenum (B-Ⅰ) and small intestine (B-Ⅱ) more easily, which would definitely impact the diagnostic accuracy. However, other reports[9,11,12] showed that, with a proper procedure and an appropriate cutoff value, the $^{13}$C-UBT was a reliable detection method in patients after gastrectomy.

Miwa *et al*[21] investigated the effect of different positions (supine position and sitting position) and of changing the position by rolling during the period after $^{13}$C-urea injection on the diagnostic performance of the $^{13}$C-UBT for *H. pylori* infection in infected patients with intact stomach. The study revealed that posture affected the DOB values at $T_5$ and $T_{10}$ but did not affect the results at the 15-min, 20-min, and later time points. Compared with the intact stomach, the anatomy, pH, motility, and distribution of *H. pylori* in the residual stomach are considerably altered, which makes the posture during the $^{13}$C-UBT an important clinical factor. Therefore, we conducted this study to determine the optimal posture for residual stomach subjects.

Our study showed that the DOB values in the control group were lower compared with the infection group at all time points, and the first positive results appeared at $T_5$, which might be due to the presence of urease-positive organisms in the oral cavity early in the procedure. The study by Lee *et al*[8] proved that the effect of oral bacteria was most remarkable at $T_5$ and $T_{10}$, decreased at $T_{15}$, and was weakest at $T_{30}$.

**Table 2 The reconstructive procedure and mean delta over baseline values (‰) for the subjects in the infection group**

| Subgroup | Time | DOB (B-Ⅰ) | DOB (B-Ⅱ) | *t* value | *P* value |
|---|---|---|---|---|---|
| A | $T_5$ | 9.67 ± 1.79 | 9.30 ± 3.00 | 0.314 | 0.760 |
| | $T_{10}$ | 12.57 ± 1.92 | 12.18 ± 3.06 | 0.324 | 0.752 |
| | $T_{15}$ | 12.28 ± 1.95 | 11.89 ± 3.08 | 0.319 | 0.756 |
| | $T_{20}$ | 11.50 ± 1.93 | 10.80 ± 2.94 | 0.588 | 0.568 |
| | $T_{25}$ | 10.91 ± 1.90 | 10.20 ± 2.98 | 0.592 | 0.566 |
| | $T_{30}$ | 10.58 ± 1.88 | 9.86 ± 2.93 | 0.609 | 0.555 |
| B | $T_5$ | 9.74 ± 2.33 | 8.74 ± 2.85 | 0.841 | 0.412 |
| | $T_{10}$ | 10.69 ± 2.11 | 10.01 ± 2.60 | 0.628 | 0.538 |
| | $T_{15}$ | 14.20 ± 1.56 | 13.56 ± 1.56 | 0.880 | 0.391 |
| | $T_{20}$ | 13.99 ± 1.59 | 13.35 ± 1.62 | 0.861 | 0.401 |
| | $T_{25}$ | 13.57 ± 1.60 | 12.91 ± 1.62 | 0.885 | 0.389 |
| | $T_{30}$ | 13.34 ± 1.56 | 12.69 ± 1.59 | 0.890 | 0.386 |
| C | $T_5$ | 8.96 ± 1.69 | 8.69 ± 1.71 | 0.344 | 0.735 |
| | $T_{10}$ | 10.03 ± 1.53 | 9.81 ± 1.67 | 0.288 | 0.777 |
| | $T_{15}$ | 12.33 ± 1.74 | 12.05 ± 1.73 | 0.347 | 0.733 |
| | $T_{20}$ | 11.79 ± 1.69 | 11.50 ± 1.72 | 0.364 | 0.720 |
| | $T_{25}$ | 11.26 ± 1.62 | 10.99 ± 1.69 | 0.352 | 0.729 |
| | $T_{30}$ | 10.95 ± 1.62 | 10.72 ± 1.62 | 0.295 | 0.771 |
| D | $T_5$ | 10.21 ± 1.54 | 8.39 ± 2.18 | 2.136 | 0.048 |
| | $T_{10}$ | 12.12 ± 1.57 | 9.98 ± 2.56 | 2.446 | 0.026 |
| | $T_{15}$ | 14.51 ± 1.39 | 12.91 ± 1.76 | 2.213 | 0.041 |
| | $T_{20}$ | 15.32 ± 1.37 | 13.83 ± 1.82 | 2.049 | 0.056 |
| | $T_{25}$ | 15.04 ± 1.38 | 13.56 ± 1.77 | 2.057 | 0.055 |
| | $T_{30}$ | 14.84 ± 1.37 | 13.33 ± 1.82 | 2.068 | 0.054 |

DOB: Delta over baseline.

However, Togashi *et al*[9] found that oral organisms could affect the final results in residual stomach subjects. Therefore, we suggest that a thorough cleaning of the oral cavity with a mouth wash is important in residual stomach patients after $^{13}$C-urea administration, especially if the powder form is used.

Based on comparing the different subgroups of the infection group, we found that the posture in the period after the first measurement affected the results to some degree. The DOB values peaked at a different point in each subgroup, with those in the sitting position subgroup reaching the maximum at the earliest point ($T_{10}$) and those in the left lateral recumbent position peaking at the latest point ($T_{20}$). Although the DOB values in all subgroups were similar at $T_5$, they differed substantially thereafter. Thus, the DOB values in the subgroups diverged at $T_{10}$, suggesting that they may be affected by the posture early in the test, except for the component caused by the presence of residual organisms in the oral cavity. Furthermore, during the late stage (especially at $T_{20}$ and thereafter), the DOB values were mainly affected by the posture. As the gastric antrum, the most common site of colonization by *H. pylori*, is removed during the operation, the *H. pylori* infection rate in patients after B-Ⅰ or B-Ⅱ gastrectomy is reduced by about 50%[22]. Park *et al*[23] reported *H. pylori* infection rates of 70.8% (B-I) and 45.9% (B-Ⅱ). From the viewpoint of pathophysiology, gastric emptying is faster in the absence of the gastric antrum, and the clearance of $^{13}$C-urea is further accelerated in the sitting position by the gravity force. Together, these factors reduce the time of exposure of the gastric mucosa to $^{13}$C-urea, leading to a significant decrease in DOB values during the late stage of the test. This is the main reason of the low diagnostic accuracy of the $^{13}$C-UBT in residual stomach patients. A test meal, such as citric acid solution, commonly used in the routine $^{13}$C-UBT to prolong gastric emptying and improve the diagnostic accuracy for *H. pylori* infection is ineffective in partial gastrectomy subjects[15]. The time dependence of the DOB values in the right lateral recumbent position group, which was also affected by gastric anatomy and motility, was similar to that in the sitting position group. In the left lateral recumbent position, $^{13}$C-urea clearance was delayed, which allowed better access of the substrate to *H. pylori* urease, resulting in the DOB values peaking at a later time point ($T_{20}$) and remaining relatively stable during the late stage of the test. The DOB values in subgroup D at $T_{25}$ and $T_{30}$ were higher than those in the remaining three subgroups. For clinical convenience[16,24] and to avoid the influence of intestinal bacteria during the late stage, we did not collect breath samples beyond 30 min. Urita *et al*[24] suggested that the routine $^{13}$C-UBT should be conducted for at least 20 min to diagnose *H. pylori* infection. Combined with our findings, the duration of 30 or 25 min with the subject positioned horizontally on the left side during the procedure might be optimal for residual stomach patients.

Based on the effect of posture on DOB values, we conclude that the patients' posture during breath samples collection could also influence final results. To balance the accuracy and convenience, we recommend that residual stomach patients are placed in the sitting position when collecting the first sample and in the left lateral recumbent position thereafter, including when collecting the last sample.

We found no significant differences in DOB values between the groups with different reconstruction methods (B-Ⅰ and B-Ⅱ), indicating that the anastomosis type does not affect the diagnostic value of $^{13}$C-UBT, which is consistent with the study of Togashi *et al*[9]. The differences in DOB values between the subjects with B-Ⅰ and B-Ⅱ anastomoses at $T_5$, $T_{10}$, and $T_{15}$ in infection subgroup D suggest that the late stage of the test ($T_{20}$ and thereafter) may be optimal for avoiding the effect of the operation type. Moreover, the left lateral recumbent position during the $^{13}$C-UBT procedure was most suitable for the B-Ⅰ and B-Ⅱ subjects. In this study, we selected subjects with B-I and B-Ⅱ gastrectomy to simplify the analysis and to be able to use universal conditions during the test, and other operative methods will require further research.

As the *H. pylori* load is lower and gastric emptying is faster after the operation, the $CO_2$ concentration in breath samples may be insufficient to detect positive results, and the cutoff value for the $^{13}$C-UBT in residual stomach subjects should be lower than that in the

general population[1,5,12]. Therefore, the cutoff value in this study was reduced from 3.5‰ to 2‰. According to the literature[9], the sensitivity of the $^{13}$C-UBT for gastric mucosa *H. pylori* detection is 82.2%-96.3%, and the specificity is 94.6%-100%. Based on the comparison with the results of histological examinations, Kubota *et al*[11] found that the most appropriate cutoff value in residual stomach subjects was 2.0‰ as determined using a receiver operating characteristic curve. Under these conditions, high sensitivity (96.3%), specificity (100%), and accuracy (97.1%) were achieved. Our previous study[19] found that, when the cutoff value was 2.0‰, the $^{13}$C-UBT had a high sensitivity (88.6%) and specificity (94.9%), and its accuracy (92.6%) was similar to that of the invasive test method (histological examination, 93.5%), with good consistency between the two approaches (Kappa = 0.84). Accordingly, the 2.0‰ cutoff value is more suitable for residual stomach subjects than the conventional value of 3.5‰.

Gisbert *et al*[25] suggested that values in the 2.0‰-5.0‰ range represented borderline results. We did not observe such borderline results in the present study. We suggest that a reexamination by the $^{13}$C-UBT or other tests should be performed to verify the results for residual stomach patients whose DOB values are within the above-mentioned range, and special attention should be paid in cases with DOB values around 5‰.

In conclusion, unlike in the general population, posture can influence the diagnostic accuracy of the $^{13}$C-UBT in residual stomach subjects, especially during the late stage of the test (20 min and thereafter). We suggest that the mouth should be washed after $^{13}$C-urea solution administration and that a cutoff value of 2.0‰ and the horizontal position on the left side should be used during the procedure (whose optimal time is 30 or 25 min) and when collecting the last breath sample. The modified $^{13}$C-UBT may be a simple, safe, and effective method for diagnosing *H. pylori* infection and for long-term follow-up after eradication therapy in residual stomach subjects.

## COMMENTS

### Background

Accurate detection of the presence of *Helicobacter pylori* (*H. pylori*) in patients with partial gastrectomy is of crucial importance. The efficiency of the $^{13}$C-urea breath test ($^{13}$C-UBT) in the diagnosis of *H. pylori* infection in patients after gastrectomy is still controversial.

### Research frontiers

Many factors may affect the diagnostic accuracy, and posture is especially important.

### Innovations and breakthroughs

Unlike in the general population, posture may affect the diagnostic accuracy of the $^{13}$C-UBT in residual stomach subjects, especially during the late stage of the test (20 min and thereafter). Utilization of the left lateral recumbent position during the procedure and when collecting the last breath sample may improve the diagnostic accuracy of the $^{13}$C-UBT in partial gastrectomy patients.

### Applications

The modified $^{13}$C-UBT may be a simple, safe, and effective method for diagnosing *H. pylori* infection and for long-term follow-up after eradication therapy in residual stomach subjects.

### Peer-review

This manuscript is interesting to me. Authors meticulously designed four subgroups in *H. pylori* infection and control patients with different postures for demonstrating the significance of outcome of $^{13}$C-urea breath test. The data analysis is confident and exact. I think authors need amend some information of outcome of $^{13}$C-urea breath test in the same patient with four different postures, which can provide the more trustful results.

## REFERENCES


1 **Malfertheiner P**, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, Hunt R, Rokkas T, Vakil N, Kuipers EJ. Current concepts in the management of Helicobacter pylori infection: the Maastricht III Consensus Report. *Gut* 2007; **56**: 772-781 [PMID: 17170018 DOI: 10.1136/gut.2006.101634]

2 **Chey WD**, Wong BC. American College of Gastroenterology guideline on the management of Helicobacter pylori infection. *Am J Gastroenterol* 2007; **102**: 1808-1825 [PMID: 17608775 DOI: 10.1111/j.1572-0241.2007.01393.x]

3 **Tian XY**, Zhu H, Zhao J, She Q, Zhang GX. Diagnostic performance of urea breath test, rapid urea test, and histology for Helicobacter pylori infection in patients with partial gastrectomy: a meta-analysis. *J Clin Gastroenterol* 2012; **46**: 285-292 [PMID: 22392025 DOI: 10.1097/MCG.0b013e318249c4cd]

4 **Sinning C**, Schaefer N, Standop J, Hirner A, Wolff M. Gastric stump carcinoma - epidemiology and current concepts in pathogenesis and treatment. *Eur J Surg Oncol* 2007; **33**: 133-139 [PMID: 17071041 DOI: 10.1016/j.ejso.2006.09.006]

5 **Gisbert JP**, Pajares JM. 13C-urea breath test in the management of Helicobacterpylori infection. *Dig Liver Dis* 2005; **37**: 899-906 [PMID: 16280266 DOI: 10.1016/j.dld.2005.09.006]

6 **Braden B**, Lembcke B, Kuker W, Caspary WF. 13C-breath tests: current state of the art and future directions. *Dig Liver Dis* 2007; **39**: 795-805 [PMID: 17652042 DOI: 10.1016/j.dld.2007.06.012]

7 **Campuzano-Maya G**. An optimized 13C-urea breath test for the diagnosis of H pylori infection. *World J Gastroenterol* 2007; **13**: 5454-5464 [PMID: 17907288 DOI: 10.3748/wjg.v13.i41.5454]

8 **Lee TH**, Yang JC, Lee SC, Farn SS, Wang TH. Effect of mouth washing on the. *J Gastroenterol Hepatol* 2001; **16**: 261-263 [PMID: 11339415 DOI: 10.1046/j.1440-1746.2001.02437.x]

9 **Togashi A**, Matsukura N, Kato S, Masuda G, Ohkawa K, Tokunaga A, Yamada N, Tajiri T. Simple and accurate (13)C-urea breath test for detection of Helicobacter pylori in the remnant stomach after surgery. *J Gastroenterol* 2006; **41**: 127-132 [PMID: 16568371 DOI: 10.1007/s00535-005-1731-8]

10 **Colaiocco Ferrante L**, Papponetti M, Marcuccitti J, Neri M, Festi D. 13C-urea breath test for helicobacter pylori infection: stability of samples over time. *Scand J Gastroenterol* 1999; **34**: 942-943 [PMID: 10522617]

11 **Kubota K**, Shimoyama S, Shimizu N, Noguchi C, Mafune K, Kaminishi M, Tange T. Studies of 13C-urea breath test for diagnosis of Helicobacter pylori infection in patients after partial gastrectomy. *Digestion* 2002; **65**: 82-86 [PMID: 12021481 DOI: 10.1159/000057709]

12 **Kubota K**, Hiki N, Shimizu N, Shimoyama S, Noguchi C, Tange T, Mafune K, Kaminishi M. Utility of [13C] urea breath test for Helicobacter pylori detection in partial gastrectomy patients. *Dig Dis Sci* 2003; **48**: 2135-2138 [PMID: 14705818 DOI: 10.1023/B:DDAS.0000004516.45529.ee]

13 **Adamopoulos AB**, Stergiou GS, Sakizlis GN, Tiniakos DG, Nasothimiou EG, Sioutis DK, Achimastos AD. Diagnostic value of rapid urease test and urea breath test for Helicobacter pylori

detection in patients with Billroth II gastrectomy: a prospective controlled trial. *Dig Liver Dis* 2009; **41**: 4-8 [PMID: 18606579 DOI: 10.1016/j.dld.2008.05.010]

14 **Schilling D**, Jakobs R, Peitz U, Sulliga M, Stolte M, Riemann J, Labenz J. Diagnostic accuracy of (13)C-urea breath test in the diagnosis of Helicobacter pylori infection in patients with partial gastric resection due to peptic ulcer disease: a prospective multicenter study. *Digestion* 2001; **63**: 8-13 [PMID: 11173894 DOI: 10.1159/000051866]

15 **Sheu BS**, Lee SC, Lin PW, Wang ST, Chang YC, Yang HB, Chuang CH, Lin XZ. Carbon urea breath test is not as accurate as endoscopy to detect Helicobacter pylori after gastrectomy. *Gastrointest Endosc* 2000; **51**: 670-675 [PMID: 10840298 DOI: 10.1067/mge.2000.105719]

16 **Ohara S**, Kato M, Asaka M, Toyota T. Studies of 13C-urea breath test for diagnosis of Helicobacter pylori infection in Japan. *J Gastroenterol* 1998; **33**: 6-13 [PMID: 9497214 DOI: 10.1007/PL00009968]

17 **Logan RP**, Polson RJ, Misiewicz JJ, Rao G, Karim NQ, Newell D, Johnson P, Wadsworth J, Walker MM, Baron JH. Simplified single sample 13Carbon urea breath test for Helicobacter pylori: comparison with histology, culture, and ELISA serology. *Gut* 1991; **32**: 1461-1464 [PMID: 1773948 DOI: 10.1136/gut.32.12.1461]

18 **Kwon YH**, Kim N, Lee JY, Choi YJ, Yoon K, Yoon H, Shin CM, Park YS, Lee DH. The diagnostic validity of the (13)c-urea breath test in the gastrectomized patients: single tertiary center retrospective cohort study. *J Cancer Prev* 2014; **19**: 309-317 [PMID: 25574466 DOI: 10.15430/JCP.2014.19.4.309]

19 **Yin SM**, Zhang GS, Xiang P, Xiao L, Huang YQ, Chen J, Bao ZJ, Yu XF. Application value of 13C-urea breath test in the diagnosis of Helicobacter pylori infection in gastric remnant. *Zhongguo Xiaohua Zazhi* 2012; **32**: 669-673 [DOI: 10.3760/cma.j.issn.0254-1432.2012.10.005]

20 **Edge SB**, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. *Ann Surg Oncol* 2010; **17**: 1471-1474 [PMID: 20180029 DOI: 10.1245/s10434-010-0985-4]

21 **Miwa H**, Murai T, Ohkura R, Kawabe M, Tanaka H, Ogihara T, Watanabe S, Sato N. Effect of fasting subjects' posture on 13C-urea breath test for detection of Helicobacter pylori infection. *Helicobacter* 1997; **2**: 82-85 [PMID: 9432333]

22 **Danesh J**, Appleby P, Peto R. How often does surgery for peptic ulceration eradicate Helicobacter pylori? Systematic review of 36 studies. *BMJ* 1998; **316**: 746-747 [PMID: 9529411]

23 **Park S**, Chun HJ. Helicobacter pylori infection following partial gastrectomy for gastric cancer. *World J Gastroenterol* 2014; **20**: 2765-2770 [PMID: 24659869 DOI: 10.3748/wjg.v20.i11.2765]

24 **Urita Y**, Hike K, Torii N, Kikuchi Y, Kanda E, Kurakata H, Sasajima M, Miki K. Breath sample collection through the nostril reduces false-positive results of 13C-urea breath test for the diagnosis of helicobacter pylori infection. *Dig Liver Dis* 2004; **36**: 661-665 [PMID: 15506664 DOI: 10.1016/j.dld.2004.06.008]

25 **Gisbert JP**, Olivares D, Jimenez I, Pajares JM. Long-term follow-up of 13C-urea breath test results after Helicobacter pylori eradication: frequency and significance of borderline delta13CO2 values. *Aliment Pharmacol Ther* 2006; **23**: 275-280 [PMID: 16393307 DOI: 10.1111/j.1365-2036.2006.02741.x]

**P- Reviewer**: Bener A **S- Editor**: Yu J **L- Editor**: Wang TQ
**E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12896

*World J Gastroenterol* 2015 December 7; 21(45): 12896-12953
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


SYSTEMATIC REVIEWS

# Possible association between hepatitis C virus and malignancies different from hepatocellular carcinoma: A systematic review

Sirio Fiorino, Letizia Bacchi-Reggiani, Dario de Biase, Adele Fornelli, Michele Masetti, Andrea Tura, Fabio Grizzi, Matteo Zanello, Laura Mastrangelo, Raffaele Lombardi, Giorgia Acquaviva, Luca di Tommaso, Arrigo Bondi, Michela Visani, Sergio Sabbatani, Laura Pontoriero, Carlo Fabbri, Andrea Cuppini, Annalisa Pession, Elio Jovine

Sirio Fiorino, Andrea Cuppini, Unità Operativa di Medicina Interna, Ospedale di Budrio, Budrio, 40054 Bologna, Italy

Letizia Bacchi-Reggiani, Dipartimento di Medicina Sperimentale, Istituto di Cardiologia Policlinico S. Orsola-Malpighi, Università degli Studi di Bologna, 40138 Bologna, Italy

Dario de Biase, Giorgia Acquaviva, Dipartimento di Medicina Sperimentale, Università di Bologna, Unità Operativa Biologia Molecolare, Anatomia Patologica Ospedale Bellaria, 40139 Bologna, Italy

Adele Fornelli, Arrigo Bondi, Servizio di Anatomia Patologica, Ospedale Maggiore, 40133 Bologna, Italy

Michele Masetti, Matteo Zanello, Laura Mastrangelo, Raffaele Lombardi, Elio Jovine, Unità Operativa di Chirurgia A, Ospedale Maggiore, 40133 Bologna, Italy

Andrea Tura, Institute of Neuroscience, National Research Council, 35127 Padova, Italy

Fabio Grizzi, Luca di Tommaso, Humanitas Clinical and Research Center, Rozzano, 20089 Milano, Italy

Michela Visani, Annalisa Pession, Dipartimento di Farmacia e Biotecnologie, Università di Bologna, 40139 Bologna, Italy

Sergio Sabbatani, Istituto di Malattie Infettive, Policlinico S. Orsola-Malpighi, Università degli Studi di Bologna, 40138 Bologna, Italy

Laura Pontoriero, Unità di Cura Materno-Infantile, Distretto Lametino, ASP, 88100 Catanzaro, Italy

Carlo Fabbri, U.O. Endoscopia, Ospedale Maggiore, 40133 Bologna, Italy

Author contributions: Fiorino S conceived the study and coordinated the search activity of colleagues; Visani M and Acquaviva G contributed to the design of the review and coordinated the preparation of the first draft of manuscript; Masetti M and Lombardi R independently and in a parallel manner, performed the literature search, identified and screened the articles; Bacchi-Reggiani L and Fornelli A supervised the literature search analysis; Grizzi F and di Tommaso L contributed to write the first draft of manuscript; Tura A and Pontoriero L checked the accuracy of data collection; Zanello M and Mastrangelo L independently extracted and tabulated all relevant data from included studies by means of a standardized flow path and contributed to writing the manuscript; Fabbri C and Cuppini A commented on drafts of the manuscript; Bondi A and Pession A supervised and critically reviewed the manuscript; Sabbatani S and Jovine E were responsible for the final approval of manuscript; de Biase D contributed to the design of the study and commented on drafts of the manuscript; all authors approved the final version of the manuscript.

Conflict-of-interest statement: All authors declare that they have no competing interest.

Data sharing statement: The dataset is available from the corresponding author at sirio.fiorino@ausl.bologna.it.



Correspondence to: Sirio Fiorino, MD, Unità Operativa di Medicina Interna, Ospedale di Budrio, Via Benni 44, Budrio, 40054 Bologna, Italy. sirio.fiorino@ausl.bologna.it
Telephone: +39-51-809259
Fax: +39-51-809296

Received: April 17, 2015
Peer-review started: April 19, 2015
First decision: July 14, 2015
Revised: August 5, 2015
Accepted: October 13, 2015
Article in press: October 13, 2015
Published online: December 7, 2015

## Abstract

**AIM:** To summarize the current knowledge about the potential relationship between hepatitis C virus (HCV) infection and the risk of several extra-liver cancers.

**METHODS:** We performed a systematic review of the literature, according to the Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) Statement. We extracted the pertinent articles, published in MEDLINE and the Cochrane Library, using the following search terms: neoplasm/cancer/malignancy/tumor/carcinoma/adeno-carcinoma and non-Hodgkin lymphomas, kidney/renal-, cholangio-, pancreatic-, thyroid-, breast-,oral-, skin-, prostate-, lung-, colon-, stomach-, haematologic. Case series, case-series with control-group, case-control, cohort-studies as well as meta-analyses, written in English were collected. Some of the main characteristics of retrieved trials, which were designed to investigate the prevalence of HCV infection in each type of the above-mentioned human malignancies were summarised. A main table was defined and included a short description in the text for each of these tumours, whether at least five studies about a specific neoplasm, meeting inclusion criteria, were available in literature. According to these criteria, we created the following sections and the corresponding tables and we indicated the number of included or excluded articles, as well as of meta-analyses and reviews: (1) HCV and haematopoietic malignancies; (2) HCV and cholangiocarcinoma; (3) HCV and pancreatic cancer; (4) HCV and breast cancer; (5) HCV and kidney cancer; (6) HCV and skin or oral cancer; and (7) HCV and thyroid cancer.

**RESULTS:** According to available data, a clear correlation between regions of HCV prevalence and risk of extra-liver cancers has emerged only for a very small group of types and histological subtypes of malignancies. In particular, HCV infection has been associated with: (1) a higher incidence of some B-cell Non-Hodgkin-Lymphoma types, in countries, where an elevated prevalence of this pathogen is detectable, accounting to a percentage of about 10%; (2) an increased risk of intra-hepatic cholangiocarcinoma; and (3) a correlation between HCV prevalence and pancreatic cancer (PAC) incidence.

**CONCLUSION:** To date no definitive conclusions may be obtained from the analysis of relationship between HCV and extra-hepatic cancers. Further studies, recruiting an adequate number of patients are required to confirm or deny this association.

**Key words:** Neoplasm; Cancer; Hepatitis C virus; Risk factors; Extra-hepatic malignancies; Hepatocellular carcinoma; Pancreatic cancer; Cholangiocarcinoma



**Core tip:** Hepatitis C virus (HCV) is an oncogenic virus and a well-known risk factor for hepatocellular carcinoma. Some reports suggested that its infection is associated with development of cholangiocarcinoma and some types of lymphomas, but a comprehensive assessment of the possible role of HCV in extrahepatic carcinogenesis has not been yet performed. Aim of this review is to focus on HCV infection association with extra-liver neoplasms, as lymphomas, pancreatic cancer and breast-, renal-, oral- and thyroid-cancers. Our results strongly support the need of additional studies to ensure a precise estimate of the effect of HCV on these different types of extra-hepatic cancers.

Fiorino S, Bacchi-Reggiani L, de Biase D, Fornelli A, Masetti M, Tura A, Grizzi F, Zanello M, Mastrangelo L, Lombardi R, Acquaviva G, di Tommaso L, Bondi A, Visani M, Sabbatani S, Pontoriero L, Fabbri C, Cuppini A, Pession A, Jovine E. Possible association between hepatitis C virus and malignancies different from hepatocellular carcinoma: A systematic review. *World J Gastroenterol* 2015; 21(45): 12896-12953 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12896.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12896

## INTRODUCTION

Hepatitis C virus (HCV) is a major global health problem, because it represents a very important cause of mortality, morbidity and resource utilization. Although remarkable differences are detectable in the world, depending on geographical areas and ethnicity, it is estimated that the prevalence of HCV infection is about 2% worldwide (Figure 1)[1]. Approximately 180 million people carriers this pathogen persistently[2]. HCV chronic infection can lead to a necro-inflammatory liver disease, with different pattern of severity and course. This condition is associated with an increased risk of cirrhosis, liver failure and hepatocellular carcinoma[3]. Although liver is the main target for HCV, it is now well-known that this pathogen may induce extra-hepatic pathological conditions, including mixed cryoglobulinemia, porphyria cutanea tarda, membranoproliferative glomerulonephritis, Sjögren's syndrome, thyroiditis, a high prevalence of autoantibodies[4] as well as Central and Peripheral Nervous System demyelinating disorders[5]. Several of these manifestations are thought to be caused by the host immune response to this micro-organism

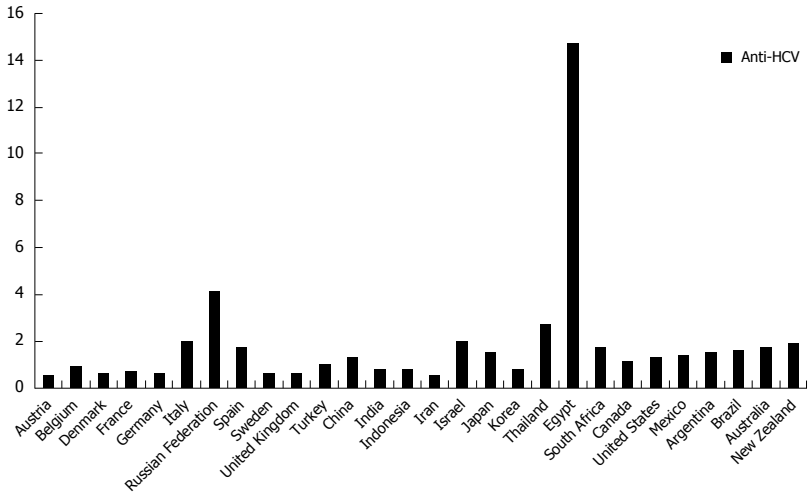



**Figure 1 Prevalence of chronic hepatitis C virus status in different coutries worldwide.** HCV: Hepatitis C virus.

and not by a direct viral cytopathic effect. In particular, chronic antigenic stimulation by HCV promotes B-lymphocyte clonal expansion, with the production and release of monoclonal and polyclonal antibodies and generation of immune complexes[6]. Their deposition in small vessels and glomerular capillary walls induces complement activation and, as consequence, tissue injury[7]. In addition, several studies have shown that HCV may infect organs and tissues other than the liver. In particular, presence of antigens, genome and/or replicative sequences of HCV have been detected in several extra-hepatic localizations, such as peripheral blood cells (*i.e.,* neutrophils, T- and B-lymphocytes)[8,9] or kidney[10], skin[11,12], oral mucosa[13], salivary glands[14] and pancreas tissues as well as, in a small number of cases, from heart, gallbladder, intestine and adrenal glands tissues[15,16].

Although HCV antigens and replicative forms have been detected in various extra-hepatic sites, the possible role on the onset of malignancies in these organs is still under investigation. Some evidences have recently suggested the possibility that this pathogen may be associated with the development of a wide spectrum of hematologic or solid cancers, such as non-Hodgkin lymphomas, biliary duct-, bladder-, renal-, pancreatic-, thyroid-, breast- and prostate-carcinomas. Here we summarize the current knowledge about the potential link between HCV infection and risk of these malignancies and we performed a systematic review of the literature that reports the prevalence of HCV infection in patients, suffering from above mentioned malignancies.

## MATERIALS AND METHODS

### *Search strategy and selection of studies*

See supplementary Material and Methods for further information.

A systematic computer-based search of published articles, according to the Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) Statement, issued in 2009, was conducted through Ovid interface, in order to identify relevant studies on the potential association between HCV infection and malignancies other than hepatocellular carcinoma (HCC). The literature review was performed in February 2015. The following electronic databases were used: MEDLINE (1950 to February, 2015) and the Cochrane Library (until the fourth quarter of 2014) for all relevant articles. The search strategy and the search terms were developed with the support of a professional research librarian. The search text words were identified by means of controlled vocabulary, such as the National Library of Medicine's MESH (Medical Subject Headings) and Keywords. In our review, assessing the possible association between HCV infection and risk of malignancies other than HCC, we focused on the following malignancies: (1) lymphomas and, in particular, non-Hodgkin lymphomas; (2) biliary ducts-/gallbladder-; (3) renal-/kidney-; (4) pancreatic-; (5) thyroid-; (6) breast-; (7) lung-; (8) stomach-; (9) colon-; (10) skin-/oral-; (11) bladder-; and (12) prostate-carcinomas. The inclusion criteria for our analysis were: (1) study designs by considering data from all published case series, case-control-, hospital-based case-control-, population-based case-control- as well as cohort-studies; (2) articles which were reported in English, as peer-reviewed, full-text publications, whereas papers that were not published as full reports, such as conference abstracts, case reports, and editorials were excluded; (3) clinical series or studies evaluating histological specimens, that included at least 15 patients, therefore reports with fewer than 15 subjects were not considered; and (4) papers describing the type of tests used to assess HCV presence; in particular, in all studies virus search was performed by means of second- or third-generation enzyme-linked immunosorbent assay

(ELISA) or recombinant immunoblot assay (RIBA) for confirmation as well as in a large part of available trials HCV-RNA presence was also tested.

### *Study selection*

**Data extraction:** Two authors (Masetti M and Bacchi-Reggiani L), independently and in a parallel manner, performed the literature search, identified and screened relevant articles, based on title or title and abstract. If a study was considered potentially eligible by either of the 2 reviewers, the full article of this research was collected for further assessment. Other two authors (Zanello M and Mastrangelo L) independently extracted and tabulated all relevant data from included studies by means of a standardized flow path, according to the Cochrane handbook section 7.3a checklist of domains. The following information was obtained from each study, by means of a predefined data extraction form, including: first author's name, study design, inclusion and exclusion criteria, year of publication, country of origin, ethnicity, matching criteria, number of cases and controls, diagnostic methods to detect each malignancy, HCV detection assays. The accuracy of data collection was checked by Tura A and any disagreements concerning the results were settled by consensus between all authors. With the purpose to prevent multiple inclusions of the same data, we searched the presence of possible duplicates, examining the first author's name as well as the place and the period of subjects' enrolment. When different versions of the same study were detected, only the most recent one was considered.

## RESULTS

The search of MEDLINE and Cochrane Library produced the following citations: (1) haematopoietic malignancies: 1424; (2) biliary ducts-/cholangio-: 616; (3) renal-/kidney-: 891; (4) pancreatic-: 244; (5) thyroid-: 126; (6) breast-: 180; (7) lung-: 247; (8) stomach-: 141; (9) colon-: 115; (10) skin-/oral-: 598; (11) bladder-: 150; and (12) prostate-carcinomas: 43.

After a preliminary review of the titles and/or abstracts with the exclusion of non-pertinent articles, we obtained these results: (1) haematopoietic malignancies, including lymphomas/non-Hodgkin lymphomas: 126; (2) biliary ducts-/cholangio-: 48; (3) renal-/kidney-: 10; (4) pancreatic-: 15; (5) thyroid-: 11; (6) breast-: 8; (7) lung-: 3; (8) stomach-: 2; (9) colon-: 5; (10) skin-/oral-: 11; (11) bladder-: 3; and (12) prostate-carcinomas: 5.

We screened the potentially relevant studies and, in accordance with predefined criteria, we identified and considered in our systematic review the following number of studies: (1) haematopoietic malignancies: 108 articles considered, 6 reviews/meta-analyses, 12 papers excluded[17-146]; (2) biliary ducts/cholangiocarcinoma: 36 articles considered, 3 reviews/meta-analyses, 9 papers excluded[147-195]; (3) renal/kidney: 8 articles considered, 2 papers excluded[118,146,196-203]; (4) pancreatic: 9 articles considered, 3 reviews/meta-analyses, 3 papers excluded[118,146,170,197,204-214]; (5) thyroid: 7 articles considered, 4 papers excluded[52,73,118,146,196,197,215-217]; (6) breast: 6 articles considered, 2 papers excluded[117,118,146,196,197,202,218,219]; (7) lung: 2 articles meeting inclusion criteria, 1 not[146,196,197]; (8) stomach: 2 articles meeting inclusion criteria[146,220]; (9) colon: 3 articles meeting inclusion criteria, 2 not[118,146,196,197,202]; (10) skin/oral: 10 articles considered, 1 paper excluded[118,146,197,221-228]; (11) bladder: 3 articles meeting inclusion criteria[118,146,197]; and (12) prostate-carcinomas: 4 articles meeting inclusion criteria, 1 not[118,146,196,197,202] (Tables 1-7). A limited number of identified studies were formally designed as "cohort-" or "case-control" trials, adequately reporting inclusion criteria for the control group, such as "odds ratios" after adjustment for the most important confounding factors, or showing that cases and controls were matched by sex and age. Whether these data were not indicated, but an acceptable series of healthy subjects or patients with different diseases were recruited for comparison and were described, we defined the considered study, as: "case series with control group".

On the basis of our results, we summarised some of the main characteristics of retrieved trials, which were designed to investigate the prevalence of HCV infection in each type of the above- mentioned malignancies. In particular, we created a main table and included a short description in the text for each of these tumours, whether at least five studies, evaluating this parameter and meeting inclusion criteria, were available in literature. In each of these tables, we reported the following data of studies considered: first author's name, study design, year of publication, country of origin, matching criteria, number of cases and controls, diagnostic methods to detect each malignancy, percentage of HCV-positive cases with 95% confidence intervals (CIs) and main conclusions. CIs for each proportion were calculated according to normal distribution or binomial distribution as appropriate. Accordingly to these pre-definite criteria, we created the following sections and the corresponding Tables: (1) HCV and haematopoietic malignancies (Table 1); (2) HCV and cholangiocarcinoma (Table 2); (3) HCV and pancreatic cancer (Table 3); (4) HCV and breast cancer (Table 4); (5) HCV and kidney cancer (Table 5); (6) HCV and skin or oral cancer (Table 6); and (7) HCV and thyroid cancer (Table 7). Furthermore, we created additional tables, reporting both the studies not considered in our systematic review and the meta-analyses, assessing the association between HCV infection and risk of each human malignancy (see Tables 1-7). A summary of number of studies and meta-analyses is shown in Figure 2. Age-standardized incidence rates of each malignancy per 100000 person-years are reported for sex and for different countries in

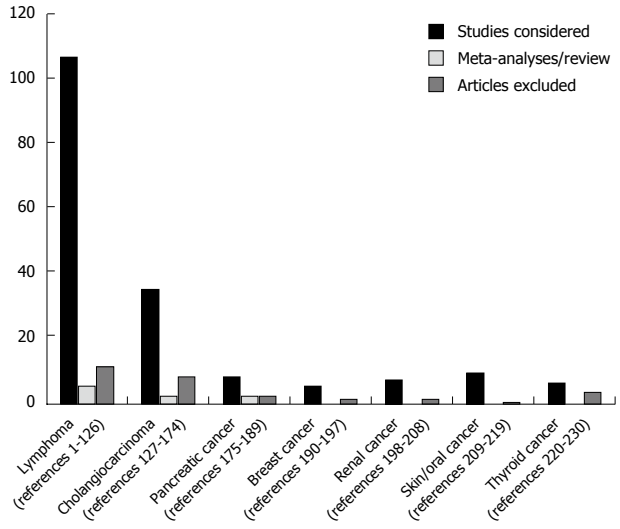



**Figure 2 Number of studies and meta-analyses, assessing the association between hepatitis C virus infection and different types of malignancies, included in the present systematic review.** References are reported in the supplementary section.

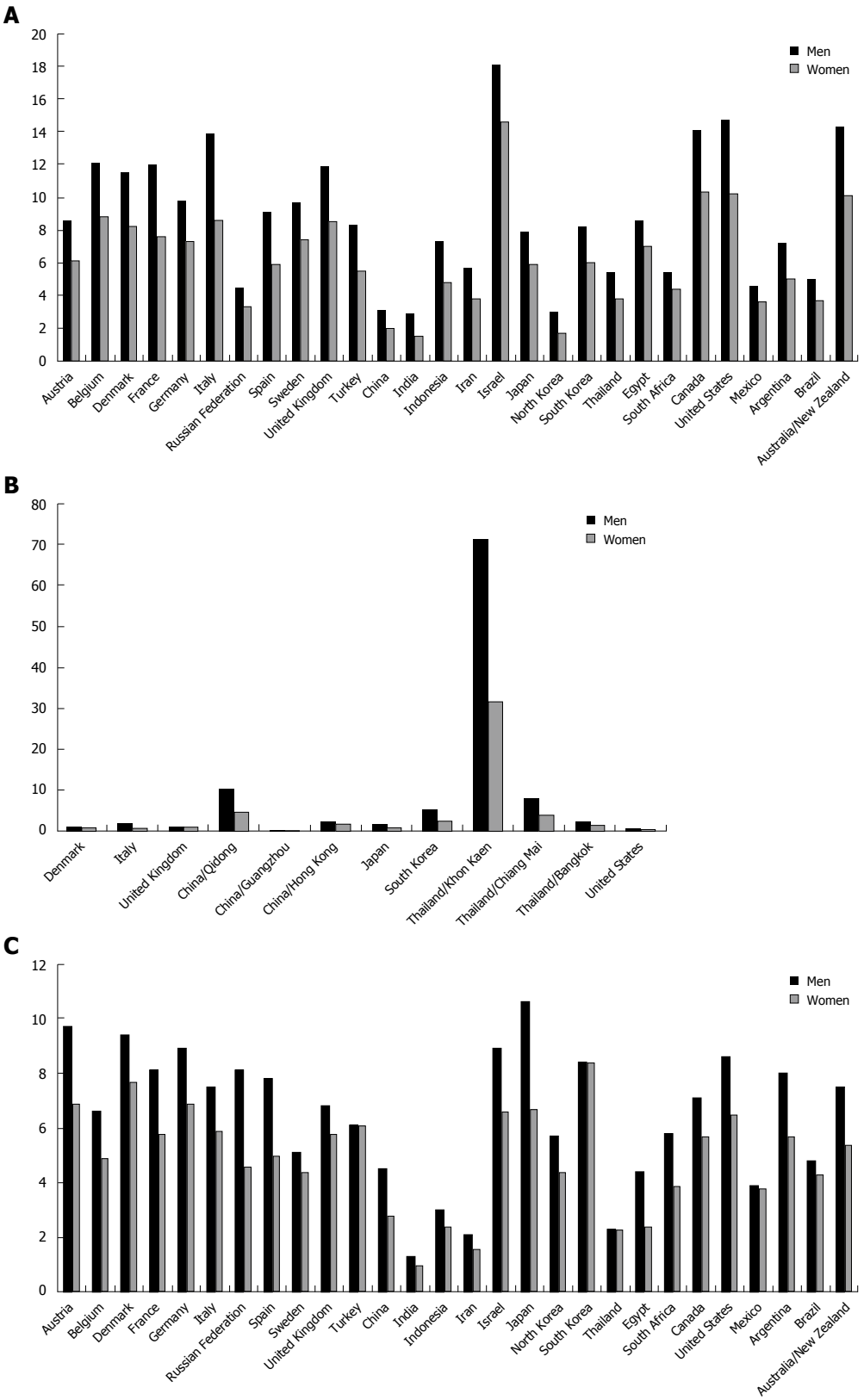
Figure 3[229].

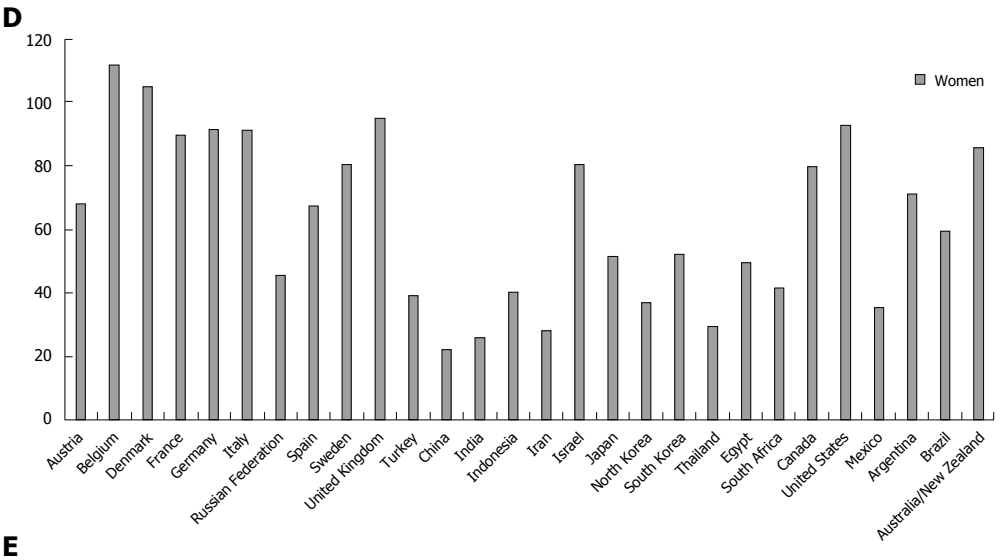
### *HCV and haematopoietic malignancies risk*

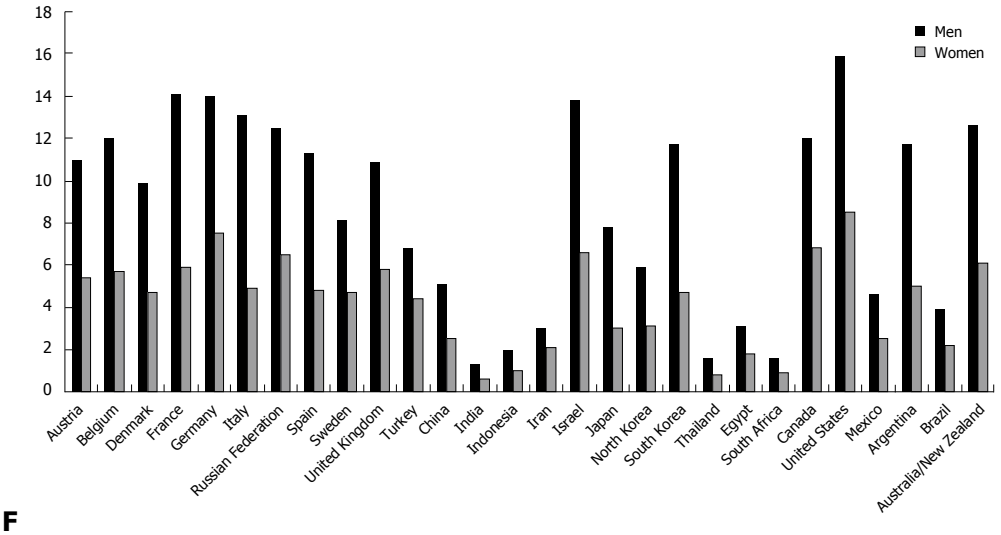
The analysis revealed that most of available studies assessed the prevalence of this pathogen in patients with non-hodgkin-lymphomas (NHLs) and, in particular, in subjects with B-lymphocyte NHLs. However, the aim, design, inclusion criteria and definition of controls widely varied among the identified trials. In particular, available studies assessed the prevalence of HCV infection either in B-, T-, NK-, subtypes of NHLs or in B-cell NHLs alone or in NHLs together with different lymphoprolipherative disorders or in lymphoprolipherative malignancies other than NHLs.

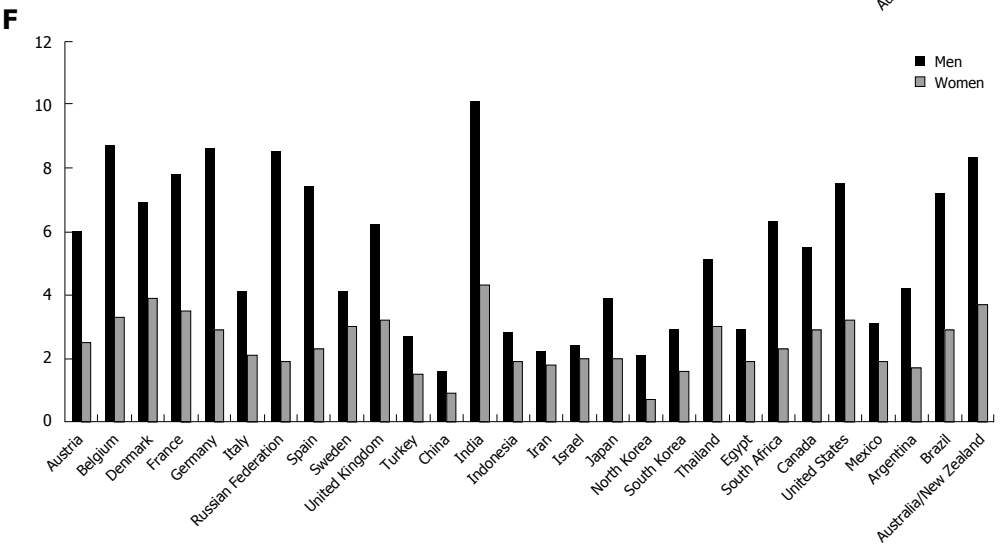
When we examined the large series of these trials, evaluating NHLs, it was necessary to take into account some problems of comparability, such as: type of NHL classification; ASSAYS used to detect HCV infection; and aim of selected studies.

Depending on year of publication, different classifications for NHLs have been used, including: Working Formulation, REAL or WHO classifications. In addition, only in some articles a detailed description of nodal or extra-nodal NHLs has been performed as well as a few studies were identifiable as formal case-control, reporting an adequate description of control groups characteristics, such as matching factors (*i.e.,* age, gender, birthplace and performance status), odds ratios adjusted for potential confounding factors. Furthermore, the sensitivity and specificity of diagnostic tests, used to detect antibodies anti-HCV or viral genome, largely differed among the trials considered in our systematic review (Table 1). In some studies the presence of anti-HCV antibodies was assayed by second-generation-, in others by third-generation-immune-enzymatic screening tests or by confirmatory tests, such as second-/third-generation-RIBA assays or by search of viral genome by means of different polymerase chain reaction (PCR)-based techniques. Furthermore, some hematologic malignancies, such as chronic lymphocytic leukemia have been classified into the category of NHLs in several trials, whereas they have been considered as entities distinct from NHLs in other reports. However, to date even if a large methodological heterogeneity exists among all these studies, the number of trials performed worldwide to assess the potential effect of HCV infection on the risk of NHLs is wide. Therefore, the available data, collected in peoples of various ethnicities as well as in populations of different geographical areas may provide a valid representation of the real situation in a large number of distinct countries. In particular, according to the results of available studies and meta-analyses, an association between HCV infection and B-lymphocyte NHLs development has emerged, with an assessed moderate risk for lymphoma development and odds ratios ranging between 2 and 3 on average. Nevertheless, this estimation differs largely, not only depending on the histological types considered but also on the geographical location and race of populations included in the different trials. An increased risk of NHLs has been described in studies performed in countries, where an elevated HCV prevalence is detectable, including Italy and Spain in Southern Europe, Japan and Taiwan in Asia as well as in Egypt and in southern United States areas. In these regions the percentage of HCV-associated NHLs can also reach a value equal to 10%. On the other hand, the association between HCV and NHLs development has not been confirmed in countries with low viral prevalence, such as countries of Northern Europe (United Kingdom, German, France, Denmark) or North America (Canada, Northern and United States regions). According to the results of a large European multicenter case-control-study as well as of available meta-analyses some subtypes of B-lymphocyte NHLs have resulted to be more frequently associated with diffuse large B-cell lymphoma (DLBCL) with an OR equal to 2.24, marginal zone lymphoma (MZL) with an OR equal to 2.47, and lymphoplasmocytic lymphoma (LPL) with an OR equal to 2.57[129]. In an additional large population-based trial in United States, an enhanced risk for follicular lymphoma (OR = 1.88), Burkitt's lymphoma (OR = 5.21), DLBCL (OR = 1.52) and MZL (OR = 2.20) was reported[119]. However, other than above mentioned lymphoprolipherative diseases, no clear relationship has emerged, concerning HCV infection and haematological malignancies. In particular, even if some trials reported a higher prevalence of HCV in patients with Multiple Myeloma[36,76,133], this observation has not been confirmed in further reports[24,119,126]. In addition, no statistically significant association has been found between anti-HCV sero-positivity and Hodgkin


A
Men
Women
Austria
Belgium
Denmark
France
Germany
Italy
Russian Federation
Spain
Sweden
United Kingdom
Turkey
China
India
Indonesia
Iran
Israel
Japan
North Korea
South Korea
Thailand
Egypt
South Africa
Canada
United States
Mexico
Argentina
Brazil
Australia/New Zealand
B
Men
Women
Denmark
Italy
United Kingdom
China/Qidong
China/Guangzhou
China/Hong Kong
Japan
South Korea
Thailand/Khon Kaen
Thailand/Chiang Mai
Thailand/Bangkok
United States
C
Men
Women
Austria
Belgium
Denmark
France
Germany
Italy
Russian Federation
Spain
Sweden
United Kingdom
Turkey
China
India
Indonesia
Iran
Israel
Japan
North Korea
South Korea
Thailand
Egypt
South Africa
Canada
United States
Mexico
Argentina
Brazil
Australia/New Zealand


D
120
100
80
60
40
20
0
Women
Austria
Belgium
Denmark
France
Germany
Italy
Russian Federation
Spain
Sweden
United Kingdom
Turkey
China
India
Indonesia
Iran
Israel
Japan
North Korea
South Korea
Thailand
Egypt
South Africa
Canada
United States
Mexico
Argentina
Brazil
Australia/New Zealand


E
18
16
14
12
10
8
6
4
2
0
Men
Women
Austria
Belgium
Denmark
France
Germany
Italy
Russian Federation
Spain
Sweden
United Kingdom
Turkey
China
India
Indonesia
Iran
Israel
Japan
North Korea
South Korea
Thailand
Egypt
South Africa
Canada
United States
Mexico
Argentina
Brazil
Australia/New Zealand


F
12
10
8
6
4
2
0
Men
Women
Austria
Belgium
Denmark
France
Germany
Italy
Russian Federation
Spain
Sweden
United Kingdom
Turkey
China
India
Indonesia
Iran
Israel
Japan
North Korea
South Korea
Thailand
Egypt
South Africa
Canada
United States
Mexico
Argentina
Brazil
Australia/New Zealand

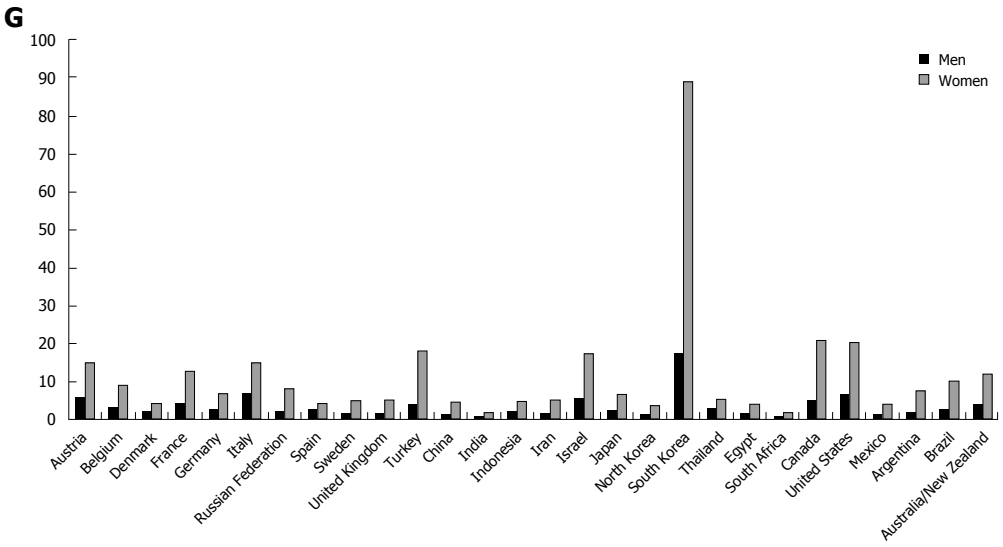



**Figure 3 Age-standardized incidence rates of each malignancy per 100000 person-years are reported for sex and for different countries.** A: Incidence rates of non-hodgkin-lymphomas (GLOBOCAN 2012); B: Incidence rates of cholangiocarcinoma (by Shin HR, *Asian Pacific Journal of Cancer Prevention* 2010; 11); C: Incidence rates of pancreatic cancer (GLOBOCAN 2012); D: Incidence rates of breast cancer (GLOBOCAN 2012); E: incidence rates of kidney cancer (GLOBOCAN 2012); F: Incidence rates of oral/skin cancers (GLOBOCAN 2012); G: Incidence rates of thyroid cancer (GLOBOCAN 2012).

Lymphoma risk. To date, no studies have evaluated whether some risk factors, such as smoking habit, alcohol use or diabetes may act in cooperation with this virus and increase the risk of lymphoprolipherative disorders. The results of our research, concerning the possible association between HCV infection and hematopoietic malignancies, studies not considered, as well as meta-analyses are summarised in Tables 1, 8 and 9. Figure 3A shows the age-standardized incidence rates of main haematopoietic malignancies per 100000 person-years.

### *HCV and cholangiocarcinoma risk*

Colangiocarcinoma (CCAs) arises from the biliary tract and can be classified into two major types with respect to location: intrahepatic cholangiocarcinoma (ICCA) and extrahepatic cholangiocarcinoma (ECCA). The latter form of malignancy may be further divided into ductal or peri-hilar cancers. The second-order bile ducts and the cystic duct represent the point of distinction between ICCAs and peri-hilar CCAs as well as respectively. The importance of this distinct classification reflects differences in clinical presentation, natural history and treatment of intra- and extra-hepatic cholangiocarcinomas.

Overall, a large series of trials, have been performed in different populations and geographical areas. Some of these studies have been carried out both in areas with an elevated prevalence of cholangiocarcinoma, including China, Korea, Japan and Egypt, and in regions with low prevalence of this cancer, including Italy, United States and Denmark. Therefore, these reports may be considered as a rather representative estimation of the real epidemiological burden of this tumour worldwide. In our systematic review we identified 36 studies. The aim, design, inclusion criteria and definition of controls widely varied among the identified trials and some of these have not distinguished among ICCs from ECCs. In particular, 6 have been carried out in United States, 3 in China, 2 in Korea, 9 in Japan, 4 in Thailand, 3 in Italy, 1 in Egypt, 1 in Iran, 1 in Greece, although a low number of studies Northern Europe. However, from the identified studies HCV infection has emerged as risk for ICC, but not for ECC, in a large number of distinct countries worldwide, even if, in some regions in Southern-East Asia, other factors are potentially involved in CCAs development. In these areas, further infectious agents, such as Opisthorchis viverrini and Clonorchis sinensis, represent risk factors for CCAs. In available meta-analyses, the presence of HCV was associated with a statistically significant increased risk of ICC incidence, with an OR ranging from 3.42 (95%CI: 1.96-5.99) to 4.84 (95%CI: 2.41-9.71)[168,190]. The results of our review, concerning the possible association between HCV infection and CCA risk, studies not considered, as well as meta-analyses are summarised in Tables 2, 10 and 11. Age-standardized incidence rates of ICC malignancies per 100000 person-years is reported in Figure 3B.

