

World Journal of *Gastroenterology*

World J Gastroenterol 2015 December 21; 21(47): 13205-13402





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).

EDITORS-IN-CHIEF

Stephen C Strom, *Stockholm*
Andrzej S Tarnawski, *Long Beach*
Damian Garcia-Olmo, *Madrid*

ASSOCIATE EDITOR

Yung-Jue Bang, *Seoul*
Vincent Di Martino, *Besancon*
Daniel T Farkas, *Bronx*
Roberto J Firpi, *Gainesville*
Maria Gazouli, *Athens*
Chung-Feng Huang, *Kaohsiung*
Namir Katkhouda, *Los Angeles*
Anna Kramvis, *Johannesburg*
Wolfgang Kruis, *Cologne*
Peter L Lakatos, *Budapest*
Han Chu Lee, *Seoul*
Christine McDonald, *Cleveland*
Nahum Mendez-Sanchez, *Mexico City*
George K Michalopoulos, *Pittsburgh*
Suk Woo Nam, *Seoul*
Shu-You Peng, *Hangzhou*
Daniel von Renteln, *Montreal*
Angelo Sangiovanni, *Milan*
Hildegard M Schuller, *Knoxville*
Dong-Wan Seo, *Seoul*
Adrian John Stanley, *Glasgow*
Jurgen Stein, *Frankfurt*
Bei-Cheng Sun, *Nanjing*
Yoshio Yamaoka, *Yufu*

GUEST EDITORIAL BOARD MEMBERS

Jia-Ming Chang, *Taipei*
Jane CJ Chao, *Taipei*

Kuen-Feng Chen, *Taipei*
Tai-An Chiang, *Tainan*
Yi-You Chiou, *Taipei*
Seng-Kee Chuah, *Kaohsiung*
Wan-Long Chuang, *Kaohsiung*
How-Ran Guo, *Tainan*
Ming-Chih Hou, *Taipei*
Po-Shiuan Hsieh, *Taipei*
Ching-Chuan Hsieh, *Chiayi county*
Jun-Te Hsu, *Taoyuan*
Chung-Ping Hsu, *Taichung*
Chien-Ching Hung, *Taipei*
Chao-Hung Hung, *Kaohsiung*
Chen-Guo Ker, *Kaohsiung*
Yung-Chih Lai, *Taipei*
Teng-Yu Lee, *Taichung City*
Wei-Jei Lee, *Taoyuan*
Jin-Ching Lee, *Kaohsiung*
Jen-Kou Lin, *Taipei*
Ya-Wen Lin, *Taipei*
Hui-kang Liu, *Taipei*
Min-Hsiung Pan, *Taipei*
Bor-Shyang Sheu, *Tainan*
Hon-Yi Shi, *Kaohsiung*
Fung-Chang Sung, *Taichung*
Dar-In Tai, *Taipei*
Jung-Fa Tsai, *Kaohsiung*
Yao-Chou Tsai, *New Taipei City*
Chih-Chi Wang, *Kaohsiung*
Liang-Shun Wang, *New Taipei City*
Hsiu-Po Wang, *Taipei*
Jaw-Yuan Wang, *Kaohsiung*
Yuan-Huang Wang, *Taipei*
Yuan-Chuen Wang, *Taichung*

Deng-Chyang Wu, *Kaohsiung*
Shun-Fa Yang, *Taichung*
Hsu-Heng Yen, *Changhua*

MEMBERS OF THE EDITORIAL BOARD



Algeria

Saadi Berkane, *Algiers*
Samir Rouabhia, *Batna*



Argentina

N Tolosa de Talamoni, *Córdoba*
Eduardo de Santibanes, *Buenos Aires*
Bernardo Frider, *Capital Federal*
Guillermo Mazzolini, *Pilar*
Carlos Jose Pirola, *Buenos Aires*
Bernabé Matías Quesada, *Buenos Aires*
María Fernanda Troncoso, *Buenos Aires*



Australia

Golo Ahlenstiel, *Westmead*
Minoti V Apte, *Sydney*
Jacqueline S Barrett, *Melbourne*
Michael Beard, *Adelaide*
Filip Braet, *Sydney*
Guy D Eslick, *Sydney*
Christine Feinle-Bisset, *Adelaide*
Mark D Gorrell, *Sydney*
Michael Horowitz, *Adelaide*

Gordon Stanley Howarth, *Roseworthy*
 Seungha Kang, *Brisbane*
 Alfred King Lam, *Gold Coast*
 Ian C Lawrence, *Perth/Fremantle*
 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, *St Leonards*
 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 Robaey, *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, *Ribeirão Preto*
 Eduardo LR Mello, *Rio de Janeiro*
 Stihela 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 Alegre*
 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 Yang, *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 Gillissen, *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, *Larisa*
 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*
 Spiliot 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 Zazos, *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 Dhimani, *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 Thirumoothy, *Coimbatore*



Indonesia

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



Iran

Arezo 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 Tikva*

Jacob Yahav, *Rehovot*
 Doron Levi Zamir, *Cedera*
 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 Aurelio, *Rome*
 Salvatore 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 Bruscianno, *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*
 Carmela Loguercio, *Naples*
 Amedeo Lonardo, *Modena*
 Carmelo Luigiano, *Catania*
 Francesco Luzzza, *Catanzaro*
 Giovanni Maconi, *Milano*
 Antonio Macri, *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 Prantero, *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*
 Lucia 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 Kiriya, *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*
 Kunihiro 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 Lucian Dumitrascu, *Cluj-Napoca*

Carmen Fierbinteanu-Braticevici, *Bucharest*

Romeo G Mihaila, *Sibiu*

Lucian Negreanu, *Bucharest*

Adrian Saftoiu, *Craiova*

Andrada Seicean, *Cluj-Napoca*

Ioan Sporea, *Timisoara*

Letiția 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, *Linköping*

Johan Christopher Bohr, *Örebro*

Mauro D'Amato, *Stockholm*

Thomas Franzen, *Norrköping*

Evangelos Kalaitzakis, *Lund*

Riadh Sadik, *Gothenburg*

Per Anders Sandstrom, *Linköping*

Ervin Toth, *Malmö*

Konstantinos Tsimogiannis, *Vasteras*

Apostolos V Tsolakis, *Uppsala*

**Switzerland**

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

**Thailand**

Thawatthai Akaraviputh, *Bangkok*
P Yoysungnoen Chintana, *Pathumthani*
Veerapol Kukongviriyapan, *Muang*
Vijitra 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*
Gülüm Ozlem Elpek, *Antalya*
Ayse Basak Engin, *Ankara*
Eren Ersoy, *Ankara*
Osman Ersoy, *Ankara*
Yusuf Ziya Erzin, *Istanbul*
Mukaddes Esrefoglu, *Istanbul*
Levent Filik, *Ankara*
Ozgur Harmanaci, *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 Koulaouzis, *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 Boorum, *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 Ciacchio, *New York*
Dahn L Clemens, *Omaha*
Yingzi Cong, *Galveston*
Laura Iris Cosen-Binker, *Boston*
Joseph John Cullen, *Iowa*
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 Osona, *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*

**EDITORIAL**

- 13205** Safety of anti-tumor necrosis factor therapy during pregnancy in patients with inflammatory bowel disease
Androulakis I, Zavos C, Christopoulos P, Mastorakos G, Gazouli M

REVIEW

- 13212** Endoscopic ultrasound elastography: Current status and future perspectives
Cui XW, Chang JM, Kan QC, Chiorean L, Ignee A, Dietrich CF

ORIGINAL ARTICLE**Basic Study**

- 13225** Hepatitis B and C virus-induced hepatitis: Apoptosis, autophagy, and unfolded protein response
Yeganeh B, Rezaei Moghadam A, Alizadeh J, Wiechec E, Alavian SM, Hashemi M, Geramizadeh B, Samali A, Bagheri Lankarani K, Post M, Peymani P, Coombs KM, Ghavami S
- 13240** Histidine decarboxylase and urinary methylimidazoleacetic acid in gastric neuroendocrine cells and tumours
Tsolakis AV, Grimelius L, Granerus G, Stridsberg M, Falkmer SE, Janson ET
- 13250** Protective role of adiponectin in a rat model of intestinal ischemia reperfusion injury
Liu XH, Yang YW, Dai HT, Cai SW, Chen RH, Ye ZQ
- 13259** Evaluation of epithelial-mesenchymal transitioned circulating tumor cells in patients with resectable gastric cancer: Relevance to therapy response
Li TT, Liu H, Li FP, Hu YF, Mou TY, Lin T, Yu J, Zheng L, Li GX
- 13268** Potential roles of EZH2, Bmi-1 and miR-203 in cell proliferation and invasion in hepatocellular carcinoma cell line Hep3B
Yang F, Lv LZ, Cai QC, Jiang Y
- 13277** Guggulsterone induces apoptosis of human hepatocellular carcinoma cells through intrinsic mitochondrial pathway
Shi JJ, Jia XL, Li M, Yang N, Li YP, Zhang X, Gao N, Dang SS
- 13288** Mast cell tryptase and carboxypeptidase A expression in body fluid and gastrointestinal tract associated with drug-related fatal anaphylaxis
Guo XJ, Wang YY, Zhang HY, Jin QQ, Gao CR

Retrospective Study

- 13294 Surgical care quality and oncologic outcome after D2 gastrectomy for gastric cancer

Mrena J, Mattila A, Böhm J, Jantunen I, Kellokumpu I

- 13302 Clinical impact of atypical endoscopic features in rectal neuroendocrine tumors

Hyun JH, Lee SD, Youk EG, Lee JB, Lee EJ, Chang HJ, Sohn DK

- 13309 Features associated with progression of small pancreatic cystic lesions: A retrospective study

Tsai HM, Chuang CH, Shan YS, Liu YS, Chen CY

- 13316 Long-term outcomes after radical gastrectomy in gastric cancer patients with overt bleeding

Wang L, Wang XA, Hao JQ, Zhang LN, Li ML, Wu XS, Weng H, Lv WJ, Zhang WJ, Chen L, Xiang HG, Lu JH, Liu YB, Dong P

- 13325 Feasible endoscopic therapy for early gastric cancer

Guo TJ, Qin JY, Zhu LL, Wang J, Yang JL, Wang YP

Clinical Trials Study

- 13332 Robotic gastrectomy with transvaginal specimen extraction for female gastric cancer patients

Zhang S, Jiang ZW, Wang G, Feng XB, Liu J, Zhao J, Li JS

- 13339 Enhanced recovery after surgery with laparoscopic radical gastrectomy for stomach carcinomas

Abdikarim I, Cao XY, Li SZ, Zhao YQ, Taupyk Y, Wang Q

Observational Study

- 13345 Colorectal resection in deep pelvic endometriosis: Surgical technique and post-operative complications

Milone M, Vignali A, Milone F, Pignata G, Elmore U, Musella M, De Placido G, Mollo A, Fernandez LMS, Coretti G, Bracale U, Rosati R

- 13352 Characteristics of symptomatic reflux episodes in Japanese proton pump inhibitor-refractory non-erosive reflux disease patients

Nakagawa K, Koike T, Iijima K, Saito M, Kikuchi H, Hatta W, Ara N, Uno K, Asano N, Shimosegawa T

- 13360 Development of *Fok-I* based nested polymerase chain reaction-restriction fragment length polymorphism analysis for detection of hepatitis B virus X region V5M mutation

Kim H, Hong SH, Lee SA, Gong JR, Kim BJ

- 13368 Epidemiological study of elderly constipation in Beijing

Zhang M, Yang XJ, Zhu HM, Tang Z, Li BY, Zhao DD

META-ANALYSIS

- 13374** Endoscopic stenting for inoperable malignant biliary obstruction: A systematic review and meta-analysis
Zorrón Pu L, de Moura EGH, Bernardo WM, Baracat FI, Mendonça EQ, Kondo A, Luz GO, Furuya Júnior CK, Artifon ELA
- 13386** Effect of intraperitoneal local anesthetic on pain characteristics after laparoscopic cholecystectomy
Choi GJ, Kang H, Baek CW, Jung YH, Kim DR

CASE REPORT

- 13396** Over-the-scope clip to close a gastrocutaneous fistula after esophagectomy
Shen SS, Zhang XQ, Li ZL, Zou XP, Ling TS

LETTERS TO THE EDITOR

- 13400** Current status of superparamagnetic iron oxide contrast agents for liver magnetic resonance imaging
Wang YXJ

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Yeun-Jun Chung, MD, PhD, Director, Professor, Integrated Research Center for Genome Polymorphism (IRCGP), Department of Microbiology, The Catholic University of Korea, College of Medicine, Seoul 137-701, South Korea

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1377 experts in gastroenterology and hepatology from 68 countries.

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

INDEXING/ABSTRACTING

World Journal of Gastroenterology is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. According to the 2014 Journal Citation Reports® released by Thomson Reuters (ISI), the 2014 impact factor for *WJG* is 2.369, ranking 41 among 76 journals in gastroenterology and hepatology, quartile in category Q2.

FLYLEAF

I-IX Editorial Board

EDITORS FOR THIS ISSUE

Responsible Assistant Editor: *Xiang Li*
Responsible Electronic Editor: *Xiao-Mei Liu*
Proofing Editor-in-Chief: *Lian-Sheng Ma*

Responsible Science Editor: *Yuan Qi*
Proofing Editorial Office Director: *Jin-Lei Wang*

NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORS-IN-CHIEF
Damian Garcia-Olmo, MD, PhD, Doctor, Professor, Surgeon, Department of Surgery, Universidad Autonoma de Madrid; Department of General Surgery, Fundacion Jimenez Diaz University Hospital, Madrid 28040, Spain

Stephen C Strom, PhD, Professor, Department of Laboratory Medicine, Division of Pathology, Karolinska Institutet, Stockholm 141-86, Sweden

Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of Medicine, Chief Gastroenterology, VA

Long Beach Health Care System, University of California, Irvine, CA, 5901 E. Seventh Str., Long Beach, CA 90822, United States

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 21, 2015

COPYRIGHT
© 2015 Baishideng Publishing Group Inc. Articles published by this Open-Access journal are distributed under the terms of the Creative Commons Attribution Non-commercial License, which permits use, distribution, and reproduction in any medium, provided the original work is properly cited, the use is non commercial and is otherwise in compliance with the license.

SPECIAL STATEMENT
All articles published in journals owned by the Baishideng Publishing Group (BPG) represent the views and opinions of their authors, and not the views, opinions or policies of the BPG, except where otherwise explicitly indicated.

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/>

Safety of anti-tumor necrosis factor therapy during pregnancy in patients with inflammatory bowel disease

Ioannis Androulakis, Christos Zavos, Panagiotis Christopoulos, George Mastorakos, Maria Gazouli

Ioannis Androulakis, Panagiotis Christopoulos, George Mastorakos, Endocrine Unit, Aretaieion Hospital, University of Athens Medical School, 11527 Athens, Greece

Christos Zavos, Maria Gazouli, Department of Basic Biological Sciences, Laboratory of Biology, School of Medicine, University of Athens, 11527 Athens, Greece

Author contributions: Androulakis I, Christopoulos P and Gazouli M wrote the paper; Zavos C and Mastorakos G performed the critical review and edited the manuscript.

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

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Maria Gazouli, Assist Professor, Department of Basic Biological Sciences, Laboratory of Biology, School of Medicine, University of Athens, Michalakopoulou 176, 11527 Athens, Greece. mgazouli@med.uoa.gr
 Telephone: +30-210-7462231
 Fax: +30-210-7462231

Received: July 7, 2015
 Peer-review started: July 8, 2015
 First decision: July 20, 2015
 Revised: July 24, 2015
 Accepted: October 12, 2015
 Article in press: October 13, 2015
 Published online: December 21, 2015

Abstract

Treatment of inflammatory bowel disease has sign-

ificantly improved since the introduction of biological agents, such as infliximab, adalimumab, certolizumab pegol, and golimumab. The Food and Drug Administration has classified these factors in category B, which means that they do not demonstrate a fetal risk. However, during pregnancy fetuses are exposed to high anti-tumor necrosis factor (TNF) levels that are measurable in their plasma after birth. Since antibodies can transfer through the placenta at the end of the second and during the third trimesters, it is important to know the safety profile of these drugs, particularly for the fetus, and whether maintaining relapse of the disease compensates for the potential risks of fetal exposure. The limited data available for the anti-TNF drugs to date have not demonstrated any significant adverse outcomes in the pregnant women who continued their therapy from conception to the first trimester of gestation. However, data suggest that anti-TNFs should be discontinued during the third trimester, as they may affect the immunological system of the newborn baby. Each decision should be individualized, based on the distinct characteristics of the patient and her disease. Considering all the above, there is a need for more clinical studies regarding the effect of anti-TNF therapeutic agents on pregnancy outcomes.

Key words: Anti-tumor necrosis factor; Pregnancy; Adverse effects; Crohn's disease; Ulcerative colitis; Inflammatory bowel disease

© **The Author(s) 2015.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Modern inflammatory bowel disease treatment includes biological therapy, such as anti-tumor necrosis factor (TNF) agents. There are concerns over the use of anti-TNF agents during pregnancy, although the data available to date are limited. No significant increases in the adverse outcomes of pregnancy have been reported in women who continued their treatment from conception to the first trimester of pregnancy.

Decision making in this case dictates that the mother's benefits of maintaining relapse of the disease through continuation of anti-TNFs exceeds the potential risks of fetal exposure.

Androulakis I, Zavos C, Christopoulos P, Mastorakos G, Gazouli M. Safety of anti-tumor necrosis factor therapy during pregnancy in patients with inflammatory bowel disease. *World J Gastroenterol* 2015; 21(47): 13205-13211 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13205.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13205>

INFLAMMATORY BOWEL DISEASE AND ANTI-TNF THERAPY

Anti-tumor necrosis factor (TNF) agents are an effective therapeutic option in patients with inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD). The highest incidence of IBD is seen between the second and fourth decades of life, which is the most fertile age for women. However, data concerning their safety during pregnancy are scarce.