### *HCV and pancreatic cancer risk*

Until few years ago, although it was well-known that several viruses, including HCV, may infect pancreas and cause the acute inflammation of this organ[230], no studies had been specifically designed to investigate the possible role of HCV in the PDAC development. Different viral and host factors have contributed to make the study of the pancreas extremely hard, including the localization of this organ in retro-peritoneum, the small size of precursor malignant lesions, the difficulty to identify HCV antigens and/or

**Table 1 Characteristics of available studies, reported in English, designed to assess the association between hepatitis C virus infection and haematopoietic malignancies**

| Author/Journal/ Publication year | Study design | Diagnosis | HCV positive HM/total HM | Control source | HCV positive controls/total controls | Percentage of HCV-positive cases with 95%CI | Main conclusions |
|---|---|---|---|---|---|---|---|
| Akdogan M *Turk J Gastroenterol* 1998 | Case series study with control group Period: NR | All lymphomas: NHLs: 30 HL: 18 NHLs NHL classification: Working Formulation | (1) NHL: 4/30 (13.3%) (2) Patients with Hodgkin Lymphoma | (2) Healthy blood donors | (1) 17/9488 (0.8%) | 13.3 (3.8-30.7) | Increased prevalence of HCV persistent infection in patients with NHL, but not in patients with HL, in comparison with general population |
| Amin J *J Hepatol* 2006 | Community-based cohort-study Period: 1990-2002 | NHLs Cohort of HCV positive patients: 75834, Cohort of HBV/ HCV positive patients: 2604 Incidence of LNHs observed in the study cohort was compared to expected incidence derived from New South Wales population cancer rates by calculating standardised incidence ratios | Individuals with HCV infection: 75834 LNH cases detected: 33 | Incidence observed in the study cohort was compared to expected incidence derived from NSW population cancer rates by calculating standardised incidence ratios (SIR) | SIR: 0.9 (0.6-1.2) | 0.04 (0.03-0.05) | In HCV infection group no increased overall risk of NHL-cell lymphoma, but, a number of B-cell NHLs (diffuse NHL, immunoproliferative malignancies and chronic lymphocytic leukaemias) had SIRs greater than one |
| Anderson LA *Epidemiol Biomarkers Prev* 2008 | Population-based nested case-control study of hematopoietic malignancies Period: 1993 and 2002 | Subjects with hematopoietic malignancies identified, using SEER-Medicare data. SEER program: a cancer surveillance program supported by the National Cancer Institute and covering about 25% of United States population NHL classification: World Health Organization classification Myeloproliferative malignancies classification: acute- and chronic myeloid leukaemia, myelodysplastic syndrome, chronic myeloproliferative disease | 195/61464 (0.3%) cases with Hemato-poietic malignancies identified NHLs: 103/33940 (0.3%) DLBCL: 34/10144 (0.3%) BL: 2/197 (1.5%) MZL: 12/1908 (0.6%) FL: 19/4491 (0.4%) CLL: 23/10170 (0.2%) LL: 2/1148 HL: 3/1155 (0.3%) PCM: 31/9995 (0.3%) Myeloid neoplasm: 47/11945 (0.4%) AML: 23/6068 (0.4%) CML: 1/1528 (0.1%) MS: 18/3084 (0.6%) CMD: 1/1346 (0.1%) | Controls were identified by means of Medicare, a federally funded program administered by the Centres for Medicare and Medicaid Services, For each included case, two controls were selected at random from the 5% random sample of Medicare beneficiaries | 264/122531 (0.2%) population-based controls identified | 0.3 (0.2-0.4) | Association between HCV and elevated risk of NHLs and acute myeloid leukemia. HCV may induce lymphoproliferative malignancies through chronic immune stimulation |
| Arcaini L *Clinical Lymphoma, Myeloma & Leukemia* 2011 | Case series study with control group Period: NR | Splenic MZLs NHL classification: World Health Organization classification | 25/92 Splenic MZL patients (27.2%) | Patients (122) with WMc 66/122 subjects with HCV markers | 6/66 WMc patients (9%) | 27.2 (18.1-36.2) | Despite similar outcomes among SMZL and WM, SMZL appears as a disease with distinct clinical and histologic characteristics, and a peculiar association with HCV infection |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Arican A *Med Oncol* 2000 | Case series Period: February-October 1997 | NHLs Low-grade: 12 (27%) Intermediate grade: 24 (55%) High-grade: 8 (18%) NHL classification: Working Formulation | 2/44 (4.5%) | NR | NR | 4.5 (0-10.7) | No association between HCV chronic infection and NHL development in this study. The prevalence of HCV infection reported to be 0.3%-1.5% in healthy Turkish-blood donors in previous studies be 1.5% in healthy Turkish-blood donors in previous |
| Aviles A *Med Oncol* 2003 | Case-control study Period: January 1997-December 1999 | B-cell NHLs: 416 Diffuse large cell: 236 Follicular: 97 Marginal B-cell zone: 83 NHL classification: World Health Organization classification | B-cell NHLs 2/416 (0.5%) | Group 1: 682 first-degree relatives (spouses, children, fathers, and brothers of the patient) living in the neighboring area of the patient. Group 2: 832 healthy blood donors, donating during the same period of time at the central blood bank. Group 3: Neoplastic disease group, with 408 patients with solid tumors, breast cancer:127 colon cancer: 94 gastric cancer; 79 lung cancer: 98 Group 4: 353 patients with HCV-positive related chronic liver disease | Prevalence of HCV equal to: (1) 0 among first-degree relatives of patients (2) 0.12 (0.02-0.88) among healthy blood donors (3) 0.56, (0.28-0.75). among patients with solid tumors (4) No patients with HCV chronic liver disease developed malignant lymphoma in a median follow-up of 7.9 yr | 0.5 (0-1.1) | Association between HCV infection and development of malignant lymphoma represents an hazardous observation, the close association reported in areas with a higher prevalence of HCV infection has to be considered with caution, because other epidemiological factors have not been considered, such as a high prevalence of HCV infection compared to other areas |
| Bauduer F *Hematol Cell Ther* 1999 | Case series Period: January 1995-June 1998 | NHLs: 136 subjects B-cell-NHLs: 110 patients NHL classification: Revised European American Lymphoma (REAL) histological scheme | 2/136 (1.5%) | NR | NR | 1.5 (0-3.4) | No evidence of relationship between HCV and NHLs |
| Besson C *J Clin Oncol* 2006 | Case control Period: March 1993-June 2002 | B-NHL (DLBCL) NHL classification: Working Formulation | 26/5586 (0.5%) | (1) HCV negative patients with DLCL enrolled in the present study (2) individuals with DLCL randomly chosen among HCV-negative patients included in the GELA program | (1) 5586 (2) 35 | 0.5 (0.29-0.64) | HCV-positive patients with DLBCL differ from other patients both at presentation and during chemotherapy. Specific protocols evaluating antiviral therapy should be designed for these patients |
| Bianco E *Haematologica* 2004 | Italian multi-center case-control study Period: January 1998 -February 2001 | All lymphomas: 637 HD: 157 CLL: 100 ALL: 54 MM: 107 AML: 140 CML: 49 T-NHL: 30 NHL classification for T-NHLs: REAL/WHO classification | 44/637 (6.9%) HD: 5/157 (3.2%) CLL: 9/100 (9%) ALL: 4/54 (7.6%) MM: 5/107 (4.7%) AML: 11/140 (7.9%) CML: 6/49 (12.2%) T-NHL 4/30 (13.8%) | Patients from other departments of the same hospitals: the departments of dentistry, dermatology, general surgery, gynecology, internal medicine, ophthalmology, orthopedics, otorhinolaryngology, and traumatology | 22/396 (5.6%) | 6.9 (4.9-8.8) | Possible association of HCV infection not only with B-NHL but also with some other lymphoid and myeloid malignancies, however no definitive significant results, due to the absence of large groups of patients to confirm this assumption |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Bronowicki JP *Hepatology* 2003 | Case records Data obtained from the hepatology gastroenterology, hematology, internal medicine and pathology departments of 64 French hospitals Period: 1992-1999 | All PLL: 31 cases, 27/31 patients with a B-cell lymphoma: -DLBCL: 22, -BL: 1, -EMZBL of mucosa-associated lymphoid tissue type: 3, unclassified, small B-cell lymphoma:1, T-cell lymphomas: 4 NHL classification: World Health Organization classification | HCV-test available for 28 subjects, HCV test available in 23 patients with B-cell PLL 1 HCV positive patient with peripheral T-cell lymphoma 5/23 (21.7%) | NR | NR | 21.7 (7.5- 43.7) | This study confirms the rarity of PLL and demonstrates an increased prevalence of HCV infection |
| Cavanna L *Haematologica* 1995 | Case- control study Period: 1985-1990 | All LPDs: 300 patients Anti-HCV positive patients 57/300 (19.7%) NHLs: 150; HL: 20 CLL: 40 Plasma cell discrasias: 90 | NHL: 38/150 (25.3%) HL: 2/20 (10%) CLL: 2/40 (5%) Plasma cell discrasias: 15/90(16%) | Blood donors | 53/3108 (1.7%) | 25.3 (18.3-32.3) | High prevalence of anti-HCV antibodies among patients with lymphoprolipherative disorders as compared with the control group of healthy blood donors |
| Caviglia GP J *Gastroenterol Hepatol* 2014 | Cohort study Period: January 2006 -December 2013 | 1313 patients with chronic HCV hepatitis 121 patients with extra-hepatic manifestations: B-NHL: 41/1323 (3.1%) MCS: 25/1323 (1.9%) MGUS: 55/1323 (4.2%) NHL classification: World Health Organization classification | B-cell NHL: 41 MZL: 15 (36.6%), had DLBCL: 10 (24.4%), FL: 4 (9.8%) LPL; 1 (2.4%), MM: 1 (2.4%), CLL: 1 (2.4%) and B-NHL not otherwise specified: 9 (22%) | Controls selected on the basis of the absence of extra-hepatic manifestation of HCV infection | 130 HCV positive subjects without extrahepatic manifestation | 3.1 (2.2-4) | Cirrhosis is an additional risk factor for the development of lymphoproliferative disorders in patients with chronic HCV infection |
| Chindamo MC *Oncol Rep* 2002 | Case series with control group Period: May 1995 -September 1998 | All lymphomas: 207 -HL: 67 -B-NHL: 87 -T-NHL: 22 -CLL: 31 NHL classification: Revised European American Lymphoma (REAL) histological scheme | B-cell NHL: 8/87 (9.2%) | (1) Blood donors (2) Other haematological malignancies (Hodgkin's disease and chronic lymphocytic leukaemia) | (1) 472/39371 (1.2%) (2) 2/98 (2%) | 9.2 (3.1-15.2) | Association between HCV infection and NHLs |
| Chuang SS *J Clin Pathol* 2010 | Case-control study Period: January 2004 -December 2008 | All malignancies: 346 -HL: 25 (3HCV+) -B-NHL: 321 (DLBCL, FC CLL, MZL, BL, others) -T- or NK/T-cell NHL: 55 NHL classification: World Health Organization classification | All NHL: 35/321 (11%) B-cell NHL: 34/266 (12.8%) (3/38 with HBV coinfection) | Healthy Taiwanese subjects | 15/824 (1.8%) | 12.8 (8.7-16.8) | The incidence of HCV infection among lymphoma patients in Taiwan was significantly higher than that for healthy controls Non-MALT (nodal and splenic) MZL was the only group significantly associated with HCV |
| Cocco P *Int J Hematol* 2008 | Case-control study Period: -February 1999 - October 2002 - January 2002 - July 2003 | All malignancies (277): -HL: 13 -NHL: 264 (DLBCL, FC CLL, MZL, MM, T-cell NHL, others) NHL classification: World Health Organization classification | (1) All B cell- NHL: 20/237 (8.4%) (2) NHLs (excluding CLL and MM): 15/177 (8.5%) | Randomly selected controls from population registrars | 9/217 (4.1%) | (1) 8.4 (4.9-11.9) (2) 8.5 (4.3-12.5) | Acute or chronic hepatitis C is associated with a consistent risk increase in all lymphoma subtypes, but follicular lymphoma |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Collier JD *Hepatology* 1999 | Case series with control group Period: February 1997 and May 1997 | B-cell NHLs: 100 NHL classification: Working Formulation | 1/100 (1%) | In-Hospital patients with nonhematologic malignancies, treated at the Princess Margaret Hospital | 1/100 (1%) | 1 (0-3) | No association between hepatitis C and B-cell lymphoma |
| Cowgill KD *Int J Epidemiol* 2004 | Case-control study Period: October 1999- and January 2003 | B-cell NHL: 220 NHL classification: NR | Total: 106/220 (48.1%) (1) anti-HCV+/RNA- 12/220 (5.4%) (2) anti-HCV+/RNA+ 94/220 (42.7%) | In-Hospital patients with fractures, treated at the Kasr El-Aini Orthopaedic Hospital, | Total: 80/222 (36%) (1) anti-HCV+/ RNA-28/222 (12.6%) (2) anti-HCV+/RNA+ 52/222 (23.4%) | 48.2 (41.5-54.7) | Strong association between chronic HCV infection and risk of developing NHL, persisting after adjustment in multivariate models and after several sensitivity analyses |
| Cucuianu A *Br J Haematol* 1999 | Case series with control group Period: December 1997 and March 1999 | All B-cell NHL: 68 NHL classification: Working Formulation | 20/68 (29.5%) | Non-hospitalized Romanian individuals | 46/943 (4.9%) | 9.1 (5.3-12.9) | Detection of high prevalence (29.5%) of anti-HCV in patients with NHL, especially in low-grade types |
| De Renzo A *Haematologica* 2002 | Case-control Period: NR | All LPDs: 227 -B-cell LPds: 127 -HL 100 NHL classification: Revised European American Lymphoma (REAL) histological scheme | B-cell LPds: 22/127 (17.3%) B-NHL 12/61(19.7%) MM 4/48 (8.3%) WM 4/9 44.4%) CLL 2/9 (22.2%) | A group of occasional blood donors from the same geographical area, studied as healthy controls | -HL 2/100 (2%) -Controls: 2/110 (1.8%) | 19.7 (9.7-29.6) | Detection, in Southern Italy, of a higher prevalence of HCV infection in patients suffering from B-LPD in comparison with healthy subjects, particularly in patients with B-cell-NHL, CLL and WMc |
| De Renzo A *Euro J Haematology* 2008 | Case series Period: 1990-2005 | All NHLs patients observed: 550 Primary hepatic lymphomas (PHL): 6 Primary splenic Lymphomas (PSL): 19 NHL classification: World Health Organization classification | PHL: 4/6 PSL: 13/19 | NR | NR | PHL 66.7 (22.3-95.7) PSL 68.4 (43.5-87.4) | High prevalence of HCV infection among patients with rare haematologic malignancies (PHL and PSL ), favourable outcome of these subjects |
| De Rosa G *Am J Hematol* 1997 | Case series with control group Period: November 1994 -November 1995 | All Lympho-prolipherative Disorders (315): (1) No-B LPD: 52 HD: 43 (1 HCV+) T-NHL: 9 (2) B LPD: 272, including; NHL-B-cell lymphoma, CLL, HCL, MGUS, WMc, MM, ( 59 HCV+) NHL classification: Working Formulation | B-cell NHL: 21/91 (23.1%) | (1) Patients with Hodgkin Lymphoma (2) Healthy blood donors | (1) 1/43 (2.3%) 0/9 (2) 30/1568 (1.9%) | 23.1 (14.4-33.7) | Detection of a higher prevalence of anti-HCV antibodies patients with B-Lymphoprolipherative disorders, as compared to the normal population and to patients with a non-B-lymphoprolipherative disorders |
| De Vita S *Br J Cancer* 1998 | Case-control study Period: January 1994-June 1997 | All malignancies 84 NHLs NHL classification: Working Formulation | 20/84 (23.8%) | Controls recruited at Aviano, with cancers in: ovary: 13 uterus:14, colon-rectum:13, pancreas:10, lung: 8, stomach: 6, oesophagus: 4 other sites: 5 HCC: 27 | Controls: 3/73 (4.1%) HCC: 11/27 (40.7%) | 23.8 (15.2-34.3) | Detection of a higher than expected prevalence of HCV infection in B-cell NHL patients |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Duberg AS *Hepatology* 2005 | Nationwide cohort of HCV-infected persons Cancer Registry used to identify all incident cancers diagnosed in the cohort malignant NHL Period: 1990-2000 | All malignancies: Patients with B-Cell NHLs, after exclusion of patients with HIV coinfection: 16 CLL: 4 MM:7 ALL: 1 HL: 1 NHL classification: NR | B-Cell NHLs: 16 in 27150 HCV positive patients included in the cohort, HCV infection diagnosis made to the Swedish Institute for Infectious Disease Control (SMI) | NR | NR | 0.06 (0.04-0.1) | A significantly increased risk of NHL and MM observed in this study, although an underestimation of the risk may have been caused by the delayed diagnosis of HCV |
| Ellenrieder VJ *Hepatol* 1998 | Case series Period: 1991- 1995 | B-cell NHLs: Low-grade B-cell NHL: 55 High- low-grade B-cell: 14 NHL classification: Kiel Classification | 3/69 (4.3%) CLL: 1/14 CC: 0/4 CB: 1/14 CCBC: 1/19 IC = 0/18 | NR | NR | 4.3 (0.9-12.2) | No aetiological role of HCV in the development of NHL in German |
| El-Serag HB *Hepatology* 2002 | Cohort study Period: 1992- 1999 | Identification of LNHs cases by means of ICD-9-CM diagnosis codes NHL classification: NR | 421/34204 (1.23%) | 34204 HCV positive patients and 136816 randomly selected patients without HCV (controls) | 1669/136816 (1.22%) | 1.23 (1.1-1.3) | Significant high association between HCV infection and NHL, after adjustment for age |
| Engels EA *Int J Cancer* 2004 | Case-control study Period: July 1998-June 2000 | All NHL subtypes: (1) 32/813 (3.9%) (2) Low-grade B-cell NHL 18/411 (4.4%) (3) Intermediate-and high-grade B-cell NHL 8/275 (2.9%) (4) T-cell NHL 2/50 (4.0%) (5) other/unknown 4/77 (5.2%) NHL classification: Revised European American Lymphoma (REAL) histological scheme | 26/686 (3.8%) | Eligible cases and controls sampled from individuals 20-74 yr old, prospectively identified by using Surveillance, Epidemiology and End Results (SEER) program of the National Cancer Institute (NCI) | 14/684 (2.1%) | 3.8 (2.3-5.2) | Detection of an association between HCV infection and NHL in the United States. HCV infection may be a cause of NHL |
| Ferri C *Br J Haematol* 1994 | Case series with control group Period: NR | B-cell NHL: 50 NHL classification: Working Formulation | B-cell NHL: 17/50 (34%) | (1) Patients with Hodgkin Lymphoma (2) Healthy subjects (3) anti-HCV negative patients with type B or delta chronic active hepatitis | 1/30 (3%) 30 15 HCV prevalence in in the healthy Italian population: 1.3% | 34 (20.8-47.1) | Presence of HCV infection in a substantial number of unselected NHL patients, particularly in comparison with HCV prevalence in control groups and in healthy Italian population |
| Franceschi S *Cancer Epidemiol Biomarkers Prev* 2011 | Nested case-control study Period: standardized lifestyle and personal history questionnaires collected between 1991 and 2000. Vital status followed up to 2004 and 2006 | All lymphomas: 1023 cases NHL: 739 MM: 238 HL: 46 HCV positive: 12/1023 (1.17%) NHL classification: World Health Organization classification | B-cell NHLs: 628/1023 (61.4%) Number of HCV positive patients in B-NHLs not reported 9/730 HCV positive patients in all NHLs 14/1454 HCV positive in controls HL: 2/46 (4.3%) MM: 1/238 (0.4%) | Lymphoid tissue Malignancies classified according to the second revision of the International Classification of Diseases for Oncology (ICD-O-2) and to the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, Third Edition | 18/2028 controls (0.9%) | 61.4 (58.4-64.3) | The present study neither weakened nor strengthened the evidence of an association between HCV and NHL or other lymphoid tissue malignancies |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Gentile G *Cancer Epidemiol Biomarkers Prev* 1996 | Hospital-based case-control study of risk factors for acute leukemias Period: 1 November 1986 - 31 March 1990 | All acute leukemias: 430 Diagnosis performed by means of: French-American-British classification of bone marrow aspirates for acute leukemias and RAEB, whereas diagnosis for CML was based on typical dinical and cytogenetic laboratory features | All acute leukemias: 27/430 (6.3%): AML: 15/172 (8.7%) ALL: 5/67 (7.5%) CML: 2/125 (1.6%) RAEB: 5/66 (7.6%) | Controls recruited in the region of the three hospitals (Rome, Bologna, Pavia) during the study period among outpatients without hematobogical malignancies who were seen in the same hospitals at which cases had been identified | 44/857 (5.1%) | 6.3 (3.9-8.5) | Association between acute leukemias, RAEB, and CML Possible association between hepatitis B virus, AML, RAEB, and CML, but further confirmation required |
| Genvresse I *Ann Hematol* 2000 | Case series Period: 1995-2000 | All lymphomas: 119 (1) B-NHLs: 105 (2) T-NHLs: 14 NHL classification: REAL histological scheme | (1) 2/105 (1.9%) (2) 0/14 | NR | NR | 1.9 (0-4.5) | Possible HCV involvement in NHLs development *via* a continuous antigenic stimulation, leading to a B-cell clonal expansion |
| Germanidis G *Blood* 1999 | Case series with control group Period: January 1994 -July 1997 | B-NHL: 201 HD: 94 NHL classification: Revised European American Lymphoma (REAL) histological scheme | B-NHL: 4/201 (2%) | Hematologic malignancies different from B-cell NHL (HD) | 1/94 (1.1%) | 2 (0-3.9) | No existence of a significant relationship between HCV infection and B-NHL in France |
| Giordano TP *JAMA* 2007 | Cohort study Period: 1997-2004 | Identification of LNH cases by means of ICD-9-CM diagnosis codes NHL classification: NHL (200, 202.0-202.2, 202.8), WMc (273.0, 273.3), HL (201), MM (203.0-203.1, 238.6), ALL (204.0), CLL (204.1) AnLLs 205.0, 206.0), CML (205.1), other leukemia (204.2, 204.8-204.9, 205.2, 205.8-205.9, 206.1-206.2, 206.8-206.9, 207.8, 208.0-208.2, 208.88.9), MGUS (273.1, 273.2) | HCV- positive cohort: 146394 patients During follow-up, 813 patients in HCV-infected cohort (0.5%) had a HIV diagnosis NHL: 319 HL: 65 MM: 95 CCL:69 ALL: 27 WMc: 67 CML: 30 | Inpatients records from more than 150 United States Veterans Affairs (VA) hospitals in the Patients' treatment file and outpatients records from any VA facility in the Output Clinic File | HCV- negative cohort: 572293 patients. During follow-up, 35696 uninfected HCV patients (6.2%) had a recorded HCV diagnosis and 1539 patients (0.3%) a HIV diagnosis NHL: 1040 HL: 295 MM: 431 CCL:343 ALL; 184 WMc: 98 CML: 163 | 0.2 (0.19-0.25) | An increased risk of: (1) non-Hodgkin lymphoma overall (20%-30%), (2) Waldenström macroglobulinemia, a low-grade lymphoma (3-fold higher risk), (3) cryoglobulinemia, in subjects with HCV infection. An etiological role for HCV, in causing lymphoproliferation and non-Hodgkin lymphoma, supported by these results |
| Goldman L *Cancer Causes Control* 2009 | Case-control study Period: October 1999 -March 2004 | All lymphomas: 139/296 (47% ) -T-NHL: 8/24 (34.8%) -DLBCL:79/146 (54.9%) -MZL: 14/24 (58.3%) -CLL: 24/58 (41.4%) -FC: 9/23 (40.9%) -MCL: 5/16 (31.3%) NHL classification: World Health Organization classification | B-NHL: 131/272 (48.2%) | Cancer-free subjects, sampled from the Kasr El Aini Faculty of Medicine Orthopaedic Hospital in Cairo | 283/786 (37.4%) | 48.2 (42.2-54.1) | HCV is a risk factor for diffuse large B cell, marginal zone, and follicular lymphomas in Egypt |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Guida M *Leukemia* 2002 | Case-control study Period: September 1999-October 2001 | All Lymphomas: 12/60 (20%) MM: 5/60 B-NHL: 55/60 NHL classification: Working Formulation | B-NHL: 12/55 (21.8%) | Control patients with non-hematological malignancies recruited from the Surgery Department the Oncology Institute of Bari (Italy) | 9/63 (14.2%) | 21.8 (10.9-32.7) | Moderate increase of prevalence of HCV infection among patients with B cell lymphoproliferative disorders in a very homogeneous population of southern Italy |
| Hanley J *Lancet* 1996 | Case series Period: NR | All LPDs: 72. B-cell NHLs: 38 MM: 24 MGUS: 10 NHL classification: Working Formulation | 0/72 | NR | NR | 0 (0-4.9) | No association between chronic HCV infection and risk of NHLs development |
| Harakati MS *Saudi Med J* 2000 | Case series with control group Period | B-cell NHLs: 56 patients NHL classification | B-NHL: 12/56 (21.4%) | (1) Blood donors and general medical patients) (2) Other hematologic malignancies other than B-cell NHL | (1) 3/104 (3%), (2) 2/41 (5%) | 21.4 (10.6-32.7) | Higher prevalence of Hepatitis C virus infection in Saudi Arab patients with B-cell non-Hodgkin's lymphoma than in the control groups |
| Hausfater P *Am J Hematol* 2001 | Prospective controlled study Period: June to September 1998 | All LPD: 394 B-NHL: 164 HD: 34 CLL: 107 MM: 54 WMc:12 NHL classification: NR | B-NHL: 3/164 (1.8%) | (1) In-Hospital patients without cancers (2) Nonmalignant hematological diseases (3) Hematological malignancies other than B-cell NHL | (1) 3/694 (0.43%) (2) 8/224 (3.6%) (3) 9/425 (2.1%) | 1.8 (0-3.8) | No increased prevalence of HCV infection in patients admitted to the Hematology department for B-NHL. No major pathophysiologic role of HCV in lymphoproliferative disorders in Paris |
| Hwang JP *J Oncol Pract* 2014 | Cohort-study Period: January 2004 -April 2011 | Patients'data, obtained from four institutional sources: Tumor registry: to assess patients' demographic characteristics Pharmacy informatics: to evaluate chemotherapy drugs and dates administered. Patient accounts: to identify study patients' International Classification of Diseases (ninth edition; ICD-9) codes Laboratory informatics: to determine HCV antibody (anti-HCV) and ALT test dates and results | 141877 patients with cancer, who were newly registered at MD Anderson Cancer during the study period. Patients considered in the study: 16, 773. HCV screened subjects: 1628/16773 (9.7%) with NHLs, 1400 patients with anti-HCV test 42 NHLs antiHCV-positive (3%) | NR | NR | 3 (2.1-3.9) | HCV screening rates were low, even among patients with risk factors, and the groups with the highest rates of screening did not match the groups with the highest rates of a positive test result |
| Imai Y *Hepatology* 2002 | Cohort study Period: February 1992 -July 1992 | NHLs: 187 B-cell NHLs: 156 T-cell NHL:31 NHL classification: World Health Organization classification | 21/156 (13.5%) | Use of screening data of 197600 first-time voluntary blood donors to the Osaka Red Cross Blood Center | Expected numbers of anti-HCV-positive patients with NHL categorized by gender and phenotype in general population: 4.64 117 | 13.5 (8.1-18.8) | A significantly higher frequency of HCV infection in B cell NHL in comparison with that in birth cohort- and sex-matched blood donors; chronic HCV infection may be associated with B-cell NHL in Japan |
| Isikdogan A *Leuk Lymphoma* 2003 | Case series with control group Period: December 1997-September 2001 | NHLs: 119 High-grade NHLs: 10 Intermediate-grade: 64 Low-grade: 45 NHL classification: Working formulation | 0/119 | Subjects admitted as outpatients at Internal Medicine of Dicle Univeristy, Diyarbakir, without history of haematological disordres, during the same period | | 0 (0-3) | No relationship between HCV and NHLs in the Southeastern Anatolia of Turkey |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Iwata H *Haematologica* 2004 | Hospital-based case control Study Period: 1995-2001 | All NHLs: 145, 140 with anti-HCV test NHL classification: World Health Organization classification | 16/140 (11.4%) | Randomly selected controls from patients admitted to the (1) orthopedics (290 patients, 286 with anti-HCV markers) or (2) ear, nose and throat (284 patients, 282 with anti-HCV markers) departments of the hospital | (1) 9//286 (3.1%) (2) 20/282 (7%) | 11.4 (6.1-16.7) | Significant association between HCV infection, and malignant lymphoma by multivariate analysis |
| Izumi T *Blood* 1996 | Case series Period: 1992-1997 | All lymphomas: 83 patients, B-cell NHLs: 54 Non-B-cell NHLs: 20 HLs: 9 NHL classification: NR | B-cell NHLs: 12/54 (22.2%) Non-B-cell NHLs: 0/20 HLs: 0/9 | NR | NR | 22.2 (11.2-33.3) | Direct causal relationship between the occurrence of PHSL and chronic HCV infection |
| Izumi T *Leukemia* 1997 | Case series Period: NR | B-cell LPDs: 50 patients B-cell NHLs: 25 MM: 21 WMc: 4 NHL classification: NR | 4/25 (16%) | NR | NR | 16 (4.5-36.1) | Association between HCv infection and B-cell NHLs |
| Karavattathayyil SJ *Am J Clin Pathol* 2000 | Case series Period: January 1993-December 1996 | Patients with B-cell NHLs: 31 NHL classification: World Health Organization classification | Positive HCV-RNA strands: 8/31 (25.8%) Negative HCV-RNA strands: 6/31 (19.4%) | (1) T-cell NHLs: 2 cases (2) HL: 2 cases (3) Patients with lymph nodes removed for reasons other than lymphoma: 28 | 0/32 | Positive HCV-RNA strands: 25.8 (10.4-41.2) Negative HCV-RNA strands 19.4 (5.4-33.2) | Presence of HCV infection in a significant percentage of paraffin-embedded tissue from B-cell NHLs patients, compared with control subjects; detection of negative-strand RNA suggests HCV replication in these tissues, excluding the possibility of contamination with viral RNA or blood |
| Kashyap A *Ann Intern Med* 1998 | Case series with control group Period: February 1992-December 1995 | All NHLs: 312 36 HCV positive patients NHL classification: NR | NHLs: 36/312 (11.5%) | (1) Healthy United States blood donors (2) Black and Hispanic patient population at City of Hope National Medical Center | (1) (0.4%) (2) approximately 25% | 11.5 (8-15.1) | Prevalence of HCV positivity is still much higher than expected, even after adjustment for differences in patient demographic characteristics |
| Kaya H *Clin Lab Haematol* 2002 | Case-control study Period: NR | All NHLs: 70 patients Low-grade NHLs: 22, Intermediate- grade NHLs: 17 high-grade NHLs: 31 NHL classification: Working Formulation | 1/70 (1.4%) | Healthy-subjects admitted at Departments of Haematology, Ataturk University, Erzurum | 1/70 (1.4%) | 1.4 (0-4.2) | No aetiologic role of HCV in NHL development |
| Kim JH *Jpn J Cancer Res* 2002 | Case-control study Period: January 1997 -December 1998 | NHLs: 233 patients 214 patients with anti-HCV positivity NHL classification: Working Formulation | 7/214 (3.3%) | Control groups comprised patients with (1) non-hematological malignancy (control group 1) and subjects with (2) non-malignant conditions (control group 2) diagnosed at Seoul National University Hospital during the same period. For each case, four controls selected | (1) 7/426 (1.6%) (2) 12/439 (2.7%) | 3.3 (0.8-5.6) | No association between NHL and HCV infection |
| King PD *Clin Lab Haematol* 1998 | Case series series with control group Period: June 1995-May 1997 | All lymphomas: 93 patients. NHLs: 73 patients HL: 20 patients 438 HCV positive patients NHL classification: Working Formulation | 1/73 (1.4%) | Patients with HL admitted at Department of Gastroenterology, University of Missouri Hospital | 0/20 1/438 (0.22%) patients developed NHL | 1.4 (0-4) | No association between NHL and HCV infection |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Kocabaş E *Eur J Epidemiol* 1997 | Case series with control group Period: October 1993-March1994 | 137 Children with malignancies: Acute leukemia: 48 Lymphoma: 51 Solid tumours: 38 NHL classification: | 8/137 children were anti HCV positive, 129 patients were anti- HCV negative, but 7/129 were HCV-RNA positive | Children admitted, at Balcah Hospital, Adana, during the same period with diseases other than malignancies | 1/45 | 5.8 (1.9-9.7) | HCV infection is common among Turkish children with different types of cancer |
| Kuniyoshi M *J Gastroenterol Hepatol* 2001 | Case-control Study Period:January 1990-March 1998 | NHLs 348 patients 20/348 (8.1%) HCV positive patients with NHLs NHL classification: Working Formulation | B-cell NHLs: 15/348 (4.3%) | 1658234 blood donors, representing general population in the area (Fukuoka, Japan) | 11922/1658234 (0.72%) | 4.3 (2.1-6.4) | Involvement of HCV infection in the development of a subgroup of NHL, in males |
| Luppi M *Ann Oncol* 1998 | Case series Period: January 1989-August 1993 | B-cell NHLs: 157 patients NHL classification: Revised European American Lymphoma (REAL) histological scheme | 35/157 (22.3%) HCV positive B-cell NHLs: LDBCL 8/35 (23%) FC: 14/35 (40%) LPL: 2/35 (6%) 122/157 (67.7%) HCV negative B-cell NHLs | NR | NR | 22.3 (15.8-28.8) | Association of HCV infection with the malignant proliferation of defined B-cell subsets other than the immunoglobulin Mk B-cell subset involved in the pathogenesis of mixed cryoglobulinemia type II and associated lymphoplasmacytoid lymphoma type |
| Markovic *Hepato-Gastroenterology* 1999 | Case-series Period: January 1991-April 1996 | All lymphomas: 305 patients NHLs: 300 patients HL: 5 patients 181 patients with anti-HCV test NHL classification: NR | 3/181 (1.6%) | NR | NR | 1.7 (0-3.5) | No association between HCV infection and non-Hodgkin's lymphomas, because of low HCV prevalence in Slovenia |
| Mazzaro C *Br J Haematol* 1996 | Case-series with control group Period: NR | All lymphomas: 199 patients Low-grade NHLs: 105 (52.7%) Intermediate grade NHLs: 48 (24.1%) High-grade: 39 (19.6%) MALT: 5 (2.5%) T-cell NHLs: 2 (1%) NHL classification: Working Formulation | 57/199 (28.6%) Low-grade NHLs: 40/110 (36.47%) Intermediate grade NHLs: 6/48 (12.5%) High-grade: 9/39 (23.1%) | (1) Patients with other haematological malignancies, including HL (21 patients), CLL (41), myelodysplastic syndrome (72), plasma cell myeloma (19); (2) general population of two towns in the same geographical area (Cormons and Campogalliano) in the cohort study called Dyonisos project | (1) 5/153 (3.1%) (2) 199/6917 (2.9%) | 28.6 (22.4-34.9) | Important role of HCV in the development of low-grade non-Hodgkin's lymphomas |
| McColl MD *Leuk Lymphoma* 1997 | Case series Period: NR | B-Cell NHL: 72 patients Low-grade: 41 Intermediate-grade: 23 High grade: 8 NHL classification: Working Formulation | 0/72 | Patients with CLL, recruited at two Hospital in the West of Scotland | 0/38 | 0 (0-9.2) | Possible role of HCV infection in the aetiology of certain subgroups of NHLs, although this effect may be regional |
| Mele A *Blood* 2003 | Multicenter case-study with control group Period: 1998-2001 | B-Cell NHL: 400 patients NHL classification: REAL/ World Health Organization classifications | 70/400 (17.5%) Aggressive B-NHL: 43/230 (18.7%) Indolent NHL:27/170 (15.9%) | Patients recruited n other departments of the same Hospitals: the departments of dentistry, dermatology, general surgery, gynecology, internal medicine, ophthalmology, orthopedics, otorhinolaryngology and traumatology | 22/396 (5.6%) | 17.5 (13.8-21.2) | Detection of an association between HCV and B-NHL |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Mizorogi F *Intern Med* 2000 | Case series with control group Period: January 1993-December 1998 | Patients with LPDs: 161, subdivided into 2 groups: (1) patients with B-cell LPDs, including B-cell-NHLs: 100 MM: 17 CLL: 4 (2) patients with non B-cell LPDs: 38 NHL classification: Working Formulation | B-cell NHLs: 17/100 (17%) | Subjects with miscellaneous diseases other than liver diseases or LPDs, used as controls | nonB-cell LPDs: 0/25 34/516 (6.6%) | 17 (9.6-24.3) | Higher prevalence of HCV infection in patients with B-cell NHL than in those with non-B-cell NHL and the control group, frequent primary liver involvement and liver-related causes of death in HCV-positive patients with B-cell NHL |
| Montella M *Leuk Res* 2001 | Case-control study Period: January 1997 and December 1999 | -B-cell-NHLs: 101 -HL: 63 -T-cell NHLs: 10 -MM: 41 NHL classification: Working Formulation/REAL | 25/101 (24.8%) | Controls: patients with no history of malignant tumor, admitted to the National Cancer Institute and Cardarelli Hospital of Naples, in the same period | -Controls: 17/226 (8%) -HL: 6/63 (10%) -T-cell NHLs: 3/10 (30%) -MM: 13/41 (32%) | 24.8 (16.3-33.1) | Detection of a significant association between HCV infection and B-cell NHLs in the extranodal localization, and also indicate an association for the nodal seat |
| Morton LM *Cancer Epidemiol Biomarkers Prev* 2004 | Population-based case-control study of women in Connecticut The Yale Comprehensive Cancer Center's Rapid Case Ascertainment Shared Resource (RCA), a part of the Connecticut Tumor Registry (CTR), a population-based tumor registry Period: 1995-2001 | All lymphomas: B cell 362 T cell 34 Others: 60 NHL classification: World Health Organization classification Incident cases of NHL identified by means of (ICD)-O: M-9590-9595, 9670-9687, 9690-9698, 9700-9723 | B cell 7/362 (1.9%) T cell 0/4 Others 1/60 (1.6%) Total: 8/464 (2%) | A population-based control group of female residents of Connecticut, aged 21-84, assembled using two methods:- Random digit dialing used to contact women less than 65 yr of age,-random selection from the files of the Centers for Medicare and Medicaid Services for women aged 65 yr and older | 5/534 (1%) | 1.9 (0.5-3.3) | Indirect HCV involvement in the development of B-NHL, this risk varying by B-NHL subtype among women |
| Musolino C *Haematologica* 1996 | Case series Period: NR | All-NHLs:24 HCV positive: 2 patients HCV-RNA positive: 5 patients NHL classification: Working Formulation | 5/24 HCV-RNA positive/ NHLs | NR | NR | 20.8 (7.1-42.2) | Possible HCV involvement in NHL development |
| Musto P *Blood* 1996 | Case series with control group Period: NR | B-LPDs B-NHL: 150 HCL: 9 CLL: 41 MM: 90 WMc:13 MGUS: 47 NHL classification: NR | B-NHLs: 40/150 (26.7%) HCL:1/9 (11.1%) CLL: 8/41 (19.5%) MM: 10/90 (11.1%) WMc: 3/13 (23%) MGUS: 6/47 (12.8%) | Patients hospitalized for acute trauma | 25/466 (5.4%) | 26.7 (19.6-33.7) | A significantly higher prevalence of anti-HCV in patients with B-NHLs than in controls and independent of age |
| Nicolosi *Guidicelli S Hematol Oncol* 2012 | Case-control study Period: July 2001 to March 2002 | All lymphomas: 137 NHL classification: World Health Organization's classification | 6/137 (4.4%) | Patients observed in Hospital Clinic, Barcelona and San Giovanni Hospital, Bellinzona, (ideally in traumatology and orthopaedic divisions | 7/125 (5.6%) | 4.4 ( 0.9-7.8) | Existence of marked geographic differences in the prevalence of HCV in NHL but no significant evidence for an association between HCV and B-cell NHLs |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Nieters A *Gastroenterology* 2006 | European Multicenter Case-Control Study Period: 1998-2004 | Total Lymphomas: 1807 NHL classification: World Health Organization's classification | 53/1807 (2.9%) | Controls drawn randomly from population registers of the study regions in Germany and Italy. In the remaining countries, controls recruited from the same hospital as cases | 41/1788 (2.3%) | 2.9 (2.1-3.7) | Positive association between HCV infection and B-cell lymphoma and a role of viral replication in lymphomagenesis |
| Ogino H *Hepatol Res* 1999 | Case-control sudy Period: 1991-1997 | All LPDs: 43 patients NHLs: 33 ALL: 10 NHL classification: Working Formulation | 4/33 (12.1%) | (1) 45 patients, undergoing colonscopy from July 1995 to June 1996 (2) 10599 healthy subjects, receiving a general medical check-up in Toyama prefecture from April 1996 to March 1997 | 2/45 (4.4%) | 12.1 (3.4-28.2) | High prevalence of HCV infection in patients with NHL in Toyama prefecture in Japan |
| Ohsawa M *Int J Cancer* 1999 | Cohort-study Period: 1957-1997 | Patients with HCV chronic infection, included in the present study: 2162 NHL classification: World Health Organization's classification | Patients developing B-cell NHLs: 4/2162 During follow-up | Expected number of cases of NHLs in the sex-, age- and calendar year-matched general population: 1.90 | NR | 0.2 (0-0.3) | Chronic HCV infection moderately associated with increased risk of NHL |
| Okan V *Int J Hematol* 2008 | Case series with control group Period: NR | All Lymphomas: 334 NHL classification: World Health Organization's classification | 9/334 (2.7%) MM: 1/67 (3.1%) CLL: 2/78 (2.5%) DLBCL: 4/67(6%) Follicular 0/9 Mantle: 1/11 (9%) Other: 0/26 T-cell lymphoid tumors: 1/16 (6.2%) HL: 0/60 | Controls recruited, using records from the University blood center in Gaziantep | 9/802 (1.1%) | 2.7 (0.9-4.4) 6 (0.3-11.6) | Higher HCV- seropositivity rate in patients with DLBCL in comparison with controls. No significant differences in the prevalence of HCV seroposity between patients with lymphoproliferative disorders and controls |
| Omland LH *Int J Cancer* 2012 | Cohort-study Period:1991-2006 Patients and subjects with HCV infection identified by means of: -Danish HCV cohort (DANVIR). -Civil registration system (CRS)-Danish cancer registry (DCR). -Danish national patient registry (DNPR) | 10 digit civil registration number assigned to all individuals in Denmark Analysis of the association between HCV and risk of NHL (ICD-10 codes: C82.0-85.9 and C96) NHL classification: Cancers classified according to the "International Classification of Diseases" 7$^{th}$ revision (ICD-7) for the period 1943-1977 and the 10$^{th}$ revision (ICD-10) for the period 1978-2006 | -11975 anti- HCV-positive patients LNH cases detected: 12 12/11975: 0.1% | Comparison cohort, which consisted of 6 age- and gender-matched individuals (without a HCV diagnosis) from the general population randomly selected from the CRS, on the day HCV-infection was diagnosed in the corresponding DANVIR cohort member | -71850 anti- HCV-positive patients LNH cases detected: 24 | 0.1 (0.04-0.15) | Possible increased risk of NHLs in patients with chronic HCV infection |
| Panovska I *Br J Haematol* 2000 | Case-series with control group Period: NR | B-cell-NHLs: 112 NHL classification: REAL histological scheme | 1/112 (0.9%) | Patients with other B-cell malignancies HL: 38 CLL: 43, ALL: 9 MM: 26 WMc: 1 Prevalence of HCV carriers in Republic of Macedonia within the general population is equal to 2.0% | 1/137 (0.72%) | 0.9 (0-2.6) | Low prevalence of HCV infection in patients with B-cell NHL from Macedonia and a lack of association between the two disorders |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Park SC *J Med Virol* 2008 | Case-control study Period: January 1998-December 2001 | 235 patients with NHLs, B-cell subtypes: 168 T-cell subtypes: 57 not identified subtypes: 10 NHL classification: NR | 5/235 (2.1%) No information about number of patients with HCV infection and B-NHL cases | Patients with advanced gastric cancer diagnosed at the Korea Cancer Center Hospital | 7/235 (3%) | 2.1 (0.3-3.9) | No association between HCV infection and non-Hodgkin's lymphoma |
| Paydas S *Br J Cancer* 1999 | Case series Period: NR | LPDs: 228 patients NHL: 98 CLL: 38 MM: 47 HD: 36 ALL: 9 NHL classification: NR | NHL: 9/98 (9.2%) CLL: 4/38 (10.5%) MM: 5/47 (10.6%) HD: 7/36 (19.4%) ALL: 1/9 (11.1%) | NR | NR | 9.2 (3.4-14.9) | HCV infection as a causative and/or contributing factor in lymphoproliferation in this study |
| Pellicelli *World J Gastroenterology* 2011 | Case-series Period: January 2008 -January 2009 | 125 patients with B-cell NHLs NHL classification: World Health Organization's classification | 24/125 (19.2%) | NR | NR | 19.2 (12.3-26.1) | HCV genotypes and duration of HCV infection differed between B-NHL subtypes. Indolent lymphomas can be managed with antiviral treatment, while DLBCL is not affected by the HCV infection |
| Pioltelli P *Lancet* 1996 | Case-series with control groups Period: January-June 1995 | All Lymphomas: 204 NHLs: 126 HL: 78 28HCV positive lymphomas NHL classification: Working Formulation | 26/126 (20.6%) | (1) HL (2) candidated blood donors (3) elderly people | (1) 2/78 (2) 9/832 (3) 9/94 | 20.6 (13.5-27.7) | High prevalence of HCV infection in NHLs, in the absence of an increased risk for HCV infection and of a clinical history of MC |
| Pioltelli P *Am J Hematol* 2000 | Case-control study Period: 01/01/96-30/06/97 | Patients with B-cell NHLs: 300 NHL classification: Working Formulation (WF) and REAL histological scheme | 48/300 (16%) | Individuals consecutively recruited during routine visits at medicine, surgery, or traumatology departments during the recruitment period of the study population (1) Patients with internal and surgical diseases (2) Patients with solid neoplasm (3) Patients with autoimmune disorders | (1) 51/600 (2) 15/247 (3) 6/122 | 16 (11.8-20.1) | The prevalence of HCV infection is higher in patients with NHLs than in non-neoplastic people and in patients with non-lymphoproliferative malignancies or receiving immunosuppressive treatment, but the small difference among these groups, the identical genotype pattern between NHL and controls do not support the hypothesis that HCV plays a role in lymphomagenesis |
| Pivetti S *Br J Haematol* 1996 | Case-series with control group Period: NR | Patients with LPDs: 167 patients (30 HCV positive) HL: 30 NHLs: 47 CLL: 29 MM: 18 MGUS: 31 WMc: 12 NHL classification: NR | 7/47 (14.9%) | (1) Patients with connective tissue diseases (2) Patients with idiopathic thrombocytopenic purpura | (1) 26/100 (26%) (2) 12/33 (36.4%) | 14.9 (4.7-25) | HCV may link lymphoid malignancies and autoimmune diseases by skewing the activity of the immune system toward the production of autoAbs |
| Pozzato G *Blood* 1994 | Case series Period: NR | 31 patients with MC. 12 patients/31 with low-grade NHLs 26/31 HCV positive NHL classification: Working Formulation | 10/12 patients with low-grade NHLs were anti-HCV positive | NR | NR | 83.3 (51.6-97.9) | HCV associated with a high prevalence of low-grade non-Hodgkin's lymphomas |
| Prati D *Br J Haematol* 1999 | Case series Period: January 1989 -August 1998 | Primary cutaneous B-cell NHL. NHL classification: European Organisation for Research and Therapy of Cancer (EORTC) | 1/34 (2.9%) | NR | NR | 2.9 (0-8.6) | Primary cutaneous B-cell NHL might represent a distinctive group among B-cell NHLs |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Rabkin CS *Blood* 2002 | Cohort study Period: June 1959 and September 1966 | All LPDs: 95 B-cell NHL: 57 MM: 24 HL: 14. NHL classification: Tumors classified according to the International Classification of Diseases for Oncology, second edition, as NHL (histologic classifications 9590 through 9642 and 9670 through 9698), multiple myeloma (9730 through 9732), or Hodgkin disease (9650 through 9667) | 4/95 (4.2%) 0/95 at RIBA 0/95 at HCV-RNA | Study subjects (48 420 individuals) recruited from the Child Health and Development Study (CHDS) cohort established in 1959 at the Kaiser Foundation Health Plan, Oakland, CA | 1/48.420 at ELISA 0/48420 at RIBA | 4.2 (0.1-8.2) | Not substantial role of chronic HCV infection in the etiology of B-cell neoplasia |
| Ramos-Casals M *J Rheumatol* 2004 | Case series Period: 1994-2000 | NHL classification: World Health Organization's classification | 6/98 | NR | NR | 6.1 (1.3-10.8) | Description concerning a triple association of HCV infection, autoimmune diseases and NHLs |
| Salem AK *Gulf J Oncol* 2009 | Case series with control-group Period: January 2005-January 2007 | All NHLs: 192 patients NHL classification: NR | 29/192 (15.1%) | Patients checked for HCV infection with several acute medical conditions and coming from different parts of the country | 814/20329 (4%) | 15.1 (10-20.1) | Higher prevalence of HCV infection among Yemeni patients with NHL than among persons in the control group |
| Salem Z *Eur J Epidemiol* 2003 | Case-series with control group Period: NR | B-cell NHL: 35 patients. NHL classification: NR | 0/35 | (1) Patients with different malignancies (malignant myeloproliferative disorders: 12, malignant lymphoproliferative disorders: 28, non haematological cancers: 23 patients) (2) Healthy blood donors and patients without malignant conditions, attending General Medicine of American university, Beirut | (1) 0/63 (2) 0/220 | 0 (0 -10) | No association between HCV infection and B-cell NHLs development in Lebanese patients |
| Sansonno D *Blood* 1996 | Case series Period: January 1991 to December 1995 | 12 HCV-positive patients with MC and 2 HCV-positive patients with reactive lympho-adenopaties NHL classification: Working Formulation | 3/12 (25%) | NR | NR | 25 (0.5-49.5) | These data emphasize that lymphoid organs may be a site of HCV infection. The demonstration of HCV-related proteins in a nonmalignant condition, namely HRL, indicates that HCV infection precedes the neoplastic transformation and possibly plays a major role in lymphomagenesis in MC |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Schöllkopf C *Int J Cancer* 2008 | Nation-wide Danish-Swedish case-control study (Scandinavian Lymphoma Etiology study, SCALE) Period: The SCALE study population includes the entire Danish population between June 1, 2000 - August 30, 2002, and the Swedish population between October 1, 1999-April 15, 2002 | All lymphomas: 2819 NHLs: 2353 HL: 466 NHL classification: World Health Organization's classification | HCV positive NHLs: 57 (2.4%) HL: 6 (1%) at III G ELISA test,only NHLs: 7/2353 (0.7%) HL: 0 positive at ELISA test and positive or intermediate at RIBA test for anti-HCV antibodies | Controls randomly sampled from the entire Danish and Swedish populations using continuously updated, computerized population registers | 21/1856 (1%) | 2.4 (1.8-3) | Positive association between HCV and risk of NHL, in particular of B-cell origin |
| Seve P *Eur J Gastroenterol Hepatol* 2004 | Cross-sectional study Period: January 1997-December 1998 | B-NHL:212 patients BL 6 DLBCL 109 FC 31 LL 7 LPL 5 MALT 17 MCL 21 MZL16 NHL classification: Revised European American Lymphoma (REAL) classification | (1) 6/212 (2.8%) (2) MALT 3/17 | Transfusion patients from surgical emergency, internal medicine pneumology, endocrinology, gastroeterology, nephrology, oncology, general surgery, orthopaedics, rheumatology, obstetrics and gynaecology, and intensive care wards | 20/974 (2.05%) | (1) 2.8 (0.6-5) (2) 17.6 (3.8-43.4) | Possible association between HCV and MALT lymphoma |
| Shariff S *Ann Oncol* 1999 | Case series with control group Period: 1996 and part of 1997 | patients with B-cell NHL NHL classification: Working Formulation/Revised European American Lymphoma (REAL) classification | 2/88 (2.3) | (1) patients with a T-cell NHL (2) second control group, including health-care workers, recruited between 1995 and 1997 | 0/37 11/1085 (1%) | 2.3 (0-5.3) | Chronic HCV infection as a risk factor for B-cell NHL in certain populations or with certain genotypes of the virus, no significant association in British Columbia |
| Shirin H *Isr Med Assoc J* 2002 | Case control group Period: May 1997 -September 1999 | B-NHL (DLCL FC CLL) NHL classification: Revised European American Lymphoma (REAL) classification | Total: 212 patients Lymphoproliferative disorders: 10/128 (7.8%) | (1) Patients with Myeloprolipherative and myelodisplastic disorders: (2) Israeli blood donors | (1) 1/84 (1.1%) (2) HCV prevalence equal to 0.64% | 7.8 (3.1-12.4) | Significant association between HCV infection and diffuse large B cell lymphoma |
| Silvestri F *Bood* 1996 | Case series with control group Period: NR | 537 unsekected patients with LPDs B-cell NHLs: 311 T-cell NHLs: 57 MM: 78 HL:88 ALL: 23 NHL classification: Kiel classification/Revised European American Lymphoma (REAL) classification | 29/311 (9%) | NR | T-cell NHLs: 2/57 (4%) MM: 3/78 (4%) HL:0/88 ALL: 1/23 (4%) | 9 (6-12.5) | High prevalence of HCV infection in patients with B-cell NHL |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Silvestri F *Haematologica* 1997 | Case series Period: NR | B-cell NHLs NHL classification: Revised European American Lymphoma (REAL) classification | 42/470 (8.9%) 21/22 (95.4%) B cell-NHLs patients with cryo-globulinemia 21/448 (4.6%) B cell-NHLs patients without cryo-globulinemia | NR | NR | 8.9 (6.3-11.5) | Close association between HCV infection and B-cell NHLs |
| Singer IO *Leuk Lymphoma* 1997 | Case-series with control group Period: NR | All Lymphomas: 50 unselected patients B-cell NHLs: 31 T-cell NHLs: 6 HL: 13 NHL classification: Working Formulation | 0/31 | No information about control groups | 0/19 | 0 (0-11.2) | No evidence supporting an association between HCV infection and LNH development |
| Sonmez M *Tumori* 2007 | Case-control study Period: 2002-2005 | B-cell NHLs: 109 DLBCL: 71 Small-cell LL: 38 NHL classification: World Health Organization's classification | 3/109 (2.8%) Low grade: 1/38 (2.6%) High grade: 2/71 (2.9%) | Patients selected from orthopedics, general surgery, urology, ophthalmology, otorhino-laryngology clinics with irrelevant diseases | 28/551 (5.1%) | 2.8 (0-5) | No difference in the incidence of HCV infection between NHL- and control-group |
| Spinelli JJ *Int J Cancer* 2008 | Population-based case-control study Period: March 2000 and February 2004 | All-NHL cases:795, from the Greater Vancouver Regional District (GVRD) and the Capital Regional District (CRD), including the city of Victoria, enrolled from the BC Cancer Registry NHL classification: World Health Organizations classification | NHLs: 19/795 (2.4%) B-cell NHLs:18/717 (2.5%) T-cell NHLs: 1/78 | Controls selected from the Client Registry of the BC Ministry of Health | 5/697 (0.7%) | 2.4 (1.3-3.4) | HCV infection contributes to increase NHL risk |
| Swart A *BMJ Open* 2012 | Cohort-study Period: 1 January 1993-31 December 2007 | Individuals registered on the Pharmaceutical Drugs of Addiction System, a record of all NSW Health Department authorities that administer methadone or buprenorphine to opioid-dependent people as opioid substitution therapy. Solid cancers classified according to the International Classification of Diseases (ICD), 10$^{th}$ revision, haematopoietic neoplasms and Kaposi sarcomas classified according to the ICD for Oncology, 3rd edition | Patients considered in the study: 29613 Subjects with HCV infection alone: 14892 Observed number of LNH in HCV-positive cohort: 75 | Calculation of expected number of incident LNHs | Expected number of LNH: 49.6 | 0.5 (0.4-0.6) | Association between HCV infection and LNHs |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Takai S *Eur J Haematol* 2005 | Case series Period: January 1996 to September 200 | All haematological malignancies: 601 NHL: 218 DLBCL: 110 FCL: 100 MCL: 3 PTCL: 5 Acute Leukemia: 246 AML: 193 ALL: 53 Adult T-cell Leukaemia: 13 MM:124 | 37/601 patients were anti-HCV positive NHL: 22/218 (10.1%) DLBCL: 13/110 (11.8%) FCL: 8/100 (8%) MCL: 1/3 (33%) PTCL:1/5 (20%) AML: 5/193 (2.6%) ALL: 2/53 (1.8%) adult T-cell Leukaemia: 0/13 MM: 8/124 | NR | NR | NHLs: 10.1 (6.1-14.1) DLBCL: 11.8 (5.8-17.8) FCL: 8 (2.7-13.3) MCL: 33 (0-86.2) PTCL:20 (0-63) AML: 2.6 (0.4-4.8) ALL: 1.8 (0-8.9) MM: 6.5 (2.2-10.8) | High prevalence of HCV infection in NHL Possible role of HCV in the pathogenesis of NHs |
| Takeshita M *Histopathology* 2006 | Case series with control group Period: NR | All-Lymphomas: 537 (1) HL: 18 -B-NHL: 400 (DLBCL, FC CLL, MALT, PCM, MCL, MZL, BL, others) -T-cell NHL: 96 -NK/T-cell NHL: 23 NHL classification: World Health Organization's classification | B-cell NHL 45/400 (11.3%) Primary Effusion Lymph: 3/6 (50%) BL: 1/7 (14.3%) DLCL:28/161 (17.4%) FL: 3/47 (6.4%) MALTOMA: 5/52 (9.6%) MM: 4/81 (4.9%) CLL, SMZL, Mantle cell Lymp: 0 | (1) Other haematological malignancies (2) Blood donors | (1) HL: 1/18 (5.6%) T-cell NHL: 5/96 (5.2%) NK-Tcell Lymphomas: 2/23 (8.7%) (2) 396/15567 (2.5%) | 11.3 (8.1-14.3) | HCV infection may play a role in lymphomagenesis of splenic and gastric DLBCL |
| Talamini R *Int J Cancer* 2004 | Case-control study Period: January 1999 -July 2002 | Total NHL: 225 cases 44/225 HCV positive patients NHL classification: International Classification of Diseases for Oncology, updated to include categories in the Revised European-American Lymphoma (REAL)/ World Heath Organization classification | 44/225 HCV positive patients 40/225 (17.8%) patients with B-cell NHLs (1) Low-grade B-cell: 24 (2) Intermediate- and high-grade B-cell: 16 (3) T-cell: 2 (4) Unknown:2 | Patients with a wide spectrum of acute conditions admitted at National Cancer Institute, Aviano; the "Santa Maria degli Angeli" General Hospital, Pordenone; the "Pascale" National Cancer Institute, Naples and 4 general hospitals, Naples | 45/504 (8.9%) | 17.8 (12.8-22.8) | HCV infection associated with an increased NHL risk |
| Teng CJ *Clinics* 2011 | Case series Period: 2003-2008 | MM: 155 patients 30 patients with chronic hepatitis MM diagnosis: International Myeloma Working Group | 14/155 (9%) 1/155 with HBV/HCV co-infection | NR | NR | 9 (4.5-13.5) | High prevalence of cytogenetic abnormalities in patients with HCV chronic hepatitis |
| Thalen DJ *Br J Haematol* 1997 | Case series Period: NR | NHLs: 115 patients B-cell NHLs: 99/115 (86%) T-cell NHLs: 15 (13%) Unclassified: 1 (1%) NHL classification: Working Formulation | B-cell-NHLs: 0/99 T cell NHLs: 0/15 | NR | NR | 0 (0-3.7) | No association between HCV infection and B-cell NHLs in the study |
| Timuragaoğlu A *Haematologia* 1999 | Case series with control group Period: NR | NHLs: 48 patients NHL classification: Working Formulation | Anti HCV positive: 0/48 HCV-RNA positive: 3/35 (8.6%) | Patients with various haematological disorders (MM, HL, acute myeloblastic leukaemia, acute lymphoblastic leukaemia, chronic myelogenous leucemia, idiopathic thrombocytopenic purpura, myelodysplastic syndrome) | 0/28 | 8.6 (1.8-23.1) | Association between HCV infection and B-cell NHLs in the study |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Tkoub EM *Blood* 1998 | Case series with control group Period: NR | 46 patients with gastric MALT: High grade: 21 Low-grade 25 37/46 patients with *Helicobacter Pylori* NHL classification: NR | 1/46 (2.2%) | Patients with gastroduodenal disease: 84 with duodenal ulcer 43 with gastric ulcer 38 with dispepsia | 4/165 (2.4%) | 2.2 (0-6.3) | No link between HCV infection and gastric MALT in France |
| Tursi A *Am J Gastroenterol* 2002 | Case series Period: NR | 25 HCV positive patients with gastric MALT. -20/25 (80%) with grade 2-5/25 (20%) with grade 3. 18/25 patients with *Helicobacter Pylori* NHL classification: World Health Organization's classification | NR | NR | NR | MALT grade 2: 80 (59.3-93.2) MALT grade 3: 20 (6.8-40.7) | HCV may colonize gastric MALT, allowing the development of a grade of acquired MALT, this represents the first step toward a MALT lymphoma |
| Udomsakdi-Auewarakul C *Blood* 2000 | Case series Period: NR | All malignancies: 130 Intermediate- to high-grade NHL: 98 Low-grade NHL 32 patients NHL classification: Working Formulation | 2/98 (2%) 1/32 (3.1%) | NR | NR | 2.(0-4.8) | No association between HCV infection and NHLs in this study from Thailand, where HCV infection is highly prevalent |
| Vajdic CM *Cancer Epidemiol Biomarkers Prev* 2006 | Population-based case-control study Period: January 2000 and August 2001 | Total Lymphomas:694 -B-cell NHLs: 659 (95%) -T-cell NHLs: 28 (4%) -Undetermined: 7 (1%) NHL classification: World Health Organization's classification | NHLs: 3/694 (0.4%) | Potential participants (both cases and controls) received a letter to inviting their participation in research about the development of NHL | 2/694 (0.3%) | 0.4 (0-0.9) | No strong evidence for an association between any infection and non-Hodgkin lymphoma risk in immunocompetent people, but increased risk between HCV infection and non-Hodgkin lymphoma in subjects with injecting drug use |
| Vallisa D *Am J Med* 1999 | Case-control study Period: 1990-1996 | B-cell-NHLs: 175 patients NHL classification: Working Formulation/Revised European American Lymphoma clasification | 65/175 (37.1%) | Subjects without lymphoma selected from: (1) inpatients (175) (2) outpatients (175) cared at Civil Hospital, Piacenza, subdivided into 2 groups | (1) 17/175 (10%) (2) 15/175 (9%) | 37.1 (30-44.3) | Possible HCV role as an etiologic agent in non-Hodgkin's B-cell lymphoma |
| Varma S *Hepatol Int* 2011 | Case-control study Period: NR | B-NHLs: 57 patients High-grade disease (DLBCL): 44 (77.2%) Intermediate-disease (FC): 6 (10.5%) Low grade disease: (small lymphocytic): 7 (12.3%) NHL classification: World Health Organizations classification | 1/57 (1.7%) | Patients with non-hematological conditions admitted to Departments of Ophthalmology, Otorhinolaryngology, Dermatology, and Internal Medicine in the Hematology Clinic, Institute of Medical Education and Research, Chandigarh | 2/171 (1.2%) | 1.7 ( 0-5.1) | No significant association between HCV infection and NHL in Northern India |
| Veneri D *Am J Hematol* 2007 | Case series Period: January 1995 -December 2006 | 947 patients with lymphoproliferative disorders: DLBCL: 361 MM: 139 B-cell MZL: 62 HL: 103 B-CLL: 186 FL. 96 NHL classification: World Health Organization's classification | 55/947 patients were HCV positive DLBCL: 27/361 (7.5%) MM: 1/139 (0.7%) B-cell MZL: 15/62 (24.2%) HL: 4/103 (3.9%) B-CLL: 4/186 (2.1%) FL: 4/96 (4.2%) | NR | NR | DLBCL: 7.5 (4.7-10.2) B-cell MZL: 24.2 (13.5-34.8) | Confirmed association between a subset of B-cell lymphomas and HCV infection |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Yamac K *Eur J Epidemiol* 2000 | Case series Period: August 1996-June 1998, | All Lymphomas: 92 NHLs: 73 HL 19 NHL classification: Revised European American Lymphoma clasification | 1/92 (1.1%) | NR | NR | 1.1 (0-3.2) | No significant association between HCV and NHL in the study |
| Yenice N *Turk J Gastroenterol* 2003 | Case series with control group | All Lymphomas: 134 B cell NHLs: 84 HLs: 50 | B-cell NHLs: 6/84 (7.1%) HLs: 1/50 (2%) | Healthy blood donors | 1/100 (1%) | 7.1 (1.6-12.6) | HCV may play a role in the development of B-cell non-Hodgkin lymphoma, but not in Hodgkin lymphoma |
| Yoshikawa M *J Clin Gastroenterol* 1997 | Case series with control group Periood: NR | All Lymphomas: 100 B-NHLs: 55 T-NHLs: 10 HL: 5 MM: 25 B-CLL: 2 MGUS: 3 NHL classification: Working Formulation | B-NHLs: 9/55 (16.4%) MM:5/25 (20%) MGUS: 1/3 (33.3%) | Patients with any cancer in digestive organs except liver enrolled at Nara Medical University | 1/25 (4%) | 16.4 (6.5-26.1) | High rates of HCV infection detected in B-NHL and MM |
| Yu SC *Kaohsiung J Med Sci* 2013 | Case series Period: 1988-2011 | All lymphomas: 74 patients: -B-cell lymphomas: 69 -T-cell lymphomas: 3 -Lymphoblastic Lymphoma: 1 -Unspecified high-grade lymphoma: 1 41/74 patients with serology for HCV infection NHL classification: World Health Organization's classification | Patients with B-cell-NHL and with serology for HCV infection: 39 Patients with B-cell-NHL and HCV positive 10/39 (25.6%) | NR | NR | 25.6 (11.9-39.3) | High HCV sero-prevalence in patients with early-stage DLBCL suggests a role of HCV in the pathogenesis of primary DLBCL |
| Zucca E *Haematologica* 2000 | Case series Period: 1990 and 1995 | B-cell NHLs: 180 Anti-Helicobacter antibodies detected in 81/180 (45%) patients. NHL classification: REAL histological scheme | 17/180 (9.4%) Gastric lymphoma: 2 Non gastric lymphoma: 15 | A survey of 5424 subjects new blood donors from the same area tested between 1992 and 1997 (Swiss Red Cross Transfusional Medicine Service for Canton Ticino) | 49/5424 (0.9%) | 9.4 (5.1-13.7) | High prevalence of HCV infection detected in NHL lymphoma patients and associated with a shorter time to lymphoma progression. HCV infection not correlated with primary gastric presentation or with MALT-type histology |
| Zuckerman E *Ann Intern Med* 1997 | Controlled, cross-sectional study. Period: October 1994 and May 1996 | B-cell NHLs: 120 patients NHL classification: Working Formulation | B-cell NHLs 26/120 (22%) | (1) Patients with hematologic malignancies other than B-cell NHLs; (2) Patients without hematologic malignancies, attending the general medicine clinic at LAC-USC and with: systemic hypertension or ischemic heart disease: 69 diabetes mellitus: 35 primary hypothyroidism: 10 | 268 patients 7/154 (4.5%) (2) 6/114 (5%) | 21.7 (14.3-29) | Increased prevalence of HCV infection among patients from the United States with B-cell lymphoma, but uncertain generalizability to other populations, because of high number of patients, belonging to Hispanic ethnicity |

ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; AnLLs: Acute non-lymphocitic leukemia; B-LPD: B-cell lymphoproliferative disorders; BL: Burkitt lymphoma; CC: Centrocitic; CB: Centroblastic; CBCC: Centroblastic/centrocitic; CLL: Chronic lymphocytic leukemia; CML: Chronic myeloid leukemia; CMD: Chronic myeloprolipherative disease; DLBCL: Diffuse large B-Cell lymphoma; CLISA: Chemiluminescence immunoassay; ELISA: Enzyme-linked immunosorbent assay; EIA: Enzyme–immunoassay; EMZBL: Extranodal marginal zone B-cell lymphoma; FC: Follicular lymphoma; FCL: Follicle center lymphoma; HCL: Hairy cell leukemia; HL: Hodgkin lymphoma; IC: Immunocytoma; LAC-USC: Los Angeles County-University of Southern California; LL: Lymphocitic lymphoma; LPds: Lymphoproliferative diseases; LPD: Lymphoproliferative disorders; LPL: Lymphoplasmocitoid lymphoma; MCL: Mantle Cell Lymphoma; MGUS: Monoclonal Gammopathy of uncertain significance; MEIA: Microparticle enzyme immunoassy; MCS: Mixed cryoglobulinemia syndrome; MGUS: Monoclonal gammopathy of undetermined significance; MM: Multiple myeloma; MS: Myelodisplastic syndrome; MZL: Marginal zone lymphoma; PCM: Plasma cell myeloma; PHL: Primary hepatic non-Hodgkin's lymphoma; PLL: Primary Liver Lymphoma; PSL: Primary splenic non-Hodgkin's lymphoma; PTCL: Peripheral T-cell lymphoma; RAEB: Refractory anemia with excess of blasts; SIR: Standardised incidence ratio; WMc: Waldenström's microglobulinemia; Y: Determined; N: Not determined; NR: Not reported.

**Table 2 Characteristics of available studies, reported in English, assessing the association between hepatitis C virus infection and cholangiocarcinomas (A) or bile duct dysplasia (B)**

| Author/Journal/ Publication year | Study design study period | CCA diagnosis | HCV positive colangiocarcinoma (*n*)/ total colangiocarcinoma cases (*n*) | Total patients enrolled and control source | HCV positive controls (*n*)/controls (*n*) | Percentage of HCV-positive cases with 95%CI | Main conclusion |
|---|---|---|---|---|---|---|---|
| (A) | | | | | | | |
| Abdel Wahab M 2007 | Case series Period: Januiary 1995-October 2004 | Histologic confirmation/CT/MRI/ ERCP/PTD | 440 patients with hilar cholangiocarcinoma 238 anti-HCV positive patients 238/440 (54%) | NR | NR | 54.1 (49.4-58.7) | Liver cirrhosis and HCV may be risk factors For hilar cholangiocarcinoma in Egypt |
| Barusrux S *Asian Pacific J Cancer Prev* 2012 | Case series with control group Period: NR | Histologic confirmation | 8/295 (2.7%) | Total patients: 6120 Controls randomly selected from people in 4 provinces in Thailand, representing 4 geographically distinct areas and thus, populations in the North, North-east, South and Center of the country, respectively | 125/5825 (2.15%) HCV-Ab prevalence in Thailand ranging from 1.5% to 2.15%. Sunanchaikarn S, Theamboonlers A, Chongsrisawat V *et al* (2007). Seroepidemiology and genotypes of hepatitis C virus in Thailand. *Asian Pac J Allergy*, 25, 175-182 | 2.7 (0.8-4.5) | No significant association between CAA and HCV in northeast Thailand, with prevalence of HCV infection comparable among CCA and general population |
| Chantajitr S *J Hepatobiliary Pancreat Surg* 2006 | Case series with control group Period: 2000-2004 | Histologic confirmation | HCC-CCA = 25 15 patients with test for anti- HCV 2/15 (13.3%) | Total patients: 75. 50 individuals, diagnosed with HCC at Ramathibodi Hospital | HCC = 50 32 patients with test for anti- HCV 1/32 (3.1%) | 13.3 (1.6-40.5) | No significant differences in presence of hepatitis C virus (HCV) antibody (13% *vs* 3%) as etiologic risk factor between HCC-CC and HCC patients |
| Donato F *Cancer Causes and control* 2001 | Hospital-based case-control study Period: January 1, 1995-July 31, 2000 | Histologic confirmation | 6/24 (25%) | Total individuals: 848. Subjects unaffected by liver diseases or malignant neoplasms, admitted to the Department of Ophtalmology, Dermatology, Urology, Surgery, Cardiology, Internal Medicine in the two main Hospitals in Brescia, enrolled as controls | 50/824 (6%) | 25 (7.7-42.3) | HCV as possible risk factor for ICC in Western countries |
| El-Serag H *Hepatology* 2009 | Cohort study Period: October 1, 1988, and September 30, 2004 | Identification of PAC cases by means of ICD-9-CM diagnosis codes (157.0, 157.1, 157.2, 157.3, 157.8, 157.9) Identification of HCV infected subjects by means of ICD-9-CM diagnosis codes (070.41, 070.44, 070.51, 070.54 and V02.62) | HCV-infected cohort: 146394 patients ICC = 14 ECC = 15 | 718687 patients (146394 HCV-infected cohort, 572293 HCV-uninfected cohort) , ICC: 37 and ECC: 75 (14 ICC and 15 ECC in HCV infected patients, 23 ICC and 60 ECC in HCV uninfected subjects) | HCV-uninfected cohort: 572293 patients ICC = 23 ECC = 60 | ICC: 0.01 (0-0.15) ECC: 0.01 (0-0.15) | A more than twofold elevated risk of ICC in patients with HCV infection, absence of an association with ECC |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Hai S *Dig Surg* 2005 | Case series with control group Period: January 1997 - December 2002 | Histologic confirmation | 19/50 (38%) | Total patients: 50 Subjects admitted to the Osaka City University Hospital or the Osaka City General Hospital | 31/50 (62%) | 38 (24.5-51.4) | Possibility to detect a small ICC or a hepatocellular carcinoma by means of a follow-up for patients with chronic HCV by imaging series at regular intervals |
| Hsing AW *Int J Cancer* 2008 | Population-based case-control study Period: June 1997 - May 2001, | Histologic confirmation or by means of ERCP | 3/234 (2%) with gallbladder cancers 2/134 (1.5%) with extrahepatic bile duct cancers 1/49 (2%) with Ampulla of Vater carcinomas | Total patients: 1696 Controls represented by biliary stone case patients and by healthy subjects without a history of cancer, randomly selected from all permanent residents listed in the Shanghai Resident Registry | 2/301 (0.7%) patients with gallbladder stones, 5/216 (2.3%) with bile duct stones and 15/762 (2%) healthy individuals | 1.5 (0-3.5) | Low prevalence of HCV infection in this population (2%), therefore limited ability to detect an association with biliary diseases |
| Kobayashi M *Cancer* 2000 | Case series with control group Period: 1980-1997 | Cirrhosis confirmation by means of liver biopsy, peritoneoscopy, or both | 14/600 (2.3%) developed CCA 11/14 patients with CCA 3/14 patients with CCA-HCC | 600 HCV positive patients in follow-up between 1980 to 1997 | 206/600 (34.3%) patients developed HCC in the same period | 2.3 (1.1-3.5) | HCV-related cirrhosis as a major risk factor for primary CCA in Japanese patients |
| Kuper H *Soz Praventivmed* 2001 | Case-control study Period: January 1995-December 1998 | Histologic confirmation | 0/6 with CCA | Total subjects: 699 Controls represented by patients with injuries or eye, ear, nose and throat conditions admitted to three teaching Hospitals in Athens | 52/333 (16%) with HCC 1/360 (0.3%) controls | 0 (0-45.9) | No HCV positivity in CCA patients |
| Lee CH *Br J Cancer* 2009 | Case-control study Period: 1991-2005 | Histologic confirmation | 21/160 (13.1%) | Individuals generally surveyed for any disease Chang Gung Memorial Hospital at the Lin-Ko Medical Center | 10/160 (6.3%) | 13.1 (7.9-18.3) | HCV-associated ICC and HCC shared common disease process for carcinogenesis and, possibly, both arose from the hepatic progenitor cells |
| Lee TY *Am J Gastroenterol* 2008 | Hospital-based case-control study Period: 2000-2004 | Histologic confirmation | 12/622 (1.9%) | Total subjects:3110 2488 healthy controls selected from 192655 individuals undergoing routine health examinations at the health promotion center at Asan Medical Center, Seoul | 47/2488 (1.9%) | 1.9 (0.8-3) | No significant association between ICC and HCV |
| Lee WS *Surg Today* 2006 | Case series with control group Period: November 1994-December 2003 | Histologic confirmation | ICC = 3/79 (3.8%) HCC-CCA = 4/33 (12.1%) | Total patients: 952, subjects, undergoing surgical resection at Samsung Medical Center, because of: HCC-CCA = 33 ICC = 79 HCC = 832 | HCC = 61/832 (6.5%) | 3.8 (0-8) | Significantly poorer survival rates of patients with transitional type HCC-CCA in comparison with HCC after hepatic resection |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Matsumoto K *Intern Med* 2014 | Case series with control group Period: NR | Histologic confirmation | 145 patients undergoing surgical resection because of ICC: 50 ECC: 95 (1) ECC: 7/95 (7.4%) (2) ICC: 10/50 (20%) | General Japanese population (individuals ⩾ 20 yr of age) | HCV-Ab prevalence equal to 1.2% in the Japanese individuals ⩾ 20 yr of age | (1) 7.4 (2.1-12.6) (2) 20 (8.9-31) | HCV infection as a possible risk factor for the development of CCA. Surveillance of ICC and ECC required in HCV carriers |
| Mohammad-Alizadeh AH *Asian Pac J Cancer Prev* 2012 | Case series with control group Period: 2004-2011 | Histologic confirmation ERCP MRCP | CCA: 43/283 (15.2%) No distinction between HCV and number of ICC and ECC cases | Total subjects: 566 Patients with the primary or final diagnosis of CAA, admitted to gastroenterology ward of a tertiary academic center in Tehran-Iran | Gallstones 72/283 (25.4%), diabetes 70/283 (24.6%), HBV infection 52/283 (18.3%), primary sclerosing cholangitis 16/283 (5.6%) smoking 120/283 (42.3%) | 15.2 (11-19.3) | In current study smoking, opiate and alcohol use as the most common risk factors in CCA patients, chronic hepatitis C infection and cirrhosis represent further risk factors |
| Nuzzo G *Updates Surg* 2010 | Case series with control group Period: 1997-2008 | Histologic confirmation | 8/55 (14.5%) (2 patients with HBV coinfection), undergoing surgical resection at Policlinico Gemelli, Rome | Total subjects: 55 | 47/55 (76.5%) | 14.5 (5.2-23.8) | ICC associated with chronic HCV infection in 14.5% of patients |
| Perumal V *Human Pathology* 2006 | Case series with control group Period: NR | Histologic confirmation | 2/11 (18.2%) | 10 liver specimens from anti-HCV negative individuals and 13 liver specimens from individuals who were negative for HBV surface antigen by serologic testing, used as negative controls HCV RNA-positive liver tissues from HCV positive cases used as positive controls for HCV RNA detection, at Johns Hopkins Hospital, Baltimore | Total subjects: 21 | 18.2 (2.2-51.8) | Possible etiologic role of HCV in some cases of ICC |
| Portolani N *Annals of Surgical Oncology* 2008 | Case series with control group Period: 1990-2006 | Histologic confirmation or typical findings on ultrasound, CT-, MRI-examination | ICC = 33 patients undergoing resection and 16 not resected 6/33 (18.1%) | Total subjects: 51 Patients diagnosed with ICC-HCC at the Surgical Clinic of Brescia University, Italy | ICC-HCC = 18 patients undergoing resection 11/18 (61.1%) | 18.1 (5-31.3) | HCV infection and cirrhosis as a risk condition for ICC and combined HCC-ICC |
| Qu Z *Asia-Pacific Journal of Clinical Oncology* 2012 | Case series with control group Period: January 1990 - June 2001 | Histologic confirmation of ECC | ECC: 305, 139 with test for anti- HCV ECC: 6/139 (4.3%) | Total subjects: 353 Patients with BBD with cholelithiasis or acute cholangitis, undergoing surgical intervention selected as controls at Tianjin Nankai Hospital, Tianjin Third Central Hospital, Tianjin Medical University General Hospital and The Second Hospital of Tianjin Medical University hospitals in the corresponding time period | BBD:480, 214 with test for anti-HCV BBD:12/214 (5.6%) | 4.3 (0.9-7.6) | No association between chronic HCV infection and ECC |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Shaib YH *Gastroenterology* 2005 | Hospital-Based Case-Control Study Period: 1993-1999 | Histologic confirmation HCV defined by using ICD-9 codes for HCV (ICD-9 codes 070.41, 070.44, 070.51, 070.54, and V02.62) or for unspecified hepatitis (ICD-9 codes 070.9, 571.4, 571.8, and 571.9) | Data obtained from the National Cancer Institute (NCI)'s Surveillance, Epidemiology and End Results program SEER-Medicare database, linking SEER registry information with Medicare claims data, it is a program of the NCI to collect population-based cancer incidence and survival data, including population-based cancer registries in 5 states and 6 metropolitan areas ( about 14% of the United States population). ICC cases: 625 (3) HCV-specific codes: 5/625 (0.8%) (1) HCV (including unspecified hepatitis):35/625 (5.6%) | Controls included in the study derived from the 5% random sample of Medicare-enrolled beneficiaries with no cancer of any type residing in the geographic regions of SEER registries | 90834 controls (1) HCV (including unspecified hepatitis): 940 (1%) (3) HCV-specific codes: 161 (0.2%) | 0.8 (0.1-1.4) | Chronic HCV infection as possible risk factors for ICC |
| Shaib YH *Am J Gastroenterol* 2007 | Hospital-Based Case-control Study Period: 1992-2002 | Histologic confirmation | 246 patients undergoing surgical resection because of ICC: 5/83 (6%) ECC: 6/163 (3.7%) | Total patients: 482 Controls randomly selected from an existing database of healthy individuals at M.D. Anderson | 2/236 (0.8%) | ICC: 6 (0.9-11.1) ECC: 3.7 (0.8-6.5) | Chronic HCV infection as possible risk factors for ICC but not ECC |
| Shin RH *Int J Epidemiol* 1996 | Case-control study Period: August 1990-August 1993 | Histologic confirmation or typical findings on ultrasound, CT-, MRI-examination | 41 patients with CCAs 203 patients with HCC (1) 29/41 patients with tests for antiHCV/HBV status. 4/29 (13.8%) HCV positive (2) 128/203 patients with test for antiHCV/HBV status 17/128 (13.3%) HCV positive | (1) Inpatients without liver disease, systemic disease, and malignant disorders from the Departments of Ophthalmology or Otorhinolaryngology (2) healthy people who had visited the Non-Communicable Disease Control Center All subjects were visited at the Tnje University Pusan Paik Hospital | (3) 203 (4) 203 394/406 subjects with tests for anti-HCV status. 23/394 (6.6%) HCV positive | (1) 13.8 (1.2-26.3) (2) 13.3 (7.4-19.1) | No association between chronic HCV infection and CCA |
| Songsivilai S *Trans R Soc Trop Med Hyg* 1996 | Case series with control group Period: July 1993 - June 1995 | Histologic confirmation | 0/30 | Total subjects: 110 Patients with HCC, undergoing surgical resection at Siriraj Hospital, Mahidol University, Bangkok | 9/80 (11.2%) | 0 (0-11.6) | No association between chronic HCV infection and CCA |
| Srivatanakul P *Asian Pacific J Cancer Prev* 2010 | Case-control study Period: September 1999 -2001 | Histology, or typical findings on ultrasound examination with an elevated titre (⩾ 40 units/mL) of CA 19-9 and normal level of alpha-fetoprotein (AFP < 20 ng/mL) | 7/103 (6.8%) | Total subjects: 206 Community hospitals in Nakhon Phanom Province and Nakhon Phanom Provincial Hospital | 0/103 | 6.8 (1.9-11.6) | Possible role of HCV infection in the development of CCA in northeast Thailand |
| Taguchi J *J Gatroenterol Hepatol* 1996 | Case series with control group Period: January 1988-July 1995 | Histologic confirmation | 14/20 (70%) | Total subjects: 367 HCC-CCA: 23/367, 20 patients with anti-HCV markers | 6/20 (30%) | 70 (49.9-90) | HCC-CCA associated with chronic HCV infection in 70% of patients |
| Tanaka M *J Viral Hepat* 2010 | Cohort study Period: 1991-1993 | ICC cases identified by the ICD-10 code (C22.1). diagnosis of ICC was based on histological examination and/or combined clinical, radiological (echography, CT and endoscopic retrograde cholangio-pancreatography) and laboratory findings | ICC: 11 cases 1/11 (9.1%) | 154814 study subjects voluntary blood donors | 1927/154814 (1.2%) | 9.1 (0.2-41.3) | No association between HCV infection and ICC development |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Tomimatsu M *Cancer* 1993 | Case series with control group Period: January 1985 - December 1990 | Histologic confirmation | (1) CCA: Anti-HCV +: 4/13 (30.8%) $HBsAg^+$: 3/13 (23.1%) Anti-HCV-/ $HBsAg^-$: 6/13 (46.1%) (2) CCA-HCC: Anti-HCV +: 5/7 (71.4%) $HBsAg^+$: 1/7 (14.3%) Anti-HCV- / $HBsAg^-$: 1/7 (14.3%) | Total subjects: 141 Patients with HCC, undergoing surgical resection at the Institute of Gastroenterology of Tokyo Women's Medical College | Anti-HCV +: 85/121 (70.3%), Anti-HCV+ /$HBsAg^+$: 5/121 (4.1%) $HBsAg^+$: 16/121 (13.2%) $HBsAg^-$/anti-HCV -: 15/121 (12.4%) | (1) 30.8 (9-61.4) (2) 71.4 (29-96.3) | The anti-HCV-positive rate was high in combined HCC-CC as well as in HCC |
| Uenishi T *Journal of Surgical Oncology* 2014 | Case series with control group Period: January 2000 - December 2011 | Histological confirmation | 33/90 (36.7%) | Total subjects: 90 Patients enrolled at Hirakata and Osaka University Hospital | 57/90 (63.4%) | 36.7 (26.7-46.6) | HCC-related death often occurred in patients undergoing curative resection for HCV-related ICC. HCV as adverse prognostic factor after curative resection for mass-forming ICC |
| Yamamoto M *Cancer* 1998 | Case-series Period: February 1990 - March 1996 | Histologic confirmation | 50 patients with ICC Anti-HCV positive: 16/50 (32%) $HBsAg^+$/ Anti-HCV positive: 1 (2%) | NR | NR | 32 (19-44.9) | Minute nodular ICC appears to be related to hepatitis viral infection and could be detected at an early stage, similar to hepatocellular carcinoma, by following up cases of chronic hepatitis or cirrhosis |
| Yamamoto S *Cancer Sci* 2004 | Hospital case-control based study Period: January 1991 - December 2002 | Histologic confirmation | 18/50 (36%) | Total subjects: 255 Control patients enrolled at the two major medical centers of Osaka City | 7/205 (3%) | 36 (22.7-49.3) | HCV infection as a possible etiology of ICC in Japan |
| Yano Y *Jpn J Clin Oncol* 2003 | Case-control study Period: January 1978 - December 1998 | Histologic confirmation | HCV alone: (1) HCC-CCA = 10/26 (38.5%) (2) CCA = 5/53 (9.4%) HCV + HBV: 1/53 (2%) | Total subjects: 1172 Patients with HCC, undergoing surgical resection at the Department of Surgery, National Cancer Center Hospital, Tokyo | HCV alone: HCC = 526/1093 (48%) HCV + HBV: 16/1093 (1%) | (1) 38.5 (19.8-57.1) (2) 9.4 (1.5-17.3) | HCC-CCA represents a variant of ordinary HCC with cholangiocellular features, rather than an intermediate disease entity between HCC and CCA |
| Wahab A *M Hepatogastro-enterology* 2007 | Case series Period: January 1995 - October 2004 | Histologic confirmation or typical findings on CT, ERCP, MRI and PTD | Total patients: 440238/440 (54.1%) | NR | NR | 54.1 (49.4-58.7) | HCV chronic infection as possible risk factor for hilar CCA in Egypt |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Welzel TM *Clin Gastroenterol Hepatol* 2007 | Population-based case-control study Period: 1993-1999 | Identification of CAA cases from the Surveillance, Epidemiology and End Results-Medicare databases by means of ICD-9-CM diagnosis codes: (C22.0, C22.1, C24.0, 8010, 8020, 8041, 8070, 8140, 8144, 8160, 8161, 8260, 8310, 8480, 8490, 8560). Identification of HCV infection by means of ICD-9-CM diagnosis codes 070.41, 070.44, 070.51, 070.54 and 070.7 | (1) ICC = 5/535 (0.9%) (2) ECC = 5/549 (0.9%) | 102782 cancer-free controls identified using the Surveillance, Epidemiology and End Results-Medicare databases | 142/102782 | ICC: 0.9 (0.1-1.7) ECC: 0.9 (0.1-1.7) | Association between HCV infection and ICC |
| Zhou HQ *Hepatobiliary Pancreat Dis Int* 2007 | Case-series Period: January 1996 - November 2005 | Histologic confirmation | (1) HCC: 132 patients Anti-HCV positive: 26/132 (19.7%) (2) CCA: 44 patients Anti-HCV positive: 4/44 (9.1%) (3) HCC-CCA: 15 anti-HCV positive: 3/15 (20%) | NR | NR | (1) 19.7 (12.9-26.4) (2) 9.1 (0.6-17.5) (3) 20 (4.3-48) | Percentage of cHCC-CC patients with serum anti-HCV antibodies were similar to those of HCC patients but different from CC patients |
| Zhou YM *World J Gastroenterol* 2008 | Hospital-based-case control Study Period: February 2004 - May 2006 | Histologic confirmation | 9/312 (2.9%) | Total patients: 750 Controls were selected from patients who were unaffected by liver diseases in the Changhai Hospital of the Second Military Medical University | 6/438 (1.4%) | 2.9 (0.9-4.7) | No significant difference between cases and controls in the prevalence of anti-HCV seropositivity |
| (B) | | | | | | | |
| Torbenson M *Am J Surg Pathol* 2007 | Review of liver explants with control group from 3 transplant centers Period: 1995 -2005 | Histologic confirmation in explanted livers | (1) HCV alone = 10/511 (2%) (2) HCV + alcohol = 4/85 (5%) | 1058 total liver explants Control groups included: (1) alcohol cirrhosis, (2) chronic hepatitis B infection, (3) nonviral causes of cirrhosis such as cryptogenic cirrhosis, (4) noncirrhotic livers that were transplanted for fulminant liver failure | (1) Alcohol cirrhosis = 5/112 (4%) (2) HBVchronic hepatitis = 0/67 (0%) (3) Cirrhosis from nonviral and non alcohol causes = 0/149 (0%) (4) Noncirrhotic =/134 (0%) | (1) 2 (0.7-3.1) (2) 4.7 (0.2-9.2) | Dysplasia detectable within the intrahepatic bile ducts in chronic HCV cirrhosis; chronic HCV, alone or in association with alcohol, as major risk factor for ICC |
| Wu TT *Cancer* 2009 | Review of liver explants with control group at Mayo Clinic Rochester, Minnesota Period: 1995 - 2007 | Histologic confirmation in explanted livers | (1) Alcohol-related and HCV-related cirrhosis: 24/26 (92%) (2) HCV-related cirrhosis: 27/44 (61%) | 244 total liver explants Causes: 94 alcohol-related cirrhosis, 44 HCV-related cirrhosis, 26 alcohol- and HCV-related cirrhosis, 28 massive hepatic necrosis, 24 correction of metabolic conditions, 16 primary or metastatic tumors, 8 nodular regenerative hyperplasia, 2 subacute Budd- Chiari syndrome, 2 liver failure during the first week after transplantation | Noncirrhotic 27/80 (34%) alcohol-related cirrhosis 86/94 (91%) | (1) 92.3 (74.9-99) (2) 61.4 (46.9-75.7) | Epidemiologic role of HCV and alcohol in the development of CCA |

BIN: Biliary intraepithelial neoplasia; BBD: Benign biliary disease; CT: Computed tomography; ERCP: Endoscopic retrograde cholangiopancreatography; HBsAg: Hepatitis surface antigen; cHCC-CC patients: Combined HCC and CCA; ICC: Intrahepatic colangiocarcinoma; ECC: Extrahepatic colangiocarcinoma colangiocarcinoma; CCA: Colangiocarcinoma; HCC: Hepatocellular carcinoma; MRI: Magnetic resonance imaging; PTD: Percutaneous transhepatic cholangiography; NR: Not reported; NA: Not available.

**Table 3 Characteristics of available studies, reported in English, designed to assess the association between hepatitis C virus infection and pancreatic cancer risk**

| First author/ Journal/ Publication year | Study design/ study period | PAC diagnosis | HCV positive PAC (*n*)/ total PAC cases (*n*) | Control source | HCV positive controls (*n*)/ controls (*n*) | Percentage of HCV-positive cases with 95%CI | Main conclusions |
|---|---|---|---|---|---|---|---|
| Amin J *J Hepatol* 2006 | Community-based cohort-study Period: 1990-2002 | Identification of pancreatic cancer cases by means of ICD-10- diagnosis codes | -Individuals with HCV infection: 75834 PAC detected: 17/75834 (0.02%) | Incidence observed in the study cohort was compared to expected incidence derived from NSW population cancer rates by calculating standardised incidence ratios | SIRs: 1.4 (0.8-2.2) | 0.02 (0.01-0.03) | No evidence supporting an association between HCV infection and PAC development |
| Chang MC *World J Gastroenterol* 2014 | Case-control study Period: 2000-2013 | Histological or citological | 22/585 (3.8%) | Controls were individuals recruited from a free screening program in a community located in Northern Taiwan | 45/1716 (2.6%) | 3.8 (2.2-5.3) | HCV infection not associated with higher risk of PAC development, after adjustement for age, sex, diabetes and smoking (independent risk factors for PAC) |
| El Serag *Hepatology* 2009 | Cohort study Cohort: 718687 patients PAC detected: 617 Period: 1988-2004 | Identification of PAC cases by means of ICD-9-CM diagnosis codes (157.0, 157.1, 157.2, 157.3, 157.8, 157.9) Identification of HCV infected subjects by means of ICD-9-CM diagnosis codes (070.41, 070.44, 070.51, 070.54 and V02.62) | 146394 patients in HCV-infected cohort PAC detected:140/146,394 (0.09%) | Sources included inpatients records from more than 150 of USA Veterans Affairs (VA) hospitals in the Patients treatment file and outpatients records from any VA facility in the Output Clinic File | 572293 patients in HCV-uninfected cohort PAC detected: 477 | 0.09 (0.08-0.11) | Higher risk of PAC in patiens of HCV-infected cohort , but this association was attenuated after adjustement for alcohol use, pancreatitis, choledocholitiasis, cholelithiasis or primary sclerosing cholangitis |
| Hassan MM *J Clin Oncol* 2008 | Hospital-based case-control study Period: 2000-2007 | Histological confirmation | 6/474 (1.5%) | Community-based (healthy genetically unrelated family members of patients with cancer other than pancreatic, GI, lung or head cancers) | 9/872 (1%) | 0.8 (0.02-1.6) | HCV infection not associated with higher risk of PAC development |
| Huang J *Br J Cancer* 2013 | Retrospective Nationwide cohort study 197208 participants: Period: 1990-2006 | Identification of PAC cases from the Swedish Cancer Register (International Classification of Disease ICD-7: 157) and from the Cause of Death Register (ICD-9: 157; ICD-10: C25) | Individuals in HCV reference cohort: 39442 PAC detected: 34/39442 (0.09%) | Control population obtained from the national surveillance database at the Swedish Institute for Infectious Disease Control. The expected numbers of calculated PAC from the observed person-time in each 5-yr age group by sex and the corresponding Swedish population incidence rates. | Expected number of PAC: 16.5 | 0.09 (0.05-0.11) | Statistically significant increased risk of PAC development |
| Omland LH *Clinical Epidemiology* 2010 | Cohort-study Period: 1994 - 2003 | Patients and subjects with HCV infection identified by means of: -The Danish National Hospital Registry (DNHR) -The Danish Cancer Registry People listed in DNHR with at least one diagnosis of acute or chronic HCV infection (ICD-10 B17.1 and 18.2) were included Cancer diagnoses based on the Danish version of the international classification of diseases, 8$^{th}$ revision (ICD-8) until Dec 31, 1993, and 10$^{th}$ version (ICD-10) thereafter | 4349 patients with HCV infection in the DNHR 4/4349 PAC detected (0.1%) | The expected number of cases of cancer after a diagnosis of HCV infection using Danish incidence rates of first cancer diagnoses according to sex, age, and year of diagnosis in 1-yr intervals was calculated | Expected number of PAC: 1.01 | 0.1 (0-0.18) | Association between HCV infection and higher risk of PAC development |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Qiwen Ben *Pancreas* 2012 | Double-centre ongoing hospital-based case-control study. Period: January 1, 2004-August 31, 2008 January 1, 2003-Octobet 31, 2009 | Histological or citological confirmation | 14/943 (1.5%) | Patients admitted to the same Hospitals (Ruijin Hospital and Changai Hospital, Shangai for any acute conditions) | 12/1128 (1.1%) | 1.5 (0.7-2.2) | No higher HCV prevalence in patients with PAC in comparison with controls |
| Swart A *BMJ Open* 2012 | Cohort-study Patients considered in the study: 29613 1 January 1993 - 31 December 2007 | Individuals registered on the Pharmaceutical Drugs of Addiction System, a record of all NSW Health Department authorities that administer methadone or buprenorphine to opioid-dependent people as opioid substitution therapy. Solid cancers classified according to the International Classification of Diseases (ICD), 10th revision, haematopoietic neoplasms and Kaposi sarcomas classified according to the ICD for Oncology, 3rd edition | Subjects with HCV infection alone: 14892 Observed number of PAC in HCV-positive cohort: 20/14892 (0.1%) | Calculation of expected number of incident PAC | Expected number of PAC: 7.12 | 0.13 (0.08-0.21) | Increased risk of PAC in patients with HCV infection |
| Woo SM *J Korean Med Sci* 2013 | Case-control study Period: 2001-2011 | Histological or radiological/clinical confirmation | 753 patients with PAC 724/753 with available anti-HCV test 21/724 (2.8%) | Individuals subjected to routine health examination in the Cancer Screening Cohort | 36/3012 (1.2%) | 2.9 (1.7-4.1) | Seropositivity for anti-HCV, infection, may increase the risk of developing PC in Korea |

NR: Not reported; SIR: Standardised incidence ratio; HCV: Hepatitis C virus; PAC: Pancreatic cancer.

genome in normal and/or cancerous pancreatic tissue specimens. Nevertheless, since 2008, this topic has gained a progressive interest, and an increasing number of case-control-, cohort-studies and meta-analyses with different design have been carried out with this purpose. To date the number of available studies, concerning the association between HCV and PAC risk, is still limited, but among these trials, some have suggested that HCV infection represents a risk factor for PDAC, whereas others have not confirmed this association[205-207]. Two trials have been carried out in Chinese population, 2 in United States, 2 in Australia and 1 in Sweden and 1 in Korea. Among these studies, the largest-sized one has been performed in United States. On the basis of these reports, up to now, three meta-analyses have been carried out. One of these reported no statistically significant relationship between anti-HCV positivity and PDAC risk because of the small number of studies available, although a borderline value was detected in this comparison (RR = 1.16 with 95%CI ranging from 0.99 to 1.3)[211], but the others found an increased risk of this malignancy in HCV-infected patients, in comparison to controls (RR = 1.21 with 95%CI ranging from 1.02 to 1.44 or RR = 1.26 with 95%CI ranging from 1.03 to 1.5)[204,212]. However, none of these studies, available to date, included the Swedish-, the Korean-, the Australian-reports as well as the last Chinese case-control research. The majority of these trials have been published only recently, in particular, between the end of 2013 and the beginning of 2014, therefore their results were not included in the described meta-analyses. They showed that HCV infection is associated with an increased risk to develop PDAC. The characteristics and number of studies included in the reported meta-analyses vary among the 3 identified meta-analyses, depending on the different selection criteria used by each author. Furthermore, although most studies included as matching factors smoking habit, alcohol use or diabetes, no research has assessed whether these variables may act in cooperation with HCV and increase the risk of PAC. The results of our review, concerning the possible association between HCV infection and PAC risk, studies not considered, as well as meta-analyses are summarised in Tables 3, 12 and 13. Age-standardized incidence rates of pancreatic cancer per 100000 person-years is shown in Figure 3C.

**Table 4 Characteristics of available studies, reported in English, designed to assess the association between hepatitis C virus infection and breast cancer risk**

| Author/Journal/ Publication year | Country | Study design/ study period | Diagnosis | Sample size (HCV positive breast cancer cases) | Control source | HCV positive controls/ controls | Matching factors | Percentage of HCV-positive cases with 95%CI | Main conclusions |
|---|---|---|---|---|---|---|---|---|---|
| Amin J *J Hepatol* 2006 | Australia | Community-based cohort-study Period: 1990-2002 | Patients'data obtained from: -New South Wales (NSW) Australia Health Department's Notifiable Diseases Database (NDD) for notification of newly diagnosed HCV infection -NSW Central Cancer Registry (CCR) for notification of incident cancer cases -National Death Index (NDI) database, containing records of all deaths in Australia since 1980 Identification of breast cancer cases by means of ICD-10-diagnosis codes | Individuals with HCV infection: 75834 Breast cancers detected: 50 50/75834 | Incidence observed in the study cohort was compared to expected incidence derived from NSW population cancer rates by calculating standardised incidence ratios | SIR: 0.3 (0.4-0.5) | NR | (0.05-0.09) | No evidence supporting an association between HCV infection and breast cancer development |
| Hwang JP *J Oncol Pract* 2014 | United States | Cohort-study Period: January 2004 - April 2011 | Patients'data, obtained from four institutional sources: Tumor registry: to assess patients' demographic characteristics Pharmacy informatics: to evaluate chemotherapy drugs and dates administered. Patient accounts: to identify study patients' International Classification of Diseases (ninth edition; ICD-9) codes Laboratory informatics: to determine HCV antibody (anti-HCV) and ALT test dates and results | 141877 patients with cancer, who were newly registered at MD Anderson Cancer during the study period. Patients considered in the study: 16773. HCV screened subjects: 2330/16773 (13.9%) HCV screened females: 1038 HCV-positive patients with cancers: 35/2330 (1.5%) HCV-positive females with cancers: 12 (1) HCV-positive females with breast cancers: 3/12 (2) HCV-negative females with breast cancer: 102/1026 | NR | NR | NR | (1) 25 (5.5-57.2) (2) 9.9 (8.1-11.8) | HCV screening rates were low, even among patients with risk factors, and the groups with the highest rates of screening did not match the groups with the highest rates of a positive test result |
| Larrey D *World J Gastroenterol* 2010 | France | Case serie with control gorup Period: NR | Females with history of HCV-related chronic infection, observed in Liver Unit of Montpellier School of Medicine, France, for chronic liver diseases in several occasions for a period longer than 1 yr. Chronic hepatitis proved by liver biopsy and/or biological markers of inflammation and fibrosis | 17/294 (5.8%) | Females sequentially and prospectively seen during the same period with chronic liver disease over 1 yr, with well defined clinical, radiological and histological characteristics [chronic-HBV, alcoholic-liver disease, auto-immune hepatitis, hemochromatosis, non alcoholic fatty liver disease (NAFLD), cholangitis] | 5/107 (4.7%) | NR | 5.8 (3.1-8.4) | Chronic HCV infection is not a strong promoter of breast carcinoma in adult females of any age |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Omland LH *Clinical Epidemiology* 2010 | Denmark | Cohort-study Period: 1994-2003 | Patients and subjects with HCV infection identified by means of: The Danish National Hospital Registry (DNHR) -The Danish Cancer Registry People listed in DNHR with at least one diagnosis of acute or chronic HCV infection (ICD-10 B17.1 and 18.2) were included Cancer diagnoses based on the Danish version of the international classification of diseases, 8th revision (ICD-8) until Dec 31, 1993, and 10th version (ICD-10) thereafter | 4349 patients with HCV infection in the DNHR 2 breast cancer detected 2/4349 (0.05%) | The expected number of cases of cancer after a diagnosis of HCV infection using Danish incidence rates of first cancer diagnoses according to sex, age, and year of diagnosis in 1-yr intervals was calculated | Expected number of breast cancers 8.05 | NR | 0.05 (0-0.1) | No association between HCV infection and higher risk of breast cancer development |
| Su FH *BMC Cancer* 2011 | Taiwan | Population-based study Period: 2000-2008 | Data retrieved from National Health Insurance Research Database (NHIRD), which is maintained by the National Health Research Institute (NHRI), Taiwan. Newly diagnosed breast cancer identified from the registry for Catastrophic Illness Patients Database (ICD-9-CM code 174 and 175). Identification of HCV infected subjects by means of ICD-9-CM diagnosis codes (ICD-9-CM 070.41, 070.44, 070.51, 070.54, and V02.62) | 56/1958 (2.9%) | Randomly selected and matched individuals without a history of breast cancer (control to patient ratio was 4:1) | 178/7832 (2.3%) | age- and sex | 2.9 (2.1-3.5) | HCV infection associated with early onset risk of breast cancer in areas endemic for HCV |
| Swart A *BMJ Open* 2012 | Australia | Cohort-study 1 January 1993 - 31 December 2007 | Individuals registered on the Pharmaceutical Drugs of Addiction System, a record of all NSW Health Department authorities that administer methadone or buprenorphine to opioid-dependent people as opioid substitution therapy. Solid cancers classified according to the International Classification of Diseases (ICD), 10th revision, haematopoietic neoplasms and Kaposi sarcomas classified according to the ICD for Oncology, 3rd edition | Patients considered in the study: 29613 Subjects with HCV infection alone: 14892 Observed number of breast cancer in HCV-positive cohort: 48 48/14892 (0.03%) | Calculation of expected number of incident breast cancer | Expected number of breast cancers: 101 | NR | 0.03 (0.02-0.04) | No evidence supporting an association between HCV infection and breast cancer development |

NR: Not reported; SIR: Standardised incidence ratio; HCV: Hepatitis C virus.

### *HCV and breast cancer risk*

On the basis of the observation that the wide geographical differences in the age-standardized incidence of breast cancer[229] cannot be entirely explained by variations in known risk factors among countries, it was hypothesized, since 1997, that a relationship might exist between this malignancy and late exposure to a common virus[231]. It has been reported that some viruses may be involved in the occurrence of this malignancy[232], but, in particular, in 1999 an anecdotal report suggested, for the first time, the HCV might play a role in the development of some solid tumors other than liver, including breast cancer. Since then, some studies investigated the possible involvement of HCV in breast carcinogenesis. Among the 6 trials identified in our review, 5 were cohort- and 1 case-control-studies. Two of them were performed in Australia, 1 in United States, 1 in Denmark, 1 in Taiwan and 1 in France, respectively. Two studies, 1 case-control performed in Taiwan and 1 cohort trials in France were designed with the primary aim to assess the potential relationship between HCV and breast cancer risk, whereas the others were not. Only the research carried out in Taiwan found an association with early onset risk of breast cancer.

**Table 5 Characteristics of available studies, reported in English, assessing the association between hepatitis C virus infection and renal cancer**

| Author/Journal/ Publication year | Country | Study design/ study period | Diagnosis | Sample size (HCV positive RCC cases) | Control source | HCV positive controls/controls | Matching factors | Percentage of HCV-positive cases with 95%CI | Main conclusions |
|---|---|---|---|---|---|---|---|---|---|
| Amin J *J Hepatol* 2006 | Australia | Community-based cohort-study Period: 1990-2002 | Identification of renal cancer cases by means of ICD-10- diagnosis codes | Individuals with HCV infection: 75834 RCC detected: 19 19/75834 | Incidence observed in the study cohort was compared to expected incidence derived from NSW population cancer rates by calculating standardised incidence ratios | SIR: 0.9 (0.6-1.4) | NR | 0.02 (0.01-0.03) | No evidence supporting an association between HCV infection and kidney cancer development |
| Budakoğlu B *Med Oncol* 2012 | Turkey | Case series with control group 2005-2010 | Histological confirmation | 15/903 (1.7%) | Data collected in previous prevalence studies in healthy subjects in three different geographical areas of the Turkey, used as control group | 81/5267 (1.5%) | NR | 1.7 (0.8-2.4) | No higher frequency of HCV positivity in RCC patients in comparison with healthy people |
| Gonzalez HC *Dig Dis and Sci* 2015 | United States | Case series with control group January 2011 - August 2013 | Histological confirmation | Anti-HCV positive: 11/140 (7.9%) (2) HCV-RNA positive: 9/140 (6.4%) | Consecutive individuals newly diagnosed with colon cancer. The control group recruited simultaneously and from the same health care system (Henry Ford Health System in Detroit, Michigan) | Anti-HCV positive: 1/100 (1%) HCV-RNA positive: 0/100 | NR | (1) 7.9 (3.4-12.3) (2) 2.3 (10.5) | Increased risk of RCC in subjects with HCV chronic infection |
| Gordon SC *Cancer Epidemiol Biomarkers Prev* 2010 | United States | Cohort study Period: 1997-2006 | Use of administrative data from Henry Ford Hospital, an integrated healthcare delivery system serving southeastern Michigan. Cancer diagnosis codes in administrative databases [International Classification of Diseases, 9th ed., Clinical Modification (ICD-9-CM) codes in the range of 140 through 208.9] | 72487 patients tested for anti-HCV 3057/72487 anti-HCV positive patients 17/3057 (0.6%) with RCC | Control cohort of patients who tested negative for anti-HCV | 64006/72487 anti-HCV negative patients 177/64006 (0.3%) with RCC | NR | 0.6 (0.3-0.8) | Chronic infection with HCV confers an increased and independent risk for developing RCC |
| Hofmann JN *Eur J Cancer Prev* 2011 | Sweden | Nationwide register-based cohort- study Period: 1990-2008 | HCV diagnosis extracted from the national surveillance database at the Swedish Institute for Infectious Disease Control (SMI). Cancer diagnoses were coded using the seventh revision of the International Classification of Diseases (ICD-7) (ICD-7 codes 180.0 and 180.9) | 43000 Lag period after HCV notification (1) None: 38, Expected: 27.1 (2) Three months 33 Expected: 26.5 (3) One year: 29 Expected: 24.9 | A non-HCV-infected cohort selected from the general population | 215000 | Year of birth, sex, and county of residence in Sweden, five subjects never diagnosed with HCV infection were matched to each HCV-infected subject | (1) 0.06 (0.09-0.21) (2) 0.05 (0.08-0.21) (3) 0.05 (0.07-0.21) | In the cohort of HCV-infected subjects, no increased risk of developing kidney cancer but an enhanced risk of non-cancer chronic kidney disease, particularly among women |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Malaguarnera M *Eur J Int Medicine* (2006) | Italy | Case-control study Period: NR | All cancer patients: 236 HCV diagnosis performed with II G ELISA test. Cancers diagnosed at Garibaldi Hospital | 15 patients with RCC 8/15 (53%) HCV positive patients | Elderly volunteers evaluated at Garibaldi Hospital, Catania | 30/300 (10%) | Age, sex and previous blood transfusions | 53.3 (26.5-78.7) | High prevalence of anti-HCV antibodies in patients with renal cancer |
| Omland LH *Clinical Epidemiology* 2010 | Denmark | Cohort-study Period: 1994-2003 | Patients and subjects with HCV infection identified by means of: -The Danish National Hospital Registry (DNHR) -The Danish Cancer Registry People listed in DNHR with at least one diagnosis of acute or chronic HCV infection (ICD-10 B17.1 and 18.2) were included Cancer diagnoses based on the Danish version of the international classification of diseases, 8th revision (ICD-8) until Dec 31, 1993, and 10th version (ICD-10) thereafter | 4349 patients with HCV infection in the DNHR 4 renal cancer detected 4/4349 | The expected number of cases of cancer after a diagnosis of HCV infection using Danish incidence rates of first cancer diagnoses according to sex, age, and year of diagnosis in 1-year intervals was calculated | Expected number of kidney cancers: 1.11 | NR | 0.1 (0-0.2) | Association between HCV infection and higher risk of renal cancer development |
| Swart A *BMJ Open* 2012 | Australia | Cohort-study 1 January 1993 - 31 December 2007 | Individuals registered on the Pharmaceutical Drugs of Addiction System, a record of all NSW Health Department authorities that administer methadone or buprenorphine to opioid-dependent people as opioid substitution therapy. Solid cancers classified according to the International Classification of Diseases (ICD), 10th revision, haematopoietic neoplasms and Kaposi sarcomas classified according to the ICD for Oncology, 3rd edition | Patients considered in the study: 29613 Subjects with HCV infection alone: 14892 Observed number of RCCs in HCV-positive cohort: 20 20/14892 | Calculation of expected number of incident RCCs | Expected number of RCCs: 18.1 | NR | 0.1 (0.08-0.20) | No evidence supporting a strong association between HCV infection and RCC development |

NR: Not reported; SIR: Standardised incidence ratio; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

The results of our review, concerning the possible association between HCV infection and renal cancer risk as well as studies not considered are summarised in Table 4 and 14. To date, no meta-analysis has been published on this topic. Age-standardized incidence rates of breast cancer per 100000 person-years is reported in Figure 3D.