CD usually affects people between the second and third decades of life, thereby having a negative impact on pregnancy outcomes. Simultaneously, pregnancy itself can negatively affect the outcome of CD^[1]. Approximately 25% of women with IBD can achieve normal conception and fertilization. It is already known that one-third of patients with active CD present a relapsing course during pregnancy, and, if conception occurs while the disease is active, a deterioration in two-thirds of the cases is observed^[2].

Likewise, UC is a chronic, inflammatory disease that predominantly occurs during reproductive age. Although controversial, studies indicate that UC may increase the risk for preterm births, suspension of full fetal development, and perinatal mortality. The controversy surrounding these studies arises from differences in their design and their usually small sample size^[3].

Two studies to date have reported an increased incidence of congenital abnormalities in fetuses of women with UC^[4-6]. Dominitz *et al*^[4] underscored that it is not possible to distinguish between small or large abnormalities and that chromosomal aberrations should also be taken into consideration. In another case series, it was reported that women with UC present with a 30% increase (though non-significant, 95%CI: 0.9-1.8) in congenital abnormalities of fetuses and a significantly higher risk of certain abnormalities, such as lesions in the extremities, obstruction of the urinary tract, and multiple genetic abnormalities.

Modern IBD treatment armamentarium includes biological therapy, such as anti-TNF agents, accompanied with concerns over their use during gestation. In

general, the Food and Drug Administration has categorized all drugs to five categories, A, B, C, D, and X, based on the incurring fetal risk. According to the Food and Drug Administration (FDA), category A consists of drugs for which controlled studies in women have failed to demonstrate a risk to the fetus in the first trimester, and, thus, fetal harm appears remote. Category B consists of either animal reproduction studies that have not demonstrated a fetal risk but have no corresponding human studies or animal studies that have shown adverse effects that have not been confirmed in controlled studies of women in the first trimester (or subsequent trimesters). Category C means that animal studies have revealed adverse fetal effects but that no controlled studies in women have been done or that no studies in women or animals are available. Category D consists of drugs for which human studies and/or adverse reaction data have shown evidence of fetal risk, but the benefits of use may outweigh the potential risks. Category X encompasses drugs in which human and animal studies have demonstrated fetal abnormalities, and the risk of using the drug in a pregnant woman clearly outweighs any benefit.

In particular, although there are limited data to date concerning the use of anti-TNF agents during pregnancy, no significant increases in the adverse outcomes of pregnancy have been reported in women who continued their treatment from conception to the first trimester of pregnancy^[7,8]. The FDA has classified these factors in category B. Most researchers, however, recommend discontinuing anti-TNF therapy prior to pregnancy^[7].

Decision making, however, in this case scenario dictates that the mother's benefits of maintaining relapse of the disease through continuation of anti-TNFs exceeds the potential risks of fetal exposure. The fact that antibodies transfer through the placenta at the end of the second and during the third trimesters suggests that the scheduled dosing of anti-TNF agents may continue in the first 20 wk of pregnancy and then should stop. However, Mishkin *et al*^[9] studied pregnancy outcomes in 92 to 122 cases and reported that the incidence of miscarriage and dead embryos was not significantly different than the general population and patients that are not under any biological treatment therapy.

Although biological drugs do not seem to possess any teratogenic properties, at least in animal models, they may inhibit maturation of the immunological system of the newborn child. Comparative studies between women with IBD exposed to anti-TNF agents during pregnancy and the general population did not demonstrate a high risk to the fetus incurred from anti-TNF use. It is important to note that there have been published cases of pregnant women who received anti-TNF drugs that did not report any side effects. Nevertheless, the FDA recommends that doctors

should evaluate each case with increased caution and prudence and not to encourage anti-TNF use during pregnancy unless necessary^[7].

With respect to IBD patients under biological treatment who become pregnant or if a pregnancy occurs while the disease is active, requiring immediate biological treatment, decisions should be made on an individual basis, as the short- and long-term effects of fetal exposure to these specific factors are still under investigation. Furthermore, there are as yet no clear guidelines regarding the best decision, which is a balance between the risks and benefits for the mother and the child, separately. Therefore, the decision whether or not to use biological treatment during pregnancy should be made carefully, especially because the levels of anti-TNF drugs in the maternal serum are unknown and it is uncertain whether this treatment is safe for the fetus^[10].

Adverse pregnancy outcomes, such as premature birth, miscarriages, preeclampsia, and low birth weight embryos, have been observed in certain cases of fetal exposure to biological agents. However, they tend to be balanced by the need for clinical remission of the pregnant woman, and also, they may be an outcome of the autoimmune disease itself. Information regarding birth defects caused by anti-TNF agents is scarce, and to our knowledge, only one study has reported on the incidence of birth abnormalities in the offspring of women who were under anti-TNF therapy at some stage during gestation^[11].

Therefore, there is a need for more clinical studies regarding the effect of anti-TNF therapeutic agents on pregnancy outcomes. The decision whether or not the agent compensates for the risk of fetal exposure should be taken carefully by the clinician (*e.g.*, gastroenterologist) in collaboration with the gynecologist. Finally, psychological support for the pregnant woman and scheduled monitoring throughout pregnancy are recommended.

ANTI-TNF AGENTS

Anti-TNF biological agents are either fully chimeric or humanized antibodies for ligands or soluble receptors, or recombinant receptor antagonists. Their mode of action concerns inhibition of TNF- α , based on the pleiotropic function of the molecule in cellular proliferation, cell adhesion, migration, and inflammatory cytokine induction response. Their main purpose, therefore, is interference on the inflammation pathway. However, the way they achieve this differs among monoclonal antibodies and soluble receptors^[12].

Monoclonal antibodies were the first molecules developed for the inhibition of TNF agents, and they are widely used today in clinical practice. The production of monoclonal antibodies from mice was originally developed for research purposes in the 1980s. Mouse monoclonal antibodies are derived from

cell lines, known as hybridomas^[13]. A few years after the production of monoclonal antibodies as therapeutic targets, soluble forms of the TNF receptor (TNF-R) were isolated from human urine. The unique form of soluble TNF-R was one subunit, but it was immediately abandoned because of reduced binding of the ligand. The design and production of the soluble dimeric TNF-R form was an important step for the development of biological therapies in medicine^[14].

As biological agents are protein molecules, they are quickly degraded inside the stomach. For this reason, they are parenterally administered, *i.e.*, either subcutaneously or intravenously at regular intervals^[15].

The administration of biological agents has been associated with a number of side effects that should be known to clinicians treating these patients. Selecting the correct patients to receive biological agents as well the constant and close monitoring of them are essential fundamentals for administering anti-TNF therapy safely^[16,17].

Currently, four anti-TNF agents, namely infliximab, adalimumab, certolizumab pegol, and golimumab, are FDA-approved for treatment of IBD.

SAFETY OF ANTI-TNF TREATMENT DURING PREGNANCY

Infliximab

It is currently unknown whether infliximab causes damage to the fetus when administered to the mother or even if it can affect the reproductive capacity of women. However, it is certain that it should be given to pregnant women only when necessary. Because it does not interact with TNF- α factor of other species besides humans and chimpanzees, there have been no reproductive studies conducted in laboratory animals using infliximab. No indication of maternal toxicity, embryotoxicity, or teratogenesis has been observed in any study conducted in mice, using a similar antibody, which has the ability to selectively inhibit the activity of murine TNF- α agent. As an immunoglobulin antibody, infliximab crosses the placenta and has been detected for more than 6 mo in neonatal serum from children born to mothers who received the treatment during pregnancy. Therefore, these newborns, may be at increased risk for infection, and their vaccinations should be carried out with special attention (FDA, 2015).

Multiple case reports refer to women with IBD who received infliximab during pregnancy, four of which were CD patients. The first described a 26-year-old CD patient who became pregnant while receiving infliximab infusions. She gave birth to a 681 g baby at 24 wk of gestation. The neonate developed internal bleeding and was disconnected from artificial support on the third day of birth and subsequently died. However, the severity of her disease and the fact that the patient conceived at the moment when

IBD was still active and that she was also under other medications could have affected the pregnancy outcome. In the remaining three pregnancies, two full-term and one premature, the newborns were still alive months after birth^[18,19].

More recently, the Crohn's Therapy, Resource, Evaluation and Assessment Tool (TREAT) database has provided information for more patients. TREAT is a progressive record database, associated with studying patients with CD and evaluating safety of various treatments, including infliximab. These patients can be treated with infliximab or not. Of the 5807 patients who participated, there were 66 pregnancies, 36 of which were women under infliximab therapy prior to conception. Embryonic abnormalities were not observed in any of the embryos. Comparing patients who received infliximab vs those who did not, there were no significant differences in the rate of abortions (11.1% vs 7.1%, $P = 0.53$) and complications in newborns (8.3% vs 7.1%, $P = 0.78$). Patients under infliximab were more likely to exhibit severe form of the disease and to use steroids and immunomodulatory drugs^[20].

In 2004, Katz *et al.*^[20], elaborated a larger volume of information over the use of infliximab before and after gestation and published the first large-scale study on the probable outcome of pregnancy in women with CD. Data collection was based on drug indications, administration time in relation to conception, and course and outcome of pregnancy. The percentage of live embryos, miscarriages, and therapeutic abortions for women who were exposed to infliximab before or during gestation was comparable to the general population of pregnant women and to those of pregnant women with CD who had not received this specific therapy.

Of 146 identified pregnancies, 131 were exposed to infliximab, and data concerning their pregnancy outcome were provided for 96 cases. From these cases with known outcome, 64 (67%) resulted in live newborns, 14 (15%) in miscarriages, and 18 (19%) in termination for medical reasons. There were five reports of newborns born with complications: the first was born at 24 wk and expired, the second underwent a complex period of complications, the third was born with Fallot's tetralogy, the fourth developed an intestinal twist, and the last showed delayed growth and hypothyroidism. These results were similar to those of the general population of pregnant women and pregnant women with CD who did not receive infliximab. Based on these results, researchers concluded that the overall safety of the drug during gestation was not significantly different between women who received treatment and women who did not. However, an increased risk to the fetus was observed^[20].

The first study, concerning the intentional use of infliximab during pregnancy to induce and maintain remission of CD was conducted in 2005 by Mahadevan

et al.^[21]. All previous reports concerned unintentional or forced treatment for seriously ill women^[18-20]. This study included 10 women, eight of whom were receiving maintenance therapy with infliximab and one who had started drug administration at the third trimester of pregnancy due to a severe relapse of her CD. One of them started infliximab during the first trimester of pregnancy due to steroid-dependent disease but was not compliant and refused further treatment. Five pregnant women were also receiving mercaptopurine treatment, and another four were under corticosteroids, with the last one using steroids during the first trimester.

According to the results of the study^[21], four women exhibited no symptoms of the disease in the period from conception to labor (in the first two cases remission was observed and in the remaining two there was moderate disease activity). Two women showed disease improvement and four relapsed (two of them were in remission, although they relapsed after labor; the third showed mild relapse during the second trimester, but infliximab administration had just begun at that time; and the last developed a severe relapse and, therefore, started steroid administration). From those 10 women, eight gave birth by cesarean section (four due to active perianal disease, two due to active CD, one due to preterm birth, and the last due to a previous cesarean section). All 10 pregnancies resulted in live newborns. Congenital abnormalities were not observed in any of the fetuses, with an average follow up of 6 mo. Finally, there were three premature deliveries and two neonates with some embryonic disease not associated with infliximab. These data, in combination with earlier reports of accidental use of the drug during pregnancy, suggest that the benefit of infliximab use in inducing and maintaining remission in CD pregnant women usually outweighs fetal risk due to exposure to the agent^[21].

The most recent review^[22] concerning infliximab administration in IBD patients during gestation includes data until June 2014. It reported that infliximab crosses the placenta at the end of the second trimester of gestation. The use of the agent after the second trimester leads to intrauterine exposure. Although infliximab administration during gestation appears to be safe in the short term, there are concerns about the drug's effects on the development of the immune system of the fetus. Researchers argue that the administration of the drug should be discontinued, at least at the second trimester, when the mother is in remission, an approach considered safe for the mother that also minimizes the risk of fetal exposure to the agent. Infliximab has been detected in minor quantities in breast milk. Case reports do not suggest toxicity^[18,19], but the long-term effects of exposure are currently under investigation^[22].

Adalimumab

In 2002, the FDA has conducted an embryotoxicity

study^[23] of adalimumab in experimental animals and showed no harm incurring from this agent to the fetus for doses exceeding 100 mg/kg. However, in humans there are not sufficient data over its toxicity to the embryo. Since developmental and reproductive studies in laboratory animals are not always representative of the human response, it is reasonable to suggest that adalimumab should be administered during gestation only if considered necessary.

The first case report of maintenance treatment with adalimumab on IBD during pregnancy was conducted by Vesga *et al.*^[24]. This report described the pregnancy course of a 34-year-old woman with chronic and active CD during conception. Infliximab administration was initially successful, but over time, the response was lost. Subsequently, adalimumab was introduced and continued until 1 mo before conception. During gestation, the patient received a total of 38 doses of 40 mg of the drug, subcutaneously. She continued the drug administration after labor and during breast-feeding.

In detail, the mother had active disease at the time of conception, mild symptomatic improvement in the first trimester of gestation, and mild disease activity during the third trimester. After labor, she showed mild to disease in remission. Pregnancy occurred without any complications, and the fetal prenatal ultrasound showed normal growth without visible congenital anomalies. Due to the history of perianal disease, a cesarean section was performed without complications at 38.5 wk. No neonatal abnormalities were detected. The newborn was monitored up to 6 mo and showed normal development and function^[24].

The next case report concerning the use of adalimumab during IBD pregnancy was published 1 year later by Mishkin *et al.*^[9]. This particular case involved a 35-year-old female with persistent CD who did not respond to 5-aminosalicylic acid, antibiotics, or thiopurines. Seven years later, infliximab was introduced to the patient, initially with good clinical response. However, over time, she began requiring larger doses and smaller intervals between injections (every 4 or 6 wk), and she did not respond to the addition of corticosteroids. Adalimumab was then suggested at 80 mg initially and then 40 mg, subcutaneously, every second week. Her condition improved, but seven months after adalimumab initiation, she became pregnant.

By her own decision, she continued administration of the drug during gestation. She was admitted to a hospital at week 20 for unexplained fever and abdominal pain, but symptoms improved after a 2-wk course of steroids. She gave birth normally to a healthy neonate. Moreover, she continued treatment after labor and during breast-feeding. The newborn was monitored until 6 mo and showed normal development, in accordance to the previous study^[25].

Another case report described the pregnancy course of a 34-year-old woman with CD treated with

budesonide and then prednisone between weeks 6 and 20 of gestation. At this time, azathioprine was initiated at 100 mg/d, and adalimumab, initially, at 80 mg (one dose) and then at 40 mg every week. Labor was induced at 38 wk. The patient gave birth to a healthy newborn (2.89 kg) who developed normally until the age of 1 year^[25].

A subsequent case report described a 32-year-old woman with CD. Treatment with infliximab was successful in the first 17 mo of pregnancy. Then, her condition deteriorated, and she received adalimumab with an initial dose of 160 mg, followed by maintenance therapy, with a dose of 80 mg every other week. Remission was once again induced, and, therefore, medication was reduced to 40 mg every other week for further maintenance therapy, for a total of 18 doses. Overall, three non-intentional doses of adalimumab were given during the first trimester of gestation. At that time, treatment with adalimumab was automatically discontinued, and the patient and fetus were closely monitored for any side effects. Finally, the patient gave birth to a healthy newborn (3360 g), who remained healthy until the age of 2 years old^[26].

In a recent study by Schnitzler *et al.*^[27], pregnancy outcome was recorded and risk-to-benefit ratio was assessed in 212 women with IBD under infliximab or adalimumab. Specifically, 42 pregnancies with direct exposure to anti-TNF agents (35 infliximab, seven adalimumab) were compared to 23 pregnancies before IBD diagnosis, 78 pregnancies before infliximab administration, 53 pregnancies with direct exposure to infliximab, and 56 pregnancies of healthy women.

The results of this study showed that 32 of the 42 pregnancies resulted in live fetuses, with an average gestational duration of 38 wk. Three resulted in premature birth, six were low weight newborns, and one had not been completed at that time. One of them (1640 kg) was born the 33rd week and died 13 d later due to necrotic colitis. A total of eight miscarriages was observed. A trisomy 18 was diagnosed in a mother with CD (aged 37 years) under adalimumab therapy, and the pregnancy was terminated. The outcomes of pregnancies with direct exposure to anti-TNF agents was not different from those that had discontinued therapy before pregnancy or from those with indirect exposure to the agent, but it was worse compared to pregnancies before IBD diagnosis^[27].

In a subsequent study by Zelinkova *et al.*^[28], the focus was on the pregnancy of women with mild IBD who discontinued anti-TNF treatment. The investigators analyzed the levels of biological agents in omphalo-placenta samples. In total, 31 pregnancies were studied, with 28 of them exposed to anti-TNF agents (18 received infliximab and 13 adalimumab). Enzyme-dependent immunoprecipitation was used to measure the levels of factors in umbilical cord blood, collected from 18 newborns (12 of whom exposed to infliximab and six to adalimumab).

Among the patients who received infliximab, 12 (71%) discontinued treatment on the 30th week of gestation, and all patients remained in remission. All patients who received adalimumab discontinued treatment before 30th the week, which resulted in two relapses. Finally, 28 live newborns were born, there was one miscarriage in a patient receiving infliximab (on the 6th week of gestation), and two miscarriages among patients receiving adalimumab (on the sixth and eighth week). No congenital anomalies were observed^[28].