### *HCV and kidney cancer risk*

Although some authors have reported, several years ago, that HCV is associated with an higher probability to develop chronic end-stage renal diseases[233] or that a relationship may exist between this virus and a major risk of kidney cancer, as reported in some case reports[234,235] or in very small series of patients[236], only in the last 5 years, the interest for this topic has progressively increased and adequately powered studies have been carried out in different geographical areas worldwide. To date 4 cohort-, 1 case-control- and 2 case series with control groups studies available in literature, have been considered in our systematic review. Two among cohort-trials were performed in Australia, 1 in United States and 1 in Sweden, whereas the case-control trial in Italy. One case series study was carried out in United States[199] and one in Turkey[198]. The results of these reports are not univocal. In particular, only 1 of these cohort studies as well as 1 case series with control group research and

**Table 6 Characteristics of available studies, reported in English, assessing the association between hepatitis C virus infection and oral or skin cancer**

| Author/Journal/ Publication year | Study design/study period | Diagnosis | Sample size (cases/ controls) | Control source | HCV positive controls/ controls | Percentage of HCV-positive cases with 95%CI | Main conclusions |
|---|---|---|---|---|---|---|---|
| Amin J *J Hepatol* 2006 | Community-based cohort-study Period: 1990-2002 | Identification of skin/oral cancer cases by means of ICD-10- diagnosis codes | Individuals with HCV infection: 75834 Skin/oral cancer: 19 including , mouth (7 cases), tongue (6 cases), tonsil (6 cases) no skin cancers described | Incidence observed in the study cohort was compared to expected incidence derived from NSW population cancer rates by calculating standardised incidence ratios | SIR: Mouth: 1.5 (0.7-3.2) Tongue: 1.1 (0.5-2.4) Tonsil: 2.1 (1-4.8) | 0.02 (0.01-0.03) | No evidence supporting an association between HCV infection and skin/oral cancer development, low increased risk for tonsil cancer |
| Eftekharian A *Eur Arch Otorhinolaryngol* 2012 | Case-series 107 patients with SCCHN Period: October 2008-June2010 | Histological confirmation: SCCHN | 1/107 (0.9%) | NR | NR | 0.9 (0-2.7) | HCV at least in Iran not a risk factor for SCCHN |
| Gandolfo S *Oral Oncol* 2004 | Case-series 402 patients with OLP Patients with available HCV test: 357 HCV positive patients: 69/357 (19.3%) Period: January 1988 - July 1999 | During the follow-up period: 9 patients developed an oral squamous cell carcinoma Histological confirmation: OSCC | HCV positive patients with OSCC: 4/9 (44.5%) | NR | NR | 44.5 (11.9-76.9) | Possible increased risk for OSCC in HCV-related infection in patients oral lichen planus (OLP) |
| Nagao Y *J Oral Pathol Med* 1995 | Case-series 100 patients with oral cancer enrolled Period: January 1989-October 1993 | Histological confirmation: Different histotypes | 24/100 (24%) | Patients with non-malignant disease receiving dental treatment at the Department of Oral Surgery of the Kurume University Patients with gastric cancer | (1) 11/104 (10.6%); (2) 12/113 (10.6%) | 24 (15.6-32.3) | HCV causing pathologic changes in the oral cavity, with HCV involved in cancerization |
| Nagao Y *J Oral Pathol Med* 2000 | Biopsies of 36 patients, including: (1) OLP: 19; (2) Oral cancer: 17 Period: NR | Histological confirmation: Well-differentiated SCCHN | (1) 14/19 (73.7%); (2) 7/17 (41. 2%) | Biopsies of 10 patients, including: (3) Non-malignant disease with HCV (4) Non-malignant disease without HCV | (3): 6 (4): 4 | (1) 73.7 (53.8-93.4); (2) 41.2 (17.8-64.5) | HCV causing pathologic changes in the oral cavity, with HCV involved in cancerization |
| Nobles J *Laringoscope* 2004 | Case-series 100 patients with SCCHN enrolled. Period: June 1991-December 2002 | Histological confirmation: SCCHN | 21/100 (21%) | NR | NR | 21 (13-28.9) | A large number of patients (21%) with SCCHN, included in this study, coinfected with HCV. This prevalence is significantly increased when compared with the general population (1.4%) or the population at VA hospitals (9.9%) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Omland LH *Clinical Epidemiology* 2010 | Cohort-study Period: 1994-2003 | Patients and subjects with HCV infection identified by means of: -The Danish National Hospital Registry (DNHR) -The Danish Cancer Registry People listed in DNHR with at least one diagnosis of acute or chronic HCV infection (ICD-10 B17.1 and 18.2) were included Cancer diagnoses based on the Danish version of the international classification of diseases, 8th revision (ICD-8) until Dec 31, 1993, and 10th version (ICD-10) thereafter | 4349 patients with HCV infection in the DNHR 4 oropharyngeal cancers detected | The expected number of cases of cancer after a diagnosis of HCV infection using Danish incidence rates of first cancer diagnoses according to sex, age, and year of diagnosis in 1-yr intervals was calculated | Expected number of oropharyngeal cancers: 1.73 | 0.1 (0-0.2) | No sssociation between HCV infection and higher risk of oropharyngeal cancer development |
| Su FH *PlosOne* 2012 | Nationwide Population-Based Cohort Study HCV positive patients: 5311 HCV and HBV positive patients: 3519 Period: 1996-2008 | Data obtained from the Taiwan National Health Insurance Research Database (NHIRD). HCV cases identified by means of ICD-9-CM diagnosis codes (ICD-9-CM: 070.41, 070.44, 070.51, 070.54, V02.62) | (1) 21/5311; (2) 9/3519 | Controls identified by means of a systematic random sampling method to select 4 insured people without viral hepatitis for every insured person with viral hepatitis during the same period | 147/84796 | (1) 0.4 (0.2-0.5); (2) 0.3 (0.09-0.4) | HCV infection is a risk factor for oral cavity cancer. In addition, subjects with HCV infection tend to be at early onset risk for oral cavity malignancy |
| Swart A *BMJ Open* 2012 | Cohort-study 1 January 1993 - 31 December 2007 | Individuals registered on the Pharmaceutical Drugs of Addiction System, a record of all NSW Health Department authorities that administer methadone or buprenorphine to opioid-dependent people as opioid substitution therapy. Solid cancers classified according to the International Classification of Diseases (ICD), 10th revision, haematopoietic neoplasms and Kaposi sarcomas classified according to the ICD for Oncology, 3rd edition | Patients considered in the study: 29613 Subjects with HCV infection alone: 14892 Observed number of following cancer in HCV-positive cohort: (1) Tonsil: 10; (2) Mouth:8; (3) Salivary gland: 4; (4) Tongue: 9 Total: 31 | Calculation of expected number of incident tonsil/ mouth/salivary gland/ tongue cancers | Expected number of oral cancers: Tonsil: 2.96 Mouth; 3.54 Salivary gland: 2.75 Tongue:5.35 | 0.2 (0.1-0.3) | Possible association between HCV and tonsil/mouth cancers. No association between HCV infection and tongue/ salivary cancers |
| Takata Y *Oral Diseases* 2002 | Case series Patients with anti-HCV antibodies: 2613 HCV positive patients: 151/2613 (5.8%) Period: January 1989 -December 1998 | Histological confirmation Histotype not reported | 25/245 (10.2%) | NR | NR | 10.2 (6.4-13.9) | High HCV antibody prevalence in patients with oral cancer. Possible no important association between oral cancer and HCV infection, with increased prevalence, depending on higher age of anti-HCV positive patients |

NR: Not reported; SIR: Standardised incidence ratio; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

**Table 7 Characteristics of available studies, reported in English, assessing the association between hepatitis C virus infection and thyroid cancer**

| Author/Journal/ Publication year | Study design/study period | Diagnosis | Sample size | Control source | Controls | Percentage of HCV-positive cases with 95%CI | Main conclusions |
|---|---|---|---|---|---|---|---|
| Amin J *J Hepatol* 2006 | Community-based cohort-study Period: 1990-2002 | Identification of thyroid cancer cases by means of ICD-10- diagnosis codes | Individuals with HCV infection: 75834 Thyroid cancers detected: 9 | Incidence observed in the study cohort was compared to expected incidence derived from NSW population cancer rates by calculating standardised incidence ratios | SIR: 0.3 (0.2-0.7) | 0.01 (0-0.02) | No evidence supporting an association between HCV infection and thyroid cancer development |
| Antonelli A *Clin Exp Rheumat* 2002 | Case-control study Period: 1999-2001 | FNA PTC | 94 patients with HCV-associated MC Patients with PTC and HCV-associated MC/patients with HCV-associated MC: 2/94 (2.1%) | Control group obtained from a sample (2401 individuals) of the general population, 5 controls were randomly associated with each MC patient | 0/470 | 2.1 (0-5) | Possible association between HCV-related MC and thyroid cancer, careful monitoring of the thyroid opportune, during the clinical follow-up of HCV-associated MC patients |
| Antonelli A *Thyroid* 2007 | Case-control study Period: January 1995 - December 2001 | FNA PTC | 308 HCV positive patients PTC and HCV positive cases/all HCV positive cases: 6/308 (1.9%) | (1) subjects from an iodine deficient area; (2) subjects from an iodine-sufficient area | PTC cases/all HCV negative controls: (1) 0/616; (2) 1/616 | 1.9 (0.4-3.4) | High prevalence of thyroid papillary cancer in HCV+ patients, overall in presence of thyroid autoimmunity; careful thyroid monitoring is indicated during the follow-up of these patients |
| Giordano TP *JAMA* 2007 | Cohort study Period: 1997-2004 | Identification of HCV infected subjects by means of ICD-9-CM , diagnosis codes of HCV infection (070.41, 70.44, 070.51, 070.54, V02.62) Identification of thyroid cancer by menas of ICD-9-CM diagnosis codes: 193 | HCV-positive cohort: 146394 patients During follow-up, 813 patients in HCV-infected cohort (0.5%) had a HIV diagnosis. 46 patients developed thyroid cancer | Inpatients records from more than 150 United States Veterans Affairs (VA) hospitals in the Patients' treatment file and outpatients records from any VA facility in the Output Clinic File | HCV- negative cohort: 572293 patients. During follow-up, 35696 uninfected HCV patients (6.2%) had a recorded HCV diagnosis and 1539 patients (0.3%) a HIV diagnosis 274 patients developed thyroid cancer | 0.03 (0.02-0.04) | No increased, risk for thyroid cancer in HCV-positive cohort |
| Montella M *Oncol Rep* 2003 | Case-control study Period 1997-1999 | Histological confirmation PTC | HCV positive PTC cases/all PTC cases: 16/130 (12.3%) | Control group including subjects, operated for benign diseases. Cases and controls selected from the hospital tumor registry | 242 controls and 311 surgical procedures. HCV positive controls/ total controls 18/311 | 12.3 (6.6-17.9) | Association between HCV and thyroid cancer. This malignancy more readily detectable in countries with a high prevalence of HCV |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Omland LH *Clinical Epidemiology* 2010 | Cohort-study Period: 1994-2003 | Patients and subjects with HCV infection identified by means of: -The Danish National Hospital Registry (DNHR) -The Danish Cancer Registry People listed in DNHR with at least one diagnosis of acute or chronic HCV infection (ICD-10 B17.1 and 18.2) were included Cancer diagnoses based on the Danish version of the international classification of diseases, 8th revision (ICD-8) until Dec 31, 1993, and 10th version (ICD-10) thereafter | 4349 patients with HCV infection in the DNHR 1 thyroid cancer detected | The expected number of cases of cancer after a diagnosis of HCV infection using Danish incidence rates of first cancer diagnoses according to sex, age, and year of diagnosis in 1-yr intervals was calculated | Expected number of thyroid cancers: 0.46 | 0.02 (0-0.06) | No association between HCV infection and higher risk of thyroid cancer development |
| Swart A *BMJ Open* 2012 | Cohort-study 1 January 1993 - 31 December 2007 | Individuals registered on the Pharmaceutical Drugs of Addiction System, a record of all NSW Health Department authorities that administer methadone or buprenorphine to opioid-dependent people as opioid substitution therapy. Solid cancers classified according to the International Classification of Diseases (ICD), 10th revision, haematopoietic neoplasms and Kaposi sarcomas classified according to the ICD for Oncology, 3rd edition | Patients considered in the study: 29613 Subjects with HCV infection alone: 14892 Observed number of thyroid cancer in HCV-positive cohort: 48 | Calculation of expected number of incident thyroid cancer | Expected number of thyroid cancers: 34.4 | 0.3 (0.2-0.4) | No evidence supporting an association between HCV infection and thyroid cancer development |

FNA: Fine needle-aspiration; MC: Mixed cryoglobulinemia; PTC: Papillary thyroid cancer; SIR: Standardised incidence ratio; HCV: Hepatitis C virus.

case-control study have detected an association between HCV infection and risk of kidney malignancy[196,199,200], whereas the remaining articles did not. However, it has to be underlined that 2 of these cohort studies were designed to assess the incidence for all cancer types following HCV infection and not specifically the renal one [118,146]. In addition, a significant relationship between renal carcinoma and viral hepatitis has been described in a further research, but it has not been considered, because no data on the type viral hepatitis infection were reported[203]. The results of our review, concerning the possible association between HCV infection and renal cancer risk as well as studies not considered are summarised in Tables 5 and 15. To date, no meta-analysis has been published on this topic. Age-standardized incidence rates of renal cancer per 100000 person-years is reported in Figure 3E.

### *HCV and oral/skin cancer risk*

A relationship between HCV and oral squamous cell carcinoma (OSCC) was described, for the first time, in 1995[224] and then it was also reported in 1997, when a high prevalence of anti-HCV antibodies and of viral genome was showed in patients with head and neck squamous cell carcinomas[237]. Since then, cases of OSCC and verrucous carcinoma have been described in anti-HCV positive subjects with or without associated oral lichen planus (OLP)[238,239]. The prevalence of this inflammatory mucocutaneous condition varies largely among different geographical areas, with the highest rates observed in countries with HCV hyperendemia[240]. According to one study performed in 1557 patients with OLP, a more elevated HCV prevalence was observed in individuals, suffering from this disease, than that in the control group (1.9%, 0.4% respectively, $P < 0.001$)[241]. The association between HCV infection and OLP has been recently confirmed by three independent meta-analyses and it emerged across throughout the world. However, this relationship was most frequently detected in East- and South-East Asia as well as in South American- and Mediterranean-regions[242-244]. HCV might be involved in this type of process[245]. It has been suggested that OLP is a precancerous lesion, although the degree of risk of this disease for development of oral cancer is controversial[225,239]. Additional studies have shown a significant higher prevalence of HCV infection in patients with squamous cell carcinoma of head and neck (SCCHN) than that described in controls[225], whereas others did not[227]. To date, 10 studies, concerning relationship between HCV infection and oral/skin cancers have been published. Our findings, concerning the possible association between HCV infection and oral/skin cancer risk as well as studies not

**Table 8 Main findings of studies, concerning the association between hepatitis C virus infection and lymphomas, not considered in the present systematic review because of not reported in English or as full-text or including incomplete data or assessing lymphoproliferative disorders other than B-cell lymphomas**

| Studies (First author/Journal/ Year of publication) | Study title | Main findings for exclusion | Study conclusion |
|---|---|---|---|
| Arcaini L *Cancer* 2004 | Splenic and nodal marginal zone lymphomas are indolent disorders at high hepatitis C virus seroprevalence with distinct presenting features but similar morphologic and phenotypic profiles | Duplicate | High HCV seroprevalence in patients with MZL |
| Catassi C *Rec Prog Med* 1998 | High prevalence of hepatitis C virus (HCV) infection in patients with non-Hodgkin lymphoma at the onset: preliminary results of a multicentre Italian study | Full-text in Italian | Possible causative role of the HCV in lymphomagenesis |
| Dal Maso L *Haematologica* 2004 | Hepatitis B and C viruses and Hodgkin lymphoma: a case-control study from northern and southern Italy | Evaluation of the association between HCV infection and HL risk | No role of HCV in the etiology of HL |
| de Sanjose S *Int J Cancer* 2004 | Role of hepatitis C virus infection in malignant lymphoma in Spain | Duplicate | HCV infection is associated with an increased risk of lymphoma in Spain |
| Domingo JM *Med Clin* (Barc) 2001 | Hepatitis C virus infection in patients with non Hodgkin's lymphoma | Full-text in Spanish | Possible association between HCV infection and NHLs |
| Ferri C *JAMA* 1994 | Non-Hodgkin's lymphoma: possible role of hepatitis C virus | Duplicate | Possible role of HCV infection in B-cell NHLs development |
| Ferri C *QJM* 1996 | Chronic hepatitis C and B-cell non-Hodgkin's lymphoma | Duplicate | Possible role of HCV infection in B-cell NHLs development |
| Gasztonyi B *Orv Hetil* 2000 | Hepatitis C virus infection and B-cell non-Hodgkin's lymphoma | Full-text in Humgarian | HCV might have an aetiological role in the lymphoproliferation leading to B-cell NHL |
| Grudeva-Popova J *BUON* 2013 | Non-Hodgkin lymphomas and carrier state of viral hepatitis B and C | Incomplete data concerning the association between HCV infection and NHLs | Hepatitis virus carrier state did not alter significantly the clinical course and disease prognosis |
| Izumi T *Leukemia* 1997 | B cell malignancy and hepatitis C virus infection | Duplicate | Association between persistent HCV infection and the occurrence of B- cell malignancy |
| Montella M *Liver* 2001 | HCV and cancer: a case-control study in a high-endemic area | Duplicate | Expected increases not only in liver cancer, but also in tumors associated with the immune system |
| Sánchez Ruiz AC *Med Clin* (Barc) 2001 | Prevalence of hepatitis C virus infection in patients with non-Hodgkin's lymphoma | Full-text in Spanish | Higher prevalence of HCV in our B-NHL patients |

MZL: Marginal zone lymphoma.

considered are summarised in Tables 6 and 16. To date, no meta-analysis has been published on this topic. Age-standardized incidence rates of oral-cancer per 100000 person-years is reported in Figure 3F.

### *HCV and thyroid cancer risk*

The first description of an association between HCV and risk of thyroid cancer development dates back to 1999, when Antonelli *et al*[246] reported a high prevalence (2.2%) of thyroid cancer in a series of 139 patients with chronic hepatitis C infection in comparison to no case among 835 control subjects, who were long-term residents of an iodine-deficient area[246]. Since then some case-control studies have been carried out to assess this finding[73,74,215-217,247]. These trials have been performed in Italy and all have confirmed the previous above-mentioned assumption, although, some of these represent duplicates[73,74,215-217,247]. Afterwards, three cohort studies have been published to assess this subject in the last years, but no evidence supporting an association between HCV infection and thyroid cancer development has emerged from these reports. Two of these trials were designed to assess the incidence for all cancer types following HCV infection and not specifically the thyroid one[118,146]. Our findings, concerning the possible association between HCV infection and renal cancer risk as well as studies not considered are summarised in Table 7 and 17. To date, no meta-analysis has been published on this topic. Age-standardized incidence rates of thyroid cancer per 100000 person-years is reported in Figure 3G.

## DISCUSSION

To our knowledge, this is the first study aimed to review systematically the prevalence of HCV infection in a wide spectrum of human malignancies, to summarise the retrieved data and to discuss the possible role of this pathogen in the genesis of the discussed tumours. Since several years the possible involvement of different viruses in human carcinogenesis has been reported, with increasing frequency, in a large series of epidemiological studies. Recently, the International Agency for Research on Cancer (IARC) has comprehensively assessed and confirmed the human carcinogenicity of 7 viral

**Table 9 Characteristics of available systematic review and/or meta-analyses, reported in English, assessing the association between hepatitis C virus infection and lymphomas**

| First author/ Country | Title | Number of studies considered | Main conclusion | Matching factors considered |
|---|---|---|---|---|
| Gisbert JP, 2003 | Prevalence of hepatitis C virus infection in B-cell non-Hodgkin's lymphoma: systematic review and meta-analysis | 23 | HCV prevalence in patients with B-NHL is approximately 15%, higher than that reported not only in general population (1.5%) but also in patients with other hematologic malignancies (2.9%), suggesting a role of HCV in the etiology of B-NHL | Age, sex, smoking, race, when available |
| Matsuo K, 2004 | Effect of hepatitis C virus infection on the risk of non-Hodgkin's lymphoma: a meta-analysis of epidemiological studies | 23 | Strongly positive association between anti-HCV seropositive test subjects and risk of NHL. Individualists with anti-HCV positive test have approximately five times higher risk of NHL. This association is consistent regardless of the endemic status of HCV, as well as subgroup analysis for B-/T-NHL | Age and sex, when available |
| Negri E, 2004 | B-cell non-Hodgkin's lymphoma and hepatitis C virus infection: a systematic review | 15 | A high HCV prevalence in B-NHL was found in southern and eastern Europe, Japan and the southern United States, but not in central and northern Europe, Canada, northern United States, or a few Asian countries. The odds ratio of B-NHL for HCV infection was relatively weak, ranging from 2 to 4 in most studies. Thus, even if the observed association were causal, the percentage of cases of B-NHL attributable to HCV infection would be relatively low (10%) also in countries with a high prevalence of HCV infection in the general population, and extremely low in other countries | Age and sex, whether available |
| Dal Maso L, 2006 | Hepatitis C Virus and Risk of Lymphoma and Other Lymphoid Neoplasms: A Meta-analysis of Epidemiologic Studies | 18 | The pooled relative risks (RR) were consistently increased for all major B-NHL subtypes, T-NHL, and primary sites of NHL presentation. The etiologic fraction of NHL attributable to HCV varies greatly by country, and may be upward of 10% in areas,where HCV prevalence is high .Associations weaker than with NHL were found between HCV infection and Hodgkin's lymphoma | Age and sex, when available |
| de Sanjose S, 2008 | Hepatitis C and non-Hodgkin lymphoma among 4784 cases and 6269 controls from the International Lymphoma Epidemiology Consortium | 7 | The results of the present study confirm the association between HCV infection and NHL and specific B-NHL subtypes (diffuse large B-cell lymphoma, marginal zone lymphoma and lymphoplasmacytic lymphoma). This research has sufficient statistical power to confirm these associations in populations with low HCV prevalence | Age, sex, county of residence, study site, geographic area, when available |
| Libra M, 2010 | Extrahepatic disorders of HCV infection: A distinct entity of B-cell neoplasia? | 18 Review of Italian studies | The results of the study confirm the association between HCV infection and NHL and specific B-NHL subtypes. The higher prevalence of anti-HCV Abs was observed among lymphoplasmacytoid/ lymphoplasmacytic/immunocytoma histotype whereas the lowest was among small lymphocytic lymphoma/chronic lymphocytic leukemia (SLL/CLL). Overall, these studies strongly support the notion that HCV-associated lymphomas may be a distinct entity and further characterization of the mechanisms by which HCV infection contributes to B-cell NHL development may improve its diagnosis, classification and treatment | NR |

HCV: Hepatitis C virus; NHL: Non-hodgkin-lymphoma.

agents, including HCV. In particular, it has been recognised that HCV acts as an indirect carcinogen, by promoting and maintaining a state of chronic inflammation in infected sites[248]. This event is now a well-known condition that is involved in the process of hepatic carcinogenesis. However, even if liver is the main target of HCV, its tropism for this organ is not exclusive. In particular, viral antigens and genomes have been also detected in extra-hepatic tissues[249]. All these evidences have contributed to consider the possible role of this pathogen as a pro-cancerous agent also in organs other from liver. On the basis of this assumption, as it has been observed for the strong relationship between HCV and hepatocellular carcinoma (HCC) development, it is conceivable to think that a similar ecological correlation might exist between HCV infection and a major risk of some extra-hepatic cancers in regions where the prevalence of this pathogen is high in comparison with geographical areas with a lower one. However, to date, the possibility that this virus may be involved in the carcinogenesis of organs other than liver has been systematically investigated only for a very limited number of malignancies. In particular, available studies have been mainly focused on hematopoietic malignancies and on cholangiocarcinomas, also on the basis of some epidemiological observations, reporting a major risk of mixed cryoglobulinemia and monoclonal gammopathy in HCV positive patients[250] as well as of a higher incidence of cholangiocarcinoma

**Table 10 Main findings of studies concerning the association between hepatitis C virus infection and cholangiocarcinomas with no complete data or not reported as full-text**

| Studies (First author/Journal/ Year of publication) | Study title | Main findings | Study conclusion |
|---|---|---|---|
| Choi D *J Hepatology* 2006 | Cholangiocarcinoma and Clonorchis sinesis infection: A case-control study in Korea | Assessment of Clonorchis sinensis role in the risk of developing CCA, including extrahepatic CCA | HCV infection detected in 1/51 (2%) patients in CCA group and 1/51 (2%) in control gorup |
| Jarnagin WR *Cancer* 2002 | Combined hepatocellular and cholangiocarcinoma: demographic, clinical, and prognostic factors | No distinction between HBV/HCV infected patients | The demographic and clinical features of patients with combined tumors were most similar to those of patients with CC. Most important, combined tumors were not found to be associated with chronic liver disease |
| Liu X *Zhonghua Wai Ke Za Zhi* 2002 | Pathogenesis of hilar cholangiocarcinoma and infection of hepatitis virus | Full-text in Chinese | HCV-core protein may play an important role in the pathogenesis of hilar cholangiocarcinoma. HCV-infected patients with hilar cholangiocarcinoma infected may have a high grade malignancy and a poor prognosis |
| Lu H *Chin Med J* (Engl) 2000 | Detection of hepatitis C virus RNA sequences in cholangiocarcinomas in Chinese and American patients | Only 12 patients included | High rate of HCV-RNA detection in CCA cases, mainly in Chinese patients as compared to United States subjects |
| Shirakawa H *Hokkaido Igaku Zasshi* 1996 | Analysis of hepatitis C virus (HCV) genotypes in hepatocellular carcinoma | Full-text in Japanese | 2/11 HCV seropositive Japanese patients with cholangiocarcinoma |
| Tao LY *Liver International* 2009 | Risk factors for intrahepatic and extrahepatic cholangiocarcinoma: a case-control study in China | Shortage of information on HCV infection | No possible assessment of significant association between HCV infection and ICC or ECC |
| Yin F *Chin Med J* (Engl) 1998 | Detection of hepatitis C virus RNA sequences in hepatic portal cholangiocarcinoma tissue by reverse transcription polymerase chain reaction | Only 6 patients included | High rate of HCV-RNA detection in CCA cases |
| Zhang H *Zhonghua Bing Li Xue Za Zhi* 1996 | Detection of hepatitis B virus DNA and hepatitis C virus RNA in human hepatocellular carcinoma by polymerase chain reaction | Full-text in Chinese | HCV may play an important role in hepatic carcinogenesis because of its high positive rate |
| Zou SQ *Zhonghua Wai Ke Za Zhi* 2003 | The retrospective analysis of HBV and HCV infection in cholangiocarcinoma | Full-text in Chinese | The HCV infection is associated with hilar cholangiocarcinoma, in particular with the proximal bile duct. The hilar cholangiocarcinoma in HCV-infected patients presents higher malignant degree and a poor prognosis |

**Table 11 Characteristics of available meta-analyses, reported in English, assessing the association between hepatitis C virus infection and cholangiocarcinoma**

| First author | Title | Number of studies considered | Main conclusion | Matching factors considered |
|---|---|---|---|---|
| Shin HR, 2010 | Epidemiology of cholangiocarcinoma: An update focusing on risk factors | 11 | HCV infection is associated with an increased risk for CCA, but its possible role in development of this malignancy requires further investigation | NR |
| Palmer WC, 2012 | Are common factors involved in the pathogenesis of primary liver cancers? A meta-analysis of risk factors for intrahepatic cholangiocarcinoma | 8 | HCV infection is associated with an increased risk for intrahepatic cholangiocarcinoma | NR |
| Zhou Yanming, 2012 | Hepatitis viruses infection and risk of intrahepatic cholangiocarcinoma: evidence from a meta-analysis | 16 | HCV infection is associated with an increased risk of ICC | NR |

NR: Not reported; HCV: Hepatitis C virus.

and other types of human cancer other than liver in subjects with cirrhosis, irrespective of etiology[251]. In this study, enrolled patients suffered from alcoholic-, primary biliary-, and chronic-hepatitis-related cirrhosis, whereas in a group of patients the causes of this pathological condition were non-specified. Unfortunately, no detailed information was available, concerning the HCV status in the cohort of

**Table 12 Main findings of available studies, not reported in English, evaluating hepatitis C virus infection and pancreatic cancer risk (A) and characteristics of studies, reported in English, assessing hepatitis C virus infection and pancreatic cancer risk, with no complete data or not reported as full-text (B)**

| Studies (First author/Journal/Year of publication) | Study title | Main findings | Study conclusion |
|---|---|---|---|
| (A) | | | |
| Hong SG, 2010 *Korean J Hepatol* 2010; 16: 1 | The relationship between hepatitis B virus infection and the incidence of pancreatic cancer: a retrospective case-control study | Full-text in Korean | Absence of significant association between anti-HCV positivity and pancreatic cancer |
| Xu P, 2011 *Cancer* (Chinese J) 2011; 31: 653-657 (52) | Risk factors for pancreatic cancer: a case-control study | Full-text in Chinese | Absence of significant relationship between anti-HCV positivity and pancreatic cancer |
| (B) | | | |
| *Fang Zhu Asian Pacific J Cancer Prev* 2011 | Chronic hepatitis virus infection and pancreatic cancer: a case-control study in southern China | No detailed description of number of patients with HCV-infection in case- and control group | Increased prevalence of anti-HCV antibodies in patients with pancreatic cancer |

HCV: Hepatitis C virus.

**Table 13 Characteristics of available meta-analyses, reported in English, assessing the association between hepatitis C virus infection and pancreatic cancer risk**

| First author/ Country | Title | Number of studies considered | Main conclusion | Adjustment for diabetes, alcohol, cigarette |
|---|---|---|---|---|
| Fiorino S, 2013 Italy | Association between hepatitis B or hepatitis C virus infection and risk of pancreatic adenocarcinoma development: a systematic review and meta-analysis | 3 studies available for assessment of HCV infection and PAC risk | No statistically significant relationship between anti-HCV positivity and PAC risk, although a borderline value was detected in this comparison (RR = 1.16 (95%CI: 0.99-1.3) | Y Y Y |
| Xing S, 2013 China | Chronic hepatitis virus infection increases the risk of pancreatic cancer: a meta-analysis | 7 studies included for assessment of HCV infection and PAC risk | Higher PAC risk in anti-HCV positive patients: RR = 1.21 (95%CI: 1.02-1.44) | Y Y Y |
| Xu JH, 2013 China | Hepatitis B or hepatitis C virus infection and risk of pancreatic cancer: a meta-analysis of observational studies | 5 studies available for assessment of HCV infection and PAC risk | Higher risk of pancreatic cancer in subjects with past-exposure to HCV: RR = 1.26 (95%CI: 1.03-1.5) | Y Y Y |

HCV: Hepatitis C virus; PAC: Pancreatic cancer; Y: Yes.

**Table 14 Characteristics of available studies, assessing the association between hepatitis C virus infection and breast cancer, not considered and causes of exclusion**

| Studies (First author/ Journal/Year of publication) | Study title | Main findings for exclusion | Study conclusion |
|---|---|---|---|
| Bruno G *Clin Ter* 1999 | Hepatitis C virus: a high risk factor for a second primary malignancy besides hepatocellular carcinoma. Fact or fiction? | Very small number of patients considered in the present study | HCV could have played an important role not only in the development of HCC but of the second primary malignancy |
| Malaguarnera M *Eur J Int Medicine* 2006 | Hepatitis C virus in elderly cancer patients | No available information concerning number of HCV positive patients with breast cancer | No higher prevalence of breast cancer in patients with HCV infection |

HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

subjects with persistent viral hepatitis. Even if this observation was very interesting, it has not stimulated the achievement of studies investigating specifically the possible association between hepatitis viruses (in particular HCV) and human cancers other than HCC. Only in the last years this idea has gained interest an increasing number of trials have been designed and carried out with the purpose. Nevertheless, to date, few data are available yet and no final or univocal conclusions may be drawn. However, putting together the results of published studies on the possible association between HCV and risk of NHLs,

**Table 15 Characteristics of available, assessing the association between hepatitis C virus infection and renal cancer, not considered and causes of exclusion**

| Studies (First author/ Journal/Year of publication) | Study title | Main findings for exclusion | Study conclusion |
|---|---|---|---|
| Macleod LC *J Urol* 2013 | Risk Factors for Renal Cell Carcinoma in the VITAL Study | No data on the type of viral hepatitis infection reported in VITAL study | A significant association of RCC with viral hepatitis |
| Bruno G *Clin Ter* 1999 | Hepatitis C virus: a high risk factor for a second primary malignancy besides hepatocellular carcinoma. Fact or fiction? | Very small number of patients considered in the present study | HCV could have played an important role not only in the development of HCC but of the second primary malignancy |

RCC: Renal cell carcinoma.

**Table 16 Characteristics of available studies, assessing the association between hepatitis C virus infection and oral or skin cancer, not considered and causes of exclusion**

| Studies (First author/ Journal/Year of publication) | Study title | Main findings for exclusion | Study conclusion |
|---|---|---|---|
| Hunt J *Laringoscope* 2005 | Outcome Analysis of Patients with Squamous Cell Carcinoma of the Head and Neck and Hepatitis C Virus | Duplicate | HCV positive with SCCHN patients have not a worse outcome than their HCV negative counterparts |

SCCHN: Squamous cell carcinoma of the head and neck.

**Table 17 Characteristics of available studies, assessing the association between hepatitis C virus infection and thyroid cancer, not considered and causes of exclusion**

| Studies (First author/ Journal/Year of publication) | Study title | Main findings for exclusion | Study conclusion |
|---|---|---|---|
| Antonelli A *JAMA* 1999 | Thyroid cancer in patients with hepatitis C infection | Duplicate | Higher prevalence of thyroid cancer in a series of patients with chronic hepatitis C infection in comparison to no case in controls |
| Montella M *Int J Cancer* 2000 | Is HCV infection associated with thyroid cancer? A case-control study | Duplicate | Possible oncogenetic role of HCV for thyroid cancer, possible association more detectable in countries with a high prevalence of HCV |
| Montella M *Liver* 2001 | HCV and cancer: a case-control study in a high-endemic area | Duplicate | Expected increases not only in liver cancer, but also in tumors associated with the immune system |
| Malaguarnera M *Eur J Int Medicine* 2006 | HCV in elderly cancer patients | No available information concerning number of HCV positive patients with thyroid cancer | No higher prevalence of thyroid cancer in patients with HCV infection |

HCV: Hepatitis C virus.

cholangio-, pancreatic-, breast-, renal-, skin/oral- and thyroid-cancers, the reports concerning the estimates of prevalence of HCV infection worldwide as well as the tables on the age-standardized incidence rates of the mentioned malignancies per 100000 person-years in different geographical areas, some interesting consideration may be made (Figures 2 and 3). Actually, a clear correlation between regions of HCV prevalence and risk of extra-liver cancers has emerged only for a very small group of types and histological subtypes of malignancies. In particular, HCV infection has resulted to be associated with: (1) a higher incidence of some B-cell NHLs types, in countries, where an elevated prevalence of this pathogen is detectable, accounting to a percentage of about 10%. Furthermore, an additional factor potentially confirming the causal role between HCV and lymphomas, in particular B-cell NHLs, is represented by the observation of the regression of a significant percentage of low-grade B-cell NHLs after HCV eradication by means of an efficacious antiviral treatment. Early evidences, concerning this assumption, date back to the end of Twentieth Century with anecdotal reports and the beginning of the following Century, when further studies, enrolling a wider number of patients, were carried out for the first time[252]; and (2) an increased

risk of intra-hepatic cholangiocarcinoma development in subjects with anti-HCV positivity, as reported by a large number of available studies with OR ranging from 3.42 to 4.84. According to an epidemiological view-point, it has to be considered the following evidence: liver flukes are associated with an increased risk both for ICC and ECC, but in endemic-nations (such as Taiwan, Singapore and Korea) and in other Eastern areas with low rates of this type of infection, the incidence of ICC is more elevated in comparison with ECC. On the other hand, in the western areas ECC incidence is higher than that of ICC. Taking into account HCV epidemiology, the prevalence of this virus presents a wide variability worldwide. More elevated percentages are detectable in developing areas, such as in South-Eastern-Asia and Egypt, intermediate ones in Southern Europe and the lowest ones in Northern Europe and America. Therefore, a correlation between HCV prevalence and ICC incidence seems to emerge from these observations.

Concerning the possible association between the infection caused by this hepato-tropic virus, surprisingly, although a higher risk of PADC in HCV-positive patients has been observed in some trials and it has been reported in the available meta-analyses, age-standardized rates of this cancer present interesting geographical variations. In particular, PADC incidence worldwide is 3-4 times higher in more economically developed countries as well as in northern area of the world, where HCV prevalence is lower in comparison with less developed countries. Different reasons may explain these results, including the accuracy of diagnostic methods used to diagnose pancreatic malignancies in distinct geographical regions worldwide and of data to assess the rates of incidence of PDAC. However, it is well-known that on the basis of a morphogenetic viewpoint, pancreas and liver share several characteristics in their embryological development, arising from common multi-potent cells of endoderm origin. Therefore, HCV might replicate also in pancreatic cells as it does in hepatocytes[253,254]. Furthermore, according to epidemiological studies, type 2 diabetes represents a risk factor for PAC[255,256] with chronic hyperglycemia and hyperinsulinemia as proposed pathogenetic mechanisms involved in the promotion of this type of malignancy. Both conditions may induce proliferation, decrease apoptosis and promote invasion ability of pancreatic cancer cells[257-259]. A recent and interesting systematic review and dose-response meta-analysis, assessing blood glucose concentration and risk of pancreatic cancer, has shown that every 0.56 mmol/L (10 mg/dL) increase in fasting blood glucose , a 14% enhancement in the rate of PADC occurs[260]. On the other hand, it is now accepted that HCV infection is associated with an increased risk of type 2 diabetes[261,262]. Therefore, HCV infection, promoting and diabetes might act in cooperation with hyperinsulinemia, hyperglycemia in the promotion of PAC. This consideration may contribute to explain the epidemiological evidence of a more elevated incidence of this cancer in the most economically developed countries, where high rates of obesity, metabolic syndrome and diabetes are observed, in comparison with less developed regions in the world.

On the other hand, up to now no definitive and univocal conclusions may be obtained from the analysis of relationship between HCV and breast-, renal-, skin/oral- and thyroid-cancers. Most of the available studies, in particular a large part of cohort trials, have not confirmed the existence of these associations. However, the lack of similar evidences may be only apparent and some elements potentially limiting the conclusions of these reports have to be taken into account. In particular, most of available data have been obtained from some retrospective cohort-trials. Although the large size and the lengthy follow-up of this type of research provides the statistical power to obtain adequate information on cancer risk in the investigated population, the retrospective nature of these studies has to be considered. The use of routinely collected administrative data, based on population registries may represent a potential limiting factor in these trials. Possible errors in diagnosis, in codes of cancers or infective diseases and in reported data may affect the hospitalization records in some case/control subjects as well as the possibility that in several countries worldwide only a part of general population is included in national cancer registry may influences the obtained results. In addition, some of these trials have evaluated the incidence of a wide spectrum of malignancies and they have been not designed to assess a specific type of human tumors. It has to be also underlined that, according to age-standardized incidence rates of these neoplasms, remarkable temporal changes in human cancer trends have been observed worldwide. The combined analysis of these figures, as performed some years ago for HCV-related liver cancer, has induced some authors to hypothesize a possible role of HCV in the development of these malignancies. For example, in Japan, Tanaka *et al* have examined temporal trends for HCC incidence rates in a period ranging from 1981 and 2003 in Osaka Prefecture. They provided an explanation in the context of HCV infection rates. According to these findings, they noted that in that span of time the incidence peak of HCC was detectable in men during their 50s, 60s and 70s of age in 1986, 1995 and 2000 and then it was progressively decreasing. He postulated that the observed trend was due to the restriction of virus transmission[263]. As previously suggested, it should be taken into account that an enhanced incidence of ICC has been observed in the most developed regions in the years, ranging from 1980 to 2000, has been considered as caused by the parallel increase of HCV infection in these areas. This hypothesis has been definitively shown in United States[264]. In addition, the relationship between HCV and risk

of ICC has been assessed in patients with different degree of hepatic damage. It has been demonstrated that the probability of ICC development increases as the hepatic damage impairs[167]. Furthermore, a previous Italian research had suggested that HCV might have a role in thyroid cancer. This represents a rather rare malignancy, but its standardized incidence ratio has progressively increased between the end of '80s and the beginning of '90s in several well-developed countries, including Italy. In this country, in the same period of time an increasing prevalence of HCV infection was observed[247]. Unfortunately, no additional trials have been performed, with the purpose to assess the possible impact of HCV infection worldwide on the age-standardized incidence rates of the aforementioned malignancies in different geographical areas and to distinguish the potential contribution of this pathogen as pro-carcinogenic agent from other risk factors. Therefore this field of research still remains largely unexplored. In addition, it has to be taken into account that HCV represents a common cause of cirrhosis development and this condition itself is a well-known independent risk factor for the development of a wide spectrum of human malignancies different from liver. Several mechanisms may be responsible for carcinogenetic role of this pathogen. It may act indirectly as well as directly. Both these activities have been proposed for this virus and plausible evidences reported in literature. To date the existence of an indirect action of this microorganism is the most convincing pathogenic mechanism. In particular, as widely reported for liver, HCV might promote in extra-hepatic organs a persistent inflammation and induce the cancerous transformation, as a consequence of a progressive rearrangement of their structure. This event might play a role in the development of all the types of the aforementioned malignancies and not only involved in hepatic carcinogenesis. This process is characterized by the interaction and the cooperation among viral-related proteins, homing-cells (specific-tissue-cells, depending on the considered organ, endothelial cells, and stem cells), not homing cells (lymphomononuclear and polymorfonuclear specific and nonspecific cells), cytokines, costimulatory molecules and additional biological mediators (*i.e.,* PGs and oxidants). This situation promotes a self-maintaining and amplifying loop, in which HCV stimulate, in turn, PGE2, enzymes (such as cyclo-oxygenase-2 or COX-2), growth factors, interleukins production and cellular signaling pathway function. The complex cooperation and interaction among these mediators is one of the main factors responsible for final outcome: viral control with recovery or its persistence in the infected-organs, with progressive development of a tissue necro-inflammatory process, potentially evolving toward malignant transformation. Presence of inflammation favours genetic instability in cells and increases the probability which further genetic and epigenetic alterations arise. Perturbance of homeostasis in adult-cells may re-modulate activity and expression of genes as well as of transcription factors that govern their regeneration and/or differentiation programs as well as the processes involved in energy production. In addition, alteration of function of some intracellular pathways, such as K-RAS, Hedgehog-, Jak-STAT-, Notch-and TGF-β signalling-cascades, may also play a key role in carcinogenesis. In particular, this events may be induced not only by inflammation itself, but also by the direct action of some viral proteins such as: core- and NS5A on the intracellular cascades pathways function. These viral elements may affect the levels of activities of the afore-mentioned signalling-cascades and contribute to deregulation of cell-cycle checkpoint controls. To date a few studies have been performed with the aim to specifically investigate the pathogenetic mechanisms, potentially involved in the development of extra-liver malignancies in anti-HCV positive patients. The majority of reports upon this topic concerns lymphoproliferative malignancies. A study has described an increased expression of genes associated with lymphomagenesis in peripheral blood B cells of chronic HCV positive patients[265].

Furthermore, it has been shown that the telomere deletion of 1p36.3 in B-cell NHLs is significantly more frequent in patients with HCV infection in comparison with anti-HCV negative individuals[266]. Deletion of the 1p36 genomic locus is associated with the loss of p73, a tumour suppressor gene, that may be inactivated both in lymphomas and in other human cancers[267,268]. On the basis of available studies, this topic is progressively acquiring an increasing importance, but to date only some definitive conclusions have been obtained, while a large number of questions still remain unanswered. Further well-designed trials, enrolling an adequate number of patients as well as focusing on populations of different geographical areas and involving larger series of patients are required to confirm or deny this association as well as to identify the pathogenetic mechanisms, potentially involved in HCV-associated human carcinogenesis.

## ACKNOWLEDGMENTS

The authors thank Dr. Simonetta Righi, Biblioteca Centralizzata, Policlinico S Orsola-Malpighi, Università di Bologna, Bologna, Italy, for her support in the search of scientific bibliography and Mr. Fabio Castellini, Dipartimento di Emergenza-Urgenza, Ospedale di Budrio, Budrio, Bologna, Italy, for his active support in clinical research.

## COMMENTS

### *Background*

Hepatitis C virus (HCV) is an oncogenic virus and a well-known risk factor for hepatocellular carcinoma. In the last years, some studies have shown that its antigens and replicative sequences are detectable also in organs other than

liver. However, the significance of this feature is uncertain. Some reports and meta-analyses suggested that its infection is associated with development of cholangiocarcinoma and some types of lymphomas, but a comprehensive assessment of the possible role of HCV in extrahepatic carcinogenesis has not been yet performed.

### Innovations and breakthroughs

Actually, a clear correlation between regions of HCV prevalence and risk of extra-liver cancers has emerged only for a very small group of types and histological subtypes of malignancies. In particular, HCV infection has resulted to be associated with a higher incidence of: (1) some B-cell NHLs types, in countries, where an elevated prevalence of this pathogen is detectable, accounting to a percentage of about 10%; (2) intra-hepatic-, but not extrahepatic-cholangiocarcinoma; and (3) pancreatic cancer development. No definitive and univocal conclusions may be obtained from the analysis of relationship between HCV and breast-, renal-, skin/oral- and thyroid-cancers, although a possible association between renal-, skin/oral- and thyroid-malignancies and HCV infection has been suggested by some studies. These results strongly supports the need of additional studies with large sample size to ensure a precise estimate of the effect of HCV on these different types of cancers to improve our knowledge on carcinogenetic potential of HCV for extra-hepatic organs and on possible pathogenetic mechanisms. Few published studies available on the association of HCV and some types of human malignancies, such as breast, kidney, oral/skin and thyroid cancers and mainly enrolling populations of Asian ethnicity. Substantial variation by different geographical areas in serum prevalence of HCV antibodies and genotypes.

### Peer-review

This paper is very interesting because it clarifies a neglected aspect of HCV infection: the role of virus in pathogenesis of extrahepatic neoplasms. The data analysis is very accurate as well as the description of pathophysiological mechanisms of HCV-mediated cancerogenesis.

## REFERENCES


1 **Gower E**, Estes C, Blach S, Razavi-Shearer K, Razavi H. Global epidemiology and genotype distribution of the hepatitis C virus infection. *J Hepatol* 2014; **61**: S45-S57 [PMID: 25086286 DOI: 10.1016/j.jhep.2014.07.027]

2 **Alter MJ**. Epidemiology of hepatitis C virus infection. *World J Gastroenterol* 2007; **13**: 2436-2441 [PMID: 17552026]

3 **Younossi ZM**, Kanwal F, Saab S, Brown KA, El-Serag HB, Kim WR, Ahmed A, Kugelmas M, Gordon SC. The impact of hepatitis C burden: an evidence-based approach. *Aliment Pharmacol Ther* 2014; **39**: 518-531 [PMID: 24461160 DOI: 10.1111/apt.12625]

4 **Cacoub P**, Poynard T, Ghillani P, Charlotte F, Olivi M, Piette JC, Opolon P. Extrahepatic manifestations of chronic hepatitis C. MULTIVIRC Group. Multidepartment Virus C. *Arthritis Rheum* 1999; **42**: 2204-2212 [PMID: 10524695 DOI: 10.1002/1529-0131]

5 **Mariotto S**, Ferrari S, Monaco S. HCV-related central and peripheral nervous system demyelinating disorders. *Inflamm Allergy Drug Targets* 2014; **13**: 299-304 [PMID: 25198705]

6 **Pawlotsky JM**, Roudot-Thoraval F, Simmonds P, Mellor J, Ben Yahia MB, André C, Voisin MC, Intrator L, Zafrani ES, Duval J, Dhumeaux D. Extrahepatic immunologic manifestations in chronic hepatitis C and hepatitis C virus serotypes. *Ann Intern Med* 1995; **122**: 169-173 [PMID: 7810933]

7 **Ko HM**, Hernandez-Prera JC, Zhu H, Dikman SH, Sidhu HK, Ward SC, Thung SN. Morphologic features of extrahepatic manifestations of hepatitis C virus infection. *Clin Dev Immunol* 2012; **2012**: 740138 [PMID: 22919404 DOI: 10.1155/2012/740138]

8 **Crovatto M**, Pozzato G, Zorat F, Pussini E, Nascimben F, Baracetti S, Grando MG, Mazzaro C, Reitano M, Modolo ML, Martelli P, Spada A, Santini G. Peripheral blood neutrophils from hepatitis C virus-infected patients are replication sites of the virus. *Haematologica* 2000; **85**: 356-361 [PMID: 10756359]

9 **Zignego AL**, Macchia D, Monti M, Thiers V, Mazzetti M, Foschi M, Maggi E, Romagnani S, Gentilini P, Bréchot C. Infection of peripheral mononuclear blood cells by hepatitis C virus. *J Hepatol* 1992; **15**: 382-386 [PMID: 1332999]

10 **Sansonno D**, Lauletta G, Montrone M, Grandaliano G, Schena FP, Dammacco F. Hepatitis C virus RNA and core protein in kidney glomerular and tubular structures isolated with laser capture microdissection. *Clin Exp Immunol* 2005; **140**: 498-506 [PMID: 15932511 DOI: 10.1111/j.1365-2249.2005.02778.x]

11 **Arrieta JJ**, Rodriguez-Inigo E, Casqueiro M, Bartolomé J, Manzarbeitia F, Herrero M, Pardo M, Carreno V. Detection of hepatitis C virus replication by In situ hybridization in epithelial cells of anti-hepatitis C virus-positive patients with and without oral lichen planus. *Hepatology* 2000; **32**: 97-103 [PMID: 10869295 DOI: 10.1053/jhep.2000.8533]

12 **Kurokawa M**, Hidaka T, Sasaki H, Nishikata I, Morishita K, Setoyama M. Analysis of hepatitis C virus (HCV) RNA in the lesions of lichen planus in patients with chronic hepatitis C: detection of anti-genomic- as well as genomic-strand HCV RNAs in lichen planus lesions. *J Dermatol Sci* 2003; **32**: 65-70 [PMID: 12788531]

13 **Carrozzo M**, Quadri R, Latorre P, Pentenero M, Paganin S, Bertolusso G, Gandolfo S, Negro F. Molecular evidence that the hepatitis C virus replicates in the oral mucosa. *J Hepatol* 2002; **37**: 364-369 [PMID: 12175632]

14 **Toussirot E**, Le Huédé G, Mougin C, Balblanc JC, Bettinger D, Wendling D. Presence of hepatitis C virus RNA in the salivary glands of patients with Sjögren's syndrome and hepatitis C virus infection. *J Rheumatol* 2002; **29**: 2382-2385 [PMID: 12415596]

15 **De Vita S**, De Re V, Sansonno D, Sorrentino D, Corte RL, Pivetta B, Gasparotto D, Racanelli V, Marzotto A, Labombarda A, Gloghini A, Ferraccioli G, Monteverde A, Carbone A, Dammacco F, Boiocchi M. Gastric mucosa as an additional extrahepatic localization of hepatitis C virus: viral detection in gastric low-grade lymphoma associated with autoimmune disease and in chronic gastritis. *Hepatology* 2000; **31**: 182-189 [PMID: 10613744 DOI: 10.1002/hep.510310127]

16 **Yan FM**, Chen AS, Hao F, Zhao XP, Gu CH, Zhao LB, Yang DL, Hao LJ. Hepatitis C virus may infect extrahepatic tissues in patients with hepatitis C. *World J Gastroenterol* 2000; **6**: 805-811 [PMID: 11819700]

17 **Akdoğan M**, Mert A, Tabak F, Özdemir S, Sonsuz A, Sentürk H. Hepatitis C infection in non-Hodgkin's lymphoma. *Turk J Gastroenterol* 1998; **1**: 73-75

18 **Arcaini L**, Paulli M, Boveri E, Vallisa D, Bernuzzi P, Orlandi E, Incardona P, Brusamolino E, Passamonti F, Burcheri S, Schena C, Pascutto C, Cavanna L, Magrini U, Lazzarino M. Splenic and nodal marginal zone lymphomas are indolent disorders at high hepatitis C virus seroprevalence with distinct presenting features but similar morphologic and phenotypic profiles. *Cancer* 2004; **100**: 107-115 [PMID: 14692030 DOI: 10.1002/cncr.11893]

19 **Arcaini L**, Varettoni M, Boveri E, Orlandi E, Rattotti S, Zibellini S, Merli M, Lucioni M, Rizzi S, Gotti M, Morello L, Pascutto C, Paulli M. Distinctive clinical and histological features of Waldenström's macroglobulinemia and splenic marginal zone lymphoma. *Clin Lymphoma Myeloma Leuk* 2011; **11**: 103-105 [PMID: 21454204 DOI: 10.3816/CLML.2011.n.020]

20 **Arican A**, Sengezer T, Bozdayi M, Bozkaya H, Uçgül E, Dinçol D, Uzunalimoğlu O. Prevalence of hepatitis-G virus and hepatitis-C virus infection in patients with non-Hodgkin's lymphoma. *Med Oncol* 2000; **17**: 123-126 [PMID: 10871818 DOI: 10.1007/BF02796207]

21 **Avilés A**, Valdez L, Halabe J, Neri N, Nellen H, Huerta-Guzmán J, Nambo MJ. No association between lymphoma and hepatitis C virus. *Med Oncol* 2003; **20**: 165-168 [PMID: 12835519 DOI: 10.1385/MO:20:2:165]

22 **Bauduer F**, Katsahian S, Blanchard Y, Oui B, Capdupuy C, Renoux M. Descriptive epidemiology of non-Hodgkin lymphomas in a southwestern French hematology center: absence of significant relationship with hepatitis C virus infection. *Hematol Cell Ther* 1999; **41**: 191-193 [PMID: 10651117]

23 **Besson C**, Canioni D, Lepage E, Pol S, Morel P, Lederlin P, Van Hoof A, Tilly H, Gaulard P, Coiffier B, Gisselbrecht C, Brousse N, Reyes F, Hermine O. Characteristics and outcome of diffuse large B-cell lymphoma in hepatitis C virus-positive patients in LNH 93

and LNH 98 Groupe d'Etude des Lymphomes de l'Adulte programs. *J Clin Oncol* 2006; **24**: 953-960 [PMID: 16418500 DOI: 10.1200/JCO.2005.01.5016]

24 **Bianco E**, Marcucci F, Mele A, Musto P, Cotichini R, Sanpaolo MG, Iannitto E, De Renzo A, Martino B, Specchia G, Montanaro M, Barbui AM, Nieddu R, Pagano L, Rapicetta M, Franceschi S, Mandelli F, Pulsoni A. Prevalence of hepatitis C virus infection in lymphoproliferative diseases other than B-cell non-Hodgkin's lymphoma, and in myeloproliferative diseases: an Italian Multi-Center case-control study. *Haematologica* 2004; **89**: 70-76 [PMID: 14754608]

25 **Boffetta P**, Armstrong B, Linet M, Kasten C, Cozen W, Hartge P. Consortia in cancer epidemiology: lessons from InterLymph. *Cancer Epidemiol Biomarkers Prev* 2007; **16**: 197-199 [PMID: 17301250 DOI: 10.1158/1055-9965]

26 **Bronowicki JP**, Bineau C, Feugier P, Hermine O, Brousse N, Oberti F, Rousselet MC, Dharancy S, Gaulard P, Flejou JF, Cazals-Hatem D, Labouyrie E. Primary lymphoma of the liver: clinical-pathological features and relationship with HCV infection in French patients. *Hepatology* 2003; **37**: 781-787 [PMID: 12668970 DOI: 10.1053/jhep.2003.50121]

27 **Catassi C**, Fabiani E, Coppa GV, Gabrielli A, Centurioni R, Leoni P, Barbato M, Viola F, Martelli M, De Renzo A, Rotoli B, Bertolani P, Federico M, Carroccio A, Iannitto E, Baldassarre M, Guarini A, Guariso G, Favaretto G, Caramaschi P, Ambrosetti A. [High prevalence of hepatitis C virus infection in patients with non-Hodgkin's lymphoma at the onset. Preliminary results of an Italian multicenter study]. *Recenti Prog Med* 1998; **89**: 63-67 [PMID: 9558907]

28 **Chindamo MC**, Spector N, Segadas JA, Pimenta G, Vanderborght B, Morais JC, Milito C, Moraes Coelho HS. Prevalence of hepatitis C infection in patients with non-Hodgkin's lymphomas. *Oncol Rep* 2002; **9**: 657-659 [PMID: 11956646]

29 **Chuang SS**, Liao YL, Chang ST, Hsieh YC, Kuo SY, Lu CL, Hwang WS, Lin IH, Tsao CJ, Huang WT. Hepatitis C virus infection is significantly associated with malignant lymphoma in Taiwan, particularly with nodal and splenic marginal zone lymphomas. *J Clin Pathol* 2010; **63**: 595-598 [PMID: 20530156 DOI: 10.1136/jcp.2010.076810]

30 **Ohmuro H**, Tomino Y, Tsushima Y, Shimizu M, Kuramoto T, Koide H. Elevation of serum IgA1 levels in patients with diabetic nephropathy. *Nephron* 1993; **63**: 355 [PMID: 8446278 DOI: 10.1007/s12185-008-0086-3]

31 **Collier JD**, Zanke B, Moore M, Kessler G, Krajden M, Shepherd F, Heathcote J. No association between hepatitis C and B-cell lymphoma. *Hepatology* 1999; **29**: 1259-1261 [PMID: 10094973 DOI: 10.1002/hep.510290422]

32 **Cowgill KD**, Loffredo CA, Eissa SA, Mokhtar N, Abdel-Hamid M, Fahmy A, Strickland GT. Case-control study of non-Hodgkin's lymphoma and hepatitis C virus infection in Egypt. *Int J Epidemiol* 2004; **33**: 1034-1039 [PMID: 15155696 DOI: 10.1093/ije/dyh183]

33 **Cucuianu A**, Patiu M, Duma M, Basarab C, Soritau O, Bojan A, Vasilache A, Mates M, Petrov L. Hepatitis B and C virus infection in Romanian non-Hodgkin's lymphoma patients. *Br J Haematol* 1999; **107**: 353-356 [PMID: 10583224]

34 **Dal Maso L**, Talamini R, Montella M, Crovatto M, Franceschi S. Hepatitis B and C viruses and Hodgkin lymphoma: a case-control study from Northern and Southern Italy. *Haematologica* 2004; **89**: ELT17 [PMID: 15533850]

35 **De Renzo A**, Persico E, de Marino F, di Giacomo Russo G, Notaro R, di Grazia C, Picardi M, Santoro L, Torella R, Rotoli B, Persico M. High prevalence of hepatitis G virus infection in Hodgkin's disease and B-cell lymphoproliferative disorders: absence of correlation with hepatitis C virus infection. *Haematologica* 2002; **87**: 714-718; discussion 718 [PMID: 12091122]

36 **De Rosa G**, Gobbo ML, De Renzo A, Notaro R, Garofalo S, Grimaldi M, Apuzzo A, Chiurazzi F, Picardi M, Matarazzo M, Rotoli B. High prevalence of hepatitis C virus infection in patients with B-cell lymphoproliferative disorders in Italy. *Am J Hematol* 1997; **55**: 77-82 [PMID: 9209002 DOI: 10.1002/(SICI)1096-8652(199706)55:2]

37 **de Sanjose S**, Nieters A, Goedert JJ, Domingo-Domenech E, Fernandez de Sevilla A, Bosch R, Herrera P, Domingo A, Petit J, Bosch X, Kallinowski B. Role of hepatitis C virus infection in malignant lymphoma in Spain. *Int J Cancer* 2004; **111**: 81-85 [PMID: 15185347 DOI: 10.1002/ijc.11727]

38 **De Vita S**, Zagonel V, Russo A, Rupolo M, Cannizzaro R, Chiara G, Boiocchi M, Carbone A, Franceschi S. Hepatitis C virus, non-Hodgkin's lymphomas and hepatocellular carcinoma. *Br J Cancer* 1998; **77**: 2032-2035 [PMID: 9667688]

39 **Domingo JM**, Romero MS, Palomera L, Gutiérrez M. [Hepatitis C virus infection in patients with non Hodgkin's lymphoma]. *Med Clin* (Barc) 2001; **117**: 638 [PMID: 11714475]

40 **Duberg AS**, Nordström M, Törner A, Reichard O, Strauss R, Janzon R, Bäck E, Ekdahl K. Non-Hodgkin's lymphoma and other nonhepatic malignancies in Swedish patients with hepatitis C virus infection. *Hepatology* 2005; **41**: 652-659 [PMID: 15723449 DOI: 10.1002/hep.20608]

41 **Ellenrieder V**, Weidenbach H, Frickhofen N, Michel D, Prümmer O, Klatt S, Bernas O, Mertens T, Adler G, Beckh K. HCV and HGV in B-cell non-Hodgkin's lymphoma. *J Hepatol* 1998; **28**: 34-39 [PMID: 9537861]

42 **El-Serag HB**, Hampel H, Yeh C, Rabeneck L. Extrahepatic manifestations of hepatitis C among United States male veterans. *Hepatology* 2002; **36**: 1439-1445 [PMID: 12447870 DOI: 10.1053/jhep.2002.37191]

43 **Engels EA**, Chatterjee N, Cerhan JR, Davis S, Cozen W, Severson RK, Whitby D, Colt JS, Hartge P. Hepatitis C virus infection and non-Hodgkin lymphoma: results of the NCI-SEER multi-center case-control study. *Int J Cancer* 2004; **111**: 76-80 [PMID: 15185346 DOI: 10.1002/ijc.20021]

44 **Ferri C**, Caracciolo F, Zignego AL, La Civita L, Monti M, Longombardo G, Lombardini F, Greco F, Capochiani E, Mazzoni A. Hepatitis C virus infection in patients with non-Hodgkin's lymphoma. *Br J Haematol* 1994; **88**: 392-394 [PMID: 7803287]

45 **Ferri C**, La Civita L, Caracciolo F, Zignego AL. Non-Hodgkin's lymphoma: possible role of hepatitis C virus. *JAMA* 1994; **272**: 355-356 [PMID: 8028163]

46 **Franceschi S**, Lise M, Trépo C, Berthillon P, Chuang SC, Nieters A, Travis RC, Vermeulen R, Overvad K, Tjønneland A, Olsen A, Bergmann MM, Boeing H, Kaaks R, Becker N, Trichopoulou A, Lagiou P, Bamia C, Palli D, Sieri S, Panico S, Tumino R, Sacerdote C, Bueno-de-Mesquita B, Peeters PH, Rodríguez L, Barroso LL, Dorronsoro M, Sánchez MJ, Navarro C, Barricarte A, Regnér S, Borgquist S, Melin B, Hallmans G, Khaw KT, Wareham N, Rinaldi S, Hainaut P, Riboli E, Vineis P. Infection with hepatitis B and C viruses and risk of lymphoid malignancies in the European Prospective Investigation into Cancer and Nutrition (EPIC). *Cancer Epidemiol Biomarkers Prev* 2011; **20**: 208-214 [PMID: 21098651]

47 **Gasparotto D**, De Re V, Boiocchi M. Hepatitis C virus, B-cell proliferation and lymphomas. *Leuk Lymphoma* 2002; **43**: 747-751 [PMID: 12153160 DOI: 10.1080/10428190290016845]

48 **Gasztonyi B**, Pár A, Szomor A, Battyány I, Nagy A, Kereskai L, Losonczy H, Mózsik G. Hepatitis C virus infection associated with B-cell non-Hodgkin's lymphoma in Hungarian patients. *Br J Haematol* 2000; **110**: 497-498 [PMID: 11023295]

49 **Gentile G**, Mele A, Monarco B, Vitale A, Pulsoni A, Visani G, Castelli G, Rapicetta M, Verani P, Martino P, Mandelli F. Hepatitis B and C viruses, human T-cell lymphotropic virus types I and II, and leukemias: a case-control study. The Italian Leukemia Study Group. *Cancer Epidemiol Biomarkers Prev* 1996; **5**: 227-230 [PMID: 8833624]

50 **Genvresse I**, Späth-Schwalbe E, Meisel H, Kaufmann O, Krüger DH, Possinger K. Primary hepatic or splenic diffuse large B-cell lymphoma and hepatitis C virus infection: a non-fortuitous association? *Ann Hematol* 2000; **79**: 530-532 [PMID: 11043427]

51 **Germanidis G**, Haioun C, Pourquier J, Gaulard P, Pawlotsky JM, Dhumeaux D, Reyes F. Hepatitis C virus infection in patients with overt B-cell non-Hodgkin's lymphoma in a French center. *Blood* 1999; **93**: 1778-1779 [PMID: 10084815]

52 **Giordano TP**, Henderson L, Landgren O, Chiao EY, Kramer

JR, El-Serag H, Engels EA. Risk of non-Hodgkin lymphoma and lymphoproliferative precursor diseases in US veterans with hepatitis C virus. *JAMA* 2007; **297**: 2010-2017 [PMID: 17488966 DOI: 10.1001/jama.297.18.2010]

53 **Gisbert JP**, García-Buey L, Pajares JM, Moreno-Otero R. Prevalence of hepatitis C virus infection in B-cell non-Hodgkin's lymphoma: systematic review and meta-analysis. *Gastroenterology* 2003; **125**: 1723-1732 [PMID: 14724825]

54 **Goldman L**, Ezzat S, Mokhtar N, Abdel-Hamid A, Fowler N, Gouda I, Eissa SA, Abdel-Hamid M, Loffredo CA. Viral and non-viral risk factors for non-Hodgkin's lymphoma in Egypt: heterogeneity by histological and immunological subtypes. *Cancer Causes Control* 2009; **20**: 981-987 [PMID: 19263231 DOI: 10.1007/s10552-009-9316-0]

55 **Grudeva-Popova J**, Nenova I, Mateva N, Ananoshtev N, Popov V, Atanasova M. Non-Hodgkin lymphomas and carrier state of viral hepatitis B and C. *J BUON* 2000; **18**: 239-244 [PMID: 23613411]

56 **Guida M**, D'Elia G, Benvestito S, Casamassima A, Micelli G, Quaranta M, Moschetta R, De Lena M, Lorusso V. Hepatitis C virus infection in patients with B-cell lymphoproliferative disorders. *Leukemia* 2002; **16**: 2162-2163 [PMID: 12357374 DOI: 10.1038/sj.leu.2402626]

57 **Hanley J**, Jarvis L, Simmonds P, Parker A, Ludlam C. HCV and non-Hodgkin lymphoma. *Lancet* 1996; **347**: 1339 [PMID: 8622540]

58 **Harakati MS**, Abualkhair OA, Al-Knawy BA. Hepatitis C Virus infection in Saudi Arab patients with B-cell non-Hodgkin's lymphoma. *Saudi Med J* 2000; **21**: 755-758 [PMID: 11423889]

59 **Hausfater P**, Cacoub P, Sterkers Y, Thibault V, Amoura Z, Nguyen L, Ghillani P, Leblond V, Piette JC. Hepatitis C virus infection and lymphoproliferative diseases: prospective study on 1,576 patients in France. *Am J Hematol* 2001; **67**: 168-171 [PMID: 11391713 DOI: 10.1002/ajh.1101]

60 **Imai Y**, Ohsawa M, Tanaka H, Tamura S, Sugawara H, Kuyama J, Fukuda K, Yonezawa T, Matsuzawa Y. High prevalence of HCV infection in patients with B-cell non-Hodgkin's lymphoma: comparison with birth cohort- and sex-matched blood donors in a Japanese population. *Hepatology* 2002; **35**: 974-976 [PMID: 11915049 DOI: 10.1053/jhep.2002.32149]

61 **Iwata H**, Matsuo K, Takeuchi K, Kishi Y, Murashige N, Kami M. High incidences of malignant lymphoma in patients infected with hepatitis B or hepatitis C virus. *Haematologica* 2004; **89**: 368-370 [PMID: 15020283]

62 **Izumi T**, Sasaki R, Miura Y, Okamoto H. Primary hepatosplenic lymphoma: association with hepatitis C virus infection. *Blood* 1996; **87**: 5380-5381 [PMID: 8652854]

63 **Izumi T**, Sasaki R, Tsunoda S, Akutsu M, Okamoto H, Miura Y. B cell malignancy and hepatitis C virus infection. *Leukemia* 1997; **11** Suppl 3: 516-518 [PMID: 9209442]

64 **Karavattathayyil SJ**, Kalkeri G, Liu HJ, Gaglio P, Garry RF, Krause JR, Dash S. Detection of hepatitis C virus RNA sequences in B-cell non-Hodgkin lymphoma. *Am J Clin Pathol* 2000; **113**: 391-398 [PMID: 10705820 DOI: 10.1309/REV9-FDTM-5NGC-HBWY]

65 **Kaya H**, Polat MF, Erdem F, Gündogdu M. Prevalence of hepatitis C virus and hepatitis G virus in patients with non-Hodgkin' s lymphoma. *Clin Lab Haematol* 2002; **24**: 107-110 [PMID: 11985556]

66 **King PD**, Wilkes JD, Diaz-Arias AA. Hepatitis C virus infection in non-Hodgkin's lymphoma. *Clin Lab Haematol* 1998; **20**: 107-110 [PMID: 9681221]

67 **Kuniyoshi M**, Nakamuta M, Sakai H, Enjoji M, Kinukawa N, Kotoh K, Fukutomi M, Yokota M, Nishi H, Iwamoto H, Uike N, Nishimura J, Inaba S, Maeda Y, Nawata H, Muta K. Prevalence of hepatitis B or C virus infections in patients with non-Hodgkin's lymphoma. *J Gastroenterol Hepatol* 2001; **16**: 215-219 [PMID: 11207904]

68 **Luppi M**, Longo G, Ferrari MG, Barozzi P, Marasca R, Morselli M, Valenti C, Mascia T, Vandelli L, Vallisa D, Cavanna L, Torelli G. Clinico-pathological characterization of hepatitis C virus-related B-cell non-Hodgkin's lymphomas without symptomatic cryoglobulinemia. *Ann Oncol* 1998; **9**: 495-498 [PMID: 9653489]

69 **Mazzaro C**, Zagonel V, Monfardini S, Tulissi P, Pussini E, Fanni M, Sorio R, Bortolus R, Crovatto M, Santini G, Tiribelli C, Sasso F, Masutti R, Pozzato G. Hepatitis C virus and non-Hodgkin's lymphomas. *Br J Haematol* 1996; **94**: 544-550 [PMID: 8790157]

70 **McColl MD**, Singer IO, Tait RC, McNeil IR, Cumming RL, Hogg RB. The role of hepatitis C virus in the aetiology of non-Hodgkins lymphoma--a regional association? *Leuk Lymphoma* 1997; **26**: 127-130 [PMID: 9250797 DOI: 10.3109/10428199709109167]

71 **Mele A**, Pulsoni A, Bianco E, Musto P, Szklo A, Sanpaolo MG, Iannitto E, De Renzo A, Martino B, Liso V, Andrizzi C, Pusterla S, Dore F, Maresca M, Rapicetta M, Marcucci F, Mandelli F, Franceschi S. Hepatitis C virus and B-cell non-Hodgkin lymphomas: an Italian multicenter case-control study. *Blood* 2003; **102**: 996-999 [PMID: 12714514 DOI: 10.1182/blood-2002-10-3230]

72 **Mizorogi F**, Hiramoto J, Nozato A, Takekuma Y, Nagayama K, Tanaka T, Takagi K. Hepatitis C virus infection in patients with B-cell non-Hodgkin's lymphoma. *Intern Med* 2000; **39**: 112-117 [PMID: 10732826]

73 **Montella M**, Crispo A, de Bellis G, Izzo F, Frigeri F, Ronga D, Spada O, Mettivier V, Tamburini M, Cuomo O. HCV and cancer: a case-control study in a high-endemic area. *Liver* 2001; **21**: 335-341 [PMID: 11589770]

74 **Montella M**, Crispo A, Frigeri F, Ronga D, Tridente V, De Marco M, Fabbrocini G, Spada O, Mettivier V, Tamburini M. HCV and tumors correlated with immune system: a case-control study in an area of hyperendemicity. *Leuk Res* 2001; **25**: 775-781 [PMID: 11489471]

75 **Morton LM**, Engels EA, Holford TR, Leaderer B, Zhang Y, Zahm SH, Boyle P, Zhang B, Flynn S, Tallini G, Owens PH, Zheng T. Hepatitis C virus and risk of non-Hodgkin lymphoma: a population-based case-control study among Connecticut women. *Cancer Epidemiol Biomarkers Prev* 2004; **13**: 425-430 [PMID: 15006919]

76 **Musto P**, Dell'Olio M, Carotenuto M, Mangia A, Andriulli A. Hepatitis C virus infection: a new bridge between hematologists and gastroenterologists? *Blood* 1996; **88**: 752-754 [PMID: 8695825]

77 **Nicolosi Guidicelli S**, Lopez-Guillermo A, Falcone U, Conconi A, Christinat A, Rodriguez-Abreu D, Grisanti S, Lobetti-Bodoni C, Piffaretti JC, Johnson PW, Mombelli G, Cerny A, Montserrat E, Cavalli F, Zucca E. Hepatitis C virus and GBV-C virus prevalence among patients with B-cell lymphoma in different European regions: a case-control study of the International Extranodal Lymphoma Study Group. *Hematol Oncol* 2012; **30**: 137-142 [PMID: 22105737 DOI: 10.1002/hon.1015]

78 **Nieters A**, Kallinowski B, Brennan P, Ott M, Maynadié M, Benavente Y, Foretova L, Cocco PL, Staines A, Vornanen M, Whitby D, Boffetta P, Becker N, De Sanjosé S. Hepatitis C and risk of lymphoma: results of the European multicenter case-control study EPILYMPH. *Gastroenterology* 2006; **131**: 1879-1886 [PMID: 17087949 DOI: 10.1053/j.gastro.2006.09.019]

79 **Ogino H**, Satomura Y, Unoura M, Yoshida T, Oguri H, Kaneko S, Kobayashi K. Hepatitis B, C and G virus infection in patients with lymphoproliferative disorders. *Hepatol Res* 1999; **14**: 187-194

80 **Ohsawa M**, Shingu N, Miwa H, Yoshihara H, Kubo M, Tsukuma H, Teshima H, Hashimoto M, Aozasa K. Risk of non-Hodgkin's lymphoma in patients with hepatitis C virus infection. *Int J Cancer* 1999; **80**: 237-239 [PMID: 9935205 DOI: 10.1002/(SICI)1097-0215(19990118)80:2]

81 **Omland LH**, Jepsen P, Krarup H, Christensen PB, Weis N, Nielsen L, Obel N, Sørensen HT, Stuver SO. Liver cancer and non-Hodgkin lymphoma in hepatitis C virus-infected patients: results from the DANVIR cohort study. *Int J Cancer* 2012; **130**: 2310-2317 [PMID: 21780099 DOI: 10.1002/ijc.26283]

82 **Panovska I**, Georgievski B, Stojanovic A, Cevreska L, Efremov DG. Low prevalence of chronic hepatitis C virus infection in B-cell non-Hodgkin's lymphoma patients from a population with a high prevalence of healthy hepatitis c virus carriers. *Br J Haematol* 2000; **109**: 249-250 [PMID: 10848806]

83 **Park SC**, Jeong SH, Kim J, Han CJ, Kim YC, Choi KS, Cho JH, Lee M, Jung HH, Ki SS, Chang YH, Lee SS, Park YH, Lee KH. High prevalence of hepatitis B virus infection in patients with B-cell non-Hodgkin's lymphoma in Korea. *J Med Virol* 2008; **80**: 960-966

[PMID: 18428141 DOI: 10.1002/jmv.21168]
84 **Paydas S**, Kiliç B, Sahin B, Buğdayci R. Prevalence of hepatitis C virus infection in patients with lymphoproliferative disorders in Southern Turkey. *Br J Cancer* 1999; **80**: 1303-1305 [PMID: 10424729 DOI: 10.1038/sj.bjc.6690522]
85 **Pioltelli P**, Gargantini L, Cassi E, Santoleri L, Bellati G, Magliano EM, Morra E. Hepatitis C virus in non-Hodgkin's lymphoma. A reappraisal after a prospective case-control study of 300 patients. Lombart Study Group of HCV-Lymphoma. *Am J Hematol* 2000; **64**: 95-100 [PMID: 10814987 DOI: 10.1002/(SICI)1096-8652(200006)64:2]
86 **Pioltelli P**, Zehender G, Monti G, Monteverde A, Galli M. HCV and non-Hodgkin lymphoma. *Lancet* 1996; **347**: 624-625 [PMID: 8596360]
87 **Prati D**, Zanella A, De Mattei C, Farma E, Boschetti C, Sirchia G, Venegoni L, Berti E. Chronic hepatitis c virus infection and primary cutaneous B-cell lymphoma. *Br J Haematol* 1999; **105**: 841 [PMID: 10354158]
88 **Rabkin CS**, Tess BH, Christianson RE, Wright WE, Waters DJ, Alter HJ, Van Den Berg BJ. Prospective study of hepatitis C viral infection as a risk factor for subsequent B-cell neoplasia. *Blood* 2002; **99**: 4240-4242 [PMID: 12010836 DOI: 10.1182/blood-2002-01-0226]
89 **Ramos-Casals M**, Trejo O, García-Carrasco M, Cervera R, De La Red G, Gil V, López-Guillermo A, Ingelmo M, Font J. Triple association between hepatitis C virus infection, systemic autoimmune diseases, and B cell lymphoma. *J Rheumatol* 2004; **31**: 495-499 [PMID: 14994393]
90 **Salem Z**, Nuwaiyri-Salti N, Ramlawi F, Ramia S. Hepatitis C virus infection in Lebanese patients with B-cell non-Hodgkin's lymphoma. *Eur J Epidemiol* 2003; **18**: 251-253 [PMID: 12800950 DOI: 10.1023/A:1023380316098]
91 **Sansonno D**, De Vita S, Cornacchiulo V, Carbone A, Boiocchi M, Dammacco F. Detection and distribution of hepatitis C virus-related proteins in lymph nodes of patients with type II mixed cryoglobulinemia and neoplastic or non-neoplastic lymphoproliferation. *Blood* 1996; **88**: 4638-4645 [PMID: 8977256]
92 **Schöllkopf C**, Smedby KE, Hjalgrim H, Rostgaard K, Panum I, Vinner L, Chang ET, Glimelius B, Porwit A, Sundström C, Hansen M, Adami HO, Melbye M. Hepatitis C infection and risk of malignant lymphoma. *Int J Cancer* 2008; **122**: 1885-1890 [PMID: 18271005 DOI: 10.1002/ijc.23416]
93 **Sève P**, Renaudier P, Sasco AJ, Dumontet C, Salles G, Coiffier B, Zoulim F, Broussolle C, Trépo C. Hepatitis C virus infection and B-cell non-Hodgkin's lymphoma: a cross-sectional study in Lyon, France. *Eur J Gastroenterol Hepatol* 2004; **16**: 1361-1365 [PMID: 15618846]
94 **Shariff S**, Yoshida EM, Gascoyne RD, Le N, Connors JM, Middleton PJ, Shenkier TN. Hepatitis C infection and B-cell non-Hodgkin's lymphoma in British Columbia: a cross-sectional analysis. *Ann Oncol* 1999; **10**: 961-964 [PMID: 10509159]
95 **Shirin H**, Davidovitz Y, Avni Y, Petchenko P, Krepel Z, Bruck R, Meytes D. Prevalence of hepatitis C virus infection in patients with lymphoproliferative disorders. *Isr Med Assoc J* 2002; **4**: 24-27 [PMID: 11802304]
96 **Silvestri F**, Barillari G, Fanin R, Pipan C, Falasca E, Salmaso F, Zaja F, Infanti L, Patriarca F, Botta GA, Baccarani M. Hepatitis C virus infection among cryoglobulinemic and non-cryoglobulinemic B-cell non-Hodgkin's lymphomas. *Haematologica* 1997; **82**: 314-317 [PMID: 9234578]
97 **Silvestri F**, Pipan C, Barillari G, Zaja F, Fanin R, Infanti L, Russo D, Falasca E, Botta GA, Baccarani M. Prevalence of hepatitis C virus infection in patients with lymphoproliferative disorders. *Blood* 1996; **87**: 4296-4301 [PMID: 8639788]
98 **Singer IO**, Cumming RL, Hogg RB. Is hepatitis C associated with non-Hodgkin's lymphoma? *Leuk Lymphoma* 1997; **26**: 633-634 [PMID: 9389373 DOI: 10.3109/10428199709050902]
99 **Sonmez M**, Bektas O, Yilmaz M, Durmus A, Akdogan E, Topbas M, Erturk M, Ovali E, Omay SB. The relation of lymphoma and hepatitis B virus/hepatitis C virus infections in the region of East Black Sea, Turkey. *Tumori* 2002; **93**: 536-539 [PMID: 18338485]
100 **Spinelli JJ**, Lai AS, Krajden M, Andonov A, Gascoyne RD, Connors JM, Brooks-Wilson AR, Gallagher RP. Hepatitis C virus and risk of non-Hodgkin lymphoma in British Columbia, Canada. *Int J Cancer* 2008; **122**: 630-633 [PMID: 17935132 DOI: 10.1002/ijc.23105]
101 **Talamini R**, Montella M, Crovatto M, Dal Maso L, Crispo A, Negri E, Spina M, Pinto A, Carbone A, Franceschi S. Non-Hodgkin's lymphoma and hepatitis C virus: a case-control study from northern and southern Italy. *Int J Cancer* 2004; **110**: 380-385 [PMID: 15095303 DOI: 10.1002/ijc.20137]
102 **Thalen DJ**, Raemaekers J, Galama J, Cooreman MP. Absence of hepatitis C virus infection in non-Hodgkin's lymphoma. *Br J Haematol* 1997; **96**: 880-881 [PMID: 9074442]
103 **Timurağlu A**, Colak D, Oğünç D, Karadoğan I, Undar L. Hepatitis C virus association with non-Hodgkin's lymphoma. *Haematologia* (Budap) 1999; **29**: 301-304 [PMID: 10438070]
104 **Tkoub EM**, Haioun C, Pawlotsky JM, Dhumeaux D, Delchier JC. Chronic hepatitis C virus and gastric MALT lymphoma. *Blood* 1998; **91**: 360 [PMID: 9414306]
105 **Tursi A**, Brandimante G, Chiarelli F, Spagnoli A, Torello M. Detection of HCV RNA in gastric mucosa-associated lymphoid tissue by in situ hybridization: evidence of a new extrahepatic localization of HCV with increased risk of gastric malt lymphoma. *Am J Gastroenterol* 2002; **97**: 1802-1806 [PMID: 12135039 DOI: 10.1111/j.1572-0241.2002.05848.x]
106 **Udomsakdi-Auewarakul C**, Auewarakul P, Sukpanichnant S, Muangsup W. Hepatitis C virus infection in patients with non-Hodgkin lymphoma in Thailand. *Blood* 2000; **95**: 3640-3641 [PMID: 10877553]
107 **Vajdic CM**, Grulich AE, Kaldor JM, Fritschi L, Benke G, Hughes AM, Kricker A, Turner JJ, Milliken S, Armstrong BK. Specific infections, infection-related behavior, and risk of non-Hodgkin lymphoma in adults. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 1102-1108 [PMID: 16775166]
108 **Vallisa D**, Bernuzzi P, Arcaini L, Sacchi S, Callea V, Marasca R, Lazzaro A, Trabacchi E, Anselmi E, Arcari AL, Moroni C, Bertè R, Lazzarino M, Cavanna L. Role of anti-hepatitis C virus (HCV) treatment in HCV-related, low-grade, B-cell, non-Hodgkin's lymphoma: a multicenter Italian experience. *J Clin Oncol* 2005; **23**: 468-473 [PMID: 15659492 DOI: 10.1200/JCO.2005.06.008]
109 **Varma S**, Menon MC, Garg A, Malhotra P, Sharma A, Chawla YK, Dhiman RK. Hepatitis C virus infection among patients with non-Hodgkin's lymphoma in northern India. *Hepatol Int* 2011; **5**: 688-692 [PMID: 21484139 DOI: 10.1007/s12072-010-9244-9]
110 **Yamac K**, Aydemir S, Ozturk G, Fen T, Senol E. Hepatitis C infection in lymphoma patients in a Turkish center. *Eur J Epidemiol* 2000; **16**: 685 [PMID: 11078127]
111 **Yoshikawa M**, Imazu H, Ueda S, Tamagawa T, Yoneda S, Yamane Y, Takaya A, Fukui H, Nakano H. Prevalence of hepatitis C virus infection in patients with non-Hodgkin's lymphoma and multiple myeloma. A report from Japan. *J Clin Gastroenterol* 1997; **25**: 713-714 [PMID: 9451705]
112 **Yu SC**, Lin CW. Early-stage splenic diffuse large B-cell lymphoma is highly associated with hepatitis C virus infection. *Kaohsiung J Med Sci* 2013; **29**: 150-156 [PMID: 23465418 DOI: 10.1016/j.kjms.2012.08.025]
113 **Zucca E**, Roggero E, Maggi-Solcà N, Conconi A, Bertoni F, Reilly I, Castelli D, Pedrinis E, Piffaretti JC, Cavalli F. Prevalence of Helicobacter pylori and hepatitis C virus infections among non-Hodgkin's lymphoma patients in Southern Switzerland. *Haematologica* 2000; **85**: 147-153 [PMID: 10681721]
114 **Zuckerman E**, Zuckerman T, Levine AM, Douer D, Gutekunst K, Mizokami M, Qian DG, Velankar M, Nathwani BN, Fong TL. Hepatitis C virus infection in patients with B-cell non-Hodgkin lymphoma. *Ann Intern Med* 1997; **127**: 423-428 [PMID: 9312998]
115 **Harris NL**, Jaffe ES, Stein H, Banks PM, Chan JK, Cleary ML, Delsol G, De Wolf-Peeters C, Falini B, Gatter KC. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. *Blood* 1994; **84**: 1361-1392 [PMID: 8068936]
116 **Herrinton LJ**. Epidemiology of the Revised European-American

Lymphoma Classification subtypes. *Epidemiol Rev* 1998; **20**: 187-203 [PMID: 9919438]

117 **Hwang JP**, Suarez-Almazor ME, Torres HA, Palla SL, Huang DS, Fisch MJ, Lok AS. Hepatitis C virus screening in patients with cancer receiving chemotherapy. *J Oncol Pract* 2014; **10**: e167-e174 [PMID: 24594679 DOI: 10.1200/JOP.2013.001215]

118 **Amin J**, Dore GJ, O'Connell DL, Bartlett M, Tracey E, Kaldor JM, Law MG. Cancer incidence in people with hepatitis B or C infection: a large community-based linkage study. *J Hepatol* 2006; **45**: 197-203 [PMID: 16684579 DOI: 10.1016/j.jhep.2006.02.014]

119 **Anderson LA**, Pfeiffer R, Warren JL, Landgren O, Gadalla S, Berndt SI, Ricker W, Parsons R, Wheeler W, Engels EA. Hematopoietic malignancies associated with viral and alcoholic hepatitis. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 3069-3075 [PMID: 18957521]

120 **Cavanna L**, Sbolli G, Tanzi E, Romanò L, Civardi G, Buscarini E, Vallisa D, Berté R, Rossi A. High prevalence of antibodies to hepatitis C virus in patients with lymphoproliferative disorders. *Haematologica* 1995; **80**: 486-487 [PMID: 8566896]

121 **Ferri C**, La Civita L, Monti M, Giannini C, Cecchetti R, Caracciolo F, Longombardo G, Lombardini F, Zignego AL. Chronic hepatitis C and B-cell non-Hodgkin's lymphoma. *QJM* 1996; **89**: 117-122 [PMID: 8729552]

122 **Isikdogan A**, Ayyildiz O, Dursun M, Tiftik N, Batun S, Muftuoglu E. Hepatitis C virus in patients with non-Hodgkin's lymphoma in southeastern Anatolia region of Turkey: a prospective case-control study of 119 patients. *Leuk Lymphoma* 2003; **44**: 1745-1747 [PMID: 14692528 DOI: 10.1080/1042819031000116724]

123 **Kocabaş E**, Aksaray N, Alhan E, Tanyeli A, Köksal F, Yarkin F. Hepatitis B and C virus infections in Turkish children with cancer. *Eur J Epidemiol* 1997; **13**: 869-873 [PMID: 9476814]

124 **Takai S**, Tsurumi H, Ando K, Kasahara S, Sawada M, Yamada T, Hara T, Fukuno K, Takahashi T, Oyama M, Onishi H, Tomita E, Takami T, Imawari M, Moriwaki H. Prevalence of hepatitis B and C virus infection in haematological malignancies and liver injury following chemotherapy. *Eur J Haematol* 2005; **74**: 158-165 [PMID: 15654908 DOI: 10.1111/j.1600-0609.2004.00376.x]

125 **Teng CJ**, Liu HT, Liu CY, Hsih CH, Pai JT, Gau JP, Liu JH, Chiou TJ, Hsu HC, Chen PM, Tzeng CH, Yu YB. Chronic hepatitis virus infection in patients with multiple myeloma: clinical characteristics and outcomes. *Clinics* (Sao Paulo) 2011; **66**: 2055-2061 [PMID: 22189730]

126 **Veneri D**, Franchini M, Zanotti R, Frattini F, Randon F, Rinaldi M, Pizzolo G. Prevalence of hepatitis C virus infection among patients with lymphoproliferative disorders: a single center survey. *Am J Hematol* 2007; **82**: 1031 [PMID: 17636475 DOI: 10.1002/ajh.20965]

127 **Yenice N**, Güllük F, Arican N, Türkmen S. HCV prevalence in Hodgkin and non-Hodgkin lymphoma cases. *Turk J Gastroenterol* 2003; **14**: 173-176 [PMID: 14655060]

128 **Dal Maso L**, Franceschi S. Hepatitis C virus and risk of lymphoma and other lymphoid neoplasms: a meta-analysis of epidemiologic studies. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 2078-2085 [PMID: 17119031]

129 **de Sanjose S**, Benavente Y, Vajdic CM, Engels EA, Morton LM, Bracci PM, Spinelli JJ, Zheng T, Zhang Y, Franceschi S, Talamini R, Holly EA, Grulich AE, Cerhan JR, Hartge P, Cozen W, Boffetta P, Brennan P, Maynadié M, Cocco P, Bosch R, Foretova L, Staines A, Becker N, Nieters A. Hepatitis C and non-Hodgkin lymphoma among 4784 cases and 6269 controls from the International Lymphoma Epidemiology Consortium. *Clin Gastroenterol Hepatol* 2008; **6**: 451-458 [PMID: 18387498 DOI: 10.1016/j.cgh.2008.02.011]

130 **Matsuo K**, Kusano A, Sugumar A, Nakamura S, Tajima K, Mueller NE. Effect of hepatitis C virus infection on the risk of non-Hodgkin's lymphoma: a meta-analysis of epidemiological studies. *Cancer Sci* 2004; **95**: 745-752 [PMID: 15471561]

131 **Merli M**, Visco C, Spina M, Luminari S, Ferretti VV, Gotti M, Rattotti S, Fiaccadori V, Rusconi C, Targhetta C, Stelitano C, Levis A, Ambrosetti A, Rossi D, Rigacci L, D'Arco AM, Musto P, Chiappella A, Baldini L, Bonfichi M, Arcaini L. Outcome prediction of diffuse large B-cell lymphomas associated with hepatitis C virus infection: a study on behalf of the Fondazione Italiana Linfomi. *Haematologica* 2014; **99**: 489-496 [PMID: 24270404 DOI: 10.3324/haematol.2013.094318]

132 **Pellicelli AM**, Marignani M, Zoli V, Romano M, Morrone A, Nosotti L, Barbaro G, Picardi A, Gentilucci UV, Remotti D, D'Ambrosio C, Furlan C, Mecenate F, Mazzoni E, Majolino I, Villani R, Andreoli A, Barbarini G. Hepatitis C virus-related B cell subtypes in non Hodgkin's lymphoma. *World J Hepatol* 2011; **3**: 278-284 [PMID: 22125661 DOI: 10.4254/wjh.v3.i11.278]

133 **Takeshita M**, Sakai H, Okamura S, Higaki K, Oshiro Y, Uike N, Yamamoto I, Shimamatsu K, Muranaka T. Prevalence of hepatitis C virus infection in cases of B-cell lymphoma in Japan. *Histopathology* 2006; **48**: 189-198 [PMID: 16405668 DOI: 10.1111/j.1365-2559.2005.02311.x]

134 **Caviglia GP**, Sciacca C, Abate ML, Olivero A, Rosso C, Touscoz GA, Ciancio A, Rizzetto M, Smedile A. Chronic hepatitis C virus infection and lymphoproliferative disorders: mixed cryoglobulinemia syndrome, monoclonal gammopathy of undetermined significance, and B-cell non-Hodgkin lymphoma. *J Gastroenterol Hepatol* 2015; **30**: 742-747 [PMID: 25351042 DOI: 10.1111/jgh.12837]

135 **De Renzo A**, Perna F, Persico M, Notaro R, Mainolfi C, de Sio I, Ciancia G, Picardi M, Del Vecchio L, Pane F, Rotoli B. Excellent prognosis and prevalence of HCV infection of primary hepatic and splenic non-Hodgkin's lymphoma. *Eur J Haematol* 2008; **81**: 51-57 [PMID: 18397390 DOI: 10.1111/j.1600-0609.2008.01081.x]

136 **Kashyap A**, Nademanee A, Molina A. Hepatitis C and B-cell lymphoma. *Ann Intern Med* 1998; **128**: 695 [PMID: 9537949]

137 **Kim JH**, Bang YJ, Park BJ, Yoo T, Kim CW, Kim TY, Heo DS, Lee HS, Kim NK. Hepatitis B virus infection and B-cell non-Hodgkin's lymphoma in a hepatitis B endemic area: a case-control study. *Jpn J Cancer Res* 2002; **93**: 471-477 [PMID: 12036441]

138 **Libra M**, Polesel J, Russo AE, De Re V, Cinà D, Serraino D, Nicoletti F, Spandidos DA, Stivala F, Talamini R. Extrahepatic disorders of HCV infection: a distinct entity of B-cell neoplasia? *Int J Oncol* 2010; **36**: 1331-1340 [PMID: 20428756]

139 **Markovic S**, Drozina G, Vovk M, Fidler-Jenko M. Reactivation of hepatitis B but not hepatitis C in patients with malignant lymphoma and immunosuppressive therapy. A prospective study in 305 patients. *Hepatogastroenterology* 1999; **46**: 2925-2930 [PMID: 10576374]

140 **Musolino C**, Campo S, Pollicino T, Squadrito G, Spatari G, Raimondo G. Evaluation of hepatitis B and C virus infections in patients with non-Hodgkin's lymphoma and without liver disease. *Haematologica* 1996; **81**: 162-164 [PMID: 8641648]

141 **Okan V**, Yilmaz M, Bayram A, Kis C, Cifci S, Buyukhatipoglu H, Pehlivan M. Prevalence of hepatitis B and C viruses in patients with lymphoproliferative disorders. *Int J Hematol* 2008; **88**: 403-408 [PMID: 18836791 DOI: 10.1007/s12185-008-0175-3]

142 **Pozzato G**, Mazzaro C, Crovatto M, Modolo ML, Ceselli S, Mazzi G, Sulfaro S, Franzin F, Tulissi P, Moretti M. Low-grade malignant lymphoma, hepatitis C virus infection, and mixed cryoglobulinemia. *Blood* 1994; **84**: 3047-3053 [PMID: 7949176]

143 **Pivetti S**, Novarino A, Merico F, Bertero MT, Brunetto MR, Bonino F, Caligaris-Cappio F. High prevalence of autoimmune phenomena in hepatitis C virus antibody positive patients with lymphoproliferative and connective tissue disorders. *Br J Haematol* 1996; **95**: 204-211 [PMID: 8857962]

144 **Salem AK**. Prevalence of HCV among Yemeni patients with non-Hodgkin's lymphoma at AI-Thawra teaching hospital. *Gulf J Oncolog* 2009; **(5)**: 22-29 [PMID: 20084782]

145 **Sánchez Ruiz AC**, Yebra Bango M, Portero F, Provencio Pulla M, Miralles Flores C, España Saz P. [Prevalence of hepatitis C virus infection in patients with non-Hodgkin's lymphoma]. *Med Clin* (Barc) 2001; **116**: 333-334 [PMID: 11333763]

146 **Swart A**, Burns L, Mao L, Grulich AE, Amin J, O'Connell DL, Meagher NS, Randall DA, Degenhardt L, Vajdic CM. The importance of blood-borne viruses in elevated cancer risk among opioid-dependent people: a population-based cohort study. *BMJ Open* 2012; **2**: e001755 [PMID: 23045358 DOI: 10.1136/

bmjopen-2012-001755]
147 **Abdel Wahab M**, Mostafa M, Salah T, Fouud A, Kandeel T, Elshobary M, Abd Allah OF, Elghawalby N, Sultan A, Ezzat F. Epidemiology of hilar cholangiocarcinoma in Egypt: single center study. *Hepatogastroenterology* 2007; **54**: 1626-1631 [PMID: 18019680]
148 **Kuper H**, Lagiou P, Mucci LA, Tamimi R, Benetou V, Trichopoulos D. Risk factors for cholangiocarcinoma in a low risk Caucasian population. *Soz Praventivmed* 2001; **46**: 182-185 [PMID: 11565447]
149 **Lu H**, Ye MQ, Thung SN, Dash S, Gerber MA. Detection of hepatitis C virus RNA sequences in cholangiocarcinomas in Chinese and American patients. *Chin Med J* (Engl) 2000; **113**: 1138-1141 [PMID: 11776153]
150 **Mohammad-Alizadeh AH**, Ghobakhlou M, Shalmani HM, Zali MR. Cholangiocarcinoma: an-eight-year experience in a tertiary-center in Iran. *Asian Pac J Cancer Prev* 2012; **13**: 5381-5384 [PMID: 23317187]
151 **Peng NF**, Li LQ, Qin X, Guo Y, Peng T, Xiao KY, Chen XG, Yang YF, Su ZX, Chen B, Su M, Qi LN. Evaluation of risk factors and clinicopathologic features for intrahepatic cholangiocarcinoma in Southern China: a possible role of hepatitis B virus. *Ann Surg Oncol* 2011; **18**: 1258-1266 [PMID: 21207172 DOI: 10.1245/s10434-010-1458-5]
152 **Shin HR**, Lee CU, Park HJ, Seol SY, Chung JM, Choi HC, Ahn YO, Shigemastu T. Hepatitis B and C virus, Clonorchis sinensis for the risk of liver cancer: a case-control study in Pusan, Korea. *Int J Epidemiol* 1996; **25**: 933-940 [PMID: 8921477]
153 **Songsivilai S**, Dharakul T, Kanistanon D. Hepatitis C virus genotypes in patients with hepatocellular carcinoma and cholangiocarcinoma in Thailand. *Trans R Soc Trop Med Hyg* 1996; **90**: 505-507 [PMID: 8944256]
154 **Taguchi J**, Nakashima O, Tanaka M, Hisaka T, Takazawa T, Kojiro M. A clinicopathological study on combined hepatocellular and cholangiocarcinoma. *J Gastroenterol Hepatol* 1996; **11**: 758-764 [PMID: 8872774]
155 **Tao LY**, He XD, Qu Q, Cai L, Liu W, Zhou L, Zhang SM. Risk factors for intrahepatic and extrahepatic cholangiocarcinoma: a case-control study in China. *Liver Int* 2010; **30**: 215-221 [PMID: 19840244 DOI: 10.1111/j.1478-3231.2009.02149.x]
156 **Tomimatsu M**, Ishiguro N, Taniai M, Okuda H, Saito A, Obata H, Yamamoto M, Takasaki K, Nakano M. Hepatitis C virus antibody in patients with primary liver cancer (hepatocellular carcinoma, cholangiocarcinoma, and combined hepatocellular-cholangiocarcinoma) in Japan. *Cancer* 1993; **72**: 683-688 [PMID: 8192727]
157 **Uenishi T**, Nagano H, Marubashi S, Hayashi M, Hirokawa F, Kaibori M, Matsui K, Kubo S. The long-term outcomes after curative resection for mass-forming intrahepatic cholangiocarcinoma associated with hepatitis C viral infection: a multicenter analysis by Osaka Hepatic Surgery Study Group. *J Surg Oncol* 2014; **110**: 176-181 [PMID: 24760391 DOI: 10.1002/jso.23611]
158 **Wu TT**, Levy M, Correa AM, Rosen CB, Abraham SC. Biliary intraepithelial neoplasia in patients without chronic biliary disease: analysis of liver explants with alcoholic cirrhosis, hepatitis C infection, and noncirrhotic liver diseases. *Cancer* 2009; **115**: 4564-4575 [PMID: 19670455 DOI: 10.1002/cncr.24471]
159 **Yamamoto M**, Takasaki K, Nakano M, Saito A. Minute nodular intrahepatic cholangiocarcinoma. *Cancer* 1998; **82**: 2145-2149 [PMID: 9610693 DOI: 10.1002/(SICI)1097-0142(19980601)82:11]
160 **Yano Y**, Yamamoto J, Kosuge T, Sakamoto Y, Yamasaki S, Shimada K, Ojima H, Sakamoto M, Takayama T, Makuuchi M. Combined hepatocellular and cholangiocarcinoma: a clinicopathologic study of 26 resected cases. *Jpn J Clin Oncol* 2003; **33**: 283-287 [PMID: 12913082]
161 **Yin F**, Chen B. Detection of hepatitis C virus RNA sequences in hepatic portal cholangiocarcinoma tissue by reverse transcription polymerase chain reaction. *Chin Med J* (Engl) 1998; **111**: 1068-1070 [PMID: 11263365]
162 **Choi D**, Lim JH, Lee KT, Lee JK, Choi SH, Heo JS, Jang KT, Lee NY, Kim S, Hong ST. Cholangiocarcinoma and Clonorchis sinensis infection: a case-control study in Korea. *J Hepatol* 2006; **44**: 1066-1073 [PMID: 16480786 DOI: 10.1016/j.jhep.2005.11.040]
163 **Donato F**, Gelatti U, Tagger A, Favret M, Ribero ML, Callea F, Martelli C, Savio A, Trevisi P, Nardi G. Intrahepatic cholangiocarcinoma and hepatitis C and B virus infection, alcohol intake, and hepatolithiasis: a case-control study in Italy. *Cancer Causes Control* 2001; **12**: 959-964 [PMID: 11808716]
164 **Parkin DM**, Srivatanakul P, Khlat M, Chenvidhya D, Chotiwan P, Insiripong S, L'Abbé KA, Wild CP. Liver cancer in Thailand. I. A case-control study of cholangiocarcinoma. *Int J Cancer* 1991; **48**: 323-328 [PMID: 1645697]
165 **Shaib YH**, El-Serag HB, Davila JA, Morgan R, McGlynn KA. Risk factors of intrahepatic cholangiocarcinoma in the United States: a case-control study. *Gastroenterology* 2005; **128**: 620-626 [PMID: 15765398]
166 **Shaib YH**, El-Serag HB, Nooka AK, Thomas M, Brown TD, Patt YZ, Hassan MM. Risk factors for intrahepatic and extrahepatic cholangiocarcinoma: a hospital-based case-control study. *Am J Gastroenterol* 2007; **102**: 1016-1021 [PMID: 17324130 DOI: 10.1111/j.1572-0241.2007.01104.x]
167 **Yamamoto S**, Kubo S, Hai S, Uenishi T, Yamamoto T, Shuto T, Takemura S, Tanaka H, Yamazaki O, Hirohashi K, Tanaka T. Hepatitis C virus infection as a likely etiology of intrahepatic cholangiocarcinoma. *Cancer Sci* 2004; **95**: 592-595 [PMID: 15245596]
168 **Zhou YM**, Yin ZF, Yang JM, Li B, Shao WY, Xu F, Wang YL, Li DQ. Risk factors for intrahepatic cholangiocarcinoma: a case-control study in China. *World J Gastroenterol* 2008; **14**: 632-635 [PMID: 18203300]
169 **Barusrux S**, Nanok C, Puthisawas W, Pairojkul C, Poovorawan Y. Viral hepatitis B, C infection and genotype distribution among cholangiocarcinoma patients in northeast Thailand. *Asian Pac J Cancer Prev* 2012; **13** Suppl: 83-87 [PMID: 23480769]
170 **El-Serag HB**, Engels EA, Landgren O, Chiao E, Henderson L, Amaratunge HC, Giordano TP. Risk of hepatobiliary and pancreatic cancers after hepatitis C virus infection: A population-based study of U.S. veterans. *Hepatology* 2009; **49**: 116-123 [PMID: 19085911 DOI: 10.1002/hep.22606]
171 **Lee CH**, Chang CJ, Lin YJ, Yeh CN, Chen MF, Hsieh SY. Viral hepatitis-associated intrahepatic cholangiocarcinoma shares common disease processes with hepatocellular carcinoma. *Br J Cancer* 2009; **100**: 1765-1770 [PMID: 19436294 DOI: 10.1038/sj.bjc.6605063]
172 **Portolani N**, Baiocchi GL, Coniglio A, Piardi T, Grazioli L, Benetti A, Ferrari Bravo A, Giulini SM. Intrahepatic cholangiocarcinoma and combined hepatocellular-cholangiocarcinoma: a Western experience. *Ann Surg Oncol* 2008; **15**: 1880-1890 [PMID: 18443881 DOI: 10.1245/s10434-008-9933-y]
173 **Qu Z**, Cui N, Qin M, Wu X. Epidemiological survey of biomarkers of hepatitis virus in patients with extrahepatic cholangiocarcinomas. *Asia Pac J Clin Oncol* 2012; **8**: 83-87 [PMID: 22369448 DOI: 10.1111/j.1743-7563.2011.01466.x]
174 **Sempoux C**, Jibara G, Ward SC, Fan C, Qin L, Roayaie S, Fiel MI, Schwartz M, Thung SN. Intrahepatic cholangiocarcinoma: new insights in pathology. *Semin Liver Dis* 2011; **31**: 49-60 [PMID: 21344350 DOI: 10.1055/s-0031-1272839]
175 **Srivatanakul P**, Honjo S, Kittiwatanachot P, Jedpiyawongse A, Khuhaprema T, Miwa M. Hepatitis viruses and risk of cholangiocarcinoma in northeast Thailand. *Asian Pac J Cancer Prev* 2010; **11**: 985-988 [PMID: 21133611]
176 **Welzel TM**, Graubard BI, El-Serag HB, Shaib YH, Hsing AW, Davila JA, McGlynn KA. Risk factors for intrahepatic and extrahepatic cholangiocarcinoma in the United States: a population-based case-control study. *Clin Gastroenterol Hepatol* 2007; **5**: 1221-1228 [PMID: 17689296 DOI: 10.1016/j.cgh.2007.05.020]
177 **Hai S**, Kubo S, Yamamoto S, Uenishi T, Tanaka H, Shuto T, Takemura S, Yamazaki O, Hirohashi K. Clinicopathologic characteristics of hepatitis C virus-associated intrahepatic cholangiocarcinoma. *Dig Surg* 2005; **22**: 432-439 [PMID: 16479112 DOI: 10.1159/000091446]
178 **Nuzzo G**, Giuliante F, Ardito F, De Rose AM, Vellone M, Clemente

G, Chiarla C, Giovannini I. Intrahepatic cholangiocarcinoma: prognostic factors after liver resection. *Updates Surg* 2010; **62**: 11-19 [PMID: 20845096 DOI: 10.1007/s13304-010-0007-x]

179 **Tanaka M**, Tanaka H, Tsukuma H, Ioka A, Oshima A, Nakahara T. Risk factors for intrahepatic cholangiocarcinoma: a possible role of hepatitis B virus. *J Viral Hepat* 2010; **17**: 742-748 [PMID: 20002305 DOI: 10.1111/j.1365-2893.2009.01243.x]

180 **Torbenson M**, Yeh MM, Abraham SC. Bile duct dysplasia in the setting of chronic hepatitis C and alcohol cirrhosis. *Am J Surg Pathol* 2007; **31**: 1410-1413 [PMID: 17721197 DOI: 10.1097/PAS.0b013e318053d122]

181 **Zuo HQ**, Yan LN, Zeng Y, Yang JY, Luo HZ, Liu JW, Zhou LX. Clinicopathological characteristics of 15 patients with combined hepatocellular carcinoma and cholangiocarcinoma. *Hepatobiliary Pancreat Dis Int* 2007; **6**: 161-165 [PMID: 17374575]

182 **Lee MH**, Yang HI, Lu SN, Jen CL, You SL, Wang LY, Wang CH, Chen WJ, Chen CJ. Chronic hepatitis C virus infection increases mortality from hepatic and extrahepatic diseases: a community-based long-term prospective study. *J Infect Dis* 2012; **206**: 469-477 [PMID: 22811301 DOI: 10.1093/infdis/jis385]

183 **Shin HR**, Oh JK, Masuyer E, Curado MP, Bouvard V, Fang YY, Wiangnon S, Sripa B, Hong ST. Epidemiology of cholangiocarcinoma: an update focusing on risk factors. *Cancer Sci* 2010; **101**: 579-585 [PMID: 20085587 DOI: 10.1111/j.1349-7006.2009.01458.x]

184 **Zhou Y**, Zhao Y, Li B, Huang J, Wu L, Xu D, Yang J, He J. Hepatitis viruses infection and risk of intrahepatic cholangiocarcinoma: evidence from a meta-analysis. *BMC Cancer* 2012; **12**: 289 [PMID: 22799744 DOI: 10.1186/1471-2407-12-289]

185 **Jarnagin WR**, Weber S, Tickoo SK, Koea JB, Obiekwe S, Fong Y, DeMatteo RP, Blumgart LH, Klimstra D. Combined hepatocellular and cholangiocarcinoma: demographic, clinical, and prognostic factors. *Cancer* 2002; **94**: 2040-2046 [PMID: 11932907 DOI: 10.1002/cncr.10392]

186 **Liu X**, Zou S, Qiu F. Pathogenesis of hilar cholangiocarcinoma and infection of hepatitis virus. *Zhonghua Wai Ke Za Zhi* 2002; **40**: 420-422 [PMID: 12139797]

187 **Shirakawa H**. Analysis of hepatitis C virus (HCV) genotypes in hepatocellular carcinoma. *Hokkaido Igaku Zasshi* 1996; **71**: 677-688 [PMID: 8996837]

188 **Zhang H**, Tang Y, Lu X. [Detection of hepatitis B virus DNA and hepatitis C virus RNA in human hepatocellular carcinoma by polymerase chain reaction]. *Zhonghua Bing Li Xue Za Zhi* 1996; **25**: 70-72 [PMID: 9206206]

189 **Zou SQ**, Liu XF, Guo RX, Li CL, Zhou XS, Zhu XG, Huang ZQ. The retrospective analysis of HBV and HCV infection in cholangiocarcinoma. *Zhonghua Wai Ke Za Zhi* 2003; **41**: 417-419 [PMID: 12895347]

190 **Palmer WC**, Patel T. Are common factors involved in the pathogenesis of primary liver cancers? A meta-analysis of risk factors for intrahepatic cholangiocarcinoma. *J Hepatol* 2012; **57**: 69-76 [PMID: 22420979 DOI: 10.1016/j.jhep.2012.02.022]

191 **Chantajitr S**, Wilasrusmee C, Lertsitichai P, Phromsopha N. Combined hepatocellular and cholangiocarcinoma: clinical features and prognostic study in a Thai population. *J Hepatobiliary Pancreat Surg* 2006; **13**: 537-542 [PMID: 17139428 DOI: 10.1007/s00534-006-1117-1]

192 **Hsing AW**, Zhang M, Rashid A, McGlynn KA, Wang BS, Niwa S, Ortiz-Conde BA, Goedert JJ, Fraumeni JF, O'Brien TR, Gao YT. Hepatitis B and C virus infection and the risk of biliary tract cancer: a population-based study in China. *Int J Cancer* 2008; **122**: 1849-1853 [PMID: 18076042 DOI: 10.1002/ijc.23251]

193 **Kobayashi M**, Ikeda K, Saitoh S, Suzuki F, Tsubota A, Suzuki Y, Arase Y, Murashima N, Chayama K, Kumada H. Incidence of primary cholangiocellular carcinoma of the liver in japanese patients with hepatitis C virus-related cirrhosis. *Cancer* 2000; **88**: 2471-2477 [PMID: 10861422]

194 **Matsumoto K**, Onoyama T, Kawata S, Takeda Y, Harada K, Ikebuchi Y, Ueki M, Miura N, Yashima K, Koda M, Sakamoto T, Endo M, Horie Y, Murawaki Y. Hepatitis B and C virus infection is a risk factor for the development of cholangiocarcinoma. *Intern Med* 2014; **53**: 651-654 [PMID: 24694471]

195 **Perumal V**, Wang J, Thuluvath P, Choti M, Torbenson M. Hepatitis C and hepatitis B nucleic acids are present in intrahepatic cholangiocarcinomas from the United States. *Hum Pathol* 2006; **37**: 1211-1216 [PMID: 16938527 DOI: 10.1016/j.humpath.2006.04.012]

196 **Malaguarnera M**, Gargante MP, Risino C, Ranno S, Berretta M, Cannizzaro MA, Costanzo M, Fricia T, Rampello E, Romano M. Hepatitis C virus in elderly cancer patients. *Eur J Intern Med* 2006; **17**: 325-329 [PMID: 16864006 DOI: 10.1016/j.ejim.2006.02.004]

197 **Omland LH**, Farkas DK, Jepsen P, Obel N, Pedersen L. Hepatitis C virus infection and risk of cancer: a population-based cohort study. *Clin Epidemiol* 2010; **2**: 179-186 [PMID: 20865115]

198 **Budakoğlu B**, Aksoy S, Arslan Ç, Üyetürk Ü, Babacan NA, Özcan MF, Yıldız R, Öven BB, Özdemir NY, Dizdar Ö, Büyükberber S, Akıncı MB, Türker I, Öksüzoğlu B, Altundag K, Zengin N. Frequency of HCV infection in renal cell carcinoma patients. *Med Oncol* 2012; **29**: 1892-1895 [PMID: 21461964 DOI: 10.1007/s12032-011-9928-6]

199 **Gonzalez HC**, Lamerato L, Rogers CG, Gordon SC. Chronic hepatitis C infection as a risk factor for renal cell carcinoma. *Dig Dis Sci* 2015; **60**: 1820-1824 [PMID: 25592719 DOI: 10.1007/s10620-015-3521-3]

200 **Gordon SC**, Moonka D, Brown KA, Rogers C, Huang MA, Bhatt N, Lamerato L. Risk for renal cell carcinoma in chronic hepatitis C infection. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 1066-1073 [PMID: 20332260]

201 **Hofmann JN**, Törner A, Chow WH, Ye W, Purdue MP, Duberg AS. Risk of kidney cancer and chronic kidney disease in relation to hepatitis C virus infection: a nationwide register-based cohort study in Sweden. *Eur J Cancer Prev* 2011; **20**: 326-330 [PMID: 21386707 DOI: 10.1097/CEJ.0b013e32834572fa]

202 **Bruno G**, Andreozzi P, Graf U, Santangelo G. Hepatitis C virus: a high risk factor for a second primary malignancy besides hepatocellular carcinoma. Fact or fiction? *Clin Ter* 1999; **150**: 413-418 [PMID: 10756661]

203 **Macleod LC**, Hotaling JM, Wright JL, Davenport MT, Gore JL, Harper J, White E. Risk factors for renal cell carcinoma in the VITAL study. *J Urol* 2013; **190**: 1657-1661 [PMID: 23665301 DOI: 10.1016/j.juro.2013.04.130]

204 **Xu JH**, Fu JJ, Wang XL, Zhu JY, Ye XH, Chen SD. Hepatitis B or C viral infection and risk of pancreatic cancer: a meta-analysis of observational studies. *World J Gastroenterol* 2013; **19**: 4234-4241 [PMID: 23864789 DOI: 10.3748/wjg.v19.i26.4234]

205 **Ben Q**, Li Z, Liu C, Cai Q, Yuan Y, Wang K, Xiao L, Gao J, Zhang H. Hepatitis B virus status and risk of pancreatic ductal adenocarcinoma: a case-control study from China. *Pancreas* 2012; **41**: 435-440 [PMID: 22422136 DOI: 10.1097/MPA.0b013e31822ca176]

206 **Chang MC**, Chen CH, Liang JD, Tien YW, Hsu C, Wong JM, Chang YT. Hepatitis B and C viruses are not risks for pancreatic adenocarcinoma. *World J Gastroenterol* 2014; **20**: 5060-5065 [PMID: 24803819 DOI: 10.3748/wjg.v20.i17.5060]

207 **Hassan MM**, Li D, El-Deeb AS, Wolff RA, Bondy ML, Davila M, Abbruzzese JL. Association between hepatitis B virus and pancreatic cancer. *J Clin Oncol* 2008; **26**: 4557-4562 [PMID: 18824707 DOI: 10.1200/JCO.2008.17.3526]

208 **Huang J**, Magnusson M, Törner A, Ye W, Duberg AS. Risk of pancreatic cancer among individuals with hepatitis C or hepatitis B virus infection: a nationwide study in Sweden. *Br J Cancer* 2013; **109**: 2917-2923 [PMID: 24178755 DOI: 10.1038/bjc.2013.689]

209 **Woo SM**, Joo J, Lee WJ, Park SJ, Han SS, Kim TH, Koh YH, Kim HB, Hong EK. Risk of pancreatic cancer in relation to ABO blood group and hepatitis C virus infection in Korea: a case-control study. *J Korean Med Sci* 2013; **28**: 247-251 [PMID: 23400555 DOI: 10.3346/jkms.2013.28.2.247]

210 **Zhu F**, Li HR, Du GN, Chen JH, Cai SR. Chronic hepatitis B virus infection and pancreatic cancer: a case-control study in southern China. *Asian Pac J Cancer Prev* 2011; **12**: 1405-1408 [PMID: 22126472]

211 **Fiorino S**, Chili E, Bacchi-Reggiani L, Masetti M, Deleonardi G,

Grondona AG, Silvestri T, Magrini E, Zanini N, Cuppini A, Nardi R, Jovine E. Association between hepatitis B or hepatitis C virus infection and risk of pancreatic adenocarcinoma development: a systematic review and meta-analysis. *Pancreatology* 2013; **13**: 147-160 [PMID: 23561973 DOI: 10.1016/j.pan.2013.01.005]

212 **Xing S**, Li ZW, Tian YF, Zhang LM, Li MQ, Zhou P. Chronic hepatitis virus infection increases the risk of pancreatic cancer: a meta-analysis. *Hepatobiliary Pancreat Dis Int* 2013; **12**: 575-583 [PMID: 24322741]

213 **Hong SG**, Kim JH, Lee YS, Yoon E, Lee HJ, Hwang JK, Jung ES, Joo MK, Jung YK, Yeon JE, Park JJ, Kim JS, Bak YT, Byun KS. The relationship between hepatitis B virus infection and the incidence of pancreatic cancer: a retrospective case-control study. *Korean J Hepatol* 2010; **16**: 49-56 [PMID: 20375642 DOI: 10.3350/kjhep.2010.16.1.49]

214 **Xu P**, Huang Q, Liu C, Xie F, Shao F, Zhu C. Risk factors for pancreatic cancer: a case-control study. *Aizheng* 2011; **31**: 653-657

215 **Antonelli A**, Ferri C, Fallahi P, Nesti C, Zignego AL, Maccheroni M. Thyroid cancer in HCV-related mixed cryoglobulinemia patients. *Clin Exp Rheumatol* 2002; **20**: 693-696 [PMID: 12412202]

216 **Antonelli A**, Ferri C, Fallahi P, Pampana A, Ferrari SM, Barani L, Marchi S, Ferrannini E. Thyroid cancer in HCV-related chronic hepatitis patients: a case-control study. *Thyroid* 2007; **17**: 447-451 [PMID: 17542674 DOI: 10.1089/thy.2006.0194]

217 **Montella M**, Pezzullo L, Crispo A, Izzo F, Amore A, Marone U, Tamburini M, Ronga D, Chiofalo MG, Chiappetta G, Mozzillo N. Risk of thyroid cancer and high prevalence of hepatitis C virus. *Oncol Rep* 2003; **10**: 133-136 [PMID: 12469158]

218 **Larrey D**, Bozonnat MC, Kain I, Pageaux GP, Assenat E. Is chronic hepatitis C virus infection a risk factor for breast cancer? *World J Gastroenterol* 2010; **16**: 3687-3691 [PMID: 20677341]

219 **Su FH**, Chang SN, Chen PC, Sung FC, Su CT, Yeh CC. Association between chronic viral hepatitis infection and breast cancer risk: a nationwide population-based case-control study. *BMC Cancer* 2011; **11**: 495 [PMID: 22115285 DOI: 10.1186/1471-2407-11-495]

220 **Amin J**, Gidding H, Gilbert G, Backhouse J, Kaldor J, Dore G, Burgess M. Hepatitis C prevalence--a nationwide serosurvey. *Commun Dis Intell Q Rep* 2004; **28**: 517-521 [PMID: 15745402]

221 **Eftekharian A**, Khajavi M, Shokoofi S, Rahmani Z, Gachkar L, Gerami H, Rajati M, Khademi B. Hepatitis C virus in patients with squamous cell carcinoma of the head and neck in Iran: is there any relation? *Eur Arch Otorhinolaryngol* 2012; **269**: 2571-2573 [PMID: 22331258 DOI: 10.1007/s00405-012-1953-3]

222 **Gandolfo S**, Richiardi L, Carrozzo M, Broccoletti R, Carbone M, Pagano M, Vestita C, Rosso S, Merletti F. Risk of oral squamous cell carcinoma in 402 patients with oral lichen planus: a follow-up study in an Italian population. *Oral Oncol* 2004; **40**: 77-83 [PMID: 14662419]

223 **Nagao Y**, Sata M, Noguchi S, Seno'o T, Kinoshita M, Kameyama T, Ueno T. Detection of hepatitis C virus RNA in oral lichen planus and oral cancer tissues. *J Oral Pathol Med* 2000; **29**: 259-266 [PMID: 10890556]

224 **Nagao Y**, Sata M, Tanikawa K, Itoh K, Kameyama T. High prevalence of hepatitis C virus antibody and RNA in patients with oral cancer. *J Oral Pathol Med* 1995; **24**: 354-360 [PMID: 7500291]

225 **Nobles J**, Wold C, Fazekas-May M, Gilbert J, Friedlander PL. Prevalence and epidemiology of hepatitis C virus in patients with squamous cell carcinoma of the head and neck. *Laryngoscope* 2004; **114**: 2119-2122 [PMID: 15564831 DOI: 10.1097/01.mlg.0000149444.30017.e8]

226 **Su FH**, Chang SN, Chen PC, Sung FC, Huang SF, Chiou HY, Su CT, Lin CC, Yeh CC. Positive association between hepatitis C infection and oral cavity cancer: a nationwide population-based cohort study in Taiwan. *PLoS One* 2012; **7**: e48109 [PMID: 23133554 DOI: 10.1371/journal.pone.0048109]

227 **Takata Y**, Takahashi T, Fukuda J. Prevalence of hepatitis virus infection in association with oral diseases requiring surgery. *Oral Dis* 2002; **8**: 95-99 [PMID: 11991310]