Certolizumab pegol and golimumab

Reproductive studies in laboratory animals are not possible to perform since certolizumab pegol is specific for humans. Likewise, golimumab effects on human pregnancies are lacking.

CONCLUSION

The decision to continue anti-TNF therapy during pregnancy is very difficult for both the clinician and the mother-patient. The aim is to maintain remission in the mother's disease while minimizing fetal exposure. It is a fact that placental transfer starts at the beginning of the second trimester of gestation and is maximized during the third. Therefore, it is preferable to avoid administering these agents in the third trimester of pregnancy. The decision should be made on an individual basis, always keeping in mind the distinct characteristics of the patient and her disease.

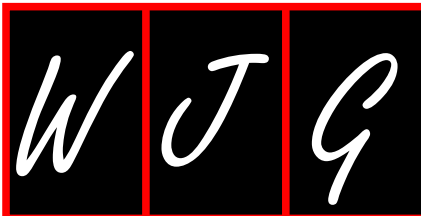
REFERENCES

- 1 **Malaguarnera M**, Cristaldi E, Romano G, Malaguarnera L. Autoimmunity in the elderly: Implications for cancer. *J Cancer Res Ther* 2012; **8**: 520-527 [PMID: 23361268]
- 2 **Caprilli R**, Gassull MA, Escher JC, Moser G, Munkholm P, Forbes A, Hommes DW, Lochs H, Angelucci E, Cocco A, Vucelic B, Hildebrand H, Kolacek S, Riis L, Lukas M, de Franchis R, Hamilton M, Jantschek G, Michetti P, O'Morain C, Anwar MM, Freitas JL, Mouzas IA, Baert F, Mitchell R, Hawkey CJ. European evidence based consensus on the diagnosis and management of Crohn's disease: special situations. *Gut* 2006; **55** Suppl 1: i36-i58 [PMID: 16481630 DOI: 10.1136/gut.2005.081950c]
- 3 **Nørgård B**, Fonager K, Sørensen HT, Olsen J. Birth outcomes of women with ulcerative colitis: a nationwide Danish cohort study. *Am J Gastroenterol* 2000; **95**: 3165-3170 [PMID: 11095336 DOI: 10.1111/j.1572-0241.2000.03290.x]
- 4 **Dominitz JA**, Young JC, Boyko EJ. Outcomes of infants born to mothers with inflammatory bowel disease: a population-based cohort study. *Am J Gastroenterol* 2002; **97**: 641-648 [PMID: 11926208 DOI: 10.1111/j.1572-0241.2002.05543.x]
- 5 **Larzilliere I**, Beau P. [Chronic inflammatory bowel disease and pregnancy. Case control study]. *Gastroenterol Clin Biol* 1998; **22**: 1056-1060 [PMID: 10051981]
- 6 **Nørgård B**, Puho E, Pedersen L, Czeizel AE, Sørensen HT. Risk of congenital abnormalities in children born to women with ulcerative colitis: a population-based, case-control study. *Am J Gastroenterol* 2003; **98**: 2006-2010 [PMID: 14499779 DOI: 10.1111/j.1572-0241.2003.07578.x]
- 7 **Furst DE**, Keystone EC, Braun J, Breedveld FC, Burmester GR, De Benedetti F, Dörner T, Emery P, Fleischmann R, Gibofsky A, Kalden JR, Kavanaugh A, Kirkham B, Mease P, Sieper J, Singer NG, Smolen JS, Van Riel PL, Weisman MH, Winthrop K. Updated consensus statement on biological agents for the treatment of rheumatic diseases, 2011. *Ann Rheum Dis* 2012; **71** Suppl 2: i2-i45 [PMID: 22460137 DOI: 10.1136/ard.2007.081430]
- 8 **Skomsvoll JF**, Wallenius M, Koksvik HS, Rødevand E, Salvesen KA, Spigset O, Kvien TK. Drug insight: Anti-tumor necrosis factor therapy for inflammatory arthropathies during reproduction, pregnancy and lactation. *Nat Clin Pract Rheumatol* 2007; **3**: 156-164 [PMID: 17334338 DOI: 10.1038/ncprheum0426]
- 9 **Mishkin DS**, Van Deinse W, Becker JM, Farraye FA. Successful use of adalimumab (Humira) for Crohn's disease in pregnancy. *Inflamm Bowel Dis* 2006; **12**: 827-828 [PMID: 16917239]
- 10 **Nielsen OH**, Loftus EV, Jess T. Safety of TNF- α inhibitors during IBD pregnancy: a systematic review. *BMC Med* 2013; **11**: 174 [PMID: 23902720 DOI: 10.1186/1741-7015-11-174]
- 11 **Carter JD**, Ladhani A, Ricca LR, Valeriano J, Vasey FB. A safety assessment of tumor necrosis factor antagonists during pregnancy: a review of the Food and Drug Administration database. *J Rheumatol* 2009; **36**: 635-641 [PMID: 19132789 DOI: 10.3899/jrheum.080545]
- 12 **McCormack G**, Moriarty D, O'Donoghue DP, McCormick PA, Sheahan K, Baird AW. Tissue cytokine and chemokine expression in inflammatory bowel disease. *Inflamm Res* 2001; **50**: 491-495 [PMID: 11713901]
- 13 **Bringham TS**, Aggarwal BB. Monoclonal antibodies to human tumor necrosis factors alpha and beta: application for affinity purification, immunoassays, and as structural probes. *Hybridoma* 1987; **6**: 489-507 [PMID: 2445655]
- 14 **Engelmann H**, Novick D, Wallach D. Two tumor necrosis factor-binding proteins purified from human urine. Evidence for immunological cross-reactivity with cell surface tumor necrosis factor receptors. *J Biol Chem* 1990; **265**: 1531-1536 [PMID: 2153136]
- 15 **Smolen J**, Landewé RB, Mease P, Brzezicki J, Mason D, Luijckens K, van Vollenhoven RF, Kavanaugh A, Schiff M, Burmester GR, Strand V, Vencovsky J, van der Heijde D. Efficacy and safety of certolizumab pegol plus methotrexate in active rheumatoid arthritis: the RAPID 2 study. A randomised controlled trial. *Ann Rheum Dis* 2009; **68**: 797-804 [PMID: 19015207 DOI: 10.1136/ard.2008.101659]
- 16 **Papamichael K**, Gazouli M, Karakoidas C, Panayotou I, Roma-Giannikou E, Mantzaris GJ. Association of TNF and Fc γ RIIIA gene polymorphisms with differential response to infliximab in a Greek cohort of Crohn's disease patients. *Ann Gastroenterol* 2011; **24**: 35-40 [PMID: 24714240]
- 17 **Tursi A**, Elisei W, Picchio M, Penna A, Forti G, Giorgetti GM, Faggiani R, Zampalà C, Pelecca G, Brandimarte G. Effectiveness of adalimumab for ambulatory ulcerative colitis patients after failure of infliximab treatment: a first "real-life" experience in primary gastroenterology centers in Italy. *Ann Gastroenterol* 2014; **27**: 369-373 [PMID: 25331091]
- 18 **James PL**. Successful treatment of pregnancy-triggered Crohn's disease complicated by severe recurrent life-threatening gastrointestinal bleeding. *Am J Gastroenterol* 2001; **96**: S295 [DOI: 10.1016/S0002-9270(01)03714-5]
- 19 **Bank LH**. Unexpected dramatic clinical response of psoriasis lesions and unexpected pregnancy in an infertile patient in response to treatment with anti-tumor necrosis factor monoclonal antibody for Crohn's disease. *Am J Gastroenterol* 2002; **97**: S260 [DOI: 10.1093/rheumatology/ke1400]
- 20 **Katz JA**, Antoni C, Keenan GF, Smith DE, Jacobs SJ, Lichtenstein GR. Outcome of pregnancy in women receiving infliximab for the treatment of Crohn's disease and rheumatoid arthritis. *Am J Gastroenterol* 2004; **99**: 2385-2392 [PMID: 15571587 DOI: 10.1111/j.1572-0241.2004.30186.x]
- 21 **Mahadevan U**, Kane S, Sandborn WJ, Cohen RD, Hanson K, Terdiman JP, Binion DG. Intentional infliximab use during pregnancy for induction or maintenance of remission in Crohn's disease. *Aliment Pharmacol Ther* 2005; **21**: 733-738 [PMID: 15771759 DOI: 10.1111/j.1365-2036.2005.02405.x]

- 22 **Chaparro M**, Gisbert JP. Letter: measurement of anti-TNF- α levels and antibodies against the drug. *Aliment Pharmacol Ther* 2013; **37**: 163-164 [PMID: 23205484 DOI: 10.1111/apt.12106]
- 23 Humira product insert. (accessed November 30, 2010). Available from: URL: <http://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications>
- 24 **Vesga L**, Terdiman JP, Mahadevan U. Adalimumab use in pregnancy. *Gut* 2005; **54**: 890 [PMID: 15888806 DOI: 10.1136/gut.2005.065417]
- 25 **Coburn LA**, Wise PE, Schwartz DA. The successful use of adalimumab to treat active Crohn's disease of an ileoanal pouch during pregnancy. *Dig Dis Sci* 2006; **51**: 2045-2047 [PMID: 17009112]
- 26 **Jürgens M**, Mahachie John JM, Cleynen I, Schnitzler F, Fidler H, van Moerkercke W, Ballet V, Noman M, Hoffman I, van Assche G, Rutgeerts PJ, van Steen K, Vermeire S. Levels of C-reactive protein are associated with response to infliximab therapy in patients with Crohn's disease. *Clin Gastroenterol Hepatol* 2011; **9**: 421-427.e1 [PMID: 21334460 DOI: 10.1016/j.cgh.2011.02.008]
- 27 **Schnitzler F**, Fidler H, Ferrante M, Ballet V, Noman M, Van Assche G, Spitz B, Hoffman I, Van Steen K, Vermeire S, Rutgeerts P. Outcome of pregnancy in women with inflammatory bowel disease treated with antitumor necrosis factor therapy. *Inflamm Bowel Dis* 2011; **17**: 1846-1854 [PMID: 21830263 DOI: 10.1002/ibd.21583]
- 28 **Zelinkova Z**, van der Ent C, Bruin KF, van Baalen O, Vermeulen HG, Smalbraak HJ, Ouwendijk RJ, Hoek AC, van der Werf SD, Kuipers EJ, van der Woude CJ. Effects of discontinuing anti-tumor necrosis factor therapy during pregnancy on the course of inflammatory bowel disease and neonatal exposure. *Clin Gastroenterol Hepatol* 2013; **11**: 318-321 [PMID: 23103819 DOI: 10.1016/j.cgh.2012.10.02]

P- Reviewer: Shehata MMM **S- Editor:** Yu J **L- Editor:** Filipodia
E- Editor: Liu XM





Endoscopic ultrasound elastography: Current status and future perspectives

Xin-Wu Cui, Jian-Min Chang, Quan-Cheng Kan, Liliana Chiorean, Andre Ignee, Christoph F Dietrich

Xin-Wu Cui, Jian-Min Chang, Christoph F Dietrich, Sino-German Research Center of Ultrasound in Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

Xin-Wu Cui, Liliana Chiorean, Andre Ignee, Christoph F Dietrich, Medical Department 2, Caritas-Krankenhaus, Bad Mergentheim, Academic Teaching Hospital of the University of Würzburg, 97980 Würzburg, Germany

Quan-Cheng Kan, Institute of Clinical Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

Liliana Chiorean, Département d'imagerie médicale, Clinique des Cévennes, 07100 Annonay, France

Author contributions: Dietrich CF established the design and conception of the paper and provided the figures; Cui XW, Chang JM, Kan QC, Chiorean L, Ignee A and Dietrich CF analyzed the literature data; Dietrich CF provided the first draft of the manuscript, which was discussed and revised critically for intellectual content by Cui XW, Chang JM, Kan QC, Chiorean L, Ignee A and Dietrich CF; all authors discussed the statement and conclusions and approved the final version to be published.

Conflict-of-interest statement: We declare that we do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Christoph F Dietrich, Professor, Sino-German Research Center of Ultrasound in Medicine, The First Affiliated Hospital of Zhengzhou University, Jianshe East Road No. 1, Zhengzhou 450052, Henan Province, China. christoph.dietrich@ckbm.de

Telephone: +86-371-66913150
Fax: +86-371-66913150

Received: March 1, 2015
Peer-review started: March 2, 2015
First decision: July 13, 2015
Revised: August 4, 2015
Accepted: September 28, 2015
Article in press: September 30, 2015
Published online: December 21, 2015

Abstract

Elastography is a new ultrasound modality that provides images and measurements related to tissue stiffness. Endoscopic ultrasound (EUS) has played an important role in the diagnosis and management of numerous abdominal and mediastinal diseases. Elastography by means of EUS examination can assess the elasticity of tumors in the proximity of the digestive tract that are hard to reach with conventional transcutaneous ultrasound probes, such as pancreatic masses and mediastinal or abdominal lymph nodes, thus improving the diagnostic yield of the procedure. Results from previous studies have promised benefits for EUS elastography in the differential diagnosis of lymph nodes, as well as for assessing masses with pancreatic or gastrointestinal (GI) tract locations. It is important to mention that EUS elastography is not considered a modality that can replace biopsy. However, it may be a useful adjunct, improving the accuracy of EUS-fine needle aspiration biopsy (EUS-FNAB) by selecting the most suspicious area to be targeted. Even more, it may be useful for guiding further clinical management when EUS-FNAB is negative or inconclusive. In the present paper we will discuss the current knowledge of EUS elastography, including the technical aspects, along with its applications in the differential diagnosis between benign and malignant solid pancreatic masses and lymph nodes, as well as its aid in the

differentiation between normal pancreatic tissues and chronic pancreatitis. Moreover, the emergent indication and future perspectives are summarized, such as the benefit of EUS elastography in EUS-guided fine needle aspiration biopsy, and its uses for characterization of lesions in liver, biliary tract, adrenal glands and GI tract.

Key words: Elastography; Endoscopic ultrasound; Characterization; Pancreas; Lymph nodes

© **The Author(s) 2015.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Endoscopic ultrasound (EUS) has played an important role in the diagnosis and management of numerous abdominal and mediastinal diseases. In the present paper, we discuss the current knowledge of EUS elastography, including the technical aspects, its applications in the differentiation between benign and malignant solid pancreatic masses and lymph nodes, and differentiation between normal pancreatic tissues and chronic pancreatitis. Moreover, the emergent indication and future perspectives are also summarized, such as the benefit of EUS elastography in EUS-guided fine needle aspiration biopsy, and its use for characterization of lesions in liver, biliary tract, adrenal glands and gastrointestinal tract.

Cui XW, Chang JM, Kan QC, Chiorean L, Ignee A, Dietrich CF. Endoscopic ultrasound elastography: Current status and future perspectives. *World J Gastroenterol* 2015; 21(47): 13212-13224 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13212.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13212>

INTRODUCTION

Elastography is an imaging modality used to demonstrate tissue stiffness. It has been investigated by ultrasound since the early 1990's. So far, two (elastography) techniques, *i.e.*, strain technique and shear wave technique, have been developed and clinically used. Endoscopic ultrasound (EUS) elastography is the application of elastography performed during EUS procedure. Currently, only the strain technique has been available for EUS examinations. Shear wave elastography has been equipped in the transrectal ultrasound transducer of Supersonic system (Aixplorer, France), but it is not one of the topics of this paper. Strain elastography (SE) is a qualitative method based on tissues response to an externally or internally generated force^[1,2]. It is based on the fact that stiffer tissues have lower strains, meaning that they deform less under compression as compared to softer tissues which deform more. With SE, the compression-induced tissues deformations within a region of interest (ROI) are comparatively assessed. The resultant strains are displayed as

transparent colors overlaid on the B-Mode image, just like for Doppler ultrasound. Different colors are used to demonstrate differences between stiffness of the ROI included tissues.

The measurement of displacement is made using a sophisticated algorithm based on the Extended Combined Autocorrelation Method^[3-5]. The characteristics of the tissues can be further analyzed either by the color-based qualitative method^[6,7] as described above, or by using a semi-quantitative method based on strain ratios between different tissues included within the selected ROI^[8,9].

SE with conventional ultrasound systems has been used for the evaluation of lesions in the breast, cervix, prostate gland and thyroid gland as well as for staging of liver fibrosis^[10-15] proving good results. It has been shown that malignant tissues are generally harder than adjacent normal tissues, therefore, it could differentiate benign from malignant lesions based on the stiffness characteristics^[10-14].

EUS, with high-frequency transducers can offer high-resolution images of the digestive tract and also of the adjacent organs. It plays an important role in the diagnosis and management of numerous abdominal and mediastinal diseases^[16-18].

The combination of EUS and elastography improves the diagnostic yield of EUS. It can assess the elasticity of tumors in the proximity of the digestive tract that are hard to reach with conventional transcutaneous ultrasound probes, such as pancreatic masses^[19-22] and lymph nodes^[23,24]. Results from early studies look promising for the differential diagnosis of solid masses [*i.e.*, lymph nodes, pancreatic or gastrointestinal (GI) tract masses].

Endoscopic ultrasound-guided fine needle aspiration biopsy (EUS-FNAB) is performed when patient management would be affected^[25,26]. However, it is an invasive technique and has the risk of bleeding and seeding of malignant cells^[27-29]. The accuracy of EUS-FNAB is affected by the selection of the targeted area within the lesion to be assessed and it is dependent upon the lesion's visibility^[30]. Although EUS elastography at present cannot replace EUS-FNAB for the diagnosis of a focal lesion located in the pancreas or for assessing lymph nodes, it still may be a useful adjunct for guiding further clinical management when EUS-FNAB is negative or inconclusive^[26,31,32]. In addition, EUS elastography can show the hardest areas within the lesion, thus being useful for the selection of the most suspicious area to be targeted for EUS-FNAB, finally improving the accuracy of the method^[25].

In the presented paper, we discuss the current knowledge of EUS elastography, including technical aspects, its current applications for benign-malignant solid pancreatic masses and lymph nodes differentiation, as well as its use for differentiation between normal pancreatic tissues and chronic pancreatitis. Moreover, the emergent indication and future perspectives are also summarized, such as the

use of elastography in EUS-FNAB, characterization of lesions in the liver, biliary tract, adrenal glands and GI tracts.

CURRENT EXAMINATION TECHNIQUES USED IN EUS ELASTOGRAPHY (QUALITATIVE AND SEMI-QUANTITATIVE METHODS)

Both qualitative and semi-quantitative methods have been developed in EUS elastography to analyze the tissues stiffness. The former, using hue histogram analysis and artificial neural networks, is the first one equipped in the early generation of ultrasound systems^[8,20]. Afterward, strain histogram (SH) and strain ratio (SR) were introduced in the second-generation EUS elastography systems as reproducible, parametric measurements. SH computes the strain values of elemental areas inside a ROI and produces a graph, while SR measures the relative strain between two selected areas inside a ROI^[25,33]. They both analyze the tissue stiffness in a (semi-) quantitative manner and, therefore, they are greatly reducing the human bias without the need for a 3rd-party software^[34].

Qualitative EUS elastography

EUS elastography detects small structural deformations caused by compression and grades the degree of relative deformation between ROI included tissues on a scale of 1-255. Each value is assigned a different shade from a hue color spectrum for further visual recognition^[25]. Most systems are using a red-green-blue color map in which stiffer tissue areas are shown in dark blue to blue, whilst softer tissue areas are displayed in hues of green to red. Because the strain (deformation) will be smaller in stiffer tissues and larger in softer tissues, the stiffness contrast can be displayed in real-time and can be superimposed as a transparent overlay on the real-time gray-scale EUS image^[30]. The ROI for the elastographic evaluation is manually selected and should include both the lesion entirety (when possible) and also normal surrounding tissue. Qualitative analysis includes a five-step score method based on the predominant color pattern inside the lesion: homogeneously hard, heterogeneously hard, mixed, heterogeneously soft or homogeneously soft^[3,7,35-38].

How to perform: As with conventional EUS, the endoscopes have to be carefully manipulated to obtain a good and a reproducible elastographical image. Both longitudinal and radial echoendoscopes can be used for elastography, however, the former has the advantage that suspicious stiffer areas can be targeted for biopsy under direct visualization^[30,39].

A ROI is used to define the area of interest in a similar manner to that used for a color Doppler exami-

nation. By manipulating the probe, the necessary pressure can be applied. Very little extra compression is seldom needed to obtain an elastographical image, since with careful placement of the endoscope, the regular pressure variation from the pulsation of adjacent vessels will normally be sufficient if the system settings are appropriately sensitive. However, the size of the ROI that defines the elastographic image is important. The ROI should be sufficiently large to include both the pathological tissue under investigation and surrounding "normal" tissue as a reference. The best image quality was recorded in phantom experiments when the lesion of interest covered 25%-50% of the ROI^[40]. In practice, for EUS elastography applications, a ratio of approximately 50% lesion, 50% normal surrounding tissue is usually achievable^[35,41]. If the ROI is too small, only the relative elasticity differences within the lesion will be measured and displayed rather than the assessment of the lesion stiffness compared to normal surrounding tissues^[1,30].

(Semi-)quantitative EUS elastography

Two quantitative techniques have been developed to improve the accuracy and reproducibility of the method and to minimize the operator bias in stiffness evaluation.

One is based on the comparison between two tissue areas within the ROI to compute a SR^[3,9,21,42]. This technique is a semi-quantitative method since the elasticity is expressed as a relative ratio but not as an absolute value^[43]. Using this technique, two non-overlapping areas are selected, usually area A is the lesion, area B is the reference zone, and SR represents the B/A quotient^[9].

Another method that has been used is the calculation of the mean value of the hue histogram SH, which represents the mean strain value within the selected area^[8,15]. The histogram is a graph that quantifies and represents a specific characteristic (*e.g.*, digitized color distribution) inside the ROI of an elastography image^[25]. SH software has been equipped in the newer ultrasound machines, and the graph can be automatically created. Within the graph, the X-axis represents the elasticity values (each value represents a pixel color) from 0 to 255, where 0 is hardest and 255 is softest, the Y-axis values represent the number of pixels of each value.

CURRENT CLINICAL APPLICATIONS OF EUS ELASTOGRAPHY

EUS elastography is only applied to assess the elasticity of solid lesions based on its principles, while cystic lesions are usually shown as an artifact, *i.e.*, BGR (blue-green-red) artifact^[4,5]. A lesion that has cystic components should not be evaluated by EUS elastography. Therefore, the current clinical indications

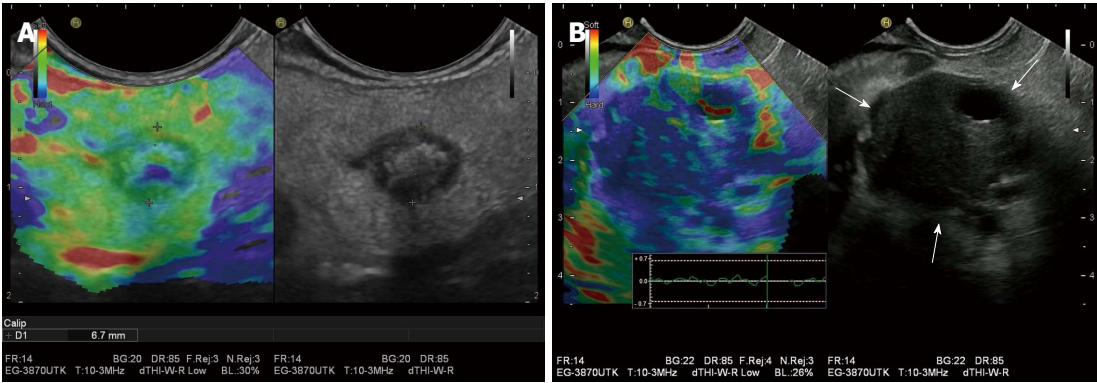


Figure 1 Benign and malignant pancreatic masses on endoscopic ultrasound elastography. A: A pancreatic teratoma is shown as heterogeneous soft (green) pattern (left: EUS elastography image; right: B-mode image); B: A pancreatic ductal adenocarcinoma appears stiffer (blue) than the adjacent normal pancreatic parenchyma, probably due to the presence of fibrosis and marked desmoplasia.

Table 1 Five score classification system for endoscopic ultrasound elastography				
Score	Color pattern	Stiffness	Histology	Ref.
1	Green	Homogeneous soft	Normal pancreatic tissue	[6,34]
2	Green, yellow and red	Soft heterogeneity	Fibrosis	[6,34]
3	Mostly blue with minimal heterogeneity	Hard	Early pancreatic adenocarcinoma	[6,34]
4	Central green hypochoic region and blue tissue outer layer	Hard	Neuroendocrine tumor, metastasis	[6,34]
5	Blue lesions with heterogeneity due to necrosis	Hard	Advanced pancreatic adenocarcinoma	[6,34]

Table 2 Four score classification system for endoscopic ultrasound elastography				
Score	Color pattern	Stiffness	Histology	Ref.
1	Homogeneous green	Soft	Normal pancreas	[38]
2	Heterogeneous, green-Predominant	Soft	Inflammatory pancreatic masses	[38]
3	heterogeneous, blue-Predominant	Hard	Pancreatic malignant tumors	[38]
4	homogeneous blue	Hard	Pancreatic neuroendocrine malignant lesions	[38]

of EUS elastography are mainly solid pancreatic lesions, submucosal GI masses, lymph nodes, focal left liver lesions and left adrenal lesions. Few other indications have been also reported^[44].

Pancreas

The normal pancreas appears elastographically soft (homogenously green) on EUS elastography in most cases, which has been shown to be highly reproducible^[6,45].

Focal pancreatic masses: Malignant pancreatic lesions are generally harder than adjacent pancreatic tissue. Therefore, measuring strain might aid classification of pancreatic masses. Meta-analyses demonstrated that EUS elastography is a reliable technique for the differentiation of solid pancreatic masses with a pooled sensitivity of 95%-97% and a specificity of 67%-76%, respectively^[46-48].

Qualitative techniques: EUS elastography has been considered a promising tool for differentiation of benign and malignant solid pancreatic masses^[6,7,34,36,38]. On EUS elastography, malignant lesions (*e.g.*, pancreatic ductal adenocarcinoma), due to the presence of fibrosis and marked desmoplasia, appears stiffer than the adjacent normal pancreatic parenchyma^[7]. Still, no evidence shows that there is a correlation between the tumor stiffness and either the tumor grading, or collagenous content^[42] (Figure 1).

Different classification of color patterns has been used to distinguish malignant masses from benign ones^[6], though this simplistic approach has been contradicted by other authors and later articles^[35]. A five score classification was firstly reported in 2006 based on the color patterns of lesions, by Giovannini *et al*^[6], with a sensitivity of 100% and a specificity of 67% (Table 1). In this scoring system, scores 3-5 were considered malignant, while 1 and 2 were considered benign. In 2009, Giovannini *et al*^[34] published their results based on a multicenter study using the same scoring system, the accuracy being 89.2%, and both sensitivity and positive predictive value (PPV) being over 90%. A four score classification has also been used by other authors (Table 2), the diagnostic sensitivity, specificity and overall accuracy of EUS elastography for diagnosing malignancy being of 100%, 85.5% and 94%, respectively based on this score^[38].

However, disappointing results of qualitative EUS elastography have also reported in two studies^[7,35]. Hirche *et al*^[35] found that the diagnostic sensitivity,

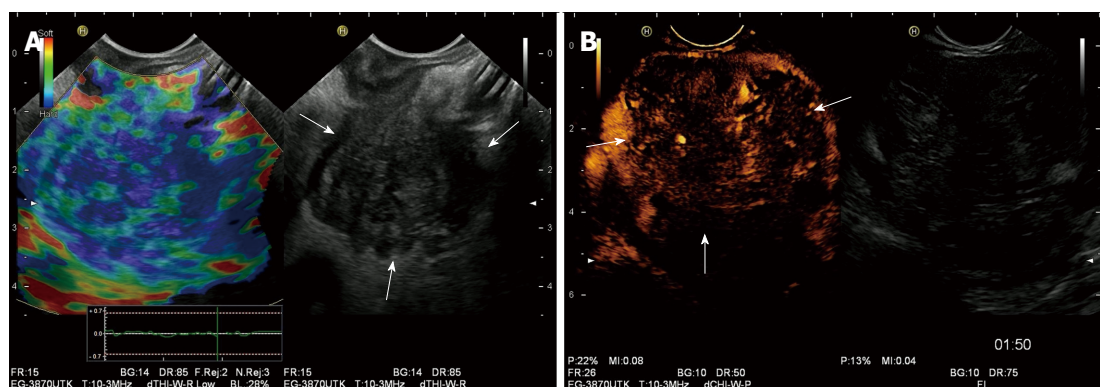


Figure 2 Combination of elastography and contrast-enhanced ultrasound in endoscopic ultrasound. A: A pancreatic ductal adenocarcinoma is demonstrated as a heterogeneous hard (blue) pattern; B: On contrast-enhanced endoscopic ultrasound, pancreatic ductal adenocarcinoma is hypo-enhancing.

specificity and accuracy for predicting the nature of pancreatic lesions were just 41%, 53% and 45%, respectively. Also, Janssen *et al.*^[7], as the result of their study, concluded that chronic pancreatitis and hard malignant tumors cannot be distinguished by elastography, probably due to their similar fibrous structure.

Therefore, the diagnostic accuracy of qualitative EUS elastography is variable among studies, probably because of the subjective interpretation of the elastographic pattern caused by perception errors and the inability of the human eye to completely characterize all color hues^[45,49]. Even though some bias cannot be avoided, one study showed very good correlation between observers by analyzing the videos recorded from 258 patients with chronic pancreatitis and pancreatic cancer^[20].

Quantitative EUS elastography: As previously described, both SR and SH are possible methods to quantitatively characterize pancreatic lesions. It is reported that there are no differences regarding the accuracy of the two techniques for the differentiation between benign and malignant pancreatic masses^[33].

SR: Iglesias-Garcia *et al.*^[9] firstly published the results of a prospective study concerning the accuracy of quantitative EUS elastography for the differential diagnosis of solid pancreatic masses on 86 consecutive patients. The results of their study showed that quantitative EUS elastography with strain ratio has higher accuracy (97.7%) and specificity (92.9%) as compared to the qualitative analysis. A SR higher than 6.04 or a mass elasticity lower than 0.05% is 100% sensitive for classification of tumors as being malignant. The specificity can be improved to 100% with a SR higher than 15.41 or a mass elasticity value below 0.03%. In addition, EUS elastography could differentiate pancreatic cancers from inflammatory masses (100% sensitivity and 96% specificity), and pancreatic cancers from neuroendocrine tumors (100% sensitivity and 88% specificity).

Using the same methods as within the above study^[9], another study retrospectively evaluated 109 patients with solid pancreatic masses^[21]. With the qualitative technique, all pancreatic cancers presented intense blue coloration, however, the inflammatory masses showed mixed colorations (green, yellow, and low-intensity blue). With the quantitative technique, the mean SR was 39.08 ± 20.54 for pancreatic cancer, and 23.66 ± 12.65 for the inflammatory masses ($P < 0.05$).

SH: Quantitative EUS elastography with SH has also been investigated in studies. Using 175 as the cut off value of the mean SH, Săftoiu *et al.*^[8] reported sensitivity, specificity, PPV, negative predictive value (NPV) and accuracy of 91.4%, 87.9%, 88.9%, 90.6%, and 89.7%, respectively, in differentiating between benign and malignant pancreatic masses. Recently, a multicenter study with 258 patients used the same cut-off value (175), and found that sensitivity, specificity, PPV, NPV and accuracy of EUS elastography were 93.4%, 66.0%, 92.5%, 68.9%, and 85.4%, respectively^[20]. In another study, Schrader *et al.*^[42] evaluated the usefulness of SH in differentiating malignant pancreatic masses from normal pancreas and found a very promising result with 100% sensitivity and 100% specificity for malignancy detection. However, a limitation of this study was the selection of the control group, since the authors used normal pancreas as control group compared with the group with malignant pancreatic diseases, but did not use patients with pancreatic masses or chronic pancreatitis as control group.

Combination of elastography and contrast-enhanced color Doppler ultrasound in EUS: Malignant solid pancreatic masses are usually hypo-vascular on (contrast enhanced) color Doppler ultrasound and hypo-enhancing on contrast-enhanced ultrasound^[50] (Figure 2). A study by Săftoiu *et al.*^[22] found that the combination between EUS elastography and contrast-enhanced color Doppler EUS could

offer important information for the decision-making process, especially in patients with negative EUS-FNAB and strong clinical suspicion of malignancy. In their study, both modalities were performed sequentially during the same EUS examination in 21 patients with chronic pancreatitis and in 33 patients with pancreatic ductal adenocarcinoma. The sensitivity, specificity, accuracy, PPV and NPV of the combined techniques for differentiation of hypo-vascular hard masses suggestive of pancreatic carcinoma were 75.8%, 95.2%, 83.3%, 96.2% and 71.4%, respectively.

Diffuse pancreatic diseases

Chronic pancreatitis: EUS has become one important method for the diagnosis of chronic pancreatitis in clinical practice. Still, there are many challenges with the criteria from EUS, especially for diagnosing non-advanced stages. EUS elastography can provide additional relevant information of tissue stiffness and thus may benefit the diagnosis of chronic pancreatitis. In a recent published prospective study^[51], quantitative EUS elastography was performed in 191 patients (from which 92 were finally diagnosed with chronic pancreatitis). The SR was measured in the head, body and tail of the pancreas and the mean value was used for analysis. For each measurement of SR, area A was the largest possible area of pancreatic parenchyma, and area B referred to a soft (red) reference area corresponding to normal surrounding gut wall. Results showed that there was a highly significant direct linear correlation between the SR and the number of EUS criteria of chronic pancreatitis ($r = 0.813$). The accuracy of EUS-elastography for diagnosing chronic pancreatitis was 91.1 % (cut-off strain ratio of 2.25). Therefore, EUS-elastography is proven to be an accurate tool for the diagnosis of chronic pancreatitis according to this study.

Pancreatic fibrosis: The usefulness of EUS elastography for diagnosing the grade of pancreatic fibrosis has been evaluated in one study^[52]. EUS elastography was performed in 58 consecutive patients before pancreatectomy for both pancreatic tumors and upstream pancreas. Quantitative technique with novel software was used to analyze the EUS elastography images, and 4 parameters (mean, standard deviation, skewness, and kurtosis) were calculated. Histological fibrosis was graded into 4 categories (normal, mild fibrosis, marked fibrosis, and severe fibrosis). The results showed that fibrosis grade was significantly correlated with all 4 quantification parameters, and that the mean was the most useful parameter for diagnosing pancreatic fibrosis. The area under the ROC curves for the diagnosis of mild or higher-grade fibrosis, marked or higher-grade fibrosis and severe fibrosis were 0.90, 0.90, and 0.90, respectively. Therefore, EUS elastography may be a useful tool for the accurate diagnosis of pancreatic fibrosis.