228 **Hunt J**, Hagan J, Nobles J, Wold C, Fazekas-May M, Gilbert J, Friedlander PL. Outcome analysis of patients with squamous cell carcinoma of the head and neck and hepatitis C virus. *Laryngoscope* 2005; **115**: 1882-1886 [PMID: 16222215 DOI: 10.1097/01.mlg.0000177991.03841.27]

229 **International Agency for Research on Cancer**. Globocan 2012: Estimated cancer incidence, mortality and prevalence worldwide in 2012. World Health Organization, 2012

230 **Alvares-Da-Silva MR**, Francisconi CF, Waechter FL. Acute hepatitis C complicated by pancreatitis: another extrahepatic manifestation of hepatitis C virus? *J Viral Hepat* 2000; **7**: 84-86 [PMID: 10718948]

231 **Richardson A**. Is breast cancer caused by late exposure to a common virus? *Med Hypotheses* 1997; **48**: 491-497 [PMID: 9247892]

232 **Yasui Y**, Potter JD, Stanford JL, Rossing MA, Winget MD, Bronner M, Daling J. Breast cancer risk and "delayed" primary Epstein-Barr virus infection. *Cancer Epidemiol Biomarkers Prev* 2001; **10**: 9-16 [PMID: 11205495]

233 **Tsui JI**, Vittinghoff E, Shlipak MG, Bertenthal D, Inadomi J, Rodriguez RA, O'Hare AM. Association of hepatitis C seropositivity with increased risk for developing end-stage renal disease. *Arch Intern Med* 2007; **167**: 1271-1276 [PMID: 17592100 DOI: 10.1001/archinte.167.12.1271]

234 **Fayek S**, Moore D, Bortecen KH, Yeh H, Markmann JF, Olthoff KM, Shaked A. Liver transplantation in the setting of extra-hepatic malignancy: two case reports. *Transplant Proc* 2007; **39**: 3512-3514 [PMID: 18089424 DOI: 10.1016/j.transproceed.2007.09.007]

235 **Garcia JH**, Coelho GR, Cavalcante FP, Valença JT, Brasil IR, Cesar-Borges G, Costa PE, Viana CF, Rocha TD, Vasconcelos JB. Synchronous hepatocellular carcinoma and renal cell carcinoma in a liver transplant recipient: a case report. *Transplantation* 2007; **84**: 1713 [PMID: 18165787 DOI: 10.1097/01.tp.0000296469.78853.83]

236 **Di Micco B**, Di Micco P. HCV and renal cell carcinoma: a new insight between HCV and oncogenesis? Report of five cases. *Experimental Oncology* 2003; **25**: 77-78

237 **Nagao Y**, Sata M, Itoh K, Chiba I, Komiyama K, Yanoma S, Eura M, Tanikawa K, Kameyama T. High prevalence of hepatitis C virus antibody and RNA in patients with head and neck squamous cell carcinoma. *Hepatology Research* 1997; **7**: 206-212

238 **Carrozzo M**, Carbone M, Gandolfo S, Valente G, Colombatto P, Ghisetti V. An atypical verrucous carcinoma of the tongue arising in a patient with oral lichen planus associated with hepatitis C virus infection. *Oral Oncol* 1997; **33**: 220-225 [PMID: 9307733]

239 **Lo Muzio L**, Mignogna MD, Favia G, Procaccini M, Testa NF, Bucci E. The possible association between oral lichen planus and oral squamous cell carcinoma: a clinical evaluation on 14 cases and a review of the literature. *Oral Oncol* 1998; **34**: 239-246 [PMID: 9813716]

240 **Campisi G**, Fedele S, Lo Russo L, Di Fede O, Aricò P, Craxì A, Mignogna MD. HCV infection and oral lichen planus: a weak association when HCV is endemic. *J Viral Hepat* 2004; **11**: 465-470 [PMID: 15357654 DOI: 10.1111/j.1365-2893.2004.00532.x]

241 **Birkenfeld S**, Dreiher J, Weitzman D, Cohen AD. A study on the association with hepatitis B and hepatitis C in 1557 patients with lichen planus. *J Eur Acad Dermatol Venereol* 2011; **25**: 436-440 [PMID: 20666875 DOI: 10.1111/j.1468-3083.2010.03809.x]

242 **Lodi G**, Olsen I, Piattelli A, D'Amico E, Artese L, Porter SR. Antibodies to epithelial components in oral lichen planus (OLP) associated with hepatitis C virus (HCV) infection. *J Oral Pathol Med* 1997; **26**: 36-39 [PMID: 9021550]

243 **Petti S**, Rabiei M, De Luca M, Scully C. The magnitude of the association between hepatitis C virus infection and oral lichen planus: meta-analysis and case control study. *Odontology* 2011; **99**: 168-178 [PMID: 21505737 DOI: 10.1007/s10266-011-0008-3]

244 **Shengyuan L**, Songpo Y, Wen W, Wenjing T, Haitao Z, Binyou W. Hepatitis C virus and lichen planus: a reciprocal association determined by a meta-analysis. *Arch Dermatol* 2009; **145**: 1040-1047 [PMID: 19770446 DOI: 10.1001/archdermatol.2009.200]

245 **Carrozzo M**, Gandolfo S. Oral diseases possibly associated with hepatitis C virus. *Crit Rev Oral Biol Med* 2003; **14**: 115-127 [PMID: 12764074]

246 **Antonelli A**, Ferri C, Fallahi P. Thyroid cancer in patients with

hepatitis C infection. *JAMA* 1999; **281**: 1588 [PMID: 10235149]
247 **Montella M**, Crispo A, Pezzullo L, Izzo F, Fabbrocini G, Ronga D, Tamburini M. Is hepatitis C virus infection associated with thyroid cancer? A case-control study. *Int J Cancer* 2000; **87**: 611-612 [PMID: 10918206 DOI: 10.1002/1097-0215(20000815)87: ]
248 **Chen CJ**, Hsu WL, Yang HI, Lee MH, Chen HC, Chien YC, You SL. Epidemiology of virus infection and human cancer. *Recent Results Cancer Res* 2014; **193**: 11-32 [PMID: 24008291 DOI: 10.1007/978-3-642-38965-8_2]
249 **Fiorino S**, Bacchi-Reggiani L, Pontoriero L, Gallo C, Chili E, Masetti M, Zanini N, Grondona A, Silvestri T, Deleonardi G, Magrini E, Motta R, Fornelli A, Bondi A, de Biase D, Baccarini P, Tallini G, Tropeano A, Cuppini A, Castellani G, Jovine E. Pancreatic carcinoma development: new etiological and pathogenetic evidence. *Italian J Med* 2013; **7**: 242-252
250 **Andreone P**, Zignego AL, Cursaro C, Gramenzi A, Gherlinzoni F, Fiorino S, Giannini C, Boni P, Sabattini E, Pileri S, Tura S, Bernardi M. Prevalence of monoclonal gammopathies in patients with hepatitis C virus infection. *Ann Intern Med* 1998; **129**: 294-298 [PMID: 9729182]
251 **Sorensen HT**, Friis S, Olsen JH, Thulstrup AM, Mellemkjaer L, Linet M, Trichopoulos D, Vilstrup H, Olsen J. Risk of liver and other types of cancer in patients with cirrhosis: a nationwide cohort study in Denmark. *Hepatology* 1998; **28**: 921-925 [PMID: 9755226 DOI: 10.1002/hep.510280404]
252 **Peveling-Oberhag J**, Arcaini L, Hansmann ML, Zeuzem S. Hepatitis C-associated B-cell non-Hodgkin lymphomas. Epidemiology, molecular signature and clinical management. *J Hepatol* 2013; **59**: 169-177 [PMID: 23542089 DOI: 10.1016/j.jhep.2013.03.018]
253 **Zaret KS**. Genetic programming of liver and pancreas progenitors: lessons for stem-cell differentiation. *Nat Rev Genet* 2008; **9**: 329-340 [PMID: 18398419 DOI: 10.1038/nrg2318]
254 **Wandzioch E**, Zaret KS. Dynamic signaling network for the specification of embryonic pancreas and liver progenitors. *Science* 2009; **324**: 1707-1710 [PMID: 19556507 DOI: 10.1126/science.1174497]
255 **Huxley R**, Ansary-Moghaddam A, Berrington de González A, Barzi F, Woodward M. Type-II diabetes and pancreatic cancer: a meta-analysis of 36 studies. *Br J Cancer* 2005; **92**: 2076-2083 [PMID: 15886696 DOI: 10.1038/sj.bjc.6602619]
256 **Li D**. Diabetes and pancreatic cancer. *Mol Carcinog* 2012; **51**: 64-74 [PMID: 22162232 DOI: 10.1002/mc.20771]
257 **Chow YW**, Pietranico R, Mukerji A. Studies of oxygen binding energy to hemoglobin molecule. *Biochem Biophys Res Commun* 1975; **66**: 1424-1431 [PMID: 6 DOI: 10.2337/dc10-066]
258 **Han L**, Ma Q, Li J, Liu H, Li W, Ma G, Xu Q, Zhou S, Wu E. High glucose promotes pancreatic cancer cell proliferation via the induction of EGF expression and transactivation of EGFR. *PLoS One* 2011; **6**: e27074 [PMID: 22087246 DOI: 10.1371/journal.pone.0027074]
259 **Li J**, Ma Q, Liu H, Guo K, Li F, Li W, Han L, Wang F, Wu E. Relationship between neural alteration and perineural invasion in pancreatic cancer patients with hyperglycemia. *PLoS One* 2011; **6**: e17385 [PMID: 21386984 DOI: 10.1371/journal.pone.0017385]
260 **Liao WC**, Tu YK, Wu MS, Lin JT, Wang HP, Chien KL. Blood glucose concentration and risk of pancreatic cancer: systematic review and dose-response meta-analysis. *BMJ* 2015; **349**: g7371 [PMID: 25556126 DOI: 10.1136/bmj.g7371]
261 **White DL**, Ratziu V, El-Serag HB. Hepatitis C infection and risk of diabetes: a systematic review and meta-analysis. *J Hepatol* 2008; **49**: 831-844 [PMID: 18814931 DOI: 10.1016/j.jhep.2008.08.006]
262 **Naing C**, Mak JW, Ahmed SI, Maung M. Relationship between hepatitis C virus infection and type 2 diabetes mellitus: meta-analysis. *World J Gastroenterol* 2012; **18**: 1642-1651 [PMID: 22529694 DOI: 10.3748/wjg.v18.i14.1642]
263 **Tanaka H**, Imai Y, Hiramatsu N, Ito Y, Imanaka K, Oshita M, Hijioka T, Katayama K, Yabuuchi I, Yoshihara H, Inoue A, Kato M, Takehara T, Tamura S, Kasahara A, Hayashi N, Tsukuma H. Declining incidence of hepatocellular carcinoma in Osaka, Japan, from 1990 to 2003. *Ann Intern Med* 2008; **148**: 820-826 [PMID: 18519928]
264 **McGlynn KA**, Tarone RE, El-Serag HB. A comparison of trends in the incidence of hepatocellular carcinoma and intrahepatic cholangiocarcinoma in the United States. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 1198-1203 [PMID: 16775181 DOI: 10.1158/1055-9965.EPI-05-0811]
265 **Ito M**, Murakami K, Suzuki T, Mochida K, Suzuki M, Ikebuchi K, Yamaguchi K, Mizuochi T. Enhanced expression of lymphomagenesis-related genes in peripheral blood B cells of chronic hepatitis C patients. *Clin Immunol* 2010; **135**: 459-465 [PMID: 20189883 DOI: 10.1016/j.clim.2010.02.002]
266 **Mosad E**, Said Abd El-Rahman Allam M, Moustafa HM, Mohammed AE, El kebeer AM, Abdel-Moneim SS. Telomeric 1p36.3 deletion and Ki-67 expression in B-Non-Hodgkin's Lymphoma patients associated with chronic hepatitis C virus infection. *J Viral Hepat* 2014; **21**: 950-955 [PMID: 25158783 DOI: 10.1111/jvh.12304]
267 **Smedley D**, Sidhar S, Birdsall S, Bennett D, Herlyn M, Cooper C, Shipley J. Characterization of chromosome 1 abnormalities in malignant melanomas. *Genes Chromosomes Cancer* 2000; **28**: 121-125 [PMID: 10738310 DOI: 10.1002/(SICI)1098-2264(200005)28:1]
268 **Stoffel A**, Filippa D, Rao PH. The p73 locus is commonly deleted in non-Hodgkin's lymphomas. *Leuk Res* 2004; **28**: 1341-1345 [PMID: 15475076 DOI: 10.1016/j.leukres.2004.04.010]

**P- Reviewer**: Xu R, Zocco MA **S- Editor**: Yu J **L- Editor**: A
**E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12954

*World J Gastroenterol* 2015 December 7; 21(45): 12954-12962
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


META-ANALYSIS

# Hybrid therapy for *Helicobacter pylori* infection: A systemic review and meta-analysis

Ping-I Hsu, Pei-Chin Lin, David Y Graham

Ping-I Hsu, Division of Gastroenterology, Department of Internal Medicine, Kaohsiung Veterans General Hospital and National Yang-Ming University, Kaohsiung 813, Taiwan

Pei-Chin Lin, Department of Pharmacy, Kaohsiung Veterans General Hospital, Kaohsiung 813, Taiwan

Pei-Chin Lin, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, Taiwan

David Y Graham, Department of Medicine/Gastroenterology, Michael E. DeBakey Veterans Affairs Medical Center, and Baylor College of Medicine, Houston, TX 77030, United States

Author contributions: Hsu PI designed the study, reviewed the articles, analyzed the data and drafted the manuscript; Lin PC reviewed the articles and analyzed the data; and Graham DY designed the study and reviewed the manuscript.

Supported by Research Foundation of the Kaohsiung Veterans General Hospital, No. VGHKS103-58.

Conflict-of-interest statement: Hsu PI and Lin PC disclose no conflict of interest; Graham DY is an unpaid consultant for Novartis in relation to vaccine development for treatment or prevention of *Helicobacter pylori* (*H. pylori*) infection; Graham DY is also a paid consultant for RedHill Biopharma regarding novel *H. pylori* therapies and for Otsuka Pharmaceuticals regarding diagnostic testing; and Graham DY has received royalties from Baylor College of Medicine patents covering materials related to $^{13}$C-urea breath test.

Data sharing statement: No additional data are available.



Correspondence to: David Y Graham, Professor, Department of Medicine/Gastroenterology, Michael E. DeBakey Veterans Affairs Medical Center, and Baylor College of Medicine, Room 3A-320, 2002 Holcombe Blvd., Houston, TX 77030, United States. dgraham@bcm.tmc.edu
Telephone: +1-713-7950232
Fax: +1-713-7901040

Received: April 29, 2015
Peer-review started: May 12, 2015
First decision: July 19, 2015
Revised: August 7, 2015
Accepted: September 30, 2015
Article in press: September 30, 2015
Published online: December 7, 2015

## Abstract

**AIM:** To compare the effectiveness of hybrid therapy with other recommended regimens using meta-analysis.

**METHODS:** Bibliographical searches for randomized trials comparing hybrid and other therapies were performed in PubMed, the Cochrane Library and relevant congresses up to February 2015 using the following keywords (all fields and/or MeSH): ("*Helicobacter pylori*" or "*H. pylori*") and ("hybrid therapy" or "sequential-concomitant therapy"). Meta-analyses were performed with Cochrane Review Manager 5.1. The random effect model proposed by DerSimonian and Laird and the Mantel-Haenszel method were used to estimate the pooled relative risk and 95%CI of the efficacy outcomes between hybrid therapy and other eradication therapies.

**RESULTS:** Eight studies (2516 subjects) met entry criteria. The antimicrobial resistance in the study groups ranged from 6.9% to 23.5%. The mean cure rates of hybrid therapy by intention-to-treat (ITT) and *per-protocol* analyses were 88.5% ($n$ = 1207; range: 80.0% to 97.4%) and 93.3% ($n$ = 1109; range: 85.7% to

99.1%), respectively. Meta-analysis showed there was no significant difference in ITT eradication rate between hybrid and sequential therapy (relative risk: 1.01; 95%CI: 0.92-1.11). Subgroup analysis revealed hybrid therapy was more effective than sequential therapy in the non-Italian populations (95%CI: 1.01-1.18) and was only less effective in one, Italian population (95%CI: 0.83-0.98). There was no significant difference in eradication rate between hybrid therapy and concomitant therapy (95%CI: 0.93-1.02). No head-to-head comparisons of hybrid therapy and standard triple therapy or bismuth quadruple therapy were found. However, a multicenter, randomized trial showed that reverse hybrid therapy was superior to standard triple therapy (95.5% *vs* 88.6% ITT; $P = 0.011$).

**CONCLUSION:** Hybrid therapy appears to be an effective, safe, and well-tolerated treatment for *H. pylori* infection in the era of increasing antibiotic resistance.

**Key words:** *Helicobacter pylori*; Concomitant therapy; Hybrid therapy; Sequential therapy; Triple therapy



**Core tip:** This article is aimed to review current evidences of hybrid therapy for *Helicobacter pylori* infection. The mean cure rates of hybrid therapy by intention-to-treat and per-protocol analyses were 88.5% and 93.3%, respectively. Meta-analysis showed that hybrid therapy was more effective than sequential therapy in the non-Italian population. In contrast, it was less effective than sequential therapy in the Italian population. There was no significant difference in eradication rate between hybrid therapy and concomitant therapy. Reverse hybrid therapy is a new one-step tow-phase treatment, achieving a higher eradication rate than standard triple therapy with similar tolerability and less pharmaceutical cost.

Hsu PI, Lin PC, Graham DY. Hybrid therapy for *Helicobacter pylori* infection: A systemic review and meta-analysis. *World J Gastroenterol* 2015; 21(45): 12954-12962 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12954.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12954

## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) treatment continues to be a challenge for physicians as antimicrobial resistance has continued to increase worldwide in part due to overuse of antibiotics in medicine and agriculture[1]. While many international guidelines still recommend standard triple therapy as a first-line therapy they often include a caveat about the problem of increasing clarithromycin resistance[2-4]. Several recent large clinical trials and meta-analyses have shown that the eradication rate of standard triple therapy has generally declined to unacceptable levels (*i.e.*, 80% or less) with some European studies reporting failure rates of 25%-60%[5-7]. Several strategies including bismuth-containing and non-bismuth-containing quadruple therapies (including sequential, concomitant and hybrid therapies) have been shown to produce acceptable cure rates in the presence of clarithromycin resistance[8-10].

The standard hybrid regimen is functionally a combination of sequential therapy and concomitant therapy as it consists of a proton pump inhibitor (PPI) and amoxicillin for 10-14 d with the addition of clarithromycin and metronidazole for the final 7 d (*i.e.*, a 2 + 4 regimen) (Figure 1). The original study[11] produced an eradication rate of 99.1% (95%CI: 97.3%-100.9%) by per-protocol (PP) analysis and 97.4% (95%CI: 94.5%-100.3%) by intention-to-treat (ITT) analysis. Several subsequent randomized trials confirmed that hybrid regimens were either comparable with or more effective than sequential therapy[12-15]. A recent large multicenter randomized trial in areas with high clarithromycin and metronidazole resistance confirmed that both 14-d hybrid and concomitant therapies cured more than 90% of *H. pylori* infections[16]. Additionally, a recent clinical trial from Taiwan tested the efficacy of a 12-d reverse hybrid therapy (a 4 + 2 regimen) (Figure 1) and achieved grade A success (≥ 95% cure rate) in an area with moderately high clarithromycin resistance (13%) and high metronidazole resistance (31%)[17]. Recent expert recommendations have proposed hybrid therapy as a treatment option for *H. pylori* in areas with moderate or high clarithromycin resistance[1,18,19].

This article reviewed the efficacy of hybrid therapy in the treatment of *H. pylori* infection and compared the treatment success of hybrid and other recommended regimens.

## MATERIALS AND METHODS

### Search strategy and data analysis

Bibliographical searches were performed in PubMed and the Cochrane Library up to February 2015 using the following keywords (all fields and/or MeSH): ("*Helicobacter pylori*" or "*H. pylori*") and ("hybrid therapy" or "sequential-concomitant therapy"). Articles published in any language were included. Reference lists from the trials selected in the electronic search were hand-searched to identify further relevant trials. We also conducted a manual search of abstract from the scientific meetings of the International Workshop of the American Digestive Disease Week, the United European Gastroenterology Week, the European Helicobacter Study Group and the Asian Pacific Digestive Week. Abstracts of the articles selected in each of these multiple searches were assessed by two reviewers. In case of duplicate reports or

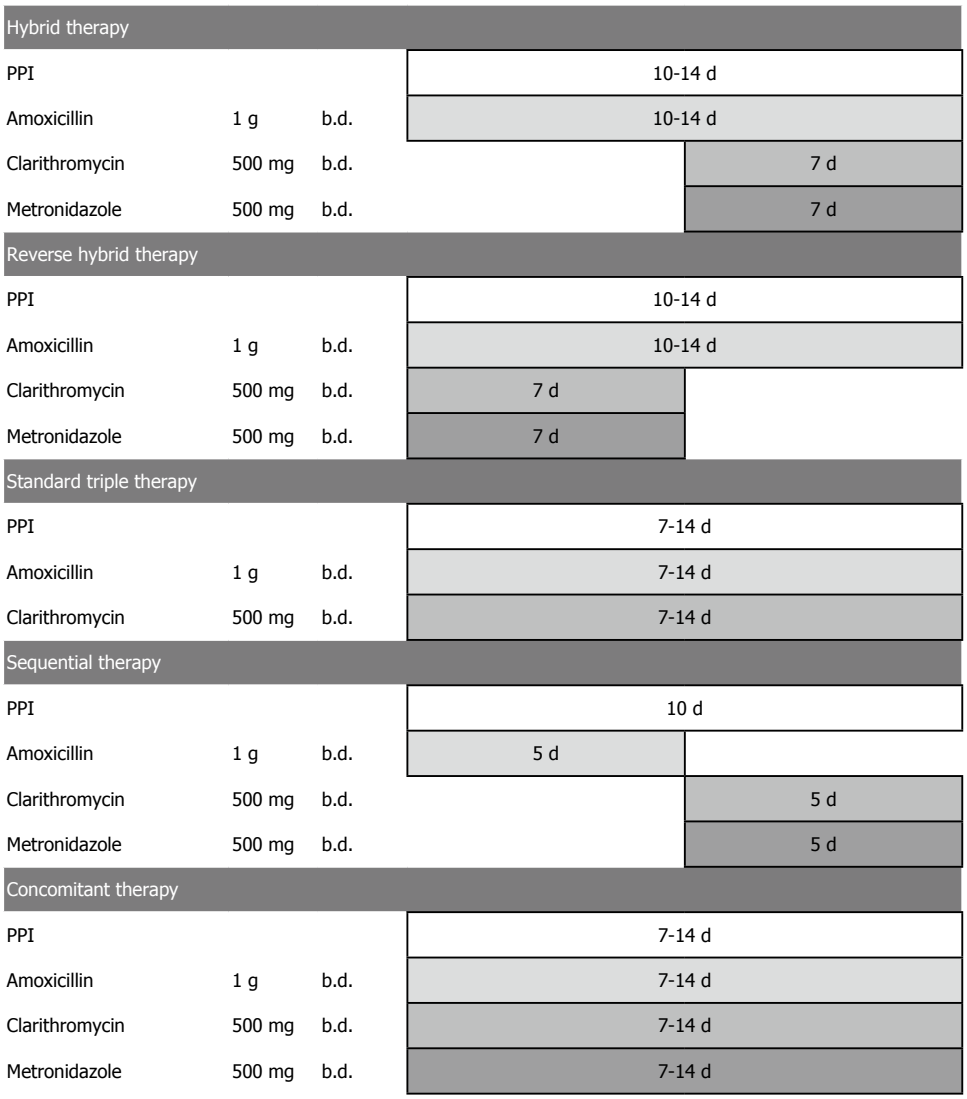



**Figure 1 Regimens of hybrid therapy, reverse hybrid therapy and other first-line anti-*Helicobacter pylori* therapies (standard triple therapy, sequential therapy, and concomitant therapy).** Standard hybrid therapy consists of a proton pump inhibitor and amoxicillin for 10-14 d, with addition of clarithromycin and metronidazole for the final 7 d; reverse hybrid therapy consists of a proton pump inhibitor and amoxicillin for 10-14 d, with addition of clarithromycin and metronidazole for the initial 7 d. PPI: Proton pump inhibitor.

studies obviously reporting results from the same study population, only the published results with largest numbers of cases were retrieved. All articles included were randomized controlled trials. All patients were *H. pylori* treatment naïve and had not used antibiotics or bismuth citrate in the preceding month. Nonrandomized studies were excluded, as were case reports, letters, editorials, commentaries, reviews, and abstracts with insufficient details to meet the inclusion criteria.

### *Statistical analysis*

Meta-analyses were performed with Cochrane Review Manager 5.1. [Review Manager (RevMan) (Computer program), Version 5.1, Copenhagen: the Nordic Cochrane Centre, the Cochrane Collaboration, 2011]. The random effect model proposed by DerSimonian and Laird[20] and the Mantel-Haenszel method[21] were used to estimate the pooled relative risk and 95%CI of the efficacy outcomes between hybrid therapy and other eradication therapies from eligible studies. The Mantel-Haenszel method was used for within-study variance and the DerSimonian and Laird method was used for incorporating both within-study and between study variances. Heterogeneity was assessed using Cochran's *Q* statistics and quantified using the $I^2$ statistics[22]. $I^2$ statistic represented the percentage of total variation attributable to between-study heterogeneity rather than sampling error. $I^2$ values of approximately 25%, 50%, and 75% were considered as representing low, moderate, and high heterogeneity, respectively.

## RESULTS

### *Rationale for use and histological perspective of the hybrid therapy*

In populations with low to absent clarithromycin

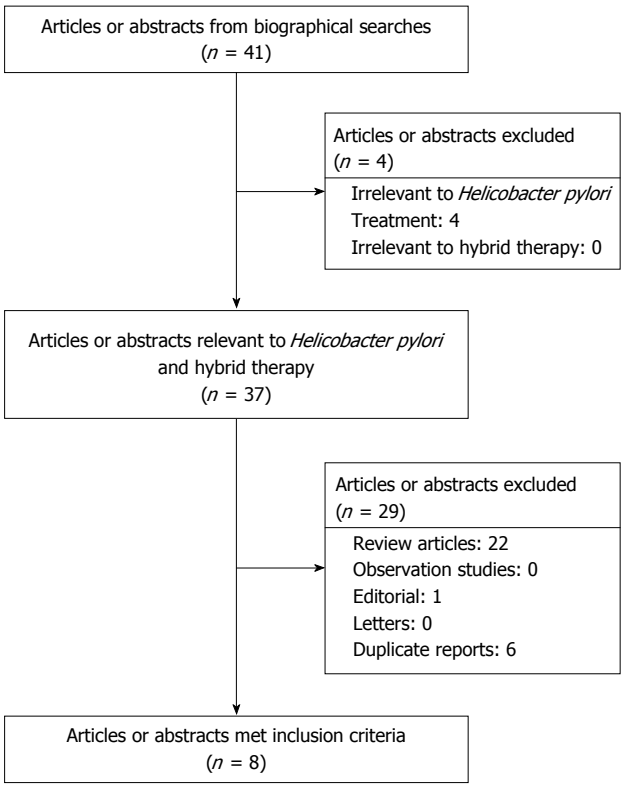



**Figure 2 Identification of eligible articles or abstracts.**

resistance, all modern clarithromycin regimens containing either 3 or 4 drugs reliably achieve cure rates of 90% or greater if given for 7 or more days. However, clarithromycin resistance compromises the efficacy of standard triple therapy and a number of studies have shown that sequential therapy (*i.e.*, a PPI plus amoxicillin for 5 d followed by a PPI plus clarithromycin and metronidazole or a 2 + 3 regimen) was more effective than standard triple therapy in areas of high clarithromycin resistance[23]. However in regions with high levels of metronidazole resistance the success rate with sequential therapy falls off significantly[24-26]. As there was no clear rationale for dropping the amoxicillin during the final portion of sequential therapy, a group of investigators from Taiwan and the United States examined whether continuing the amoxicillin through the second phase (*i.e.*, making a 2 + 4 regimen from sequential's 2 + 3 regimen) would increase the cure rate[11]. The initial study showed that 14-d hybrid therapy (a PPI plus amoxicillin for the first 7 d followed by a quadruple regimen with a PPI plus amoxicillin, clarithromycin and metronidazole for the final 7 d) achieved > 95% *H. pylori* eradication in a population with a low rate of clarithromycin resistance (7.0%). They then evaluated whether the duration of hybrid therapy could be reduced while maintaining a high eradication rate[27]. They randomized 220 *H. pylori*-infected subjects to 10-d, 12-d, or 14-d hybrid therapies consisting of esomeprazole 40 mg and amoxicillin 1 gm twice daily for 10, 12, or 14 d plus clarithromycin 500 mg, and metronidazole 500 mg twice daily for the final 7 d. The population studied had a clarithromycin resistance of 6.9% and metronidazole resistance rate of 31.0%. The eradication rates with PP analyses were similar: 95.0% for 10-d, 95.1% for 12-d, and 93.4% for 14-d hybrid therapies[27]. These results suggested that in contrast to sequential therapy, hybrid therapy was highly successful despite a high prevalence of metronidazole resistance.

Switching drugs halfway through the course increases the complexity of hybrid therapy. Reversing the sequence of drug administration of hybrid therapy simplifies the treatment and makes it become a one-step two-phase therapy (Figure 1). The study group therefore tested the efficacy of 12-d reverse hybrid therapy (*i.e.*, 4 drugs followed by 2 drugs)[17]. The 12-d reverse hybrid therapy achieved an eradication rate of 95.5% (191/200) and 95.9% (186/194) by intention-to-treat and per-protocol analysis, respectively in a population with 13% clarithromycin resistance and 32% metronidazole resistance.

### *Efficacy of the hybrid regimen for eradication of H. pylori*

Our initial search yielded 41 citations (Figure 2). Of these, four were excluded because they were irrelevant to *H. pylori* infection. Twenty-two review articles, one editorial and six duplicate reports were also excluded. Eight randomized controlled trials[11-17,27] were eligible for analysis. These eight studies were performed in Asia and Europe with clarithromycin, amoxicillin and metronidazole resistances ranging from 6.9% to 23.5%, 0% to 1.8% and 20.7% to 56.1%, respectively. Similar hybrid regimens were prescribed, with minor modifications, namely, the PPI used (esomeprazole, pantoprazole, and omeprazole), the nitroimidazole (metronidazole or tinidazole), the duration (between 10 and 14 d) and the sequence of drug administration (standard hybrid or reverse hybrid). The eradication rates in different geographic areas ranged from 85.7% to 99.1% by *per-protocol* (PP) analysis with of mean *H. pylori* cure rate of 93.3% (*n* = 1109; 95%CI: 91.8%-94.8%). The mean cure rate ITT was 88.5% (*n* = 1207; range: 80.0% to 97.4%; 95%CI: 86.7%-90.3%).

### *Adverse effects and compliance*

The prevalence of adverse effects of hybrid therapy ranged from 9.0% to 47.1%[11-17,27]. Overall, the mean prevalence of adverse effects was 24.7% (95%CI: 22.3%-27.1%). The profiles and frequencies of adverse events are listed in Table 1. The most frequent adverse effects were abdominal pain (1.4%-12.8%), diarrhea (0.5%-11.6%), taste perversion (1.0%-18.1%) and headache (0.5%-12.9%). Most adverse events were mild to moderate in severity. The frequency of adverse effects required the interruption of therapy was 3.6% (95%CI: 2.5%-4.6%; range: 2.0%-6.7%). In general,

**Table 1 List of adverse events reported in patients treated with the hybrid regimens**

| Adverse events | Frequency |
|---|---|
| Abdominal pain | 1.4%-12.8% |
| Diarrhea | 0.5%-11.6% |
| Constipation | 0%-8.6% |
| Taste perversion | 1.0%-18.1% |
| Headache | 0.5%-12.9% |
| Dizziness | 1.9%-5.1% |
| Nausea | 1.8%-7.5% |
| Vomiting | 0.5%-7.1% |
| Skin rash | 0%-2.9% |

hybrid therapy was well tolerated. The overall good compliance rate was 96.2% (95%CI: 95.1%-97.3%; range 93.8%-98.8%).

### Factors influencing the eradication rate of hybrid therapy

Antimicrobial resistance is a key factor determining *H. pylori* eradication rates[25,28-32]. Clarithromycin resistance negates the effect of clarithromycin reducing triple therapy to dual PPI amoxicillin therapy. A meta-analysis showed that with standard triple therapy clarithromycin resistance was associated with an average decline in eradication rate of approximately 60%[29]. Clarithromycin resistance also reduces the efficacy of sequential therapy but markedly less than with standard triple therapy likely because of the addition of a forth antimicrobial, metronidazole. A recent meta-analysis showed the overall eradication rate of sequential therapy in patients harboring strains resistant to clarithromycin was 72.8% (95%CI: 61.1%-82.8%)[32]. With regard to hybrid therapy, four recent prospective studies[11,16,17,27] reported that clarithromycin resistance had less effect on eradication rate of the new therapy [*i.e.*, susceptible = 99.1% (105/106) and resistant = 85.7% (12/14)], respectively. This difference did not achieve statistical significance but the number of strains with resistance was small. Importantly, the effect of metronidazole resistance on the efficacy of hybrid therapy also appeared minor 100% (68/68) and 94.2% (49/52) for clarithromycin-sensitive and resistant strains, respectively.

Sequential therapy is ineffective in patients with dual resistance (clarithromycin and metronidazole)[23]. Recently, Wu *et al*[33] investigated the effect of dual resistance on the efficacy of sequential and concomitant therapies and reported that it did not significantly influence the effectiveness of concomitant therapy. Overall, the number of clarithromycin- and metronidazole-dual resistant strains exposed to 14-d hybrid therapy was low (only 14 in the four studies with 10 being successfully eradicated). An accurate determination of the efficacy of hybrid therapy in dual resistant strains will require additional studies.

As with all antimicrobial therapies, good adherence to the regimen has also proven to be a significant predictor of successful eradication with hybrid therapy with cure rate of 91% and 50% in patients with compliance $\geqslant$ 80% and < 80%, respectively[16]. The cure rate achieved by hybrid therapy was also not significantly affected by the type of gastrointestinal disease (peptic ulcer *vs* non-ulcer dyspepsia), smoking, and *CYP2C19* genotype[11,17,26].

### Comparison of hybrid therapy and sequential therapy

Head-to-head comparisons of hybrid therapy and sequential therapy has been performed in 5 randomized trials[11-15,34]. A prospective randomized trial from Iran (a country with a high rate of clarithromycin and metronidazole resistance) demonstrated that 14-d hybrid therapy was superior to 10-d sequential therapy either by ITT (89.5% *vs* 76.7%, $P$ = 0.001) or PP analyses (92.9% *vs* 79.9%, $P$ = 0.001)[12]. The other four randomized controlled trials were done in populations with lower frequencies of resistance and reported comparable cure rates with hybrid and sequential therapies[11,13-15].

In all, 627 patients were treated 14-d hybrid therapy compared with 617 patients treated with sequential therapy lasting 10 or 14 d. Meta-analysis shows no significant differences in ITT eradication rate between 14-d hybrid and 10- to 14-d sequential therapy (Figure 3; pooled relative risk: 1.01; 95%CI: 0.92-1.11). Because high heterogeneity was noted between studies ($I^2$ = 80%, $P$ = 0.0005), we further performed subgroup analyses according to different geographic regions and treatment duration. The relative risk of eradication rate with ITT analysis was significantly different between the Italian and non-Italian populations (subgroup interaction test, $P$ = 0.0010). The hybrid therapy was more effective than sequential therapy in the non-Italian population (mean results: 89.9% *vs* 81.7%; relative risk = 1.09; 95%CI: 1.01-1.18; Figure 3). In contrast, hybrid therapy was less effective than sequential therapy in the Italian population (mean results: 81.5% *vs* 90.5%; relative risk = 0.90; 95%CI: 0.83-0.98). The PP analysis yielded similar results in the comparison of hybrid and sequential therapy for non-Italian populations (relative risk = 1.09; 95%CI: 1.01-1.18) with no significant difference in eradication rates between the two treatments in the Italian population (relative risk = 0.98; 95%CI: 0.90-1.08) by PP analysis. Possible explanations for the discrepancies included different antibiotic resistances of *H. pylori* strains, different treatment durations of sequential therapies, or variable adherence to the protocols.

With regard to the analysis by different treatment durations, there was a borderline significant trend toward higher ITT eradication rate with 14-d hybrid therapy (90.3%, 187/207) compared with 14-d sequential therapy (86.6%, 188/217) with the relative risk of 1.05 (95%CI: 1.00-1.11). However,

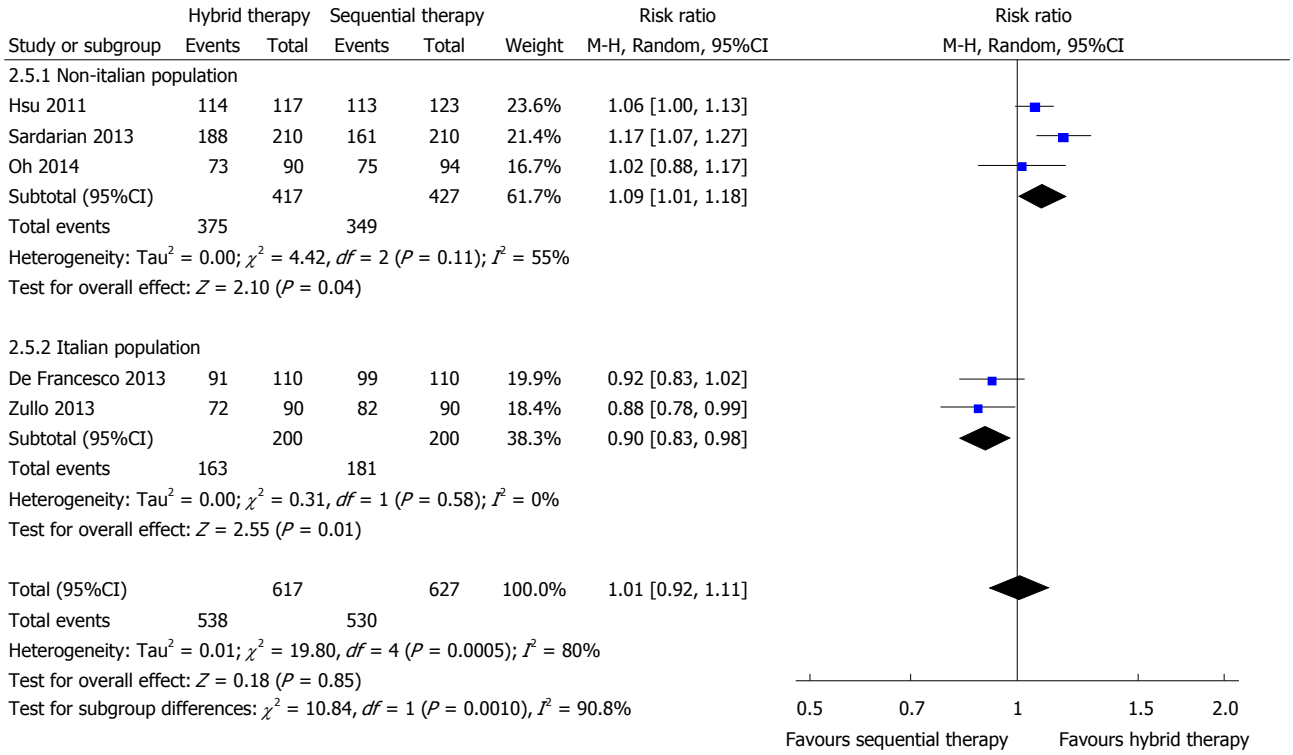



**Figure 3 Forest plot of hybrid therapy *vs* sequential therapy for *Helicobacter pylori* eradication; Estimates of odds ratio defined with the random effect model with 95%CI using intention-to-treat analysis.** The hybrid therapy was more effective than sequential therapy in the non-Italian population (relative risk = 1.09; 95%CI: 1.01-1.18). In contrast, the hybrid therapy was less effective than sequential therapy in the Italian population (relative risk = 0.90; 95%CI: 0.83-0.98).

there was no significant difference in eradication rate between 14-d hybrid therapy (83.7%, 343/410) and 10-d sequential therapy (83.4%, 342/410) (95%CI: 0.94-1.17).

Regarding tolerance, hybrid and sequential therapies exhibited comparable frequencies of adverse events (24.8% *vs* 24.4%, respectively) and drug compliance (95.4% *vs* 96.3%, respectively).

### *Comparison of hybrid therapy and concomitant therapy*

Concomitant therapy is a non-bismuth quadruple therapy proven successful in the presence of clarithromycin resistance[9,25]. It is a 4-drug regimen containing a PPI, clarithromycin, amoxicillin and metronidazole which are all given for the entire duration of therapy. A meta-analysis of 15 studies showed that a significant higher eradication rate was achieved with concomitant therapy compared to standard triple therapy in areas with high clarithromycin resistance[35]. A head-to-head non-inferiority trial of 10-d sequential and 10-d concomitant therapy showed they were equivalent (eradication rate: 92.3% *vs* 93.0%, respectively)[33]. From the perspective of clinical practice, the concomitant regimen is less complex than sequential or hybrid regimen, which is a two-step therapy.

All three randomized trials comparing the efficacies of hybrid therapy and concomitant therapy[13,14,16] showed that no differences in eradication rates, either by ITT or PP analyses, between 14-d hybrid therapy and 14-d concomitant therapy. A study from Italy[14] which compared markedly different durations of therapy (14-d hybrid therapy *vs* 5-d concomitant therapy) showed that 14-d hybrid therapy was superior by PP analysis (95.7% *vs* 85.1%). However, two meta-analyses of concomitant therapy reported a significant effect of duration on treatment success with concomitant therapy[9,34] such that the study had a significant bias.

In all, 370 patients were treated with 14-d hybrid therapy compared to 480 patients treated with concomitant therapy lasting 5 or 14 d. According to ITT analysis, the eradication rate was 85.4% (316/370; 95%CI: 81.8%-90.0%) for hybrid therapy and 86.3% (414/480; 95%CI: 83.2%-89.4%) for concomitant therapy lasting 5 or 14 d. As shown in Figure 4, there was no significant difference in eradication rate between 14-d hybrid therapy and 5 to 14-d concomitant therapy with the pooled relative risk is 0.98 (95%CI: 0.93-1.02). When the analysis excluded the 5-d concomitant studies, results with 14-d hybrid and 14-d concomitant therapy were still similar (relative risk = 0.97; 95%CI: 0.92-1.03).

Recently, a large multicenter randomized controlled trial showed that there was a borderline significant trend ($P$ = 0.05) toward higher compliance with 14-d hybrid therapy compared with 14-d concomitant therapy (98.8% *vs* 95.2%)[16]. In all, 14-d hybrid therapy and 5- to 14-d concomitant therapy had comparable compliance rates (95.9% *vs* 94.4%,

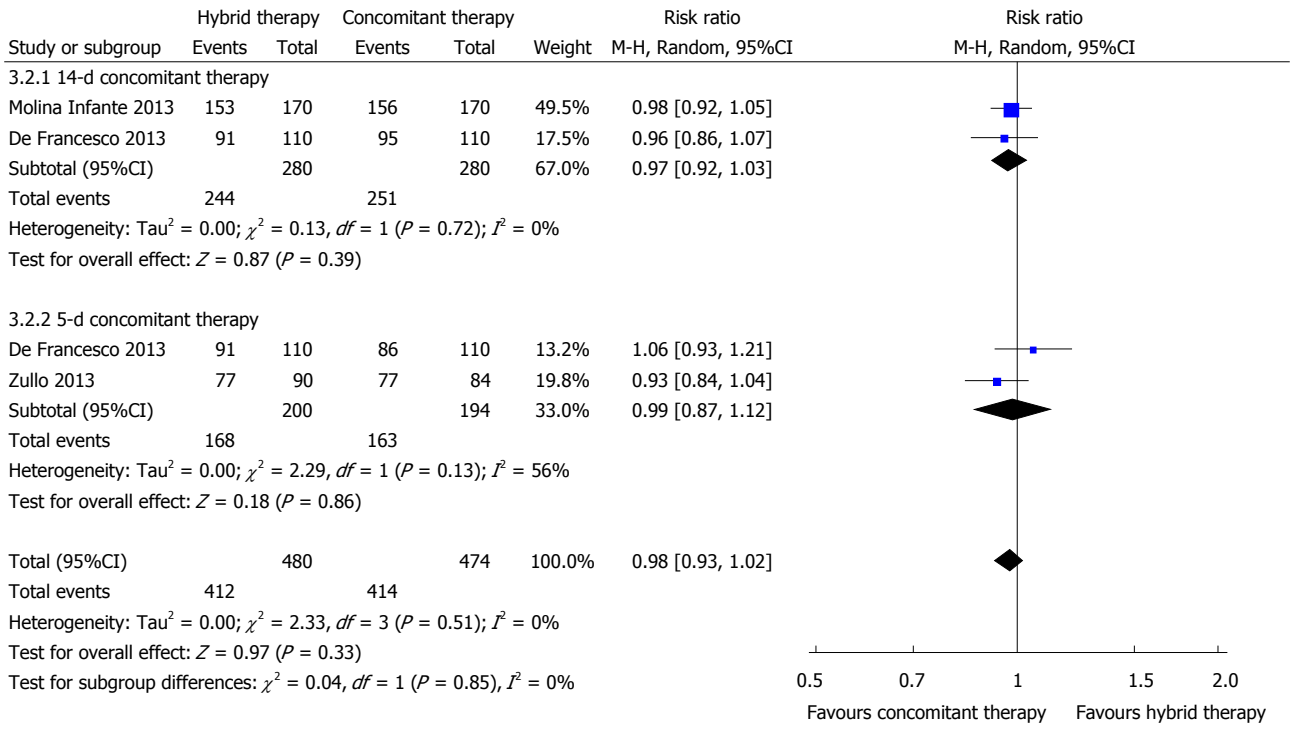



**Figure 4 Forest plot of hybrid therapy *vs* concomitant therapy according to intention-to-treat analysis.** There is no significant difference in eradication rate between 14-d hybrid therapy and 5 to 14-d concomitant therapy with the pooled relative risk is 0.98 (95%CI: 0.93-1.02).

respectively) and frequencies of adverse events (34.4% *vs* 37.1%, respectively).

### *Comparison of hybrid therapy and standard triple therapy*

Currently, there are no head-to-head studies comparing the eradication rate of hybrid therapy and standard triple therapy for *H. pylori* infection. However, the current data are consistent with the hypothesis that in regions with high clarithromycin resistance hybrid therapy should be superior to triple therapy. Hsu et al. recently conducted a multi-center, randomized controlled trial to compare the efficacies of 12-d reverse hybrid therapy and 12-d standard triple therapy in an area of moderate clarithromycin resistance (10%)[17]. The 12-d reverse hybrid therapy achieved a higher eradication rate than 12-d standard triple therapy (95.5% *vs* 88.6%) by ITT analysis. PP analysis showed similar results (95.9% *vs* 88.5%). The rates of resistance to clarithromycin, amoxicillin, and metronidazole in that study were 10%, 0% and 25%, respectively. As expected in the standard triple therapy group, the patients with clarithromycin-resistant strains had a lower eradication rate than those with clarithromycin-susceptible strains (28.6% *vs* 90.0%). In contrast, there were no significant differences in eradication rates between patients with clarithromycin-resistant and -susceptible strains (98.4% *vs* 100.0%) in the hybrid therapy group.

The ideal first-line treatment for *H. pylori* infection would be cheap and highly effective. The cost of the drugs for 12-d reverse hybrid therapy in Taiwan is nearly 6.8 dollars less than that for 12-d triple therapy (*i.e.*, $37.2 *vs* $44.0). The economic advantage is further strengthened by the consideration that 12-d reverse hybrid regimen is 6.9% more effective than 12-d standard triple therapy.

## DISCUSSION

Recently, the eradication rates of standard triple therapy have declined to less than 80% in many countries, largely owing to antimicrobial resistance. Several strategies including bismuth-containing quadruple therapy and non-bismuth-containing quadruple therapy (sequential, concomitant therapy or hybrid therapy) have been proposed to increase the eradication rates. Hybrid therapy is a novel therapeutic approach based on a combination of sequential therapy and concomitant therapy. More than 1200 patients have been treated with this regimen. The meta-analysis in this study showed that hybrid therapy was more effective than sequential therapy except in one Italian population. That conflicting result may be due to differences in antimicrobial resistances or in adherence with the regimens by the patients. Both hybrid therapy and concomitant therapy appear similarly highly effective. In conclusion, hybrid therapy appears to be an effective, safe, and well-tolerated treatment for *H. pylori* infection despite increasing antibiotic resistance.

## COMMENTS

### Background

Recently, the eradication rates of standard triple therapy have declined to less than 80% in many countries, owing to emerging organism resistances. Several strategies including sequential, concomitant therapy and hybrid therapy have been proposed to increase the eradication rate.

### Research frontiers

The pilot study of hybrid therapy showed that the novel treatment achieved an excellent eradication rate (99.1% by per-protocol and 97.4% by intention-to-treat analyses). It has therefore become a hot topic to compare the efficacy of hybrid therapy with other recommended regimens for *Helicobacter pylori* (*H. pylori*) eradication therapy.

### Innovations and breakthroughs

Meta-analysis showed there was no significant difference in eradication rate between hybrid and sequential therapy. Subgroup analysis revealed that hybrid therapy was more effective than sequential therapy in the non-Italian population. In contrast, it was less effective than sequential therapy in the Italian population. There was no significant difference in eradication rate between hybrid therapy and concomitant therapy. Reverse hybrid therapy is a new one-step tow-phase treatment, achieving a higher eradication rate than standard triple therapy with similar tolerability and less pharmaceutical cost.

### Applications

Hybrid and reverse hybrid therapies appear to be an effective, safe, and well-tolerated treatment for *H. pylori* infection in the era of increasing antibiotic resistance.

### Terminology

Hybrid therapy consists of a proton pump inhibitor and amoxicillin for 10-14 d, with addition of clarithromycin and metronidazole for the final 7 d. Reverse hybrid therapy consists of a proton pump inhibitor and amoxicillin for 10-14 d, with addition of clarithromycin and metronidazole for the initial 7 d.

### Peer-review

The authors performed a comprehensive review with meta-analysis for hybrid therapy. The results indicate that hybrid therapy can be recommended as a treatment option for the first-line therapy of *H. pylori* infection, especially in areas with moderate or high clarithromycin resistance.

## REFERENCES


1 **Rimbara E**, Fischbach LA, Graham DY. Optimal therapy for Helicobacter pylori infections. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 79-88 [PMID: 21293508 DOI: 10.1038/nrgastro.2010.210]

2 **Malfertheiner P**, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, Gensini GF, Gisbert JP, Graham DY, Rokkas T, El-Omar EM, Kuipers EJ; European Helicobacter Study Group. Management of Helicobacter pylori infection--the Maastricht IV/ Florence Consensus Report. *Gut* 2012; **61**: 646-664 [PMID: 22491499 DOI: 10.1136/gutjnl-2012-302084]

3 **Fock KM**, Katelaris P, Sugano K, Ang TL, Hunt R, Talley NJ, Lam SK, Xiao SD, Tan HJ, Wu CY, Jung HC, Hoang BH, Kachintorn U, Goh KL, Chiba T, Rani AA; Second Asia-Pacific Conference. Second Asia-Pacific Consensus Guidelines for Helicobacter pylori infection. *J Gastroenterol Hepatol* 2009; **24**: 1587-1600 [PMID: 19788600 DOI: 10.1111/j.1440-1746.2009.05982.x]

4 **Chey WD**, Wong BC. American College of Gastroenterology guideline on the management of Helicobacter pylori infection. *Am J Gastroenterol* 2007; **102**: 1808-1825 [PMID: 17608775]

5 **Graham DY**, Shiotani A. New concepts of resistance in the treatment of Helicobacter pylori infections. *Nat Clin Pract Gastroenterol Hepatol* 2008; **5**: 321-331 [PMID: 18446147 DOI: 10.1038/ncpgasthep1138]

6 **Gumurdulu Y**, Serin E, Ozer B, Kayaselcuk F, Ozsahin K, Cosar AM, Gursoy M, Gur G, Yilmaz U, Boyacioglu S. Low eradication rate of Helicobacter pylori with triple 7-14 days and quadriple therapy in Turkey. *World J Gastroenterol* 2004; **10**: 668-671 [PMID: 14991935]

7 **De Francesco V**, Margiotta M, Zullo A, Hassan C, Giorgio F, Burattini O, Stoppino G, Cea U, Pace A, Zotti M, Morini S, Panella C, Ierardi E. Prevalence of primary clarithromycin resistance in Helicobacter pylori strains over a 15 year period in Italy. *J Antimicrob Chemother* 2007; **59**: 783-785 [PMID: 17329269]

8 **Zullo A**, De Francesco V, Hassan C, Morini S, Vaira D. The sequential therapy regimen for Helicobacter pylori eradication: a pooled-data analysis. *Gut* 2007; **56**: 1353-1357 [PMID: 17566020]

9 **Essa AS**, Kramer JR, Graham DY, Treiber G. Meta-analysis: four-drug, three-antibiotic, non-bismuth-containing "concomitant therapy" versus triple therapy for Helicobacter pylori eradication. *Helicobacter* 2009; **14**: 109-118 [PMID: 19298338 DOI: 10.1111/j.1523-5378.2009.00671.x]

10 **Hsu PI**, Kao SS. Therapy of Helicobacter pylori Infection: Many Drugs for Which Association? In: Manfredi M, de'Angelis GL, editors. Helicobacter pylori. New York: Nova Science Publishers, Inc., 2013: 347-362

11 **Hsu PI**, Wu DC, Wu JY, Graham DY. Modified sequential Helicobacter pylori therapy: proton pump inhibitor and amoxicillin for 14 days with clarithromycin and metronidazole added as a quadruple (hybrid) therapy for the final 7 days. *Helicobacter* 2011; **16**: 139-145 [PMID: 21435092 DOI: 10.1111/j.1523-5378.2011.00828.x]

12 **Sardarian H**, Fakheri H, Hosseini V, Taghvaei T, Maleki I, Mokhtare M. Comparison of hybrid and sequential therapies for Helicobacter pylori eradication in Iran: a prospective randomized trial. *Helicobacter* 2013; **18**: 129-134 [PMID: 23121338 DOI: 10.1111/hel.12017]

13 **Zullo A**, Scaccianoce G, De Francesco V, Ruggiero V, D'Ambrosio P, Castorani L, Bonfrate L, Vannella L, Hassan C, Portincasa P. Concomitant, sequential, and hybrid therapy for H. pylori eradication: a pilot study. *Clin Res Hepatol Gastroenterol* 2013; **37**: 647-650 [PMID: 23747131 DOI: 10.1016/j.clinre.2013.04.003]

14 **De Francesco V**, Hassan C, Ridola L, Giorgio F, Ierardi E, Zullo A. Sequential, concomitant and hybrid first-line therapies for Helicobacter pylori eradication: a prospective randomized study. *J Med Microbiol* 2014; **63**: 748-752 [PMID: 24586031 DOI: 10.1099/jmm.0.072322-0]

15 **Oh DH**, Lee DH, Kang KK, Park YS, Shin CM, Kim N, Yoon H, Hwang JH, Jeoung SH, Kim JW, Jang ES, Jung HC. Efficacy of hybrid therapy as first-line regimen for Helicobacter pylori infection compared with sequential therapy. *J Gastroenterol Hepatol* 2014; **29**: 1171-1176 [PMID: 24955448]

16 **Molina-Infante J**, Romano M, Fernandez-Bermejo M, Federico A, Gravina AG, Pozzati L, Garcia-Abadia E, Vinagre-Rodriguez G, Martinez-Alcala C, Hernandez-Alonso M, Miranda A, Iovene MR, Pazos-Pacheco C, Gisbert JP. Optimized nonbismuth quadruple therapies cure most patients with Helicobacter pylori infection in populations with high rates of antibiotic resistance. *Gastroenterology* 2013; **145**: 121-128.e1 [PMID: 23562754 DOI: 10.1053/j.gastro.2013.03.050]

17 **Hsu PI**, Wu DC, Tsay, FW. Taiwan Acid Related Disease (TARD) Study Group. Reverse hybrid therapy versus standard triple therapy for Helicobacter pylori infection - A multi-center, randomized, controlled trial. *Medicine* 2015; in press

18 **Chuah SK**, Tsay FW, Hsu PI, Wu DC. A new look at anti-Helicobacter pylori therapy. *World J Gastroenterol* 2011; **17**: 3971-3975 [PMID: 22046084 DOI: 10.3748/wjg.v17.i35.3971]

19 **Georgopoulos SD**, Papastergiou V, Karatapanis S. Helicobacter pylori Eradication Therapies in the Era of Increasing Antibiotic Resistance: A Paradigm Shift to Improved Efficacy. *Gastroenterol Res Pract* 2012; **2012**: 757926 [PMID: 22778723 DOI: 10.1155/2012/757926]

20 **DerSimonian R**, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177-188 [PMID: 3802833]

21 **Mantel N**, Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. *J Natl Cancer Inst* 1959; **22**:

719-748 [PMID: 13655060]
22 **Higgins JP**, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med* 2002; **21**: 1539-1558 [PMID: 12111919]
23 **Vaira D**, Zullo A, Vakil N, Gatta L, Ricci C, Perna F, Hassan C, Bernabucci V, Tampieri A, Morini S. Sequential therapy versus standard triple-drug therapy for Helicobacter pylori eradication: a randomized trial. *Ann Intern Med* 2007; **146**: 556-563 [PMID: 17438314]
24 **Graham DY**, Lee YC, Wu MS. Rational Helicobacter pylori therapy: evidence-based medicine rather than medicine-based evidence. *Clin Gastroenterol Hepatol* 2014; **12**: 177-186.e3; Discussion e12-13 [PMID: 23751282 DOI: 10.1016/j.cgh.2013.05.028]
25 **Hsu PI**, Wu DC, Chen WC, Tseng HH, Yu HC, Wang HM, Kao SS, Lai KH, Chen A, Tsay FW. Randomized controlled trial comparing 7-day triple, 10-day sequential, and 7-day concomitant therapies for Helicobacter pylori infection. *Antimicrob Agents Chemother* 2014; **58**: 5936-5942 [PMID: 25070099 DOI: 10.1128/AAC.02922-14]
26 **Liou JM**, Chen CC, Chen MJ, Chen CC, Chang CY, Fang YJ, Lee JY, Hsu SJ, Luo JC, Chang WH, Hsu YC, Tseng CH, Tseng PH, Wang HP, Yang UC, Shun CT, Lin JT, Lee YC, Wu MS. Sequential versus triple therapy for the first-line treatment of Helicobacter pylori: a multicentre, open-label, randomised trial. *Lancet* 2013; **381**: 205-213 [PMID: 23158886 DOI: 10.1016/S0140-6736(12)61579-7]
27 **Wu JY**, Hsu PI, Wu DC, Graham DY, Wang WM. Feasibility of shortening 14-day hybrid therapy while maintaining an excellent Helicobacter pylori eradication rate. *Helicobacter* 2014; **19**: 207-213 [PMID: 24612093 DOI: 10.1111/hel.12113]
28 **Mégraud F**. H pylori antibiotic resistance: prevalence, importance, and advances in testing. *Gut* 2004; **53**: 1374-1384 [PMID: 15306603]
29 **Houben MH**, van de Beek D, Hensen EF, de Craen AJ, Rauws EA, Tytgat GN. A systematic review of Helicobacter pylori eradication therapy--the impact of antimicrobial resistance on eradication rates. *Aliment Pharmacol Ther* 1999; **13**: 1047-1055 [PMID: 10468680]
30 **Gisbert JP**, Calvet X, O'Connor A, Mégraud F, O'Morain CA. Sequential therapy for Helicobacter pylori eradication: a critical review. *J Clin Gastroenterol* 2010; **44**: 313-325 [PMID: 20054285 DOI: 10.1097/MCG.0b013e3181c8a1a3]
31 **Murakami K**, Fujioka T, Okimoto T, Sato R, Kodama M, Nasu M. Drug combinations with amoxycillin reduce selection of clarithromycin resistance during Helicobacter pylori eradication therapy. *Int J Antimicrob Agents* 2002; **19**: 67-70 [PMID: 11814770]
32 **Gatta L**, Vakil N, Vaira D, Scarpignato C. Global eradication rates for Helicobacter pylori infection: systematic review and meta-analysis of sequential therapy. *BMJ* 2013; **347**: f4587 [PMID: 23926315 DOI: 10.1136/bmj.f4587]
33 **Wu DC**, Hsu PI, Wu JY, Opekun AR, Kuo CH, Wu IC, Wang SS, Chen A, Hung WC, Graham DY. Sequential and concomitant therapy with four drugs is equally effective for eradication of H pylori infection. *Clin Gastroenterol Hepatol* 2010; **8**: 36-41.e1 [PMID: 19804842 DOI: 10.1016/j.cgh.2009.09.030]
34 **Hsu PI**, Wu DC, Wu JY, Graham DY. Is there a benefit to extending the duration of Helicobacter pylori sequential therapy to 14 days? *Helicobacter* 2011; **16**: 146-152 [PMID: 21435093 DOI: 10.1111/j.1523-5378.2011.00829.x]
35 **Gisbert JP**, Calvet X. Review article: non-bismuth quadruple (concomitant) therapy for eradication of Helicobater pylori. *Aliment Pharmacol Ther* 2011; **34**: 604-617 [PMID: 21745241 DOI: 10.1111/j.1365-2036.2011.04770.x]

**P- Reviewer:** Tovey FI **S- Editor**: Ma YJ **L- Editor**: A
**E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12963

*World J Gastroenterol* 2015 December 7; 21(45): 12963-12969
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


CASE REPORT

# First case report of exacerbated ulcerative colitis after anti-interleukin-6R salvage therapy

Raja Atreya, Ulrike Billmeier, Timo Rath, Jonas Mudter, Michael Vieth, Helmut Neumann, Markus F Neurath

Raja Atreya, Ulrike Billmeier, Timo Rath, Helmut Neumann, Markus F Neurath, Medical Clinic 1, Friedrich-Alexander University Erlangen-Nürnberg, D-91054 Erlangen, Germany

Jonas Mudter, Medical Department, Hospital Ostholstein, 23701 Eutin, Germany

Michael Vieth, Institute of Pathology, Klinikum Bayreuth, 95445 Bayreuth, Germany

Author contributions: Atreya R and Neurath MF designed the study; Atreya R, Billmeier U, Rath T, Mudter J, Vieth M, Neumann H and Neurath MF acquired the data; Atreya R, Billmeier U, Vieth M, Neumann H, Neurath MF analyzed the data; Atreya R, Billmeier U, Rath T, Mudter J, Vieth M, Neumann H and Neurath MF interpreted the data and wrote the paper.

Supported by Clinical Research Group 257 CEDER of the Deutsche Forschungsgemeinschaft.

Institutional review board statement: Ethics approval has been granted by the Ethics Committee of the Friedrich-Alexander Universität Erlangen-Nürnberg. It has approved analysis of the mucosal biopsies. The anti-IL-6R antibody (tocilizumab) was applied as compassionate use therapy in a patient with treatment refractory ulcerative colitis.

Informed consent statement: The patient provided informed written consent prior to study enrolment.

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



Correspondence to: Markus F Neurath, MD, Professor of Medicine, Medical Clinic 1, Friedrich-Alexander University Erlangen-Nürnberg, Ulmenweg 18, D-91054 Erlangen, Germany. markus.neurath@uk-erlangen.de
Telephone: +49-9131-8535000
Fax: +49-9131-8535209

Received: March 29, 2015
Peer-review started: March 30, 2015
First decision: June 2, 2015
Revised: June 30, 2015
Accepted: September 14, 2015
Article in press: September 14, 2015
Published online: December 7, 2015

## Abstract

We present the case of a 53-year-old woman with long-standing ulcerative colitis and severe, steroid-dependent disease course unresponsive to treatment with azathioprine, methotrexate, anti-TNF antibodies (infliximab, adalimumab) and tacrolimus, who refused colectomy as a therapeutic option. As the pro-inflammatory cytokine interleukin-6 (IL-6) had been identified as a crucial regulator in the immunopathogenesis of inflammatory bowel diseases, we treated the patient with biweekly intravenous infusions of an anti-IL-6R antibody (tocilizumab) for 12 wk. However, no clinical improvement of disease activity was noted. In fact, endoscopic, histological and endomicroscopic assessment demonstrated exacerbation of mucosal inflammation and ulcer formation upon anti-IL-6R therapy. Mechanistic studies revealed that tocilizumab treatment failed to suppress intestinal IL-6 production, impaired epithelial barrier function and induced production of pro-inflammatory cytokines such as TNF, IL-21 and IFN-γ. Inhibition of IL-6 by tocilizumab had no clinical benefit in this patient with intractable ulcerative colitis and even led to exacerbation of mucosal inflammation. Our findings suggest that anti-IL-6R antibody therapy may lead

to aggravation of anti-TNF resistant ulcerative colitis. When targeting IL-6, the differential responsiveness of target cells has to be taken into account, as IL-6 on the one side promotes acute and chronic mucosal inflammation *via* soluble IL-6R signaling but on the other side also strongly contributes to epithelial cell survival *via* membrane bound IL-6R signaling.

**Key words:** Ulcerative colitis; Interleukin-6; Epithelial barrier; Anti-interleukin-6R antibody; Inflammation; Endomicroscopy; Apoptosis; Cytokines



**Core tip:** Interleukin (IL)-6 is regarded as a pro-inflammatory cytokine in the immunopathogenesis of inflammatory bowel diseases. Unexpectedly, this first reported case describes that anti-IL-6R antibody treatment led to aggravated inflammation in a severe ulcerative colitis patient. Mechanistic studies revealed that anti-IL-6R treatment failed to suppress intestinal IL-6 production, impaired epithelial barrier function and induced production of pro-inflammatory cytokines. Our case report demonstrates that differential responsiveness of target cells has to be taken into account in therapeutic approaches, as IL-6 promotes mucosal inflammation *via* soluble IL-6R signaling, but also strongly contributes to epithelial cell survival *via* mIL-6R signaling.

Atreya R, Billmeier U, Rath T, Mudter J, Vieth M, Neumann H, Neurath MF. First case report of exacerbated ulcerative colitis after anti-interleukin-6R salvage therapy. *World J Gastroenterol* 2015; 21(45): 12963-12969 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12963.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12963

## INTRODUCTION

Ulcerative colitis (UC) is defined as a chronic relapsing inflammatory bowel disease (IBD) that is pathologically characterized by intestinal inflammation and epithelial injury. Insights into the immunopathogenesis of UC have implicated that pro-inflammatory cytokines are critically involved in the induction and perpetuation of the inflammatory process[1]. Targeted anti-cytokine therapies are therefore considered as an attractive treatment option, which is best reflected by the advent of anti-TNF antibodies as an efficacious treatment option[2]. Nevertheless, in the pivotal clinical trials for anti-TNF agents in UC, the initial response rate was approximately 60%, with a considerable proportion of these patients losing response within one year[3]. Therefore alternative cytokine targeted approaches are being sought after.

Interleukin-6 (IL-6) has been implicated to play an important role in the immunopathogenesis of IBD[4]. In agreement with this concept, mucosal IL-6 expression has been found to be elevated in active IBD[5]. Furthermore, serum-levels of IL-6 correlated with clinical disease activity in UC patients[6]. As these observations provide strong evidence for a potential functional role of IL-6 in chronic intestinal inflammation, we decided to treat an UC patient refractory to conventional therapies with a humanized anti-IL-6 receptor (IL-6R) antibody.

## CASE REPORT

The patient, a 53-year-old woman, was diagnosed with ulcerative pancolitis at the age of 28 years by histopathological criteria. She initially responded to combined therapy with oral (3 g) and local (2 g) aminosalicylates and later systemic corticosteroids, but showed recurrent inflammatory episodes in the following years. The patient developed a steroid-dependent disease course with a requirement for steroid therapy $\geqslant$ 10 mg/d. Azathioprine 100 mg (2 mg/kg) therapy was initiated in 2005, upon which clinical response was achieved for 6 mo. No endoscopic examinations were performed at that time to assess endoscopic response to azathioprine therapy. Upon subsequent relapses that required repeated prednisolone treatment, azathioprine treatment was stopped and methotrexate therapy was initiated in 2008 outside our clinic, but had to be discontinued due to severe skin reactions. Azathioprine therapy was again started thereafter, as the patient reported more aggravated disease without azathioprine therapy. Therapy with the anti-TNF antibody infliximab was initiated in 2010 in addition to azathioprine therapy due to chronic active disease. After an initial response for over one year, even an intensified therapy with infliximab (10 mg/kg every four weeks) failed to ameliorate UC activity and the treatment was stopped thereafter. Anti-TNF antibody therapy with adalimumab (initially 160 mg and 80 mg, then 40 mg every two weeks) in addition to ongoing azathioprine therapy likewise failed to ameliorate colitis activity and was stopped after 3 mo. Therapy with the calcineurin-inhibitor tacrolimus was initiated thereafter, but had to be discontinued due to impairment of renal function in 2013.

At this point the patient had up to 10 loose bowel movements per day with obvious blood. Blood count showed mild hypochromic anaemia (Hb 11.6 g/dL). C-reactive protein (CRP) levels were elevated (28.3 mg/L). The Truelove and Witts severity index indicated moderate disease. There was no tachycardia or pyrexia. Endoscopy revealed continuous colonic inflammation with enhanced granularity and isolated ulcerations (Figure 1A). The total Mayo score was 10, indicating severe disease. Endomicroscopic evaluation demonstrated dilated microvessels, leakage and disturbed crypt architecture as signs of mucosal inflammation (Figure 1C). Histopathological analysis

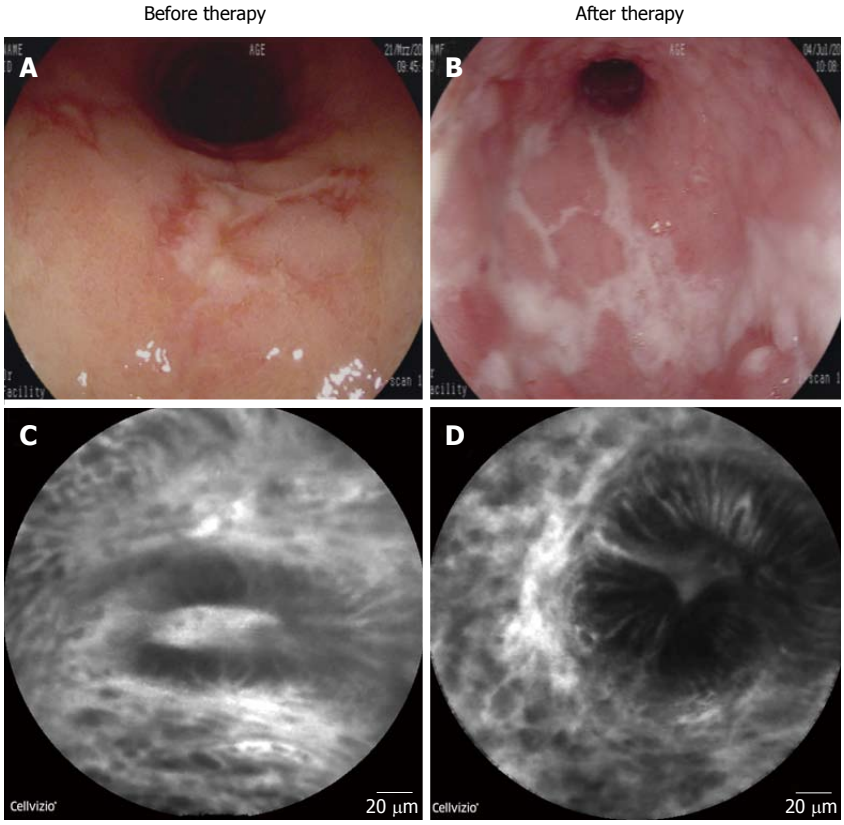



**Figure 1 Endoscopic and endomicroscopic evaluation of the mucosa before and after anti-interleukin-6R therapy.** Endoscopy of the sigmoid mucosa of the patient before anti-interleukin (IL)-6R therapy showed strong signs of mucosal inflammation (A); Mayo endoscopy score = 3; Evaluation of the sigmoid after 12 wk of tocilizumab treatment revealed augmented signs of mucosal inflammation and progressive ulcer formation. Mayo endoscopy score = 3 (B); Endomicroscopy with a probe based system (Mauna Kea System) of the sigmoid mucosa of the patient before (C); and after 12 wk of anti-IL-6R therapy (D). There was a marked increase in mucosal inflammation with increased leakage, dilatation of microvessels and disturbed crypt architecture.

of sigmoid biopsies by a pathologist resulted in a Riley histologic score[7] of 15 and a Geboes score of 0.3/1.3/2A.3/2B.3/3.2/4.3/5.4; both indicative of severe UC. An infection with cytomegalovirus (CMV) was repeatedly excluded and stool samples were always negative for infectious pathogens, including *Clostridium difficile*.

As the patient declined to undergo restorative proctocolectomy, no leucocytapheresis therapy was available nearby and the anti-adhesion molecule antibody vedolizumab had not been approved and was not available for therapy at that time, an anti-IL-6R antibody (tocilizumab) treatment was initiated with the understanding and appropriate prior informed consent of the patient. Azathioprine treatment was stopped beforehand.

Therapy with tocilizumab (Ro-Actemra, Hoffmann-La Roche, Switzerland) was intravenously administered (8 mg/kg) for 12 wk at biweekly intervals. Concomitant prednisolone therapy (10 mg/d) was unchanged during the treatment period. The therapy was well tolerated and no adverse events were recorded.

However, the patient showed no clinical improvement with persistence of 10 bloody stools per day. Blood count (Hb 12.5 g/dL) and CRP-level (1.2 mg/L) were normalised. Sigmoidoscopy up to 40 cm revealed augmented mucosal inflammation with progressive ulcer formation (Figure 1B). The total Mayo score remained at a score of 10. Endomicroscopy underlined increased signs of mucosal inflammation with enhanced leakage, impaired barrier function and disturbance of crypt architecture (Figure 1D). The Riley histologic score of sigmoid biopsies rose to 16 and the Geboes score was 0.2/1.3/2A.3/2B.3/3.2/4.3/5.4, both indicative of severe UC. As the patient refused surgical intervention and higher corticosteroid doses, she initially remained on prednisolone 10 mg/d. She later again needed intensified steroid treatment and was then put on therapy with the anti-adhesion molecule antibody vedolizumab. She showed partial clinical and endoscopic response to it. She still refused surgical intervention as a therapeutic option.

Immunohistochemistry revealed that tocilizumab treatment was not able to induce apoptosis in lamina propria mononuclear cells or in the subgroup of $CD4^+$ mucosal T cells, and there was a significantly reduced amount of apoptotic cells upon treatment (Figure 2 A-F). Quantitative gene expression analysis showed that tocilizumab application was not able to suppress mucosal IL-6 levels. Instead there were even higher IL-6 levels after completion of therapy, suggesting that blockade of IL-6 signalling may induce compensatory

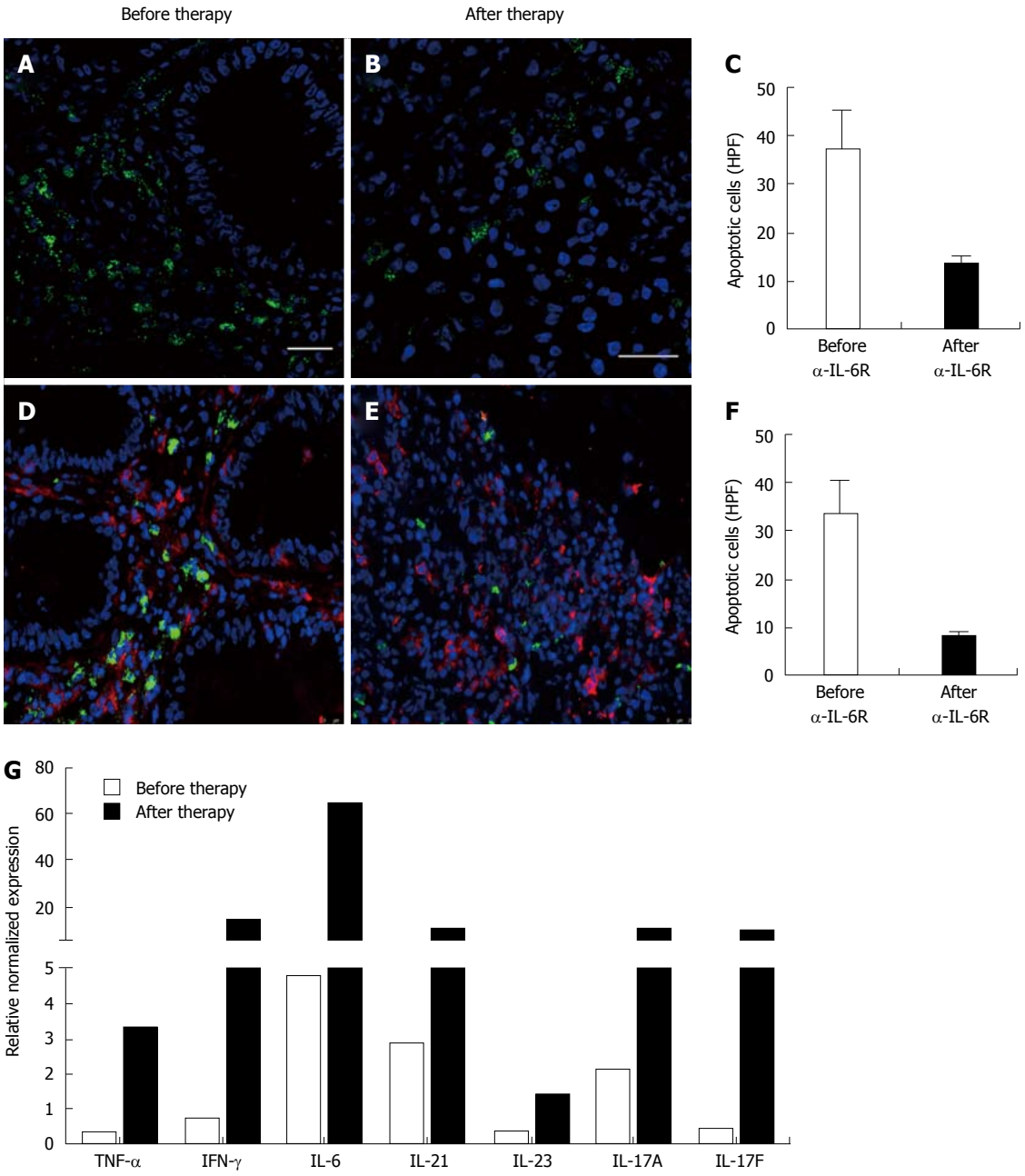



**Figure 2 Caspase positive cells and quantitative gene expression profile of mucosal biopsies before and after anti-interleukin-6R therapy.** Pairs of colonic tissue samples taken before (A) and after (B) anti-interleukin (IL)-6R therapy, were stained for caspase and assessed regarding caspase positive cells in 14 high-power fields per slide (C); Histological slides before (D) and after (E) IL-6R inhibition were also stained for caspase and CD4 and quantitatively assessed (F); There was a marked reduction of apoptotic LPMCs and mucosal T cells upon tocilizumab treatment. Nuclei were counterstained with DAPI. Scale bars represent 25 μm. Total RNA was isolated from mucosal biopsies taken before (white) and after (black) anti-IL-6R therapy and reverse transcribed (G). Gene expression profiles were analysed by quantitative PCR and normalized to the housekeeping-gene HPRT. There was a marked induction in the expression levels of pro-inflammatory cytokines upon anti-IL-6R treatment.

IL-6 production. Other pro-inflammatory cytokines such as TNF, IFN-γ, IL-21 and IL-17A/F also showed higher expression levels after completion of therapy compared to levels before commencement of anti-IL-6R treatment (Figure 2G).

Altogether, clinical, endoscopic, endomicroscopic and histologic evaluation demonstrated that IL-6R inhibition was not able to exert a therapeutic effect in our patient. Instead, signs of aggravated mucosal inflammation and ulcer formation could be observed upon anti-IL-6R treatment.

## DISCUSSION

Remarkable advances in our understanding of the immunpathogenesis of IBD have enabled the development of therapeutic agents directed at rational molecular targets. This approach is best exemplified by the selective blockade of pro-inflammatory cytokines by biological agents. The substantial

therapeutic success of anti-TNF antibodies in the treatment of UC patients has validated this concept. Nevertheless, a relevant subgroup of patients does not respond to anti-TNF therapy, as they show little or no changes of clinical symptoms[3]. Selective targeting of other cytokines profoundly involved in the immunopathogenesis of IBD has therefore been the centre of attention for designing novel therapeutic strategies. Recently completed phase 2 trials with antibodies directed against interleukin-13 however did not reach their primary therapeutic endpoints in the treatment of UC patients[8,9]. Similarly, inhibition of IL-2R signaling with the monoclonal antibody basiliximab did not result in improvement of disease activity[10].

The cytokine IL-6 has been found to be a major regulator of T cell differentiation and activation and promotes a pro-inflammatory milieu in chronic inflammatory diseases. In UC, serum levels of IL-6, which is an important factor for the synthesis of acute phase proteins like CRP, correlate well with clinical disease activity and correspondingly mucosal IL-6 and soluble IL-6R (sIL-6R) production are increased in active disease[11]. It has also been shown that effective treatment of IBD with corticosteroids resulted in an inhibition of IL-6 production in the lamina propria, while patients who suffer from intractable disease exhibit augmented mucosal IL-6 levels[12]. Finally, application of a neutralizing anti-IL6R antibody caused significant suppression of mucosal inflammation in different experimental colitis models[4,13].

As the above data indicated a pivotal role of IL-6 in chronic intestinal inflammation and targeting of IL-6 signaling had already shown therapeutic efficacy in chronic and autoimmune diseases like rheumatoid arthritis[14], a pilot study on IL-6R blockade was initiated in patients with active Crohn's disease. In this study, the anti-IL6R antibody tocilizumab or placebo was given as biweekly intravenous infusions over a treatment period of 12 wk. It was found that 80% of the tocilizumab treated patients had signs of clinical response, as compared with 31% in the placebo group. Moreover, 20% of the patients on this regimen went into remission as compared to 0% of the placebo group[15].

Based on these promising results, we initiated tocilizumab therapy in a patient with treatment-refractory UC. Unexpectedly, tocilizumab treatment did not ameliorate colitis activity and resulted in even aggravated mucosal inflammation and ulcer formation.

The complex of IL-6 and sIL-6R has been previously shown to activate gp130-positive lamina propria T cells lacking the membrane bound IL-6R in IBD. This so called trans-signalling process leads to STAT-3 activation and induction of anti-apoptotic genes like bcl-2 and bcl-xl within mucosal T cells. Augmented lamina propria T cell resistance against apoptosis then results in unrestrained accumulation of activated intestinal lymphocytes which perpetuate the inflammatory response[4]. In agreement with this anti-apoptotic role of IL-6, immunohistochemical stainings of colonic tissue samples from Crohn's disease patients treated with tocilizumab showed that inhibition of IL-6 results in the induction of apoptosis in lamina propria mononuclear cells (LPMCs)[15]. However, in our UC patient tocilizumab application did not lead to the induction of apoptosis in LPMCs or mucosal $CD4^+$ T cells, which is believed to be the central anti-inflammatory mechanism of action of anti-IL6R blockade in intestinal inflammation. Instead, we observed even a marked reduction of apoptosis in LPMCs after tocilizumab treatment compared to the staining prior to the initiation of therapy, suggesting that IL-6R blockade may predominantly cause pro-inflammatory effects in UC.

Apart from its pro-inflammatory role, IL-6 also contributes to the maintenance of epithelial cell homeostasis, as it is involved in epithelial repair and healing, resulting in mucosal reconstitution[16,17]. In contrast to IL-6, TNF has pro-inflammatory effects on the gut epithelium and may induce death of intestinal epithelial cells in IBD[18,19]. Thus, in contrast to TNF blockade, inhibition of IL-6 might have detrimental effects on the epithelial barrier function *via* blockade of the membrane bound IL-6R (mIL-6R) on intestinal epithelial cells. This adverse effect might be reflected by the reported heightened risk of gastrointestinal perforations upon tocilizumab treatment in rheumatoid arthritis patients[20]. Furthermore, one report showed the formation of multiple mucosal ulcers in the small and large intestine during tocilizumab treatment in rheumatoid arthritis[21]. Consistent with these reported clinical observations, tocilizumab treatment led to augmented mucosal ulcers in our UC patient, probably due to further impairment of epithelial barrier integrity with subsequent activation of mucosal immune cells resulting in pro-inflammatory cytokine production (TNF, IFN-γ, IL-21 or IL-17A/F) and progressive ulcer formation. The normalization of CRP levels under tocilizumab treatment do not correlate with the enhanced mucosal inflammation and might be explained by possible suppression of IL-6 levels in the blood, which may have resulted in diminished CRP blood levels. IL-6 blood levels were not measured in the patient. The discrepancy between persisting mucosal inflammation and normal CRP levels are in line with similar reports in UC patients[22].

The presented case report does not support the application of anti-IL6R antibodies in UC patients with anti-TNF refractory, severely progressed disease. When targeting IL-6, the differential responsiveness of target cells have to be taken into account, as IL-6 on the one side promotes acute and chronic mucosal inflammation *via* sIL-6R signaling but on the other side also strongly contributes to epithelial cell survival *via* mIL-6R signaling. Future therapeutic strategies in therapy refractory UC should aim to selectively target the pro-inflammatory function of IL-6, for instance by targeting sIL-6R signaling. Additionally, novel

Janus kinase inhibitors (Jak) such as tofacitinib, that block the function of multiple Jak dependent pro-inflammatory cytokines simultaneously[23], should be explored in anti-TNF refractory UC.

In summary, this is the first reported case of IL-6 inhibition in a therapy-refractory severe ulcerative colitis patient. Although a potentially pro-inflammatory role of IL-6 has been suggested in disease pathogenesis[4], our anti-IL-6R antibody treatment led to aggravated mucosal inflammation and does therefore not advocate its application in severe UC patients.

## COMMENTS

### Case characteristics

A 53-year-old woman presented with treatment-refractory ulcerative colitis.

### Clinical diagnosis

Clinical active, severe ulcerative colitis with diarrhoea and rectal bleeding.

### Differential diagnosis

Infectious colitis, *Clostridium difficile* colitis, CMV colitis.

### Laboratory diagnosis

HgB 11.6 g/dL, CRP 28.3 mg/L, kidney and liver function tests were within normal limits.

### Imaging diagnosis

Endomicroscopy and endoscopy revealed continuous colonic inflammation with isolated ulcerations.

### Pathological diagnosis

Histopathological analysis of sigmoid biopsies was indicative of severe ulcerative colitis.

### Treatment

The patient was treated with an anti-interleukin (IL)-6R antibody (tocilizumab).

### Related reports

This is the first reported case of IL-6 inhibition in severe, therapy-refractory ulcerative colitis.

### Term explanation

The anti-IL-6R antibody tocilizumab has been approved for the treatment of rheumatoid arthritis but has until now not been used in severe ulcerative colitis.

### Experience and lessons

This case report documents that anti-IL-6R antibody therapy had no clinical benefit in a patient with intractable ulcerative colitis and even led to exacerbation of mucosal inflammation.

### Peer-review

This article is the first case report of anti-IL6R antibody treatment in a patient with severe, treatment-refractory ulcerative colitis.

## REFERENCES


1 **Neurath MF**. Cytokines in inflammatory bowel disease. *Nat Rev Immunol* 2014; **14**: 329-342 [PMID: 24751956 DOI: 10.1038/nri3661]

2 **Danese S**. New therapies for inflammatory bowel disease: from the bench to the bedside. *Gut* 2012; **61**: 918-932 [PMID: 22115827 DOI: 10.1136/gutjnl-2011-300904]

3 **Danese S**, Colombel JF, Peyrin-Biroulet L, Rutgeerts P, Reinisch W. Review article: the role of anti-TNF in the management of ulcerative colitis -- past, present and future. *Aliment Pharmacol Ther* 2013; **37**: 855-866 [PMID: 23489068 DOI: 10.1111/apt.12284]

4 **Atreya R**, Mudter J, Finotto S, Müllberg J, Jostock T, Wirtz S, Schütz M, Bartsch B, Holtmann M, Becker C, Strand D, Czaja J, Schlaak JF, Lehr HA, Autschbach F, Schürmann G, Nishimoto N, Yoshizaki K, Ito H, Kishimoto T, Galle PR, Rose-John S, Neurath MF. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat Med* 2000; **6**: 583-588 [PMID: 10802717]

5 **Hyams JS**, Fitzgerald JE, Treem WR, Wyzga N, Kreutzer DL. Relationship of functional and antigenic interleukin 6 to disease activity in inflammatory bowel disease. *Gastroenterology* 1993; **104**: 1285-1292 [PMID: 7683293]

6 **Umehara Y**, Kudo M, Nakaoka R, Kawasaki T, Shiomi M. Serum proinflammatory cytokines and adhesion molecules in ulcerative colitis. *Hepatogastroenterology* 2006; **53**: 879-882 [PMID: 17153445]

7 **Riley SA**, Mani V, Goodman MJ, Dutt S, Herd ME. Microscopic activity in ulcerative colitis: what does it mean? *Gut* 1991; **32**: 174-178 [PMID: 1864537]

8 **Reinisch W**, Panés J, Khurana S, Toth G, Hua F, Comer GM, Hinz M, Page K, O'Toole M, Moorehead TM, Zhu H, Sun Y, Cataldi F. Anrukinzumab, an anti-interleukin 13 monoclonal antibody, in active UC: efficacy and safety from a phase IIa randomised multicentre study. *Gut* 2015; **64**: 894-900 [PMID: 25567115 DOI: 10.1136/gutjnl-2014-308337]

9 **Danese S**, Rudziński J, Brandt W, Dupas JL, Peyrin-Biroulet L, Bouhnik Y, Kleczkowski D, Uebel P, Lukas M, Knutsson M, Erlandsson F, Hansen MB, Keshav S. Tralokinumab for moderate-to-severe UC: a randomised, double-blind, placebo-controlled, phase IIa study. *Gut* 2015; **64**: 243-249 [PMID: 25304132 DOI: 10.1136/gutjnl-2014-308004]

10 **Sands BE**, Sandborn WJ, Creed TJ, Dayan CM, Dhanda AD, Van Assche GA, Greguš M, Sood A, Choudhuri G, Stempien MJ, Levitt D, Probert CS. Basiliximab does not increase efficacy of corticosteroids in patients with steroid-refractory ulcerative colitis. *Gastroenterology* 2012; **143**: 356-364.e1 [PMID: 22549092 DOI: 10.1053/j.gastro.2012.04.043]

11 **Neurath MF**, Finotto S. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. *Cytokine Growth Factor Rev* 2011; **22**: 83-89 [PMID: 21377916 DOI: 10.1016/j.cytogfr.2011.02.003]

12 **Ishiguro Y**. Mucosal proinflammatory cytokine production correlates with endoscopic activity of ulcerative colitis. *J Gastroenterol* 1999; **34**: 66-74 [PMID: 10204613]

13 **Yamamoto M**, Yoshizaki K, Kishimoto T, Ito H. IL-6 is required for the development of Th1 cell-mediated murine colitis. *J Immunol* 2000; **164**: 4878-4882 [PMID: 10779797]

14 **Nishimoto N**, Yoshizaki K, Maeda K, Kuritani T, Deguchi H, Sato B, Imai N, Suemura M, Kakehi T, Takagi N, Kishimoto T. Toxicity, pharmacokinetics, and dose-finding study of repetitive treatment with the humanized anti-interleukin 6 receptor antibody MRA in rheumatoid arthritis. Phase I/II clinical study. *J Rheumatol* 2003; **30**: 1426-1435 [PMID: 12858437]

15 **Ito H**, Takazoe M, Fukuda Y, Hibi T, Kusugami K, Andoh A, Matsumoto T, Yamamura T, Azuma J, Nishimoto N, Yoshizaki K, Shimoyama T, Kishimoto T. A pilot randomized trial of a human anti-interleukin-6 receptor monoclonal antibody in active Crohn's disease. *Gastroenterology* 2004; **126**: 989-996; discussion 947 [PMID: 15057738]

16 **Tebbutt NC**, Giraud AS, Inglese M, Jenkins B, Waring P, Clay FJ, Malki S, Alderman BM, Grail D, Hollande F, Heath JK, Ernst M. Reciprocal regulation of gastrointestinal homeostasis by SHP2 and STAT-mediated trefoil gene activation in gp130 mutant mice. *Nat Med* 2002; **8**: 1089-1097 [PMID: 12219085]

17 **Jin X**, Zimmers TA, Zhang Z, Pierce RH, Koniaris LG. Interleukin-6 is an important in vivo inhibitor of intestinal epithelial cell death

in mice. *Gut* 2010; **59**: 186-196 [PMID: 19074180 DOI: 10.1136/gut.2008.151175]

18 **Suenaert P**, Bulteel V, Lemmens L, Noman M, Geypens B, Van Assche G, Geboes K, Ceuppens JL, Rutgeerts P. Anti-tumor necrosis factor treatment restores the gut barrier in Crohn's disease. *Am J Gastroenterol* 2002; **97**: 2000-2004 [PMID: 12190167]

19 **Atreya R**, Zimmer M, Bartsch B, Waldner MJ, Atreya I, Neumann H, Hildner K, Hoffman A, Kiesslich R, Rink AD, Rau TT, Rose-John S, Kessler H, Schmidt J, Neurath MF. Antibodies against tumor necrosis factor (TNF) induce T-cell apoptosis in patients with inflammatory bowel diseases via TNF receptor 2 and intestinal $CD14^+$ macrophages. *Gastroenterology* 2011; **141**: 2026-2038 [PMID: 21875498 DOI: 10.1053/j.gastro.2011.08.032]

20 **Gout T**, Ostör AJ, Nisar MK. Lower gastrointestinal perforation in rheumatoid arthritis patients treated with conventional DMARDs or tocilizumab: a systematic literature review. *Clin Rheumatol* 2011; **30**: 1471-1474 [PMID: 21833686 DOI: 10.1007/s10067-011-1827-x]

21 **Iwasa T**, Nakamura K, Ogino H, Itaba S, Akiho H, Okamoto R, Iboshi Y, Aso A, Murao H, Kanayama K, Ito T, Takayanagi R. Multiple ulcers in the small and large intestines occurred during tocilizumab therapy for rheumatoid arthritis. *Endoscopy* 2011; **43**: 70-72 [PMID: 21108178 DOI: 10.1055/s-0030-1255931]

22 **Henriksen M**, Jahnsen J, Lygren I, Stray N, Sauar J, Vatn MH, Moum B. C-reactive protein: a predictive factor and marker of inflammation in inflammatory bowel disease. Results from a prospective population-based study. *Gut* 2008; **57**: 1518-1523 [PMID: 18566104 DOI: 10.1136/gut.2007.146357]

23 **Sandborn WJ**, Ghosh S, Panes J, Vranic I, Su C, Rousell S, Niezychowski W. Tofacitinib, an oral Janus kinase inhibitor, in active ulcerative colitis. *N Engl J Med* 2012; **367**: 616-624 [PMID: 22894574 DOI: 10.1056/NEJMoa1112168]

**P- Reviewer**: Herculano R **S- Editor**: Yu J
**L- Editor**: O'Neill M **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12970

*World J Gastroenterol* 2015 December 7; 21(45): 12970-12975
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


CASE REPORT

# Minimally invasive surgery for superior mesenteric artery syndrome: A case report

Si-Yuan Yao, Ryuichi Mikami, Sakae Mikami

Si-Yuan Yao, Ryuichi Mikami, Department of Surgery, Kobe City Medical Center West Hospital, Nagata-ku, Kobe 653-0013, Japan

Sakae Mikami, Department of Gastroenterology, Kobe City Medical Center West Hospital, Nagata-ku, Kobe 653-0013, Japan

Author contributions: Yao SY, Mikami S and Mikami R contributed to the manuscript writing and revision.

Institutional review board statement: The study was reviewed and approved by the Kobe City Medical Center West Hospital Institutional Review Board.

Informed consent statement: Written informed consent was obtained from the patient for publication of this case report and accompanying images.

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



Correspondence to: Si-Yuan Yao, MD, Department of Surgery, Kobe City Medical Center West Hospital, 2-4, Ichibancho, Nagata-ku, Kobe 653-0013, Japan. siyuan@kobe-nishishimin-hospi.jp
Telephone: +81-78-5765251
Fax: +81-78-5765358

Received: April 13, 2015
Peer-review started: April 15, 2015
First decision: May 18, 2015
Revised: May 25, 2015
Accepted: August 31, 2015
Article in press: August 31, 2015
Published online: December 7, 2015

## Abstract

Superior mesenteric artery (SMA) syndrome is defined as a compression of the third portion of the duodenum by the abdominal aorta and the overlying SMA. SMA syndrome associated with anorexia nervosa has been recognized, mainly among young female patients. The excessive weight loss owing to the eating disorder sometimes results in a reduced aorto-mesenteric angle and causes duodenal obstruction. Conservative treatment, including psychiatric and nutritional management, is recommended as initial therapy. If conservative treatment fails, surgery is often required. Currently, traditional open bypass surgery has been replaced by laparoscopic duodenojejunostomy as a curative surgical approach. However, single incision laparoscopic approach is rarely performed. A 20-year-old female patient with a diagnosis of anorexia nervosa and SMA syndrome was prepared for surgery after failed conservative management. As the patient had body image concerns, a single incision laparoscopic duodenojejunostomy was performed to achieve minimal scarring. As a result, good perioperative outcomes and cosmetic results were achieved. We show the first case of a young patient with SMA syndrome who was successfully treated by single incision laparoscopic duodenojejunostomy. This minimal invasive surgery would be beneficial for other patients with SMA syndrome associated with anorexia nervosa, in terms of both surgical and cosmetic outcomes.

**Key words:** Superior mesenteric artery syndrome; Anorexia nervosa; Young female patient; Single incision laparoscopic duodenojejunostomy; Minimally invasive surgery



**Core tip:** Traditional open bypass surgery has been replaced by laparoscopic duodenojejunostomy as a curative surgical approach for superior mesenteric

artery (SMA) syndrome. However, the single incision laparoscopic approach is rarely performed. Here, we show our experience of single incision laparoscopic duodenojejunostomy in a 20-year-old woman with anorexia nervosa and SMA syndrome. As both diseases are commonly seen in young women with body image concerns, the cosmetic outcome of surgery should be taken into consideration. We believe that this minimally invasive surgery is beneficial for patients with SMA syndrome associated with body image concerns, in terms of both surgical and cosmetic outcomes.

Yao SY, Mikami R, Mikami S. Minimally invasive surgery for superior mesenteric artery syndrome: A case report. *World J Gastroenterol* 2015; 21(45): 12970-12975 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12970.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12970

## INTRODUCTION

Superior mesenteric artery (SMA) syndrome is defined as a compression of the third portion of the duodenum by the abdominal aorta and the overlying superior mesenteric artery. The narrow aortomesenteric angle results in chronic, intermittent, or acute complete or partial duodenal obstruction. Patients often present with nausea, vomiting, abdominal distention, decreased appetite, and weight loss[1]. Non-surgical treatment is recommended as initial therapy, but when conservative treatment fails, surgery is often required. After the first successful laparoscopic duodenojejunostomy by Gersin *et al*[2] in 1998, the procedure became the most common surgical treatment[3].

Among these patients, eating disorder such as anorexia nervosa (AN) may coexist as an underlying pathology[4]. We describe here a young female patient with SMA syndrome successfully treated by single incision laparoscopic duodenojejunostomy. Because this patient had body image concerns associated with AN, single incision laparoscopic surgery (SILS) was selected as a "scarless" method.

## CASE REPORT

Three years prior to the surgery, a 17-year-old girl with a known history of AN presented to our emergency department with complaints of repeated vomiting and abdominal distension. She had been under the sporadic care of a psychiatrist and was not on any medications. A contrast-enhanced computed tomography scan also revealed a dilated stomach and proximal duodenum, and severe narrowing of the third portion of the duodenum (Figure 1A). A sagittal view showed the aorto-mesenteric angle was narrow at 10° (normal range, 25°-60°) and the aorto-mesenteric distance was short at 5.5 mm (normal range, 10-28 mm, Figure 1B). With these findings, the diagnosis of SMA syndrome was made. The patient was hospitalized five times over 3 years and experienced a 10-kg weight loss with persistent amenorrhea. As symptoms recurred frequently with conservative treatment, surgical treatment was suggested. Although the patient agreed to undergo surgery, she was very nervous about the residual scarring. Considering the impact on her body image, SILS was proposed. After discussion with the patient and her family, informed consent was obtained for laparoscopic duodenojejunostomy with single incision.

General anesthesia was induced and the patient was placed in the dorsal lithotomy position. The surgeon stood between the patient's legs. The first assistant handled the scope on the patient's left side. First, a trans-umbilical zigzag incision was made with the Hasson technique. After making the incision, a single-port three-channel device was inserted. Two 5-mm trocars and a 30° rigid 5-mm endoscope were used. With upward traction on the transverse colon, the dilated duodenum was easily identified. After isolation of the transverse mesocolon and duodenum, the anterior wall of second to third portion of the duodenum and the head of the pancreas were exposed. The duodenum and proximal jejunum (25 cm from the Treitz ligament) were marked with crystal violet at the planned anastomosis site, followed by a side-to-side duodenojejunostomy using a 45-mm stapler device. To insert the stapler device, one 5-mm trocar was replaced with a 12-mm trocar. The common entry hole was closed by hand in two layers, using 3-0 absorbable polyfilament. The patient's body mass index was 14.0 kg/m$^2$. The operative time was 148 min with little bleeding. No drain was placed. Intraoperative findings are summarized in Figure 2A-E.

The postoperative course was unremarkable. Oral fluid and solid food intake was allowed on postoperative day (POD) 2. An upper gastrointestinal study on POD 3 showed good patency of the anastomosis (Figure 3). The patient was discharged uneventfully on POD 6. Over the first 2 mo, she gained 3 kg. Although she continues to receive psychiatric care, she is doing well.

## DISCUSSION

SMA syndrome is an uncommon but well-recognized clinical entity. It is seen more commonly in female individuals and usually occurs in older children and adolescents. SMA syndrome associated with AN has been also recognized[4,5]. Anorexia nervosa is a complex eating disorder characterized by an abnormally low body weight, intense fear of gaining weight, and a distorted body image. It is seen most commonly in teenage girls and young adult women. The excessive weight loss can result in the loss of the fatty tissue that surrounds the superior mesenteric artery and its neurovascular pedicle. In the absence

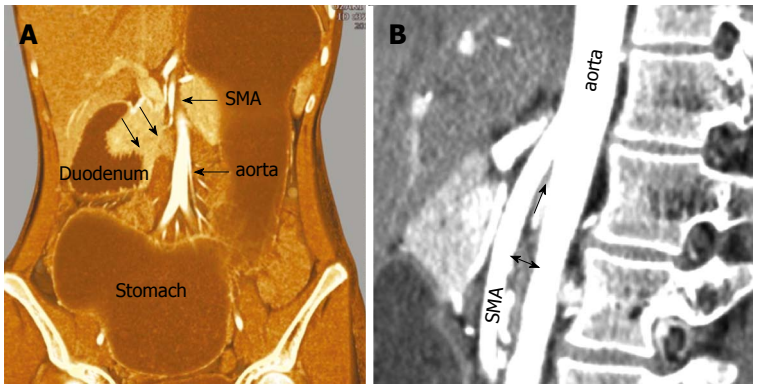



**Figure 1 Abdominal computed tomography.** A: A distended stomach and proximal duodenum were shown with caliber change at the third portion (arrows), between superior mesenteric artery (SMA) and aorta; B: On the sagittal view, the aorto-mesenteric angle was 10° (arrow) and the aorto-mesenteric distance (two-head arrow) was 5.5 mm.

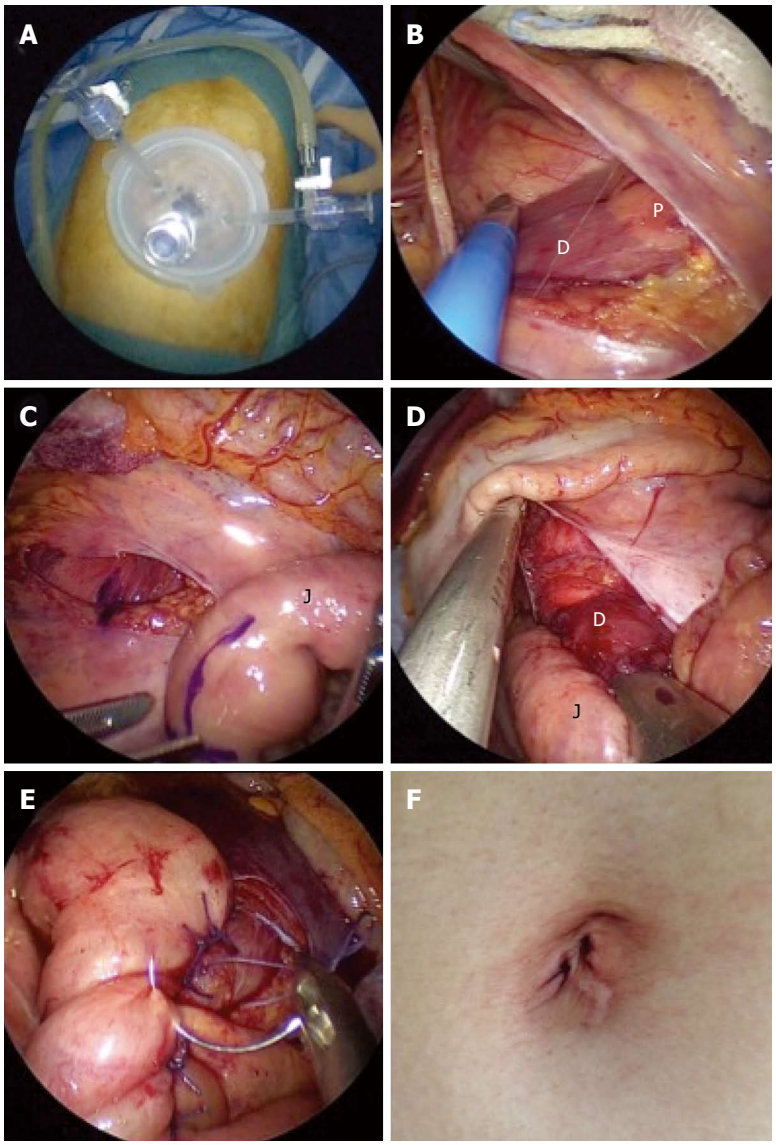



**Figure 2 Intraoperative findings.** A: Three 5-mm trocars were inserted through the umbilical incision; B: Identification of the anterior wall of the second portion of the duodenum and pancreas; C: Duodenum and proximal jejunum (25 cm from Treitz ligament) were marked with crystal violet at the planned anastomosis site; D: 45-mm linear stapler was inserted to make a side-to-side duodenojejunostomy with duodenum and jejunum; E: The common entry hole was sutured by hand; F: The umbilical incision became virtually scarless 3 mo after the operation. D: Duodenum; J: Jejunum; P: Pancreas.

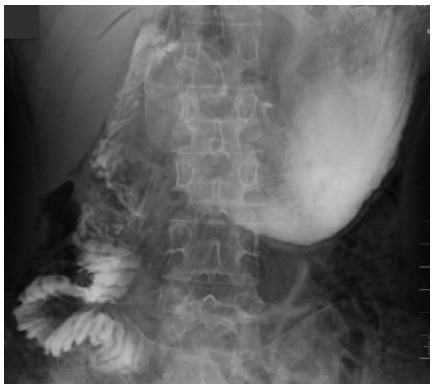


**Figure 3 Contrast study on post-operative day 3 showed smooth fluid passage through the duodenojejunostomy.**

of an appropriate fatty scaffolding, the angle at which the SMA branches from the aorta is reduced, resulting in compression of the third portion of the duodenum between the SMA and the aorta[6]. This combination of pathologies is uniquely challenging: SMA syndrome can precipitate and exacerbate AN because of the nausea associated with the small bowel obstruction and conversely, AN prevents the patient from being willing or able to ingest adequate calories to allow the SMA syndrome to resolve[7].

In addition to our patient, a literature search of the PubMed database between 1950 and 2015 retrieved 13 cases of SMA syndrome associated with AN (Table 1)[4,5,7-16]. The median age of these patients was 20 years (range, 16-47 years), indicating a greater frequency among young female patients. Most patients had low body weight with low body mass index. According to our literature search, about half of these patients were successfully managed with conservative treatment. The conservative treatment included both psychiatric and nutritional management. According to our literature search, 7 patients underwent laparotomy. While duodenojejunostomy was the most commonly performed procedure, gastrojejunostomy and Treitz ligament mobilization were also performed. One of them underwent exploratory laparotomy for a misdiagnosis of appendicitis and one had an unknown procedure. The weight gain was seen in only 4 patients, and some patients continued to lose weight despite surgical correction, indicating that AN was a refractory eating disorder. Generally, the patients who need surgical intervention would have severe symptoms because of their resistance to conservative treatment. However, the correlation between the impact of surgery and severity of anorexia nervosa is still unknown. To the best of our knowledge, this is the first reported case of successful single incision laparoscopic duodenojejunostomy performed on a patient with SMA syndrome in the setting of AN.

In recent years, SILS has been adopted for more and more cases with the innovation of surgical instruments and improvement in surgical experience and techniques. The benefits of this approach are associated with less incisional pain, less risk of incisional hernia, fewer wound complications, and improved cosmetic outcomes[17]. For example, gastrointestinal bypass with SILS has been introduced in the field of bariatric surgery. There are some reports of successful gastrojejunostomy[18,19], but only one case of single incision laparoscopic duodenojejunostomy was reported in 2014. This was performed in a 75-year-old man with SMA syndrome and resulted in a good postoperative outcome. As that report did not contain detailed images of the operation, we include intraoperative photographs here in the figures. Compared with that report, our procedure was a less invasive surgery because no drain was placed.

The benefit of selecting SILS in this case was its cosmetic outcome, which was important to the patient. To acquire a good operative field with minimal skin incision, we used a zigzag incision technique. Hachisuka *et al*[20] reported that this type of skin incision could become virtually scarless within months after an operation. In our case, the umbilical incision became virtually scarless 3 mo after surgery (Figure 2F).

Limitations that make SILS difficult to apply include internal and external conflicts between instruments and difficulty achieving traction for triangulation formation[21]. Through experience, we have learned best practices of using SILS in selected patients, including those needing cholecystectomy, appendectomy, and right hemicolectomy. Duodenojejunostomy is a simple procedure, requiring no organ resection. This resulted in fewer potential difficulties than other applications of SILS, such as cancer surgery. The loss of visceral fat, often seen in a patient with SMA syndrome or AN, resulted in a good operative field and made it easy to identify the target organs. Though the distance between the duodenum and the port site was relatively close, the interference between the scope and forceps was minimal. As SILS is often limited by the co-axial arrangement of the instruments, the close distance made the angle between the instruments wider. Using the cut mode for electrocautery instead of the coagulation mode can prevent organ injury for tensionless tissues. Dissection between the duodenum and the transverse mesocolon should be performed carefully with this method. The closure of the common entry hole after side-to-side duodenojejunostomy is an important procedure because it needs a skilled laparoscopic suturing technique. The interrupted or running suture should be performed precisely, despite the restricted operative field. To perform this operation safely, experience with SILS in other applications is recommended. Cholecystectomy or appendectomy might be a good initial experience with SILS. Right hemicolectomy is a good procedure for surgeons to learn the skills needed for duodenojejunostomy, as they have a similar operation field in the right upper

**Table 1 Clinical features of reported cases of superior mesenteric artery syndrome in the setting of anorexia nervosa**

| No. | Year | Author | Age (yr) | Sex | Boby weight (kg) | Body mass index (kg/m$^2$) | Treatment | Performed operation | Open or laparoscopic | Weight gain |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1976 | Vannatta *et al* | 17 | Female | 28 | NA | Surgery | Duodenojejunostomy | Open | Yes |
| 2 | 1978 | Froese *et al* | 16 | Male | NA | NA | Conservative | - | - | NA |
| 3 | 1981 | Sours *et al* | 17 | Female | 37.7 | NA | Conservative | - | - | NA |
| 4 | 1981 | Pentlow *et al* | 21 | Female | 32 | NA | Conservative | - | - | Yes |
| 5 | 1992 | Elbadaway *et al* | 18 | Female | 35.8 | 12.7 | Surgery | Gastrojejunostomy | Open | Yes |
| 6 | 1997 | Adson *et al* | 35 | Female | NA | NA | Surgery | Exploratory laparotomy | Open | NA |
| 7 | 1998 | da Silva *et al* | 28 | Female | NA | NA | Surgery | NA | NA | No |
| 8 | 2004 | Lo *et al* | 26 | Female | 37 | 16 | Surgery | Duodenojejunostomy | Open | No |
| 9 | 2009 | Verhoef *et al* | 16 | Female | 39 | 15 | Conservative | - | - | Yes |
| 10 | 2010 | Gwee *et al* | 17 | Female | 37.3 | 14.6 | Conservative | - | - | Yes |
| 11 | 2014 | Mearelli *et al* | 47 | Male | NA | NA | Surgery | Treitz ligament mobilization | Open | Yes |
| 12 | 2015 | Mascolo *et al* | 47 | Female | 29.1 | 10.6 | Conservative | - | - | Yes |
| 13 | 2015 | Our case | 20 | Female | 32 | 14 | Surgery | Duodenojejunostomy | Laparoscopic | Yes |

NA: Not availiable.

quadrant. With all of these procedures, this "scarless" operation could lead to early recovery and cosmetic satisfaction.

Single incision laparoscopic duodenojejunostomy may be a feasible option as a curative operation for SMA syndrome. We believe that this minimally invasive surgery is especially beneficial for patients with SMA syndrome associated with AN and its associated body image issues, in terms of both surgical and cosmetic outcomes.

## COMMENTS

### Case characteristics

A 17-year-old girl with a known history of anorexia nervosa presented with complaints of repeated vomiting and abdominal distension.

### Clinical diagnosis

The patient was diagnosed with superior mesenteric artery syndrome.

### Differential diagnosis

Anorexia nervosa, bulimia, megaduodenum or duodenal ileus.

### Laboratory diagnosis

Laboratory tests showed no abnormal value.

### Imaging diagnosis

A contrast-enhanced computed tomography scan revealed a dilated stomach and proximal duodenum, and severe narrowing of the third portion of the duodenum.

### Treatment

Three years later, after the failure of conservative treatment, laparoscopic duodenojejunostomy was performed with single umbilical incision and operative course was uneventful.

### Related reports

While laparoscopic duodenojejunostomy is widely performed as a curative surgical approach, single incision laparoscopic approach is rarely performed. The authors show the first case of a young patient with superior mesenteric artery (SMA) syndrome who was successfully treated by single incision laparoscopic duodenojejunostomy.

### Term explanation

Duodenojejunostomy is a surgical procedure for superior mesenteric artery syndrome, which includes the anastomosis between the proximal duodenum and a proximal loop.

### Experiences and lessons

Single incision laparoscopic duodenojejunostomy may be a feasible option as a curative operation for SMA syndrome, in terms of both surgical and cosmetic outcomes.

### Peer-review

The authors firstly reported a case of single incision laparoscopic duodenojejunostomy, which could both cure SMA symdrome and improve cosmetic effect. We believe this minimally invasive surgery will be suitable for the patients with SMA syndrome who do not respond to the conservative treatment.