Future developments

Future developments of EUS elastography for pancreatic related pathologies include evaluation of the role of elastography-guided biopsy of the pancreas. EUS elastography has high sensitivity for detecting even very small pancreatic masses, and in addition, can be helpful in staging pancreatic cancer and for guiding a biopsy to obtain samples for cytological or histological diagnosis^[1,46-48]. The additional value of elastography combined with other techniques such as contrast-enhanced endoscopic ultrasound (CE-EUS), fusion imaging or 3D elastography examinations might also be feasible^[30,49].

Lymph nodes

The differentiation of benign and malignant lymph nodes (LNs) is crucial for staging, for prediction of prognosis and for selection of appropriate treatment options in many cancers, such as esophageal, stomach, bronchial, and pancreatic carcinomas. LNs that are close to the gut can be imaged with EUS, but the differential diagnosis of benign and malignant nodes with EUS remains a challenge. Although there are already some established criteria on B-mode EUS for the diagnosis of malignant LNs (such as hypoechoic structure, round shape, sharp margins, > 10 mm diameter), these features overlap sometimes with benign nodes. Also, it should be considered that malignant nodes at early stages may have less or even none of these typical features^[53], the specificity and accuracy with these criteria being rather low^[25,30,54,55]. In this setting, EUS-elastography, as a minimally invasive imaging modality may be helpful for the differential diagnosis of benign and malignant LNs or to single out the more suspicious nodes to be targeted for endosonographic guided tissue sampling^[23,39].

Qualitative EUS elastography

Similar with pancreatic masses, malignant LNs are considered to be harder than benign ones, which has been studied in several publications^[6,23,24,34,56] (Figure 3). Giovannini *et al*^[6] first published their results on evaluation of the usefulness of qualitative EUS elastography in differentiating the benign from malignant LNs with different locations (cervical, celiac, mediastinal and aortocaval). A number of 31 lymph nodes from 25 patients were included in this study. The predominance of blue areas was considered to be related to malignancy, however, mostly green nodes and indeterminate ones (shown as heterogeneity), were classified as benign. The obtained sensitivity and specificity for determining malignancy were 100% and 50%, respectively. Janssen *et al*^[24] evaluated the feasibility and the usefulness of qualitative EUS elastography for characterizing the LNs in the dorsal mediastinum, regarding the histological results obtained in the same session from EUS-FNAB as the

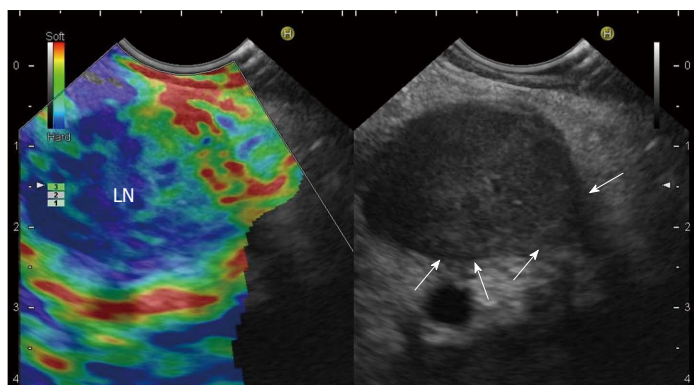


Figure 3 Malignant Lymph nodes on endoscopic ultrasound elastography. A malignant lymph node is revealed as predominantly hard (blue). Arrows indicate infiltration. LN: Lymph node.

gold standard. Benign LNs exhibited predominantly intermediate homogeneous deformation (yellow/green), while malignant LNs showed a quantitative dominance of hard (blue) units. The three examiners who participated in the study had accuracies ranging from 84.6% to 86.4% for malignant lymph nodes, and from 81.8% to 87.9% for benign ones. The interobserver agreement was good ($\kappa = 0.84$).

A five-point scoring system has also been evaluated. In a multicenter study, scores 1 and 2 were interpreted as benign, score 3 as indeterminate and scores 4 and 5 as malignant. The obtained sensitivity, specificity, PPV, NPV, and accuracy for the detection of malignancy were 91.8%, 82.5%, 88.8%, 86.8%, and 88.1%, respectively^[34]. The interobserver agreement yielded a kappa score of 0.657 for the detection of malignant LNs^[34]. Săftoiu *et al.*^[56], also using a 5-score system, evaluated LNs in cervical, mediastinal, and abdominal areas, and got better results. The obtained sensitivity, specificity, and accuracy for the distinction of benign and malignant LNs were 91.7%, 94.4%, and 92.86%, respectively.

One meta-analysis including 7 studies, 368 patients and 431 LNs, has investigated the application of EUS elastography for the differentiation between benign and malignant LNs. The pooled sensitivity and specificity of EUS elastography for the differential diagnosis of benign and malignant LNs were 88% and 85%, respectively. The area under the summary receiver operating characteristic curve was 0.9456. Authors concluded that EUS elastography is a promising, non-invasive technique for the differentiation of malignant LNs, and that it may become a valuable supplemental method to EUS-guided FNAB^[23].

Quantitative EUS elastography

The previously referred study by Săftoiu *et al.*^[56] also included a quantitative analysis, *i.e.*, a separate RGB channel histograms. With a cut-off level of 0.84, the sensitivity, specificity, and accuracy for malignancy detection were 95.8%, 94.4%, and 95.2%, respectively. A subsequent study also by Săftoiu *et al.*^[57] used quantitative EUS elastography based on SH to investigate cervical, mediastinal and abdominal LNs. With a cut off value of 166 for the

mean of the SH (between blue and green on the rainbow scale), the sensitivity, specificity, and accuracy of EUS elastography in the detection of malignancy were 85.4%, 91.9%, and 88.5%, respectively. The corresponding area under the curve was 0.928. Two recent studies using not only SH but also SR showed superior accuracy of EUS elastography as compared to conventional EUS criteria in differentiating benign and malignant LNs for the nodal staging of esophageal cancer^[58,59].

However, only one study by Larsen *et al.*^[60], using both qualitative and quantitative (with SR) EUS elastography, did not find that EUS elastography is better than EUS morphology in differentiating between benign and malignant LNs, at least for included patients with resectable upper GI cancers.

Future development (EUS elastography for EUS-FNA)

EUS-FNA offers the most reproducible results in the diagnosis of metastatic LN infiltration, with an accuracy greater than 85%^[39,54,61]. But the accuracy depends on the appropriate selection of LNs and targeting of focal infiltration within LNs for biopsy. Both B-mode diagnosis and EUS-FNAB may fail to detect the smallest LN metastases. Elastography is able to identify the smallest metastatic related changes in tissue hardness, thus it has the potential to be useful for target selection prior to endosonographic guided tissue sampling^[39] (Figure 4). The European guidelines^[4] for the use of elastography in clinical practice suggest that EUS elastography adds information to the B-mode evaluation of LNs and can better guide an EUS-FNA procedure by identifying stiffer and thus, most suspicious regions for malignant infiltration.

Subepithelial masses

Imaging the layers of the GI tract is one of the major indications of the EUS examination^[30]. EUS with high-frequency transducers of at least 7 MHz is used to improve visualization of wall layers, thickened bowel walls and target lesions. In the case of subepithelial masses, EUS elastography can provide information on stiffness, which may help increase the diagnostic confidence and accuracy of the staging. So far, there have been only few reports concerning the use of EUS

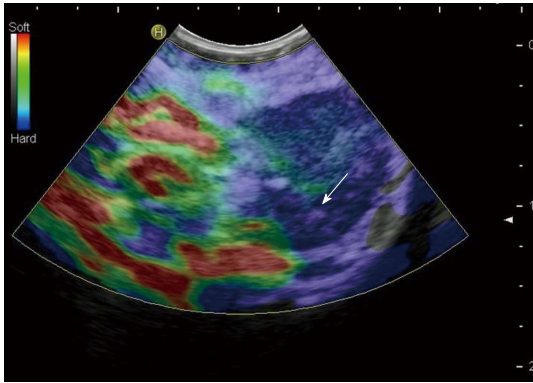


Figure 4 Endoscopic ultrasound elastography adds information to the B-mode evaluation of lymph nodes and can better guide a fine needle aspiration procedure by identifying stiffer (blue in the image) and thus, most suspicious regions for malignant infiltration. Arrow indicates needle tip.

elastography in characterizing subepithelial masses^[62-64].

On B-mode EUS, the characteristics of benign subepithelial masses include (1) specific layer location (in the case of lipoma); (2) size smaller than 3 cm; (3) a smooth contour; (4) uniform echogenicity; and (5) lack of infiltration signs. On EUS elastography, benign subepithelial masses usually are showing an intermediate stiffness with homogenous strain pattern^[30,61,63-65]. During follow-up, they usually have little to no changes in regards to the aforementioned criteria. However, the detection of some degenerative changes makes the diagnosis of benignity very difficult^[63].

Lipomas are the most common subepithelial lesions. On EUS elastography, they are usually homogeneously soft but occasionally, stiffer lipoma may also occur^[1,30,63].

Malignant subepithelial masses have the following B-mode EUS criteria: (1) size > 3-4 cm; (2) an irregular contour or ulceration; (3) heterogeneous structure; and (4) LNs infiltration^[30]. They usually show a heterogeneously stiff pattern on EUS elastography^[63,64] (Figure 5).

Gastrointestinal stromal tumors (GISTs) are difficult cases. EUS elastography does not currently provide a good enough resolution to properly assess the microfoci found in GISTs^[30,63] (Figure 6).

EMERGENT INDICATIONS AND FUTURE PERSPECTIVES

Fecal incontinence

The anal canal has the same layered structure as the rest of the GI tract and different layers have different elasticities, which is shown as different color patterns of each layer on elastography^[1,66]. Allgayer *et al.*^[66] evaluated the elastography of anal sphincters in 50 patients with fecal incontinence, and found that the inner anal sphincter and external anal sphincter had

different elastographical color distributions. However, there was no significant correlation between the elastographic appearance of sphincters and the functional and clinical parameters of the patients. Therefore, EUS elastography may not yield additional information in patients with fecal incontinence.

Liver

Although transcutaneous ultrasound elastography has been established as a modality for assessing the stiffness of liver tissue and even of focal liver lesions^[67,68], there is still a lack of data on the use of EUS elastography for the liver. A possible reason is that EUS can usually only image the left liver lobe but not the entire organ, which limits its application for the liver. Our previous publications have briefly described the use of EUS elastography for the detection and differentiation of superficial liver tumors in the left liver lobe^[1,3].

Biliary tract

Less data has been published concerning the use of EUS elastography for the diagnosis of disorders of the biliary tract^[44]. Choledocholithiasis, chronic inflammatory or sclerosing processes may cause stiffness of the bile duct walls^[30]. Our previous paper has reported that biliary papillomatosis showed a homogeneously hard pattern of the distal stenosis when the mass infiltrates beyond the wall and the stenosis caused is severe^[44].

Adrenal glands

The left adrenal gland is anatomically located near the posterior gastric body wall, and thus it can be visualized using EUS in almost every case^[69,70]. However, the right adrenal gland can be visualized more readily using the transcutaneous route^[30,69-72]. Studies regarding EUS-FNAB found that EUS-FNAB provided an accurate diagnosis of adrenal metastasis^[73]. No studies have been published regarding the use of EUS elastography in differentiating malignant adrenal masses from benign ones, except few reports^[1,3,36,65,74] (Figure 7). Malignant infiltrations tend to be stiffer than benign tumors, inflammatory processes and fatty deposits^[30].

Future perspective on techniques

Shear wave elastography, including acoustic radiation force impulse imaging and supersonic shear wave imaging, has been proven useful in assessing the stiffness of breast lesions, thyroid lesions and liver fibrosis^[4,5,68]. However, this technique is still not available with an endoscope. Supersonic shear wave imaging has been already equipped on the transrectal ultrasound transducers, and its usefulness in the distinction of benign and malignant prostate lesions has been published^[75]. It is expected that shear wave elastography could be also available on an endoscope,

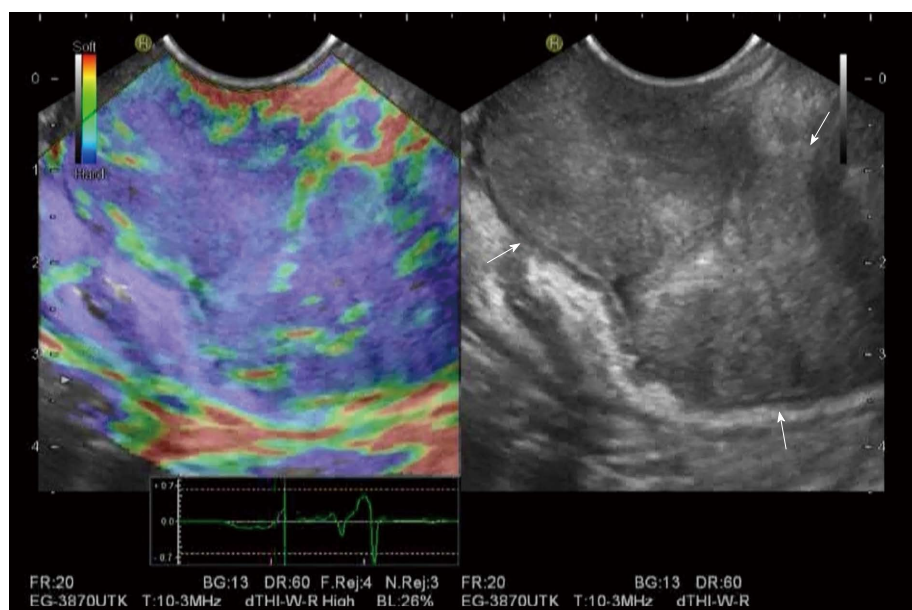


Figure 5 Gastric carcinoma is shown as heterogeneously stiff pattern (blue) on endoscopic ultrasound elastography.

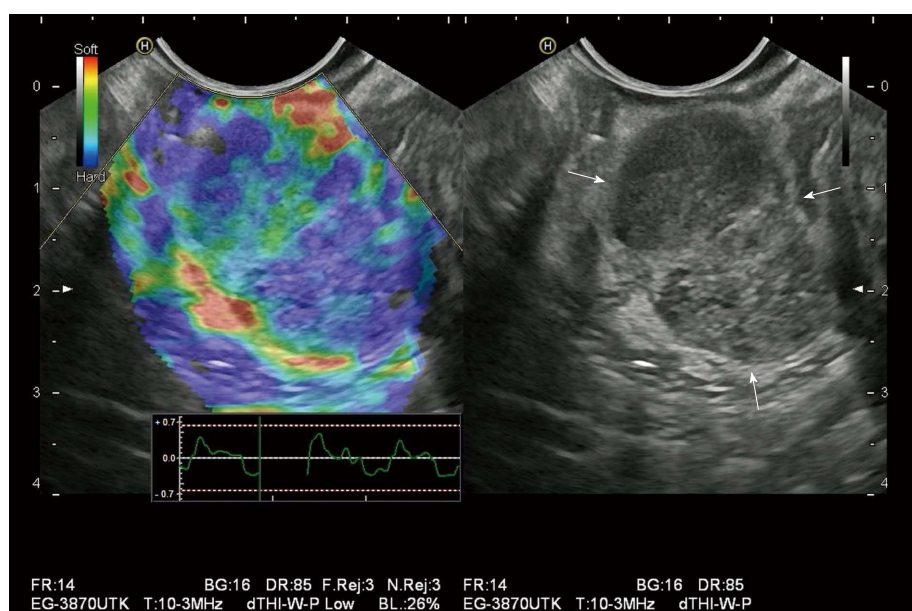


Figure 6 Gastrointestinal stromal tumor is revealed heterogeneous hard (blue) pattern on endoscopic ultrasound elastography.

which would provide a new way to assess the stiffness of pancreatic masses and adjacent LNs, etc.

LIMITATIONS

The limitations of EUS elastography are the following: (1) both qualitative and quantitative methods are observer-dependent with operator bias in the selection of ROI and areas for analysis, which could cause intra- and inter-observer variability; (2) it is difficult to control the tissue compression by the endosonographer, and excessive pressure applied to the tissues can artificially increase their strain; (3) since a high-frequency transducer is used in the EUS elastography, the depth of penetration is limited, thus only the organ or part of the organ near the GI tract can be imaged; (4) presence of motion artifacts; (5)

the strain value can be impacted by the vessels, cysts and bones in the selected ROI; and (6) the strain value may be also impacted if there is insufficient surrounding "normal tissue" as reference in the ROI, thus, the SR method may be occasionally unavailable due to this issue^[47]. Other limitations include that EUS elastography needs more training and costs more time in procedure than conventional US elastography.