## REFERENCES


1 **Gerasimidis T**, George F. Superior mesenteric artery syndrome. Wilkie syndrome. *Dig Surg* 2009; **26**: 213-214 [PMID: 19468230 DOI: 10.1159/00021930]

2 **Gersin KS**, Heniford BT. Laparoscopic duodenojejunostomy for treatment of superior mesenteric artery syndrome. *JSLS* 1998; **2**: 281-284 [PMID: 9876755]

3 **Lee TH**, Lee JS, Jo Y, Park KS, Cheon JH, Kim YS, Jang JY, Kang YW. Superior mesenteric artery syndrome: where do we stand today? *J Gastrointest Surg* 2012; **16**: 2203-2211 [PMID: 23076975 DOI: 10.1007/s11605-012-2049-5]

4 **Vannatta JB**, Cagas CR, Cramer RI. Superior mesenteric artery (Wilkie's) syndrome: report of three cases and review of the literature. *South Med J* 1976; **69**: 1461-1465 [PMID: 1019644]

5 **Sours JA**, Vorhaus LJ. Superior mesenteric artery syndrome in anorexia nervosa: a case report. *Am J Psychiatry* 1981; **138**: 519-520 [PMID: 7212113]

6 **Valdes A**, Cárdenas O, Espinosa A, Villazón O, Valdes V. Superior mesenteric artery syndrome. *J Am Coll Surg* 2005; **201**: 808 [PMID: 16256925]

7 **Verhoef PA**, Rampal A. Unique challenges for appropriate management of a 16-year-old girl with superior mesenteric artery syndrome as a result of anorexia nervosa: a case report. *J Med Case Rep* 2009; **3**: 127 [PMID: 19946542 DOI: 10.1186/1752-1947-3-127]

8 **Froese AP**, Szmuilowicz J, Bailey JD. The superior-mesenteric-artery syndrome: cause or complication of anorexia nervosa? *Can Psychiatr Assoc J* 1978; **23**: 325-327 [PMID: 679142]

9 **Pentlow BD**, Dent RG. Acute vascular compression of the

duodenum in anorexia nervosa. *Br J Surg* 1981; **68**: 665-666 [PMID: 7272696]
10 **Elbadaway MH**. Chronic superior mesenteric artery syndrome in anorexia nervosa. *Br J Psychiatry* 1992; **160**: 552-554 [PMID: 1571759]
11 **Adson DE**, Mitchell JE, Trenkner SW. The superior mesenteric artery syndrome and acute gastric dilatation in eating disorders: a report of two cases and a review of the literature. *Int J Eat Disord* 1997; **21**: 103-114 [PMID: 9062834]
12 **de Silva AP**, Molagoda A, Fernando PL, de Silva HJ. The young woman who could not stop vomiting. *Postgrad Med J* 1998; **74**: 691-692 [PMID: 10197201]
13 **Lo DY**, Yen JL, Jones MP. Massive gastric dilation and necrosis in anorexia nervosa: cause or effect? *Nutr Clin Pract* 2004; **19**: 409-412 [PMID: 16215133]
14 **Gwee K**, Teh A, Huang C. Acute superior mesenteric artery syndrome and pancreatitis in anorexia nervosa. *Australas Psychiatry* 2010; **18**: 523-526 [PMID: 20932197 DOI: 10.3109/10398562.2010.498885]
15 **Mearelli F**, Degrassi F, Occhipinti AA, Casarsa C, De Manzini N, Biolo G. Pinched: superior mesenteric artery syndrome. *Am J Med* 2014; **127**: 393-394 [PMID: 24486285 DOI: 10.1016/j.amjmed.2014.01.008]
16 **Mascolo M**, Dee E, Townsend R, Brinton JT, Mehler PS. Severe gastric dilatation due to superior mesenteric artery syndrome in anorexia nervosa. *Int J Eat Disord* 2015; **48**: 532-534 [PMID: 25639251 DOI: 10.1002/eat.22385]
17 **Bae SU**, Baek SJ, Min BS, Baik SH, Kim NK, Hur H. Reduced-port laparoscopic surgery for a tumor-specific mesorectal excision in patients with colorectal cancer: initial experience with 20 consecutive cases. *Ann Coloproctol* 2015; **31**: 16-22 [PMID: 25745622 DOI: 10.3393/ac.2015.31.1.16]
18 **Morales-Conde S**, Rubio-Manzanares Dorado M, Barranco A, Alarcón I, Pereira JL, García-Luna PP, Socas M. Single-port Roux-en-Y gastric bypass: toward a less invasive procedure with the same surgical results and a better cosmesis. *Surg Endosc* 2013; **27**: 4347-4353 [PMID: 23846366 DOI: 10.1007/s00464-013-3070-9]
19 **Pitot D**, Takieddine M, Abbassi Z, Agrafiotis A, Bruyns L, Ceuterick M, Daoudi N, Dolimont A, Soulimani A, Vaneukem P. Pure transumbilical SILS gastric bypass with mechanical circular gastrojejunal anastomosis feasibility. *Surg Endosc* 2014; **28**: 3007-3011 [PMID: 24853853 DOI: 10.1007/s00464-014-3562-2]
20 **Hachisuka T**, Kinoshita T, Yamakawa T, Kurata N, Tsutsuyama M, Umeda S, Tokunaga S, Yarita A, Shibata M, Shimizu D, Shikano T, Hattori K, Mori T, Shinohara M, Miyauchi M. Transumbilical laparoscopic surgery using GelPort through an umbilical zigzag skin incision. *Asian J Endosc Surg* 2012; **5**: 50-52 [PMID: 22776345 DOI: 10.1111/j.1758-5910]
21 **Choi SI**, Lee KY, Park SJ, Lee SH. Single port laparoscopic right hemicolectomy with D3 dissection for advanced colon cancer. *World J Gastroenterol* 2010; **16**: 275-278 [PMID: 20066750]

**P- Reviewer**: Xu L **S- Editor**: Yu J
**L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12976

*World J Gastroenterol* 2015 December 7; 21(45): 12976-12980
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


CASE REPORT

# Endoscopic fibrin sealant closure of duodenal perforation after endoscopic retrograde cholangiopancreatography

Hsin-Yeh Yang, Jui-Hao Chen

Hsin-Yeh Yang, Jui-Hao Chen, Division of Gastroenterology, Department of Internal Medicine, Shin Kong Wu Ho-Su Memorial Hospital, Taipei 11101, Taiwan

Jui-Hao Chen, Faculty of Medicine, Taipei Medical University, Taipei 11101, Taiwan

Author contributions: Both authors contributed to the acquisition of data, preparation and revision of this manuscript.

Supported by Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan.

Informed consent statement: The patient involved in this study provided written informed consent authorizing use and disclosure of his health information.

Conflict-of-interest statement: The authors declare no conflicts of interest related to this report.



Correspondence to: Jui-Hao Chen, MD, Division of Gastroenterology, Department of Internal Medicine, Shin Kong Wu Ho-Su Memorial Hospital, No. 95, Wen-Chang Road, Shih-Lin District, Taipei 11101, Taiwan. m000723@ms.skh.org.tw
Telephone: +886-2-28332211-2031
Fax: +886-2-28389335

Received: April 30, 2015
Peer-review started: May 8, 2015
First decision: July 10, 2015
Revised: August 5, 2015
Accepted: October 13, 2015
Article in press: October 13, 2015
Published online: December 7, 2015

## Abstract

Traditionally, perivaterian duodenal perforation can be managed conservatively or surgically. If a large volume of leakage results in fluid collection in the retroperitoneum, surgery may be necessary. Our case met the surgical indication for perivaterian duodenal perforation after endoscopic retrograde cholangiopancreatography (ERCP) with sphincterotomy and endoscopic papillary balloon dilatation. The patient developed a retroperitoneal abscess after the procedures, and a perivaterian perforation was suggested on computed tomography (CT). CT-guided abscess drainage was performed immediately. We unsuccessfully attempted to close the perforation with hemoclips initially. Subsequently, we used fibrin sealant (Tisseel) injection to occlude the perforation. Fibrin sealant injections have been previously used during endoscopy for wound closure and fistula repair. Based on our report, fibrin sealant injection can be considered as an alternative method for the treatment of ERCP-related type Ⅱ perforations.

**Key words:** Perivaterian duodenal perforation; Endoscopic retrograde cholangiopancreatography; Retroperitoneal abscess



**Core tip:** Perivaterian duodenal perforation can be managed conservatively or surgically. Our patient underwent endoscopic retrograde cholangiopancreatography (ERCP) with sphincterotomy and endoscopic papillary balloon dilatation, and developed a perivaterian duodenal perforation after the procedures. Computed tomography-guided abscess drainage was performed immediately but without improvement, and fibrin sealant (Tisseel) injection was then administered to occlude the perforation. The patient recovered uneventfully.

This report shows that fibrin sealant injection can be an alternative method for the treatment of ERCP-related type Ⅱ perforations.

Yang HY, Chen JH. Endoscopic fibrin sealant closure of duodenal perforation after endoscopic retrograde cholangiopancreatography. *World J Gastroenterol* 2015; 21(45): 12976-12980 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12976.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12976

## INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) with sphincterotomy (EST) and/or endoscopic papillary balloon dilatation (EPBD) is commonly used in the treatment of common bile duct (CBD) stones. However, ERCP is an invasive procedure that may lead to potential complications such as pancreatitis, hemorrhage, bowel perforation, and cholangitis. ERCP-related perforations are uncommon with a reported incidence of 0.3%-1.3%[1-3].

There are four types of ERCP-related bowel perforations[4-6]: lateral wall duodenal perforation (Type Ⅰ), perivaterian perforation (Type Ⅱ), perforation of the biliary tree (Type Ⅲ), and retroperitoneal air alone (Type Ⅳ). Traditionally, lateral wall duodenal perforations (Type Ⅰ) tend to be large and usually require surgical intervention[5,7,8]. Other types of perforation can be managed either conservatively or surgically[9,10]. If a large volume of leakage results in a fluid collection in the retroperitoneum, surgery or interventional drainage may be necessary.

Here, we describe a case of a perivaterian duodenal perforation (Type Ⅱ) after ERCP with EST and EPBD that was successfully sealed with fibrin glue.

## CASE REPORT

A 70-year-old man who had coronary artery disease and hypertension presented with symptomatic CBD stones and cholangitis. The patient had a history of gallbladder stones with acute cholecystitis and had undergone laparoscopic cholecystectomy 4 years previously. After hospital admission, he underwent ERCP with EST and EPBD (0.8 cm, 8 atm × 3 min), and a black stone was extracted with a balloon catheter (Figure 1A-C).

Fever and right flank pain were noted soon after the procedure. Initially, plain abdominal radiography and abdominal ultrasound revealed no obvious free air. Three days later, abdominal ultrasonography and abdominal computed tomography (CT) showed a retroperitoneal abscess (Figure 2A) close to the right lateral wall of the duodenum, suggesting a perivaterian perforation. CT-guided abscess drainage was performed immediately (Figure 2B).

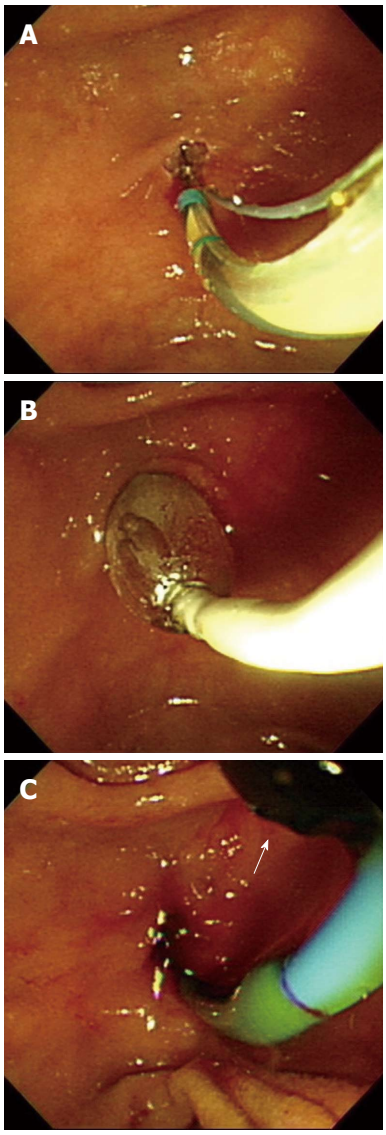



**Figure 1 The patient underwent endoscopic retrograde cholangiopancreatography with sphincterotomy and endoscopic papillary balloon dilatation.** A: Endoscopic retrograde cholangiopancreatography with sphincterotomy; B: Endoscopic papillary balloon dilatation (0.8 cm, 8 atm × 3 min); C: Black stone (white arrow) was extracted with a balloon catheter.

Another abdominal CT scan with oral contrast ingestion was performed on day 10 after ERCP due to persistent drainage of a large amount of purulent material. The scan revealed minimal contrast and air leakage from the duodenum into the right anterior pararenal space (Figure 3). On the same day, ERCP revealed a perforation of the perivaterian duodenum (Figure 4A). We unsuccessfully attempted to close the perforation with hemoclips initially. Subsequently, we used fibrin sealant (Tisseel; Baxter Healthcare, Deerfield, IL, United States) injection to occlude the perforation. Before injection of the fibrin sealant, we placed two 7F double pigtail (5 and 6 cm) plastic biliary stents into the CBD to prevent occlusion of the biliary orifice by the fibrin sealant (Figure 4B). A total of 4 mL Tisseel was injected into the perforation site (Figure 4C).

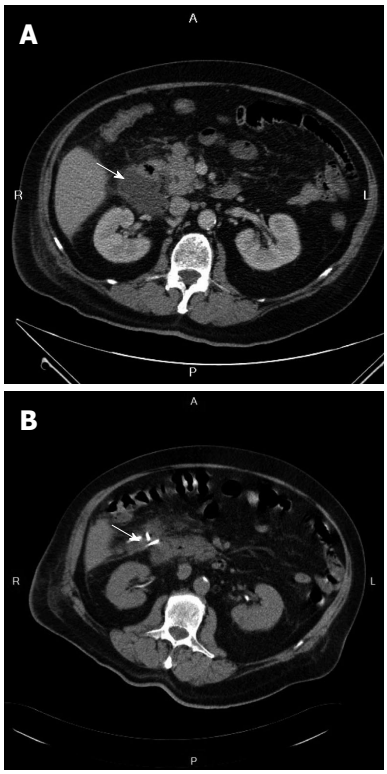



**Figure 2 Computed tomography scan.** A: Showing a hypodense abscess (arrow) between the liver and right kidney; B: Showing pig-tail drainage (arrow) of the abscess.

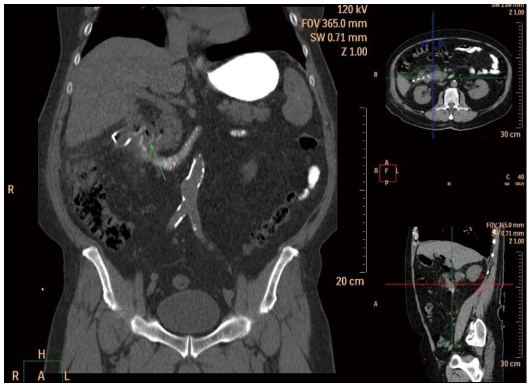



**Figure 3 Computed tomography scan of the abdomen.** Green arrow showing perforation site.

The patient's fever had subsided by the day following application of the fibrin sealant, and the amount of purulent drainage decreased from 60-70 to 20 mL/d by day 2 after the procedure. Five days later, the amount of purulent drainage had decreased to < 10 mL/d and the patient's abdominal discomfort continued to improve. He recovered uneventfully and was discharged on day 14 after fibrin sealant injection. Follow-up CT scan 2 wk later revealed an unremarkable retroperitoneal space without abscess formation or a detectable leak.

## DISCUSSION

We described a case of perivaterian duodenal perforation in a patient after undergoing ERCP with EST and EPBD. He subsequently developed a retroperitoneal abscess that required percutaneous CT-guided drainage. However, his condition did not improve because of a persistent leak from the duodenal perforation. Surgical repair was not desirable because of the patient's age and comorbidity.

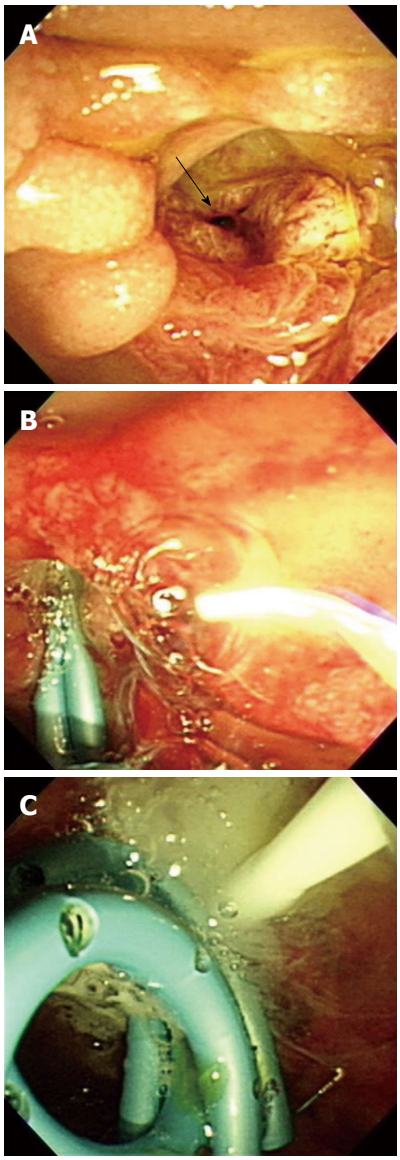



**Figure 4 Endoscopic retrograde cholangiopancreatography.** Endoscopic retrograde cholangiopancreatography revealed a perforation (arrow) in the duodenum (A); Two plastic stents were inserted into the endoscopic papillary balloon dilatation before fibrin sealant injection (B); Whitish tissue sealant was injected into the perforation in the right upper corner (C).

Several endoscopic closure techniques have been described for closure of gastrointestinal perforations including the use of endoclips, fibrin sealant[11,12], endoloops, and the over-the-scope clip system[13]. Most of these approaches have been used for Type Ⅰ perforations. For Type Ⅱ perforations, previous studies have suggested conservative treatment first, followed by surgical intervention if conservative treatment fails. In this case, endoscopic repair with hemoclips was initially

attempted, but failed. Subsequently, we were able to occlude the perforation without surgical intervention using fibrin sealant (Tisseel) injection.

Tisseel is a fibrin sealant used as an adjunct to hemostasis. It contains human fibrinogen, human factor XIII, aprotinin, polysorbate 80, human thrombin, and calcium chloride. Mixed together, the above ingredients form a cell-free clot that can block the perforation. In contrast to cyanoacrylate mixed with lipiodol, Tisseel has been shown to have tissue-healing properties and to be fully reabsorbed by macrophages and fibroblasts within 2 wk of application[14,15]. Fibrin sealant injections have been previously used during endoscopy for wound closure and fistula repair[16]. Only a few cases of duodenal perforation have been successfully treated with tissue sealant[11,12]. Based on our report, fibrin sealant (Tisseel) injection can be considered as an alternative method for the treatment of ERCP-related Type Ⅱ perforations.

## COMMENTS

### Case characteristics

A 70-year-old man who had coronary artery disease and hypertension presented with epigastralgia for 1 wk, accompanied with skin discoloration and tea color urine.

### Clinical diagnosis

Common bile duct (CBD)-stone related obstruction jaundice, underwent endoscopic retrograde cholangiopancreatography (ERCP), sphincterectomy and endoscopic papillary balloon dilatation, and stone retrieval by balloon catheter. Fever, abdominal pain and flank pain were found after the procedures. Post-ERCP bowel perforation was found with intra-abdominal abscess formation.

### Differential diagnosis

Post-ERCP pancreatitis, bowel perforation, ascending cholangitis, urine track infection or renal stone.

### Laboratory diagnosis

Leukocytosis with left shift and high C-reactive protein level after the procedure. Normal lipase level and liver function tests.

### Imaging diagnosis

Computed tomography showed periampullary duodenal microperforation-related collection/abscess at the right retroperitoneal space.

### Treatment

The authors placed two 7F double pigtail (5 and 6 cm) plastic biliary stents into the CBD to prevent occlusion of the biliary orifice, then 4 mL fibrin sealant (Tisseel) was injected into the perforation site.

### Related reports

For delayed wound healing of post-ERCP Type Ⅱ bowel perforation, surgical intervention was needed. Only a few reports have mentioned endoscopic treatment. The authors tried fibrin sealant (Tisseel) injection, which was used for surgical wound healing, and achieved a good outcome.

### Experiences and lessons

Fibrin sealant (Tisseel) injection can be considered as an alternative method for the treatment of ERCP-related Type Ⅱ perforations.

### Peer-review

A new technique for the management of post-ERCP perforation is presented. It is well written and illustrated.

## REFERENCES


1 **Cotton PB**, Lehman G, Vennes J, Geenen JE, Russell RC, Meyers WC, Liguory C, Nickl N. Endoscopic sphincterotomy complications and their management: an attempt at consensus. *Gastrointest Endosc* 1991; **37**: 383-393 [PMID: 2070995 DOI: 10.1016/S0016-5107(91)70740-2]

2 **Scarlett PY**, Falk GL. The management of perforation of the duodenum following endoscopic sphincterotomy: a proposal for selective therapy. *Aust N Z J Surg* 1994; **64**: 843-846 [PMID: 7980259 DOI: 10.1111/j.1445-2197.1994.tb04561.x]

3 **Loperfido S**, Angelini G, Benedetti G, Chilovi F, Costan F, De Berardinis F, De Bernardin M, Ederle A, Fina P, Fratton A. Major early complications from diagnostic and therapeutic ERCP: a prospective multicenter study. *Gastrointest Endosc* 1998; **48**: 1-10 [PMID: 9684657 DOI: 10.1016/S0016-5107(98)70121-X]

4 **Howard TJ**, Tan T, Lehman GA, Sherman S, Madura JA, Fogel E, Swack ML, Kopecky KK. Classification and management of perforations complicating endoscopic sphincterotomy. *Surgery* 1999; **126**: 658-663; discussion 664-665 [PMID: 10520912 DOI: 10.1016/S0039-6060(99)70119-4]

5 **Enns R**, Eloubeidi MA, Mergener K, Jowell PS, Branch MS, Pappas TM, Baillie J. ERCP-related perforations: risk factors and management. *Endoscopy* 2002; **34**: 293-298 [PMID: 11932784 DOI: 10.1055/s-2002-23650]

6 **Stapfer M**, Selby RR, Stain SC, Katkhouda N, Parekh D, Jabbour N, Garry D. Management of duodenal perforation after endoscopic retrograde cholangiopancreatography and sphincterotomy. *Ann Surg* 2000; **232**: 191-198 [PMID: 10903596 DOI: 10.1097/00000658-200008000-00007]

7 **Fatima J**, Baron TH, Topazian MD, Houghton SG, Iqbal CW, Ott BJ, Farley DR, Farnell MB, Sarr MG. Pancreaticobiliary and duodenal perforations after periampullary endoscopic procedures: diagnosis and management. *Arch Surg* 2007; **142**: 448-454; discussion 454-455 [PMID: 17515486 DOI: 10.1001/archsurg.142.5.448]

8 **Masci E**, Toti G, Mariani A, Curioni S, Lomazzi A, Dinelli M, Minoli G, Crosta C, Comin U, Fertitta A, Prada A, Passoni GR, Testoni PA. Complications of diagnostic and therapeutic ERCP: a prospective multicenter study. *Am J Gastroenterol* 2001; **96**: 417-423 [PMID: 11232684 DOI: 10.1111/j.1572-0241.2001.03594.x]

9 **Park WY**, Cho KB, Kim ES, Park KS. A case of ampullary perforation treated with a temporally covered metal stent. *Clin Endosc* 2012; **45**: 177-180 [PMID: 22866262 DOI: 10.5946/ce.2012.45.2.177]

10 **Vezakis A**, Fragulidis G, Nastos C, Yiallourou A, Polydorou A, Voros D. Closure of a persistent sphincterotomy-related duodenal perforation by placement of a covered self-expandable metallic biliary stent. *World J Gastroenterol* 2011; **17**: 4539-4541 [PMID: 22110286 DOI: 10.3748/wjg.v17.i40.4539]

11 **Mutignani M**, Iacopini F, Dokas S, Larghi A, Familiari P, Tringali A, Costamagna G. Successful endoscopic closure of a lateral duodenal perforation at ERCP with fibrin glue. *Gastrointest Endosc* 2006; **63**: 725-727 [PMID: 16564890 DOI: 10.1016/j.gie.2005.11.028]

12 **Kim KW**, Lee TH, Park SH, Son BS, Lee SH, Lee SH, Chung IK, Kim SJ. A primary repair with hemoclips and fibrin glue injection in biliary stent induced duodenal perforation. *Korean J Gastrointest Endosc* 2011; **42**: 437-441

13 **Buffoli F**, Grassia R, Iiritano E, Bianchi G, Dizioli P, Staiano T. Endoscopic "retroperitoneal fatpexy" of a large ERCP-related jejunal perforation by using a new over-the-scope clip device in Billroth II anatomy (with video). *Gastrointest Endosc* 2012; **75**: 1115-1117 [PMID: 21820111 DOI: 10.1016/j.gie.2011.05.029]

14 **Becker JC**, Beckbauer M, Domschke W, Herbst H, Pohle T. Fibrin glue, healing of gastric mucosal injury, and expression of

growth factors: results from a human in vivo study. *Gastrointest Endosc* 2005; **61**: 560-567 [PMID: 15812409 DOI: 10.1016/S0016-5107(05)00291-9]

15 **Pescatore P**, Verbeke C, Härle M, Manegold BC. Fibrin sealing in peptic ulcer bleeding: the fate of the clot. *Endoscopy* 1998; **30**: 519-523 [PMID: 9746159 DOI: 10.1055/s-2007-1001337]

16 **Petersen B**, Barkun A, Carpenter S, Chotiprasidhi P, Chuttani R, Silverman W, Hussain N, Liu J, Taitelbaum G, Ginsberg GG. Tissue adhesives and fibrin glues. *Gastrointest Endosc* 2004; **60**: 327-333 [PMID: 15332018 DOI: 10.1016/S0016-5107(04)01564-0]

**P- Reviewer**: Kate V **S- Editor**: Yu J **L- Editor**: A
**E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12981

*World J Gastroenterol* 2015 December 7; 21(45): 12981-12986
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


CASE REPORT

# Coexistence of hepatoma with mantle cell lymphoma in a hepatitis B carrier

Mu-Hsien Lee, Yu-Ching Lin, Hao-Tsai Cheng, Wen-Yu Chuang, Hsin-Chih Huang, Hsiao-Wen Kao

Mu-Hsien Lee, Hao-Tsai Cheng, Hsin-Chih Huang, Department of Gastroenterology and Hepatology, Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taoyuan 333, Taiwan

Yu-Ching Lin, Department of Intervention and Imaging, Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taipei 105, Taiwan

Wen-Yu Chuang, Department of Pathology, Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taipei 105, Taiwan

Hsiao-Wen Kao, Department of Hematology-Oncology, Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taipei 105, Taiwan

Author contributions: Lee MH wrote the paper; Cheng HT, Huang HC and Kao HW designed the study; Lin YC reviewed the images; and Chuang WY reviewed the pathological reports.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of the Chang Gung Hospital (No. 104-5688B).

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

Conflict-of-interest statement: The authors have no financial disclosures to declare and no conflicts of interest to report.



Correspondence to: Hao-Tsai Cheng, MD, Department of Gastroenterology and Hepatology, Chang Gung Memorial Hospital and Chang Gung University College of Medicine, 5 Fu-Shin Street, Kweishan, Taoyuan 333, Taiwan. howardandbetty@yahoo.com.tw
Telephone: +886-3-3281200-8108
Fax: +886-3-3272236

Received: June 28, 2015
Peer-review started: June 28, 2015
First decision: July 19, 2015
Revised: August 24, 2015
Accepted: September 30, 2015
Article in press: September 30, 2015
Published online: December 7, 2015

## Abstract

The coexistence of hepatocellular carcinoma (HCC) and non-Hodgkin's lymphoma (NHL) in the liver is rare. Reports show that these patients have cirrhotic livers or hepatitis virus infections before they develop HCC and NHL. We present a patient with hepatitis B virus infection who was transferred to our hospital with a newly detected liver mass; abdominal computed tomography examination showed one hypodense mass of 7 cm in diameter and multiple mesenteric and mediastinal lymph nodes. A liver tumor biopsy showed a hepatoma, and the pathologic findings from an inguinal lymph node excision showed mantle cell lymphoma. An immunohistochemical stain confirmed that the atypical lymphoid cells within the HCC were positive for the CD20, CD5 and cyclin D1 antigens. Taking these findings into account, the hepatic tumor was determined to be a HCC infiltrated by mantle cell lymphoma.

**Key words:** Hepatocellular carcinoma; Mantle cell lymphoma; Hepatitis B virus



**Core tip:** The coexistence of hepatocellular carcinoma

(HCC) and non-Hodgkin's lymphoma in the liver is rare. The risk factors include hepatitis virus infection, liver cirrhosis and antineoplastic therapy. This case report presents a rare case of HCC and mantle cell lymphoma in the liver. Only hepatitis B virus infection is identified. "Tumor to tumor metastasis" is noted in the pathologic findings. The literature is also reviewed.

Lee MH, Lin YC, Cheng HT, Chuang WY, Huang HC, Kao HW. Coexistence of hepatoma with mantle cell lymphoma in a hepatitis B carrier. *World J Gastroenterol* 2015; 21(45): 12981-12986 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12981.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12981

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver tumor, and it is mostly associated with hepatitis B and C virus infection[1]. Liver invasion of non-Hodgkin's lymphoma (NHL) has been the focus of several clinical investigations. Only a few publications have reported coexistence of HCC and NHL. Major risk factors include liver cirrhosis, hepatitis virus infection and chemotherapy[2-4]. Hepatitis B virus (HBV) is hepatotropic, but there were evidences that peripheral blood mononuclear cells (PBMCs) can also serve as a reservoir of this virus[5]. Therefore, it can be a causative factor of both HCC and lymphoma[6,7]. We present here a rare case of coexistent HCC and lymphoma in a HBV infected patient without liver cirrhosis.

## CASE REPORT

A 52-year-old male patient was referred to our hospital for a newly diagnosed liver tumor of the right lobe. The following symptoms were noted: easy fatigue, poor appetite and progressive weight loss of 10 kg in the past two months. He had no fever or night sweats. He had not received prior chemotherapy. A contrast-enhanced computed tomography (CT) before this referral showed one 7-cm sized hypodense mass located in Couinaud's segment 7. He was tested positive for HBV infection. A physical examination revealed multiple enlarged lymph nodes in his neck and inguinal region. Anemia, jaundice, leg edema and hepatosplenomegaly were not observed.

On admission, his laboratory tests for alanine aminotransferase (ALT), total bilirubin, albumin, leukocytes, and prothrombin time were all within normal range; but his aspartate aminotransferase was 41 U/L (0-34 U/L), hemoglobin 18.4 g/d, and red blood cell mass 6.49 × $10^6$. His platelets count decreased to 97 × $10^3$/μL (150 × $10^3$-450 × $10^3$/μL), and alpha-fetoprotein (AFP) increased to 49465.6 ng/mL (< 15 ng/mL). Viral serologic tests were negative for anti-hepatitis C virus (HCV) antibody but positive for hepatitis B surface antigen and anti-hepatitis B core antigen. HBV DNA quantitative test showed a low viral load of 0.00305 × $10^6$ IU/mL. Serum β2-microglobulin was elevated to 3370 ng/mL.

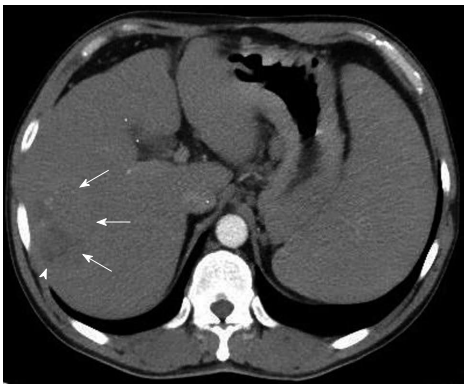


**Figure 1 Contrast-enhanced axial computed tomography during the arterial phase showed one ill-defined mass with heterogeneous enhancement (arrows) and central necrosis (arrowhead) at segment 7.**

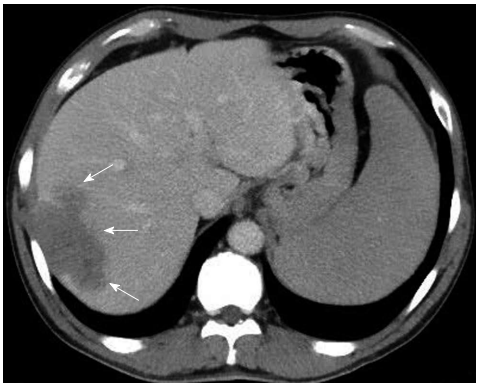


**Figure 2 Computed tomography later showed wash out during the delay phase; this is compatible with hepatocellular carcinoma (arrows) with chest wall involvement.** No obvious imaging evidence of liver cirrhosis and portal hypertension was shown.

### *Diagnostic test*

Review of CT by our radiologist revealed a 7-cm in diameter liver mass in S7 with heterogeneous enhancement in the arterial phase and wash out in the delay phase (Figures 1 and 2). It was considered a HCC with internal necrosis. There was no evidence of cirrhosis or portal vein thrombosis. Multiple mediastinal and mesenteric enlarged lymph nodes and splenomegaly were observed (Figures 3 and 4), which was not commonly seen in patients with HCC. An echo-guided core biopsy of the liver tumor was performed, and the initial pathologic finding indicated that it was an HCC (Figure 5A). Immunochemical study using an automated immunostainer BOND-MAX (Leica), however, revealed an aggregate of small to medium-sized lymphoid cells with irregular nuclei in the neighboring portal areas of normal liver tissue and also the tumor tissue (Figure 5B and C).

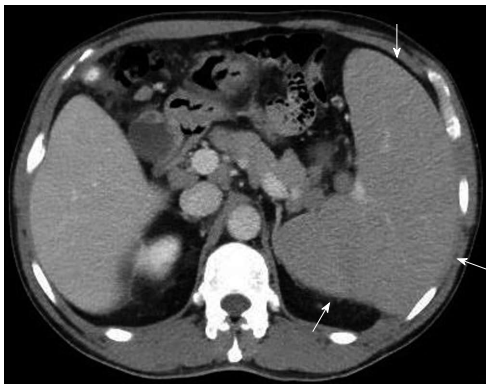


**Figure 3 Large homogenous enhanced spleen (arrows) is also noted in the upper abdomen; it is suggestive of splenomegaly.**

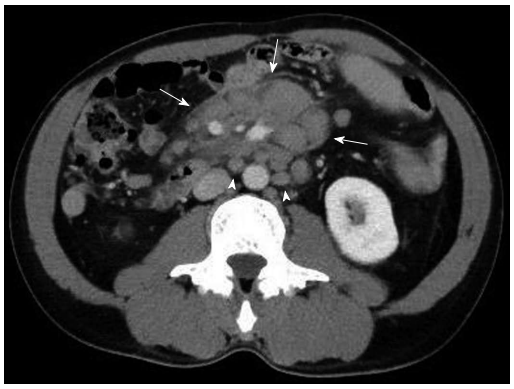


**Figure 4 Contrast-enhanced axial computed tomography showed a cluster of multiple enlarged lymph nodes in the mesenteric (arrows) and para-aortic regions (arrowheads).**

These lymphoid cells were positive for CD20 (L26; Dako; 1:1000), CD5 (4C7; Lieca; 1:200) and cyclin D1 (SP4; Zytomed; 1:25) (Figure 6). This tumor was thus an HCC infiltrated with mantle cell lymphoma. A right inguinal lymph node was excised and the result showed mantle cell lymphoma. Given these findings, this patient was diagnosed as having a coexistent HCC and mantle cell lymphoma. Whole body contrast-enhanced CT before chemotherapy revealed no gastrointestinal tract involvement. Bone marrow biopsy revealed no evidence of lymphmatous involvement. Radiofrequency tumor ablation (RFA) was performed. Chemotherapy with CHOP regimen (cyclophosphamide, hydroxydaunorubicin, oncovin, prednisolone) was initiated. He has been in remission one year after therapy.

## DISCUSSION

Mantle cell lymphoma (MCL) is a rare subgroup of B-cell NHL that occurs in approximately 6% of all NHL patients. MCL cells can enter the lymphatic channels and blood vessels, and they can also spread to other lymph nodes or tissues, such as the bone marrow, liver and gastrointestinal tract. The prognosis of MCL

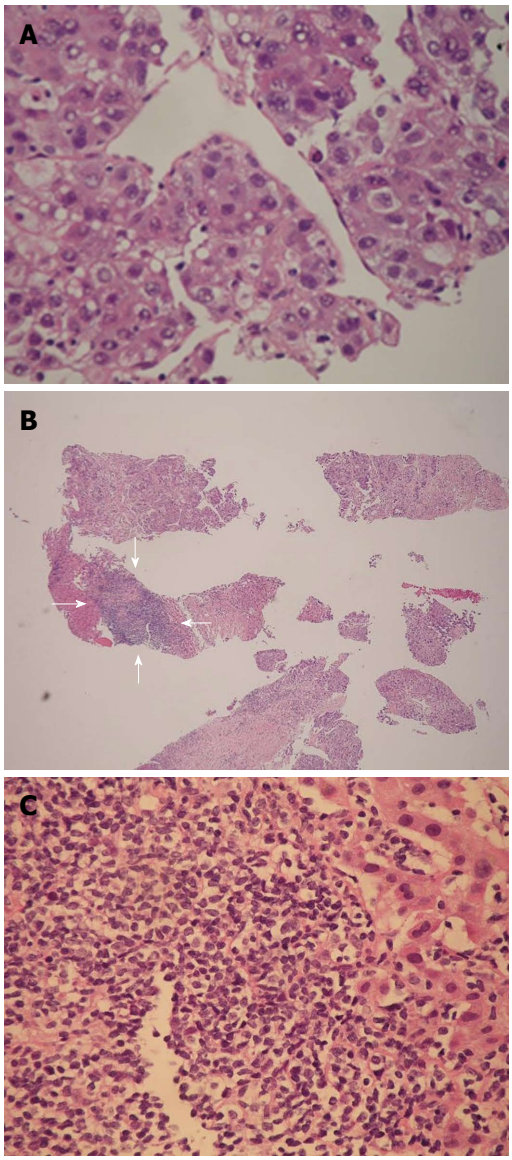



**Figure 5 Histological study of the liver tumor.** A: Neoplastic hepatocytes with pleomorphic nuclei and prominent nucleoli; they are considered diagnostic for hepatocellular carcinoma [Hematoxylin and eosin (HE) staining; original magnification × 400]; B: Tissue cores with thick trabeculae of neoplastic hepatocytes and a patchy lymphoid infiltrated (arrows) (HE staining; original magnification × 40); C: Small lymphoid cells with slightly irregular nuclei (HE staining; original magnification × 400).

is poor. Although a positive relationship has been reported between HBV infection and NHL[7], only a few reported cases of coexistent MCL and HCC, especially in patients with chronic hepatitis B but no liver cirrhosis[8-10]. Most of the patients had HCV infection and liver cirrhosis[3,11,12].

The association between HBV and HCC is well established. Worldwide, HBV accounts for more than 50% of the HCC[13]. The risk of HCC increases with higher HBV DNA level. Patients infected with HBV also have a higher risk of developing non-Hodgkin lymphoma. Ulcickas Yood *et al*[7] found that patients with HBV had a 2.8 times higher risk of NHL. However, only few cases of coexistent HCC and NHL in patients

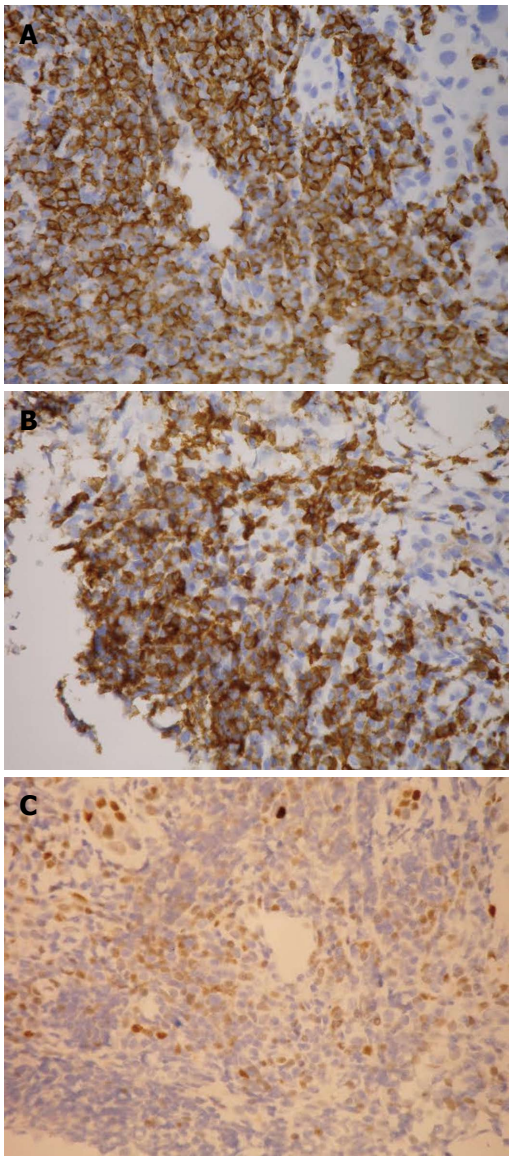



**Figure 6 Immunochemical analysis of the liver tumor.** A: The small lymphoid cells were positive for CD20 (CD20 immunostaining; original magnification × 400); B: The small lymphoid cells were positive for CD5 (CD5 immunostaining; original magnification × 400); C: The small lymphoid cells were positive for cyclin D1 (cyclin D1 immunostaining; original magnification × 400).

with HBV have been reported in the literature. Talamo *et al*[8] reported the first case of simultaneous primary hepatic lymphoma and HCC in a patient with chronic HBV. Takeshima *et al*[14] reported another patient with HBV infection developed coexistent hepatic mucosa-associated lymphoid tissue lymphoma and HCC. These two reports described primary hepatic lymphoma that did not involve extra-hepatic lymph nodes or organs. Cavanna *et al*[9] reported a case of hepatic NHL relapse without extra-hepatic involvement in a patient who had HCC. Shikuwa *et al*[10] and Monarca *et al*[15] reported two cases of HCC and malignant lymphoma with extra-hepatic involvement. These cases revealed the rare coexistence of HCC and NHL with or without extrahepatic involvement in hepatitis B carrier, but the role of HBV in HCC and NHL was not addressed. To the best of our knowledge, this is the only published report to date. Other reports include cases of HCV infection and two cases without chronic hepatitis or liver cirrhosis[16,17]. In the present case, this patient had not received any prior chemotherapy. Moreover, there are no clinical or pathologic evidences of HCV infection, cirrhosis or alcohol abuse. Therefore, the only etiology for the two tumor growth is HBV infection. This patient is a rare case of coexistent HCC and mantle cell lymphoma in HBV infection.

Only a few studies on the association between HCC and NHL have been published. Di Stasi *et al*[2] reported that 4 of the 132 patients with NHL developed HCC and that all four patients had liver cirrhosis. Tanaka *et al*[3] reported that 9 of the 592 patients with NHL developed HCC during the first 10 years following the diagnosis. Of the nine patients who developed HCC, eight had clinical or histologic evidence of cirrhosis, six were HCV antibody positive, and none had HBV infection. The study also found that patients who received chemotherapy for NHL had a significantly increased risk of HCC. Civardi *et al*[4] reported 7 cases of HCC among the 414 patients with NHL; all of these cases were HCV antibody positive. Based on these studies, hepatitis virus infection, liver damage and antineoplastic agents may play important roles in the development of concurrent HCC and NHL. A subpopulation of chemotherapy-induced lymphoma cell may selectively invade the liver. Recurrence of HCC may have resulted in the simultaneous co-localization of the two distinct tumors. Another study by Xiong *et al*[18] found that the Cdc6 G1321A polymorphism lowers the risk of developing both NHL and HCC.

There are accumulated evidences on the associations of HCV and HBV with hematologic malignancies, in particular B-cell NHL. HCV has been the most frequently studied. Chronic viral antigen stimulation that leads to proliferation of B-cells and mutations in tumor suppressor genes has been proposed as two likely pathways of HCV-induced NHL[19,20]. Conversely, the association between NHL and HBV has not been studied. Similar to HCV, HBV may induce NHL formation through the similar mechanism. Two mechanisms have been proposed[21]. In HBV-infected patients, the chronic antigen stimulation from HBV activates B-cells, which in the long term leads to subsequent DNA damage and lymphoma formation. The second mechanism involves synthesis of viral antigen and assembly of viral particles in hepatocytes and lymphocytes. HBV particles can then infect other lymphocytes that are located in lymphoid organs[5,22]. Viral DNA integrats into the host genome; this leads to overexpression of cellular oncogenes or down-regulation of tumor suppressor genes. Further investigations are needed to address these questions.

In the present case, liver tumor biopsy revealed a coexistent HCC and mantle cell lymphoma. The occurrence of one tumor spreading into another tumor, referred to as "tumor-to-tumor metastasis (TTM)", is

rare. The most frequent cancer with metastasis is lung cancer. And the most frequent recipients are renal clear cell carcinomas. To date, NHL has rarely infiltrated other tumors, especially HCC. To our knowledge, only one similar case has been reported[23].

In summary, we have presented a rare case of hepatic co-localization of HCC and mantle cell lymphoma in a patient with HBV infection. The case showed a rare pattern of "tumor-to-tumor metastasis." This case suggests that although lymph node enlargements are often considered to be reactive or metastatic lymphadenopathy in chronic hepatitis B patients who have HCC, NHL should also be considered as a differential diagnosis. Future large studies are required to establish firm evidence of the relationship between the occurrence of NHL and HCC.

## COMMENTS

### Case characteristics

A 52-year-old male with hepatitis B infection presented with easy fatigue, poor appetite and progressive weight loss of 10 kg over the past 2 mo.

### Clinical diagnosis

Multiple enlarged lymph nodes were found on the neck and in the inguinal region upon physical examination of the patient.

### Differential diagnosis

Advanced hepatocellular carcinoma (HCC) with distal lymph node metastases; lymphoma with hepatic involvement.

### Laboratory diagnosis

AST: 41 U/L; Hb: 18.4 g/dL; PLT: 97 × $10^3$ UL; alpha-fetoprotein: 49465.6 ng/mL; metabolic panel was within the normal reference limits.

### Imaging diagnosis

Contrasted computed tomography showed a 7-cm in diameter liver mass at S7 with heterogeneous enhancement in the arterial phase and wash out in the delay phase. Moreover, splenomegaly and multiple mediastinal and mesenteric enlarged lymph nodes were observed.

### Pathological diagnosis

Liver biopsy revealed HCC with an aggregate of small to medium-sized lymphoid cells that were CD20, CD5 and cyclin D1 positive.

### Terminology

Radiofrequency ablation of the liver tumor was performed, and cyclophosphamide-hydroxydaunorubicin-oncovin-prednisone chemotherapy was administered to treat the lymphoma.

### Experience and lessons

This report presents a rare case of coexistent HCC and mantle cell lymphoma in a patient with hepatitis B virus infection. The case suggests that although lymph node enlargements are often considered to be reactive or metastatic lymphadenopathy in chronic hepatitis B patients who have HCC, non-Hodgkin's lymphoma should also be considered as a differential diagnosis.

### Peer-review

The submitted manuscript represents a case report about a male patient chronically infected with HBV. The patient had enlarged lymph nodes in the neck and inguinal regions, and a tumor in the Cauinaud's segment 7. The case report describes an interesting case with lymphoid cells infiltrating the HCC. The authors should clarify or discuss whether there is an evidence of a wider infiltration into the liver tissue or exclusively into the HCC. Also, please state whether there was any evidence for a spread into the gastrointestinal tract and/or bone marrow.

## REFERENCES


1 **Schütte K**, Bornschein J, Malfertheiner P. Hepatocellular carcinoma--epidemiological trends and risk factors. *Dig Dis* 2009; **27**: 80-92 [PMID: 19546545 DOI: 10.1159/000218339]

2 **Di Stasi M**, Cavanna L, Fornari F, Civardi G, Rossi S, Sbolli G, Giacani M, Fermi S, Buscarini L. Association of non-Hodgkin's lymphoma and hepatocellular carcinoma. *Oncology* 1990; **47**: 80-83 [PMID: 2153948]

3 **Tanaka H**, Tsukuma H, Teshima H, Ajiki W, Koyama Y, Kinoshita N, Masaoka T, Oshima A. Second primary cancers following non-Hodgkin's lymphoma in Japan: increased risk of hepatocellular carcinoma. *Jpn J Cancer Res* 1997; **88**: 537-542 [PMID: 9263530 DOI: 10.1111/j.1349-7006.1997.tb00416.x]

4 **Civardi G**, Vallisa D, Bertè R, Lazzaro A, Moroni CF, Cavanna L. Focal liver lesions in non-Hodgkin's lymphoma: investigation of their prevalence, clinical significance and the role of Hepatitis C virus infection. *Eur J Cancer* 2002; **38**: 2382-2387 [PMID: 12460782]

5 **Pontisso P**, Vidalino L, Quarta S, Gatta A. Biological and clinical implications of HBV infection in peripheral blood mononuclear cells. *Autoimmun Rev* 2008; **8**: 13-17 [PMID: 18706529 DOI: 10.1016/j.autrev.2008.07.016]

6 **Giacomin A**, Cazzagon N, Sergio A, Vanin V, Farinati F. Hepatitis B virus-related hepatocellular carcinoma: primary, secondary, and tertiary prevention. *Eur J Cancer Prev* 2011; **20**: 381-388 [PMID: 21540746 DOI: 10.1097/CEJ.0b013e328346399b]

7 **Ulcickas Yood M**, Quesenberry CP, Guo D, Caldwell C, Wells K, Shan J, Sanders L, Skovron ML, Iloeje U, Manos MM. Incidence of non-Hodgkin's lymphoma among individuals with chronic hepatitis B virus infection. *Hepatology* 2007; **46**: 107-112 [PMID: 17526021]

8 **Talamo TS**, Dekker A, Gurecki J, Singh G. Primary hepatic malignant lymphoma: its occurrence in a patient with chronic active hepatitis, cirrhosis, and hepatocellular carcinoma associated with hepatitis B viral infection. *Cancer* 1980; **46**: 336-339 [PMID: 6248191]

9 **Cavanna L**, Civardi G, Fornari F, Vallisa D, Berte R, Buscarini E, Sbolli G, Paties C, Foroni R, Di Stasi M. Simultaneous relapse of liver cell carcinoma and non-Hodgkin's lymphoma in the liver. Report of a case with diagnosis by ultrasonically guided fine needle aspiration biopsy. *Acta Cytol* 1994; **38**: 451-454 [PMID: 8191841]

10 **Shikuwa S**, Ito M, Nakashima M, Hamasaki K, Naito S, Sekine I, Fujii H. Autopsy case of colocalized tumors of hepatocellular carcinoma and malignant lymphoma. *J Gastroenterol* 1996; **31**: 129-132 [PMID: 8808442 DOI: 10.1007/BF01211200]

11 **Suriawinata A**, Ye MQ, Emre S, Strauchen J, Thung SN. Hepatocellular carcinoma and non-Hodgkin lymphoma in a patient with chronic hepatitis C and cirrhosis. *Arch Pathol Lab Med* 2000; **124**: 1532-1534 [PMID: 11035591]

12 **Ohtsubo K**, Oku E, Imamura R, Seki R, Hashiguchi M, Osaki K, Yakushiji K, Yoshimoto K, Ogata H, Nagamatsu H, Ando E, Shimamatsu K, Okamura T, Sata M. Simultaneous hepatic relapse of non-Hodgkin's lymphoma and hepatocellular carcinoma in a patient with hepatitis C virus-related cirrhosis. *Acta Haematol* 2006; **116**: 266-271 [PMID: 17119328 DOI: 10.1159/000095878]

13 **Perz JF**, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol* 2006; **45**: 529-538 [PMID: 16879891 DOI: 10.1016/j.jhep.2006.05.013]

14 **Takeshima F**, Kunisaki M, Aritomi T, Osabe M, Akama F, Nakasone T, Niino D, Katayama S, Isomoto H, Omagari K, Mizuta Y, Murata I, Kohno S. Hepatic mucosa-associated lymphoid tissue lymphoma and hepatocellular carcinoma in a patient with hepatitis B virus infection. *J Clin Gastroenterol* 2004; **38**: 823-826 [PMID:

15365414]
15 **Monarca R**, Montanaro M, Stroffolini T. Simultaneous occurrence of hepatocellular carcinoma and low grade non-Hodgkin's lymphoma in a chronic hepatitis B surface antigen carrier. *Ann Hematol* 1999; **78**: 531 [PMID: 10602899 DOI: 10.1007/s002770050552]
16 **Kataoka TR**, Tsukamoto Y, Kanazawa N, Izumi T, Awata N, Nishizawa Y, Ohsawa M, Ishiguro S. Concomitant hepatocellular carcinoma and non-Hodgkin's lymphoma in a patient with nodular regenerative hyperplasia. *Pathol Int* 2006; **56**: 279-282 [PMID: 16669877]
17 **Heidecke S**, Stippel DL, Hoelscher AH, Wedemeyer I, Dienes HP, Drebber U. Simultaneous occurrence of a hepatocellular carcinoma and a hepatic non-Hodgkin's lymphoma infiltration. *World J Hepatol* 2010; **2**: 246-250 [PMID: 21161005 DOI: 10.4254/wjh.v2.i6.246]
18 **Xiong XD**, Qiu FE, Fang JH, Shen Y, Liang C, Jiang W, Zhuang SM. Association analysis between the Cdc6 G1321A polymorphism and the risk for non-Hodgkin lymphoma and hepatocellular carcinoma. *Mutat Res* 2009; **662**: 10-15 [PMID: 19101572 DOI: 10.1016/j.mrfmmm.2008.11.014]
19 **Schöllkopf C**, Smedby KE, Hjalgrim H, Rostgaard K, Panum I, Vinner L, Chang ET, Glimelius B, Porwit A, Sundström C, Hansen M, Adami HO, Melbye M. Hepatitis C infection and risk of malignant lymphoma. *Int J Cancer* 2008; **122**: 1885-1890 [PMID: 18271005 DOI: 10.1002/ijc.23416]
20 **Marcucci F**, Mele A. Hepatitis viruses and non-Hodgkin lymphoma: epidemiology, mechanisms of tumorigenesis, and therapeutic opportunities. *Blood* 2011; **117**: 1792-1798 [PMID: 20959600 DOI: 10.1182/blood-2010-06-275818]
21 **Marcucci F**, Spada E, Mele A, Caserta CA, Pulsoni A. The association of hepatitis B virus infection with B-cell non-Hodgkin lymphoma - a review. *Am J Blood Res* 2012; **2**: 18-28 [PMID: 22432084]
22 **Féray C**, Zignego AL, Samuel D, Bismuth A, Reynes M, Tiollais P, Bismuth H, Brechot C. Persistent hepatitis B virus infection of mononuclear blood cells without concomitant liver infection. The liver transplantation model. *Transplantation* 1990; **49**: 1155-1158 [PMID: 2360255 DOI: 10.1097/00007890-199006000-00025]
23 **Utsunomiya T**, Okamoto M, Tsujita E, Yamamoto M, Tsutsui S, Fujihara M, Ishida T. Hepatocellular carcinoma infiltrated with non-Hodgkin's lymphoma: report of a case. *Surg Today* 2009; **39**: 1010-1012 [PMID: 19882328 DOI: 10.1007/s00595-009-3966-0]

**P- Reviewer:** Netter HJ **S- Editor**: Ma YJ **L- Editor**: A
**E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i45.12987

*World J Gastroenterol* 2015 December 7; 21(45): 12987-12988
ISSN 1007-9327 (print) ISSN 2219-2840 (online)




# Percutaneous peritoneal drainage in isolated neonatal gastric perforation

Mustafa Aydin, Ugur Deveci, Erdal Taskin, Unal Bakal, Mehmet Kilic

Mustafa Aydin, Ugur Deveci, Erdal Taskin, Mehmet Kilic, Department of Pediatrics-Neonatology, Firat University School of Medicine, Elazig 23119, Turkey

Unal Bakal, Departments of Pediatric Surgery, Firat University School of Medicine, Elazig 23119, Turkey

Author contributions: Aydin M owned to referring article and writing of the paper; Taskin E checked paper; Deveci U provided related full text articles; Bakal U did language redaction; Kilic M advised subject for writing a letter to referring original paper.

Conflict-of-interest statement: No any conflict-of-interest to declare.



Correspondence to: Mustafa Aydin, MD, Department of Pediatrics-Neonatology, Firat University School of Medicine, Elazig 23119, Turkey. dr1mustafa@hotmail.com
Telephone: +90-424-2333555
Fax: +90-424-2388096

Received: July 4, 2015
Peer-review started: July 7, 2015
First decision: August 2, 2015
Revised: August 4, 2015
Accepted: October 13, 2015
Article in press: October 13, 2015
Published online: December 7, 2015

## Abstract

A comment on the article by He *et al*, "Idiopathic neonatal pneumoperitoneum with favorable outcome: A case report and review", published on *World Journal of Gastroenterology* that reported a case of idiopathic neonatal pneumoperitoneum, possibly due to gastric perforation, with a favorable outcome without surgical intervention.

**Key words:** Pneumoperitoneum; Gastric perforation; Intestinal perforation; Conservative management; Percutaneous peritoneal drainage; Antibiotic; Newborn infant



**Core tip:** Neonatal gastric perforation is a rare, life-threatening problem. Although surgical repair is the principal mode of managing this life-threatening disease, conservative intervention, such as percutaneous peritoneal drainage, is an alternative approach, especially under specific conditions.

Aydin M, Deveci U, Taskin E, Bakal U, Kilic M. Percutaneous peritoneal drainage in isolated neonatal gastric perforation. *World J Gastroenterol* 2015; 21(45): 12987-12988 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i45/12987.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i45.12987

## TO THE EDITOR

We read with great interest the article by He *et al*[1], which reported a case of idiopathic neonatal pneumoperitoneum, possibly due to gastric perforation, with a favorable outcome without surgical intervention. Although the principal mode of managing this serious condition is primary surgical repair, the authors concluded that conservative management is feasible for idiopathic neonatal pneumoperitoneum and that a favorable outcome could be achieved without

an exploratory laparotomy if the condition were diagnosed promptly. We recently reported a similar case of neonatal pneumoperitoneum, possibly due to isolated gastric perforation, in an extremely low birth weight infant whose clinical condition contraindicated general anesthesia and an exploratory laparotomy, and who recovered with percutaneous peritoneal drainage, along with placement of a Penrose drain and the use of wide-spectrum antibiotics[2]. Therefore, we think that a conservative approach is an alternative treatment for neonatal pneumoperitoneum, even with gastric perforation, especially when general anesthesia and surgical repair are impossible, such as in very sick, extremely low birth weight infants.

Gastric perforation in the newborn is a rare, life-threatening problem that is seen mainly in premature infants. Its reported incidence is 1 in 5000 live births, and it constitutes 7% of all gastrointestinal perforations[2-4]. The mortality rate is still high despite early diagnosis and treatment due to accompanying problems. Postoperative complications may also cause morbidity and mortality. Although surgical repair is the principal mode of managing this life-threatening disease, percutaneous peritoneal drainage is an alternative under some conditions. Supporting our report, Hesketh *et al*[5] reported seven patients with neonatal esophageal perforation who were managed non-operatively. Five patients in their series required additional interventions, such as tube thoracostomies for pneumothoraces. Four of their patients survived, and three died. Therefore, they suggest that non-operative management of esophageal perforation in newborns may be a safe initial strategy, but more aggressive interventions may ultimately be required.

In conclusion, we believe that although the principal mode of managing neonatal gastric perforation is operative, conservative intervention such as percutaneous peritoneal drainage is an alternative approach, especially under specific conditions in order to avoid intra- and postoperative complications in this vulnerable population.

## REFERENCES


1 **He TZ**, Xu C, Ji Y, Sun XY, Liu M. Idiopathic neonatal pneumoperitoneum with favorable outcome: A case report and review. *World J Gastroenterol* 2015; **21**: 6417-6421 [PMID: 26034380 DOI: 10.3748/wjg.v21.i20.6417]

2 **Aydin M**, Zenciroğlu A, Hakan N, Erdoğan D, Okumuş N, Ipek MS. Gastric perforation in an extremely low birth weight infant recovered with percutaneous peritoneal drainage. *Turk J Pediatr* 2011; **53**: 467-470 [PMID: 21980855]

3 **Jawad AJ**, Al-Rabie A, Hadi A, Al-Sowailem A, Al-Rawaf A, Abu-Touk B, Al-Karfi T, Al-Sammarai A. Spontaneous neonatal gastric perforation. *Pediatr Surg Int* 2002; **18**: 396-399 [PMID: 12415364]

4 **Attridge JT**, Clark R, Walker MW, Gordon PV. New insights into spontaneous intestinal perforation using a national data set: (2) two populations of patients with perforations. *J Perinatol* 2006; **26**: 185-188 [PMID: 16493433]

5 **Hesketh AJ**, Behr CA, Soffer SZ, Hong AR, Glick RD. Neonatal esophageal perforation: nonoperative management. *J Surg Res* 2015; **198**: 1-6 [PMID: 26055213 DOI: 10.1016/j.jss.2015.05.018]


**P- Reviewer**: Classen CF **S- Editor**: Yu J **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