CONCLUSION

As a minimally invasive method, EUS plays an important role in assessing malignancies of the GI tract and nearby organs. Elastography adds valuable information to EUS by providing a qualitative and quantitative evaluation of tissue stiffness, thus reflecting the malignant or benign nature of the disease. Pancreas

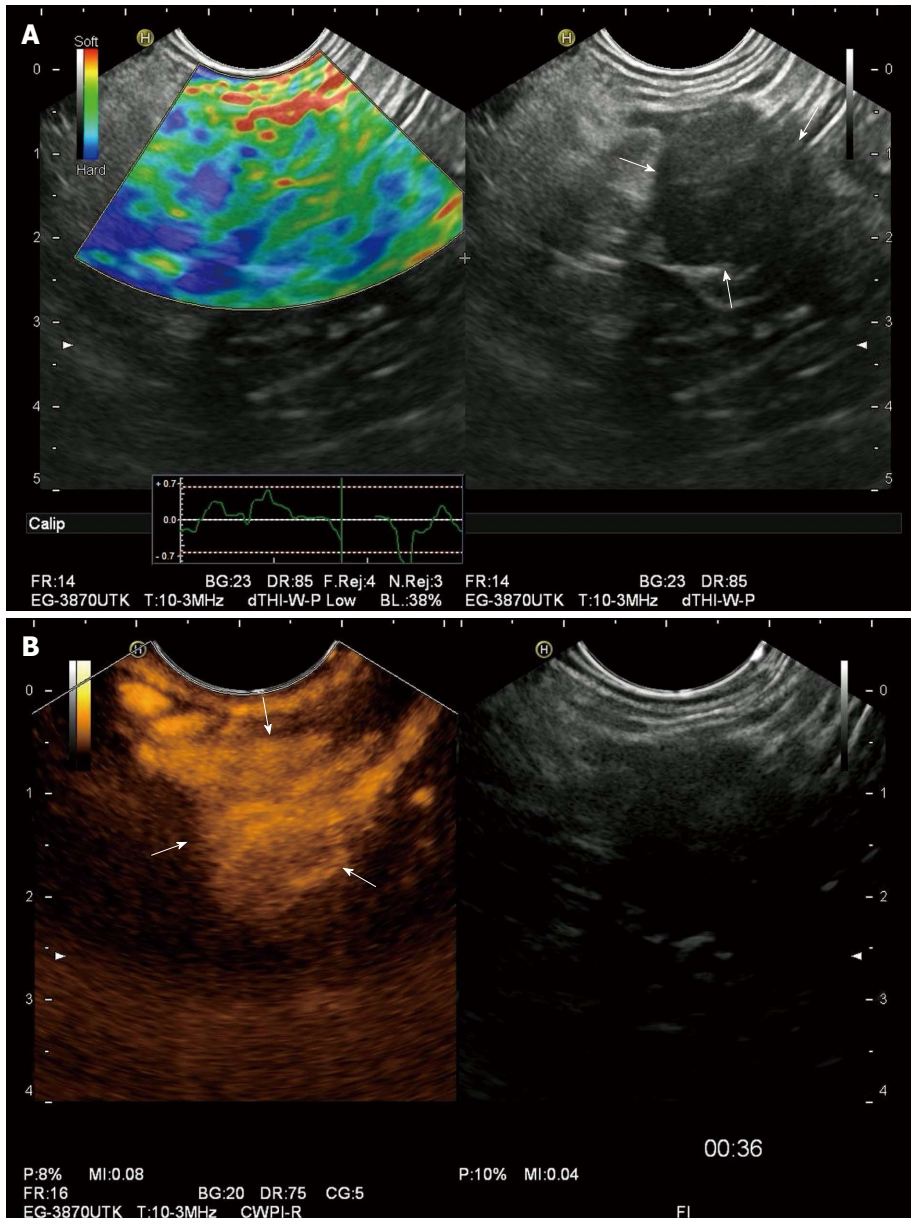


Figure 7 Benign adrenal gland tumor is typically soft (green) on endoscopic ultrasound elastography (A) and hyper-enhancing on contrast-enhanced endoscopic ultrasound (B).

and LNs are the two most investigated organs with EUS elastography. Both qualitative and quantitative techniques have been proven useful in the differentiation between benign and malignant solid pancreatic masses and lymph nodes, and differentiation between normal pancreatic tissues and chronic pancreatitis, all with a high accuracy. EUS elastography can be combined with other imaging techniques, such as CE-EUS, which may be helpful to further improve the accuracy of EUS assessment.

EUS elastography is currently not ready to replace EUS-FNAB in any of its indications. However, EUS elastography may be useful for making clinical decisions, such as whether biopsies are necessary for a patient, which LNs are most likely to be malignant and thus selected for biopsy. In addition, a suspicious

finding on EUS elastography can be helpful for guiding further clinical management when EUS-FNAB is inconclusive or negative.

Few publications have suggested that EUS elastography may be also useful to identify the hard (most likely to be malignant) areas within the pancreatic masses and lymph nodes for targeted EUS-FNAB. However, future studies are needed to further define the role of EUS elastography in this indication.

Emergent indications include the use of EUS elastography for the characterization of lesions located in the liver, biliary tract, adrenal glands, and GI tract. Still, additional evidence is required to define the role of EUS elastography in these clinical applications. Future perspectives also include monitoring treatment response in antiangiogenic therapy with elastography

technique and application of EUS elastography in pediatric patients^[76] and adequate reimbursement^[77].

ACKNOWLEDGMENTS

The authors acknowledge Bad Mergentheimer Leberzentrum e.V. for supporting Dr. Xin-Wu Cui.

REFERENCES

- 1 **Dietrich CF.** Real-time tissue elastography. Multiple clinical applications. Multiple clinical solutions. *Endo Heute* 2011; **24**: 177-212
- 2 **Dietrich CF.** [Elastography, the new dimension in ultrasonography]. *Praxis* (Bern 1994) 2011; **100**: 1533-1542 [PMID: 22161880 DOI: 10.1024/1661-8157/a000735]
- 3 **Dietrich CF.** Echtzeit-Gewebeelastographie. Anwendungsmöglichkeiten nicht nur im Gastrointestinaltrakt. *Endo Heute* 2011; **23**: 177-212
- 4 **Cosgrove D, Piscaglia F, Bamber J, Bojunga J, Correias JM, Gilja OH, Klauser AS, Sporea I, Calliada F, Cantisani V, D'Onofrio M, Drakonaki EE, Fink M, Friedrich-Rust M, Fromageau J, Havre RF, Jenssen C, Ohlinger R, Săftoiu A, Schaefer F, Dietrich CF.** EFSUMB guidelines and recommendations on the clinical use of ultrasound elastography. Part 2: Clinical applications. *Ultraschall Med* 2013; **34**: 238-253 [PMID: 23605169 DOI: 10.1055/s-0033-1335375]
- 5 **Bamber J, Cosgrove D, Dietrich CF, Fromageau J, Bojunga J, Calliada F, Cantisani V, Correias JM, D'Onofrio M, Drakonaki EE, Fink M, Friedrich-Rust M, Gilja OH, Havre RF, Jenssen C, Klauser AS, Ohlinger R, Săftoiu A, Schaefer F, Sporea I, Piscaglia F.** EFSUMB guidelines and recommendations on the clinical use of ultrasound elastography. Part 1: Basic principles and technology. *Ultraschall Med* 2013; **34**: 169-184 [PMID: 23558397 DOI: 10.1055/s-0033-1335205]
- 6 **Giovannini M, Hookey LC, Bories E, Pesenti C, Monges G, Delpero JR.** Endoscopic ultrasound elastography: the first step towards virtual biopsy? Preliminary results in 49 patients. *Endoscopy* 2006; **38**: 344-348 [PMID: 16680632 DOI: 10.1055/s-2006-925158]
- 7 **Janssen J, Schlörner E, Greiner L.** EUS elastography of the pancreas: feasibility and pattern description of the normal pancreas, chronic pancreatitis, and focal pancreatic lesions. *Gastrointest Endosc* 2007; **65**: 971-978 [PMID: 17531630 DOI: 10.1016/j.gie.2006.12.057]
- 8 **Săftoiu A, Vilman P, Gorunescu F, Gheonea DI, Gorunescu M, Ciurea T, Popescu GL, Iordache A, Hassan H, Iordache S.** Neural network analysis of dynamic sequences of EUS elastography used for the differential diagnosis of chronic pancreatitis and pancreatic cancer. *Gastrointest Endosc* 2008; **68**: 1086-1094 [PMID: 18656186 DOI: 10.1016/j.gie.2008.04.031]
- 9 **Iglesias-Garcia J, Larino-Noia J, Abdulkader I, Forteza J, Dominguez-Munoz JE.** Quantitative endoscopic ultrasound elastography: an accurate method for the differentiation of solid pancreatic masses. *Gastroenterology* 2010; **139**: 1172-1180 [PMID: 20600020 DOI: 10.1053/j.gastro.2010.06.059]
- 10 **Itoh A, Ueno E, Tohno E, Kamma H, Takahashi H, Shiina T, Yamakawa M, Matsumura T.** Breast disease: clinical application of US elastography for diagnosis. *Radiology* 2006; **239**: 341-350 [PMID: 16484352 DOI: 10.1148/radiol.2391041676]
- 11 **Cochlin DL, Ganatra RH, Griffiths DF.** Elastography in the detection of prostatic cancer. *Clin Radiol* 2002; **57**: 1014-1020 [PMID: 12409113]
- 12 **Friedrich-Rust M, Ong MF, Martens S, Sarrazin C, Bojunga J, Zeuzem S, Herrmann E.** Performance of transient elastography for the staging of liver fibrosis: a meta-analysis. *Gastroenterology* 2008; **134**: 960-974 [PMID: 18395077 DOI: 10.1053/j.gastro.2008.01.034]
- 13 **Thomas A, Kümmel S, Gemeinhardt O, Fischer T.** Real-time sonoelastography of the cervix: tissue elasticity of the normal and abnormal cervix. *Acad Radiol* 2007; **14**: 193-200 [PMID: 17236992 DOI: 10.1016/j.acra.2006.11.010]
- 14 **Lyschchik A, Higashi T, Asato R, Tanaka S, Ito J, Mai JJ, Pellot-Barakat C, Insana MF, Brill AB, Saga T, Hiraoka M, Togashi K.** Thyroid gland tumor diagnosis at US elastography. *Radiology* 2005; **237**: 202-211 [PMID: 16118150 DOI: 10.1148/radiol.2363041248]
- 15 **Săftoiu A, Gheonea DI, Ciurea T.** Hue histogram analysis of real-time elastography images for noninvasive assessment of liver fibrosis. *AJR Am J Roentgenol* 2007; **189**: W232-W233 [PMID: 17885039 DOI: 10.2214/AJR.07.2571]
- 16 **Dye CE, Waxman I.** Endoscopic ultrasound. *Gastroenterol Clin North Am* 2002; **31**: 863-879 [PMID: 12481735]
- 17 **Tamerisa R, Irisawa A, Bhutani MS.** Endoscopic ultrasound in the diagnosis, staging, and management of gastrointestinal and adjacent malignancies. *Med Clin North Am* 2005; **89**: 139-158, viii [PMID: 15527812 DOI: 10.1016/j.mcna.2004.08.010]
- 18 **Byrne MF, Jowell PS.** Gastrointestinal imaging: endoscopic ultrasound. *Gastroenterology* 2002; **122**: 1631-1648 [PMID: 12016428]
- 19 **Hocke M, Ignee A, Dietrich CF.** Advanced endosonographic diagnostic tools for discrimination of focal chronic pancreatitis and pancreatic carcinoma--elastography, contrast enhanced high mechanical index (CEHMI) and low mechanical index (CEMI) endosonography in direct comparison. *Z Gastroenterol* 2012; **50**: 199-203 [PMID: 22298098 DOI: 10.1055/s-0031-1281824]
- 20 **Săftoiu A, Vilman P, Gorunescu F, Janssen J, Hocke M, Larsen M, Iglesias-Garcia J, Arcidiacono P, Will U, Giovannini M, Dietrich C, Havre R, Gheorghe C, McKay C, Gheonea DI, Ciurea T.** Accuracy of endoscopic ultrasound elastography used for differential diagnosis of focal pancreatic masses: a multicenter study. *Endoscopy* 2011; **43**: 596-603 [PMID: 21437851 DOI: 10.1055/s-0030-1256314]
- 21 **Itokawa F, Itoi T, Sofuni A, Kurihara T, Tsuchiya T, Ishii K, Tsuji S, Ikeuchi N, Umeda J, Tanaka R, Yokoyama N, Moriyasu F, Kasuya K, Nagao T, Kamisawa T, Tsuchida A.** EUS elastography combined with the strain ratio of tissue elasticity for diagnosis of solid pancreatic masses. *J Gastroenterol* 2011; **46**: 843-853 [PMID: 21505859 DOI: 10.1007/s00535-011-0399-5]
- 22 **Săftoiu A, Iordache SA, Gheonea DI, Popescu C, Maloş A, Gorunescu F, Ciurea T, Iordache A, Popescu GL, Manea CT.** Combined contrast-enhanced power Doppler and real-time sonoelastography performed during EUS, used in the differential diagnosis of focal pancreatic masses (with videos). *Gastrointest Endosc* 2010; **72**: 739-747 [PMID: 20674916 DOI: 10.1016/j.gie.2010.02.056]
- 23 **Xu W, Shi J, Zeng X, Li X, Xie WF, Guo J, Lin Y.** EUS elastography for the differentiation of benign and malignant lymph nodes: a meta-analysis. *Gastrointest Endosc* 2011; **74**: 1001-1009; quiz 1115.e1-1115.e4 [PMID: 22032315 DOI: 10.1016/j.gie.2011.07.026]
- 24 **Janssen J, Dietrich CF, Will U, Greiner L.** Endosonographic elastography in the diagnosis of mediastinal lymph nodes. *Endoscopy* 2007; **39**: 952-957 [PMID: 18008203 DOI: 10.1055/s-2007-966946]
- 25 **Popescu A, Săftoiu A.** Can elastography replace fine needle aspiration? *Endosc Ultrasound* 2014; **3**: 109-117 [PMID: 24955340 DOI: 10.4103/2303-9027.123009]
- 26 **Hawes RH.** Indications for EUS-directed FNA. *Endoscopy* 1998; **30** Suppl 1: A155-A157 [PMID: 9765112 DOI: 10.1055/s-2007-1001503]
- 27 **Chong A, Venugopal K, Segarajasingam D, Lisewski D.** Tumor seeding after EUS-guided FNA of pancreatic tail neoplasia. *Gastrointest Endosc* 2011; **74**: 933-935 [PMID: 21951481 DOI: 10.1016/j.gie.2010.10.020]
- 28 **Topazian M.** Endoscopic ultrasonography in the evaluation of indeterminate biliary strictures. *Clin Endosc* 2012; **45**: 328-330 [PMID: 22977829 DOI: 10.5946/ce.2012.45.3.328]
- 29 **Yasuda K.** Imaging alone is sufficient in most circumstances--making the case for limited need for FNA. *Gastrointest Endosc* 2009;

- 69: S155-S156 [PMID: 19179145 DOI: 10.1016/j.gie.2008.12.039]
- 30 **Dietrich CF**, Săftoiu A, Jenssen C. Real time elastography endoscopic ultrasound (RTE-EUS), a comprehensive review. *Eur J Radiol* 2014; **83**: 405-414 [PMID: 23643030 DOI: 10.1016/j.ejrad.2013.03.023]
 - 31 **Yamao K**, Sawaki A, Mizuno N, Shimizu Y, Yatabe Y, Koshikawa T. Endoscopic ultrasound-guided fine-needle aspiration biopsy (EUS-FNAB): past, present, and future. *J Gastroenterol* 2005; **40**: 1013-1023 [PMID: 16322944 DOI: 10.1007/s00535-005-1717-6]
 - 32 **Erickson RA**. EUS-guided FNA. *Gastrointest Endosc* 2004; **60**: 267-279 [PMID: 15278063]
 - 33 **Iglesias-Garcia J**, Lindkvist B, Lariño-Noia J, Domínguez-Muñoz JE. Endoscopic ultrasound elastography. *Endosc Ultrasound* 2012; **1**: 8-16 [PMID: 24949330 DOI: 10.7178/eus.01.003]
 - 34 **Giovannini M**, Thomas B, Erwan B, Christian P, Fabrice C, Benjamin E, Geneviève M, Paolo A, Pierre D, Robert Y, Walter S, Hanz S, Carl S, Christoph D, Pierre E, Jean-Luc VL, Jacques D, Peter V, Andrian S. Endoscopic ultrasound elastography for evaluation of lymph nodes and pancreatic masses: a multicenter study. *World J Gastroenterol* 2009; **15**: 1587-1593 [PMID: 19340900 DOI: 10.3748/wjg.15.1587]
 - 35 **Hirche TO**, Ignee A, Barreiros AP, Schreiber-Dietrich D, Jungblut S, Ott M, Hirche H, Dietrich CF. Indications and limitations of endoscopic ultrasound elastography for evaluation of focal pancreatic lesions. *Endoscopy* 2008; **40**: 910-917 [PMID: 19009483 DOI: 10.1055/s-2008-1077726]
 - 36 **Saftoiu A**, Vilman P. Endoscopic ultrasound elastography-- a new imaging technique for the visualization of tissue elasticity distribution. *J Gastrointest Liver Dis* 2006; **15**: 161-165 [PMID: 16802011]
 - 37 **Dietrich CF**, Jenssen C, Allescher HD, Hocke M, Barreiros AP, Ignee A. [Differential diagnosis of pancreatic lesions using endoscopic ultrasound]. *Z Gastroenterol* 2008; **46**: 601-617 [PMID: 18537088 DOI: 10.1055/s-2008-1027523]
 - 38 **Iglesias-Garcia J**, Larino-Noia J, Abdulkader I, Forteza J, Dominguez-Munoz JE. EUS elastography for the characterization of solid pancreatic masses. *Gastrointest Endosc* 2009; **70**: 1101-1108 [PMID: 19647248 DOI: 10.1016/j.gie.2009.05.011]
 - 39 **Jenssen C**, Dietrich CF. Endoscopic ultrasound-guided fine-needle aspiration biopsy and trucut biopsy in gastroenterology - An overview. *Best Pract Res Clin Gastroenterol* 2009; **23**: 743-759 [PMID: 19744637 DOI: 10.1016/j.bpg.2009.05.006]
 - 40 **Havre RF**, Elde E, Gilja OH, Odegaard S, Eide GE, Matre K, Nesje LB. Freehand real-time elastography: impact of scanning parameters on image quality and in vitro intra- and interobserver validations. *Ultrasound Med Biol* 2008; **34**: 1638-1650 [PMID: 18524458 DOI: 10.1016/j.ultrasmedbio.2008.03.009]
 - 41 **Dietrich CF**, Hirche TO, Ott M, Ignee A. Real-time tissue elastography in the diagnosis of autoimmune pancreatitis. *Endoscopy* 2009; **41**: 718-720 [PMID: 19618344 DOI: 10.1055/s-0029-1214866]
 - 42 **Schrader H**, Wiese M, Ellrichmann M, Belyaev O, Uhl W, Tannapfel A, Schmidt W, Meier J. Diagnostic value of quantitative EUS elastography for malignant pancreatic tumors: relationship with pancreatic fibrosis. *Ultraschall Med* 2012; **33**: E196-E201 [PMID: 21630184 DOI: 10.1055/s-0031-1273256]
 - 43 **Giovannini M**. Endoscopic ultrasound elastography. *Pancreatol* 2011; **11** Suppl 2: 34-39 [PMID: 21464585 DOI: 10.1159/000323496]
 - 44 **Cui XW**, Ignee A, Braden B, Woenckhaus M, Dietrich CF. Biliary papillomatosis and new ultrasound imaging modalities. *Z Gastroenterol* 2012; **50**: 226-231 [PMID: 22298103 DOI: 10.1055/s-0031-1281967]
 - 45 **Lee TH**, Cha SW, Cho YD. EUS elastography: advances in diagnostic EUS of the pancreas. *Korean J Radiol* 2012; **13** Suppl 1: S12-S16 [PMID: 22563282 DOI: 10.3348/kjr.2012.13.S1.S12]
 - 46 **Hu DM**, Gong TT, Zhu Q. Endoscopic ultrasound elastography for differential diagnosis of pancreatic masses: a meta-analysis. *Dig Dis Sci* 2013; **58**: 1125-1131 [PMID: 23306838 DOI: 10.1007/s10620-012-2428-5]
 - 47 **Mei M**, Ni J, Liu D, Jin P, Sun L. EUS elastography for diagnosis of solid pancreatic masses: a meta-analysis. *Gastrointest Endosc* 2013; **77**: 578-589 [PMID: 23199646 DOI: 10.1016/j.gie.2012.09.035]
 - 48 **Li X**, Xu W, Shi J, Lin Y, Zeng X. Endoscopic ultrasound elastography for differentiating between pancreatic adenocarcinoma and inflammatory masses: a meta-analysis. *World J Gastroenterol* 2013; **19**: 6284-6291 [PMID: 24115828 DOI: 10.3748/wjg.v19.i37.6284]
 - 49 **Gheonea DI**, Săftoiu A. Beyond conventional endoscopic ultrasound: elastography, contrast enhancement and hybrid techniques. *Curr Opin Gastroenterol* 2011; **27**: 423-429 [PMID: 21844751 DOI: 10.1097/MOG.0b013e328349cfab]
 - 50 **Piscaglia F**, Nolsøe C, Dietrich CF, Cosgrove DO, Gilja OH, Bachmann Nielsen M, Albrecht T, Barozzi L, Bertolotto M, Catalano O, Claudon M, Clevert DA, Correas JM, D'Onofrio M, Drudi FM, Eyding J, Giovannini M, Hocke M, Ignee A, Jung EM, Klausner AS, Lassau N, Leen E, Mathis G, Saftoiu A, Seidel G, Sidhu PS, ter Haar G, Timmerman D, Weskott HP. The EFSUMB Guidelines and Recommendations on the Clinical Practice of Contrast Enhanced Ultrasound (CEUS): update 2011 on non-hepatic applications. *Ultraschall Med* 2012; **33**: 33-59 [PMID: 21874631 DOI: 10.1055/s-0031-1281676]
 - 51 **Iglesias-Garcia J**, Domínguez-Muñoz JE, Castiñeira-Alvarino M, Luaces-Regueira M, Lariño-Noia J. Quantitative elastography associated with endoscopic ultrasound for the diagnosis of chronic pancreatitis. *Endoscopy* 2013; **45**: 781-788 [PMID: 24019131 DOI: 10.1055/s-0033-1344614]
 - 52 **Itoh Y**, Itoh A, Kawashima H, Ohno E, Nakamura Y, Hiramatsu T, Sugimoto H, Sumi H, Hayashi D, Kuwahara T, Morishima T, Funasaka K, Nakamura M, Miyahara R, Ohmiya N, Katano Y, Ishigami M, Goto H, Hirooka Y. Quantitative analysis of diagnosing pancreatic fibrosis using EUS-elastography (comparison with surgical specimens). *J Gastroenterol* 2014; **49**: 1183-1192 [PMID: 24026103 DOI: 10.1007/s00535-013-0880-4]
 - 53 **Cui XW**, Jenssen C, Saftoiu A, Ignee A, Dietrich CF. New ultrasound techniques for lymph node evaluation. *World J Gastroenterol* 2013; **19**: 4850-4860 [PMID: 23946589 DOI: 10.3748/wjg.v19.i30.4850]
 - 54 **Janssen J**. [(E)US elastography: current status and perspectives]. *Z Gastroenterol* 2008; **46**: 572-579 [PMID: 18537085 DOI: 10.1055/s-2008-1027379]
 - 55 **Bhutani MS**, Hawes RH, Hoffman BJ. A comparison of the accuracy of echo features during endoscopic ultrasound (EUS) and EUS-guided fine-needle aspiration for diagnosis of malignant lymph node invasion. *Gastrointest Endosc* 1997; **45**: 474-479 [PMID: 9199903]
 - 56 **Săftoiu A**, Vilmann P, Hassan H, Gorunescu F. Analysis of endoscopic ultrasound elastography used for characterisation and differentiation of benign and malignant lymph nodes. *Ultraschall Med* 2006; **27**: 535-542 [PMID: 17160759 DOI: 10.1055/s-2006-927117]
 - 57 **Săftoiu A**, Vilmann P, Ciurea T, Popescu GL, Iordache A, Hassan H, Gorunescu F, Iordache S. Dynamic analysis of EUS used for the differentiation of benign and malignant lymph nodes. *Gastrointest Endosc* 2007; **66**: 291-300 [PMID: 17643702 DOI: 10.1016/j.gie.2006.12.039]
 - 58 **Knabe M**, Günter E, Ell C, Pech O. Can EUS elastography improve lymph node staging in esophageal cancer? *Surg Endosc* 2013; **27**: 1196-1202 [PMID: 23093233 DOI: 10.1007/s00464-012-2575-y]
 - 59 **Paterson S**, Duthie F, Stanley AJ. Endoscopic ultrasound-guided elastography in the nodal staging of oesophageal cancer. *World J Gastroenterol* 2012; **18**: 889-895 [PMID: 22408347 DOI: 10.3748/wjg.v18.i9.889]
 - 60 **Larsen MH**, Frstrup C, Hansen TP, Hovendal CP, Mortensen MB. Endoscopic ultrasound, endoscopic sonoelastography, and strain ratio evaluation of lymph nodes with histology as gold standard. *Endoscopy* 2012; **44**: 759-766 [PMID: 22752891 DOI: 10.1055/s-0032-1309817]
 - 61 **Dietrich CF**. Endoscopic ultrasound. An introductory manual and atlas. 2nd ed. Stuttgart: Thieme, 2011
 - 62 **Kocaman O**, Sentürk H, Danalıoğlu A, Türkdoğan K, Arabacı

- E, Yildiz K, Ince AT. Endosonography and elastography in the diagnosis of esophageal tuberculosis. *Turk J Gastroenterol* 2013; **24**: 290-291 [PMID: 24226724]
- 63 **Jenssen C**, Dietrich CF. Endoscopic ultrasound of gastrointestinal subepithelial lesions. *Ultraschall Med* 2008; **29**: 236-256; quiz 257-264 [PMID: 18516768 DOI: 10.1055/s-2008-1027388]
- 64 **Dietrich CF**, Jenssen C, Hocke M, Cui XW, Woenckhaus M, Ignee A. Imaging of gastrointestinal stromal tumours with modern ultrasound techniques - a pictorial essay. *Z Gastroenterol* 2012; **50**: 457-467 [PMID: 22581701 DOI: 10.1055/s-0031-1282076]
- 65 **Dietrich CF**, Jenssen C. [Evidence based endoscopic ultrasound]. *Z Gastroenterol* 2011; **49**: 599-621 [PMID: 21544753 DOI: 10.1055/s-0029-1246021]
- 66 **Allgayer H**, Ignee A, Dietrich CF. Endosonographic elastography of the anal sphincter in patients with fecal incontinence. *Scand J Gastroenterol* 2010; **45**: 30-38 [PMID: 20001748 DOI: 10.3109/00365520903383251]
- 67 **Cui XW**, Pirri C, Ignee A, De Molo C, Hirche TO, Schreiber-Dietrich DG, Dietrich CF. Measurement of shear wave velocity using acoustic radiation force impulse imaging is not hampered by previous use of ultrasound contrast agents. *Z Gastroenterol* 2014; **52**: 649-653 [PMID: 25026006 DOI: 10.1055/s-0034-1366036]
- 68 **Cui XW**, Friedrich-Rust M, De Molo C, Ignee A, Schreiber-Dietrich D, Dietrich CF. Liver elastography, comments on EFSUMB elastography guidelines 2013. *World J Gastroenterol* 2013; **19**: 6329-6347 [PMID: 24151351 DOI: 10.3748/wjg.v19.i38.6329]
- 69 **Jenssen C**, Dietrich CF. [Ultrasound and endoscopic ultrasound of the adrenal glands]. *Ultraschall Med* 2010; **31**: 228-247; quiz 248-50 [PMID: 20517815 DOI: 10.1055/s-0029-1245449]
- 70 **Dietrich CF**, Wehrmann T, Hoffmann C, Herrmann G, Caspary WF, Seifert H. Detection of the adrenal glands by endoscopic or transabdominal ultrasound. *Endoscopy* 1997; **29**: 859-864 [PMID: 9476771 DOI: 10.1055/s-2007-1004322]
- 71 **Dietrich CF**, Ignee A, Barreiros AP, Schreiber-Dietrich D, Sienz M, Bojunga J, Braden B. Contrast-enhanced ultrasound for imaging of adrenal masses. *Ultraschall Med* 2010; **31**: 163-168 [PMID: 19401979 DOI: 10.1055/s-0028-1109357]
- 72 **Trojan J**, Schwarz W, Sarrazin C, Thalhammer A, Vogl TJ, Dietrich CF. Role of ultrasonography in the detection of small adrenal masses. *Ultraschall Med* 2002; **23**: 96-100 [PMID: 11961722 DOI: 10.1055/s-2002-25190]
- 73 **Uemura S**, Yasuda I, Kato T, Doi S, Kawaguchi J, Yamauchi T, Kaneko Y, Ohnishi R, Suzuki T, Yasuda S, Sano K, Moriwaki H. Preoperative routine evaluation of bilateral adrenal glands by endoscopic ultrasound and fine-needle aspiration in patients with potentially resectable lung cancer. *Endoscopy* 2013; **45**: 195-201 [PMID: 23299524 DOI: 10.1055/s-0032-1325988]
- 74 **Dietrich CF**, Hocke M, Jenssen C. [Interventional endosonography]. *Ultraschall Med* 2011; **32**: 8-22, quiz 23-25 [PMID: 21305436 DOI: 10.1055/s-0029-1246017]
- 75 **Ahmad S**, Cao R, Varghese T, Bidaut L, Nabi G. Transrectal quantitative shear wave elastography in the detection and characterisation of prostate cancer. *Surg Endosc* 2013; **27**: 3280-3287 [PMID: 23525883 DOI: 10.1007/s00464-013-2906-7]
- 76 **Schreiber-Dietrich D**, Dietrich CF. Contrast enhanced ultrasound (CEUS) and off-label use (in children). *Ultraschall Med* 2012; **33**: 295-296 [PMID: 22891364]
- 77 **Dietrich CF**, Riemer-Hommel P. Challenges for the German Health Care System. *Z Gastroenterol* 2012; **50**: 557-572 [PMID: 22660990 DOI: 10.1055/s-0032-1312742]

P- Reviewer: Velayos B **S- Editor:** Ma YJ **L- Editor:** Filopodia
E- Editor: Ma S



Basic Study

Hepatitis B and C virus-induced hepatitis: Apoptosis, autophagy, and unfolded protein response

Behzad Yeganeh, Adel Rezaei Moghadam, Javad Alizadeh, Emilia Wiechec, Seyed Moayed Alavian, Mohammad Hashemi, Bitá Geramizadeh, Afshin Samali, Kamran Bagheri Lankarani, Martin Post, Payam Peymani, Kevin M Coombs, Saeid Ghavami

Behzad Yeganeh, Martin Post, Department of Physiology and Experimental Medicine, Hospital for Sick Children Research Institute, University of Toronto, Toronto M5G 0A4, Canada

Adel Rezaei Moghadam, Young Researchers and Elite Club, Ardabil Branch, Islamic Azad University, Ardabil 156131-56491, Iran

Javad Alizadeh, Saeid Ghavami, Department of Human Anatomy and Cell Science, College of Medicine, Faculty of Health Sciences, University of Manitoba, Winnipeg R3E 0J9, Canada

Javad Alizadeh, Kevin M Coombs, Saeid Ghavami, The Children's Hospital Research Institute of Manitoba, University of Manitoba, Winnipeg R3E 0J9, Canada

Emilia Wiechec, Department of Clinical and Experimental Medicine, Division of Otorhinolaryngology, Linköping University, 581-85 Linköping, Sweden

Seyed Moayed Alavian, Baqiyatallah Research Center for Gastroenterology and Liver Diseases (BRCGL), 1417-613151 Tehran, Iran

Mohammad Hashemi, Department of Clinical Biochemistry, School of Medicine, Zahedan University of Medical Sciences, Zahedan 43181-98167, Iran

Bitá Geramizadeh, Department of Pathology and Organ Transplant Research Center, Shiraz University of Medical Sciences, Shiraz 71348, Iran

Afshin Samali, Department of Biochemistry, National University of Ireland Galway, 000 Galway, Ireland

Kamran Bagheri Lankarani, Payam Peymani, Saeid Ghavami, Health Policy Research Centre, Shiraz University of Medical Sciences, Shiraz 71348-45794, Iran

Kevin M Coombs, Manitoba Center for Proteomics and Systems Biology, University of Manitoba, Winnipeg R3E 0J9, Canada

Kevin M Coombs, Department of Medical Microbiology and

Infectious Diseases, The Children's Hospital Research Institute of Manitoba, University of Manitoba, Winnipeg R3E 0J9, Canada

Author contributions: Yeganeh B and Post M performed fluorescence immunohistochemistry, tissue array; Yeganeh B interpreted the data, drafted the whole primary version of manuscript; Rezaei Moghadam A and Alizadeh J analyzed the fluorescence immunohistochemistry results, participated in preparing the apoptosis and autophagy parts of manuscript, respectively; Wiechec E analyzed the fluorescence immunohistochemistry and tissue array results, drafted of the autophagy and apoptosis part of manuscript; Alavian SM diagnosed and got liver biopsy of HBV patients; Hashemi M contributed PCR or samples for HBV and HCV virus titer, and analyzed the fluorescence immunohistochemistry results; Geramizadeh B contributed pathology diagnosis, scoring, and graded of HBV and HCV biopsies; Samali A contributed unfolded protein response, and proof final versions of the manuscript; Bagheri Lankarani K diagnosed and got liver biopsy of HCV patients; Peymani P prepared liver biopsy sample, biochemical liver function, statistical analysis; Coombs KM and Ghavami S designed the project, and proofed final versions of the manuscript; Ghavami S prepared primary draft of UPR part of manuscript; Peymani P, Coombs KM and Ghavami S had equal senior authorship.

Supported by University of Manitoba Start-up funds and an award from the Manitoba Medical Service Foundation to Ghavami S; University of Manitoba Start-up Funds to Alizadeh J.

Institutional review board statement: This retrospective study was approved by local research ethics committee of Health policy research Center (Protocol number HP-101-91). All patients were informed about the study and gave verbal informed consent prior to enrollment.

Conflict-of-interest statement: All authors do not have any conflict of interest.

Data sharing statement: This is an open access work and the researcher can use the data for any further investigations.

Open-Access: This article is an open-access article which was

selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Saeid Ghavami, PhD, Assistant Professor, Department of Human Anatomy and Cell Science, College of Medicine, Faculty of Health Sciences, University of Manitoba, Winnipeg R3E 0J9, Room 104, Canada. saeid.ghavami@umanitoba.ca
Telephone: +1-204-2723061
Fax: +1-204-7893920

Received: August 29, 2015
Peer-review started: August 30, 2015
First decision: September 29, 2015
Revised: October 14, 2015
Accepted: November 13, 2015
Article in press: November 13, 2015
Published online: December 21, 2015

Abstract

AIM: To investigate the co-incidence of apoptosis, autophagy, and unfolded protein response (UPR) in hepatitis B (HBV) and C (HCV) infected hepatocytes.

METHODS: We performed immunofluorescence confocal microscopy on 10 liver biopsies from HBV and HCV patients and tissue microarrays of HBV positive liver samples. We used specific antibodies for LC3 β , cleaved caspase-3, BIP (GRP78), and XBP1 to detect autophagy, apoptosis and UPR, respectively. Anti-HCV NS3 and anti-HBs antibodies were also used to confirm infection. We performed triple blind counting of events to determine the co-incidence of autophagy (LC3 β punctuate), apoptosis (cleaved caspase-3), and unfolded protein response (GRP78) with HBV and HCV infection in hepatocytes. All statistical analyses were performed using SPSS software for Windows (Version 16 SPSS Inc, Chicago, IL, United States). *P*-values < 0.05 were considered statistically significant. Statistical analyses were performed with Mann-Whitney test to compare incidence rates for autophagy, apoptosis, and UPR in HBV- and HCV-infected cells and adjacent non-infected cells.

RESULTS: Our results showed that infection of hepatocytes with either HBV and HCV induces significant increase (*P* < 0.001) in apoptosis (cleavage of caspase-3), autophagy (LC3 β punctate), and UPR (increase in GRP78 expression) in the HCV- and HBV-infected cells, as compared to non-infected cells of the same biopsy sections. Our tissue microarray immunohistochemical expression analysis of LC3 β in HBV^{Neg} and HBV^{Pos} revealed that majority of HBV-infected hepatocytes display strong positive staining

for LC3 β . Interestingly, although XBP splicing in HBV-infected cells was significantly higher (*P* < 0.05), our analyses show a slight increase of XBP splicing was in HCV-infected cells (*P* > 0.05). Furthermore, our evaluation of patients with HBV and HCV infection based on stage and grade of the liver diseases revealed no correlation between these pathological findings and induction of apoptosis, autophagy, and UPR.

CONCLUSION: The results of this study indicate that HCV and HBV infection activates apoptosis, autophagy and UPR, but slightly differently by each virus. Further studies are warranted to elucidate the interconnections between these pathways in relation to pathology of HCV and HBV in the liver tissue.

Key words: Cell fate; Cell death; Hepatocyte; Viral infection; Endoplasmic reticulum stress

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: For the first time, the current study has addressed the co-incidence of apoptosis, autophagy, and unfolded protein response in the liver tissue of hepatitis B and hepatitis C (HBV, and HCV) infected patients. The results showed that all of these events are activated at the same time by HBV and HCV infection in the liver. All of these pathways probably are involved in replication and pathogenesis of HBV and HCV infection; therefore their modulation would probably be beneficial for new therapeutic approaches in these diseases.

Yeganeh B, Rezaei Moghadam A, Alizadeh J, Wiechec E, Alavian SM, Hashemi M, Geramizadeh B, Samali A, Bagheri Lankarani K, Post M, Peymani P, Coombs KM, Ghavami S. Hepatitis B and C virus-induced hepatitis: Apoptosis, autophagy, and unfolded protein response. *World J Gastroenterol* 2015; 21(47): 13225-13239 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13225.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13225>

INTRODUCTION

The liver is the primary organ infected by, and in which both hepatitis B virus (HBV) and hepatitis C virus (HCV) replicate. HBV and HCV are structurally unrelated and responsible for infection of huge numbers of individuals in the world.

It has been reported that around 2 billion people are infected with HBV, and more than 350 million are chronic carriers according to clinical definitions that identify individuals who have continuous viral and subviral particles in their blood for more than six months^[1,2]. Several studies have determined that HBV infection in adulthood might lead to a carrier stage in about 5%-10% of infected individuals. In addition,

in up to 30% of these individuals hepatitis, fibrosis, cirrhosis, and finally hepatocellular carcinoma (HCC) can develop and it is usually considered a progressive chronic liver disease (CLD)^[3]. Several groups have investigated the risk of HCC development in different populations and shown that HCC development can be increased up to 100-fold in carrier infected individuals compared with uninfected individuals, which depends on the population and different markers. The risk of developing HCC among carriers with CLD ranges from 10-fold to 100-fold greater compared to uninfected people^[2,4]. Approximately 85% of acute HCV infection leads to a chronic situation and 50% of individuals who suffer from HCV chronic infections (about 170 million worldwide) can develop CLD. Approximately 5%-20% of this population can progress to liver cirrhosis in 5-20 years after their infection, and 1%-2% of these patients will probably develop HCC per year^[3]. It is strongly believed that this can be considered one of the closest relationships between an environmental agent and a cancer that has so far been identified^[3].

Autophagy is a tightly regulated catabolic process, which is essential in many cellular events including development, differentiation, survival, and homeostasis^[5-7]. In the past few years, many investigators have focused on the involvement of autophagy in different human diseases and many aspects of this relationship have been described^[8-10]. Autophagy is usually considered a very important step for numerous virus life cycles^[11], including HBV and HCV^[12].

Apoptosis is programmed cell death initiated *via* two different pathways (1) extrinsic which is activated by ligation of death receptors; and (2) intrinsic which is activated by mitochondrial death-related proteins. These two distinct pathways crosstalk and potentiate each other to ultimately activate the caspase cascade and facilitate controlled proteolysis of cellular components^[13-15].

Synthesized and secretory proteins are correctly folded and assembled in the endoplasmic reticulum (ER)^[16]. During cellular stress, the ER loses its capacity to correct protein folding which results in the accumulation of unfolded and misfolded proteins. Following this, the unfolded protein response (UPR) targets the degradation of the accumulated proteins in the ER, inhibits global protein translation and also activates the transcription of genes that increase the protein folding capacity of the ER including lectins, chaperones, and calcium pumps^[17]. Three ER membrane sensors mediate signals from the ER upon activation of the UPR including activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 α (IRE1 α), and protein kinase RNA (PKR)-like ER-localized kinase (PERK)^[16]. Each of these molecules activates independently distinct signaling pathways to provide an integrated response to ER stress^[18]. Unfolded and misfolded proteins in the ER disrupt binding of the binding immunoglobulin protein (BIP)/glucose-

regulated protein 78 (GRP78) with ER stress sensors, leading to their activation. PERK phosphorylates eukaryotic initiation factor 2 α (eIF2 α) which results in a decrease in mRNA translation with concurrent translation increase of several mRNAs like activating transcription factor 4 (ATF4) and the CCAAT-enhancer-binding protein homologous protein (CHOP) (ATF4 downstream target)^[16].

Several previous investigations have shown that HBV^[19-24] and HCV^[25-27] infection can modulate apoptosis, autophagy, and UPR in different *in vitro* and non-human *in vivo* models. However, most of these studies did not use human samples and also have not simultaneously investigated apoptosis, autophagy, and UPR in the same infected tissue or organ. To address these gaps, we used tissue microarray, and fluorescence immunohistochemistry (IHC) in the present study to evaluate apoptosis, autophagy and UPR in human biopsy samples from patients who were infected with HBV or HCV. This study, for the first time, provides an evaluation of these events at the same time in HBV and HCV liver biopsies of infected patients.

MATERIALS AND METHODS

Materials and antibodies

The following antibodies were used in this study for immunofluorescence or IHC, or both: LC3 β antibody was obtained from Proteintech (18725-1-AP, Chicago, IL, United States). Antibody for hepatitis B surface antigen (HBsAg) was obtained from Novus Biologicals (NBP1-22568, Littleton, CO, United States). Cleaved caspase-3 (Asp175) antibody was purchased from Cell Signaling Technology (#9661, MA, United States). Anti-Hepatitis C virus NS3 (ab49486), anti-BIP (GRP78) (ab21685) and anti-XBP1 (ab37152) antibodies were purchased from Abcam (Cambridge, MA, United States). Biotin conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, United States). VECTASTAIN Peroxidase ABC kit was purchased from Vector Laboratories (Vector Laboratories, Burlingame, CA, United States). Fluorescent-conjugated (Alexa Fluor 488 and 546) secondary antibodies were purchased from Molecular Probes (Eugene, OR, United States). Type B hepatitis and normal liver tissue array (IC03001) was purchased from United States BioMax (Rockville, MD, United States).

Ethical protocol

This retrospective study was approved by local research ethics committee of Health policy research Center (Protocol number HP-101-91). All patients were informed about the study and gave verbal informed consent prior to enrollment.

Selection criteria for HBV and HCV patients

HBV- and HCV-infected individuals (10 in each group)

were diagnosed and selected based on the following criteria from the patients who had been referred to Namazi Hospital (Shiraz, Iran): (1) HBV-infected patients: Hepatitis virus surface antigen positive and HBV DNA > 2000 IU/mL in the serum; and (2) HCV-infected patients: HCV antibody positive, which was confirmed by detecting HCV RNA in the serum.

All cases had abnormal levels of liver enzymes including alanine transaminase; $2 \times$ the upper limit of normal range (Normal Range is 7-56 IU/L) in two measurements that were performed three months apart. In all cases other diagnoses were ruled out with appropriate work up and no case had mixed cause of liver function abnormality in this series. DNA was extracted using Qiagen kit (Qiagen, Hilden, Germany), as per manufacturer's instructions. HBV and HCV genome copy numbers were measured using Qiagen kit (Germany). Patients with other causes of chronic liver disease such as Wilson's hemochromatosis, drug induced liver disease, alpha-1 antitrypsin deficiency, or alcoholic liver diseases were excluded. Patients were also excluded if pregnant, or if presented with comorbid conditions including diabetes mellitus, congestive heart failure, or chronic kidney disease.

Liver biopsy and sample preparation

Biopsy of the liver was performed by the radiologist under the guide of ultrasound or computerized tomography (CT) scan using Tru-cut[®] needles (standard biopsy needle). Biopsies were fixed in formalin, and then processed as routine pathology specimens.

Histology and immunohistochemistry

Paraffin-embedded liver tissues were used for histology and IHC. Briefly, paraffin sections of 4 μ m thickness were prepared, deparaffinized, rehydrated and used for staining. Following unmasking of antigens using Heat-Induced Epitope Retrieval and citrate buffer (0.1 mol/L citric acid, 0.1 mol/L sodium citrate, pH 6.0) in a Coplin Jar for 30 min, IHC was performed by means of detecting antigens with corresponding primary antibodies followed by biotinylated secondary antibodies (Jackson ImmunoResearch Labs) and an avidin-biotin peroxidase complex technique using ABC kit. The slides were then counterstained with Mayer's hematoxylin (Sigma, H9627), dehydrated and mounted with Permount (Thermo Fisher Scientific, Ottawa, ON, Canada). For Immunofluorescence staining, after primary antibody incubation, sections were covered with fluorescent-conjugated (Alexa Fluor 488 and 546) secondary antibodies followed by nuclear staining using ProLong[®] Gold Antifade Mountant with 4',6-diamidino-2-phenylindole DAPI (Molecular Probes, Eugene, OR, United States).

As a negative control, sections were processed as above but addition of primary antibody was omitted. Images were captured using a Leica CTRMIC 6000 confocal microscope equipped with a Hamamatsu

C910013 spinning disc camera (Leica Microsystems, Inc., Concord, ON, Canada). Laser intensity and detector sensitivity settings remained constant for all image acquisitions within each experiment. Images were later analyzed with Volocity software (Perkin-Elmer, Woodbridge, ON, Canada).

Liver tissue microarray and scoring

IHC analysis was performed on commercially available tissue microarray (TMA) (cat. No. IC03001; Biomax Inc., Rockville, MD, United States) consisting of 30 cases of normal liver tissue and 10 cases of HBV-induced hepatitis. For evaluation of IHC staining, semi-quantitative scoring (H-scores) was used to assess positive staining for LC3 β protein expression in TMAs according to the method described previously^[28]. The H-score was calculated by a semi-quantitative assessment of both staining intensity (scale 0-3) and the percentage of positive cells (0%-100%), which, when multiplied, generated a score ranging from 0 to 300. The intensity score was made on the basis of the average intensity of staining where 0 = negative, 1 = weak, 2 = intermediate and 3 = strong. Statistical analysis was carried out on the H-score data obtained. Scoring of the sections was performed blindly by three independent individuals.

Classification of HBV and HCV patients based on the liver disease stage and grade

HBV and HCV patient liver biopsies were carefully assessed by two pathologists and the stage and grade of their liver disease were classified based on the following definitions:

Grade (necroinflammation grade): 0 = none, 1-6 = mild, 7-12 = moderate, 13-18 = severe; Stage (Ishak Fibrosis Score): 0 = No fibrosis, 1-2 = Fibrous expansion of some and most portal areas (+/-) short fibrous septa, 3-4 = Fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging and Fibrous expansion of portal areas with marked bridging (P-P) as well as portal to central (P-C), 5-6 = Marked bridging (P-P and/or P-C), with occasional nodules (incomplete cirrhosis), and Cirrhosis, probable or definite. The results of HBV and HCV patient classifications are shown in Table 1 (stage) and Table 2 (grade).

Statistical analysis

All statistical analyses were performed using SPSS software for Windows (Version 16 SPSS Inc, Chicago, IL, United States). *P*-values < 0.05 were considered statistically significant. Statistical analyses were performed with Mann-Whitney test for comparing autophagy marker (punctate LC3 β), apoptosis marker (cleaved caspase-3), and UPR marker (BIP) between HBV- and HCV-infected cells and adjacent non-infected cells. Statistical analyses were performed with Kruskal-Wallis test for relation of stage (ordinal variable)

Table 1 Stage of liver disease in hepatitis B and C virus infected patients

Stage	None	Mild	Moderate	Severe
HBV (<i>n</i>)	5	5	0	0
HCV (<i>n</i>)	3	5	1	1

HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 2 Grade of liver disease in hepatitis B and C virus infected patients

Grade	None	Mild	Moderate	Severe
HBV (<i>n</i>)	4	3	3	0
HCV (<i>n</i>)	4	2	1	3

HBV: Hepatitis B virus; HCV: Hepatitis C virus.

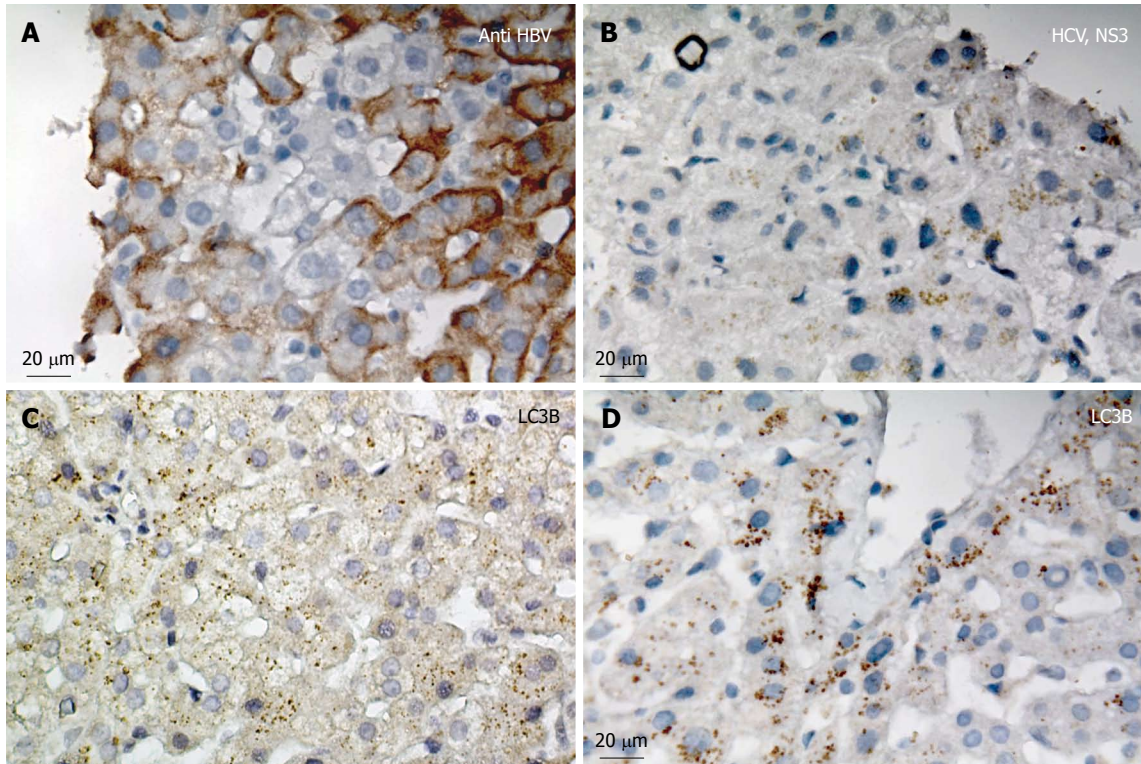


Figure 1 Confirmation of hepatitis B and C virus infection and LC3β expression in liver tissues. A: Immunohistochemistry (IHC) confirms hepatitis B virus (HBV) infection in the liver tissue; the image is representative of IHC for HBV infection in all patients; B: IHC confirms hepatitis C virus (HCV) infection (Anti NS3 HCV) in the liver tissue; the image is representative of IHC for HCV infection in all patients; C: IHC confirms LC3β expression in HBV infected liver tissue; the image is representative of IHC for all patients; D: IHC confirms LC3β expression in HCV infected liver tissue; the image is representative of IHC for all patients.

and grade (ordinal variable) liver biopsies of HBV and HCV patients with autophagy marker (punctate LC3β), apoptosis marker (cleaved caspase-3), and UPR marker (BIP). Because of our small sample size, we used the non-parametric Kruskal-Wallis test. It is noteworthy to mention that Kruskal-Wallis test is equivalent nonparametric test of “one-way analysis of variance”.

RESULTS

HBV and HCV infection induces autophagy in human liver tissue

IHC analyses confirmed HBV and HCV infection in different liver tissue samples (Figure 1A and B). LC3β expression was later investigated in different HBV- and HCV-infected liver tissues and LC3β expression was confirmed in all samples (Figure 1C and D). It has been previously shown that lipidated LC3β serves as

a reliable marker of autophagy in ICC and IHC^[5,29-31]. Because the previous immunochemistry analyses were meant to primarily confirm infection status and globally explore LC3β expression, we also used fluorescent IHC to directly identify autophagy in the liver biopsy cells from HBV and HCV positive patients (Figure 2A and B). Our results show accumulation of autophagosomes in HBV- (Figure 2A) and HCV- (Figure 2B) infected hepatocytes as determined by significantly higher number of LC3β-positive puncta in both HBV- (Figure 2C) and HCV- (Figure 2D) infected cells compared to adjacent non-infected hepatocytes ($P < 0.001$).

Tissue microarray immunohistochemical profile of LC3β in HBV^{Neg} and HBV^{Pos} hepatocytes

Immunostaining for LC3β was performed on the commercially available TMA slide (see Materials and Methods). A total of 40 samples were analyzed. Ten of the samples were type B hepatitis and 30 were normal

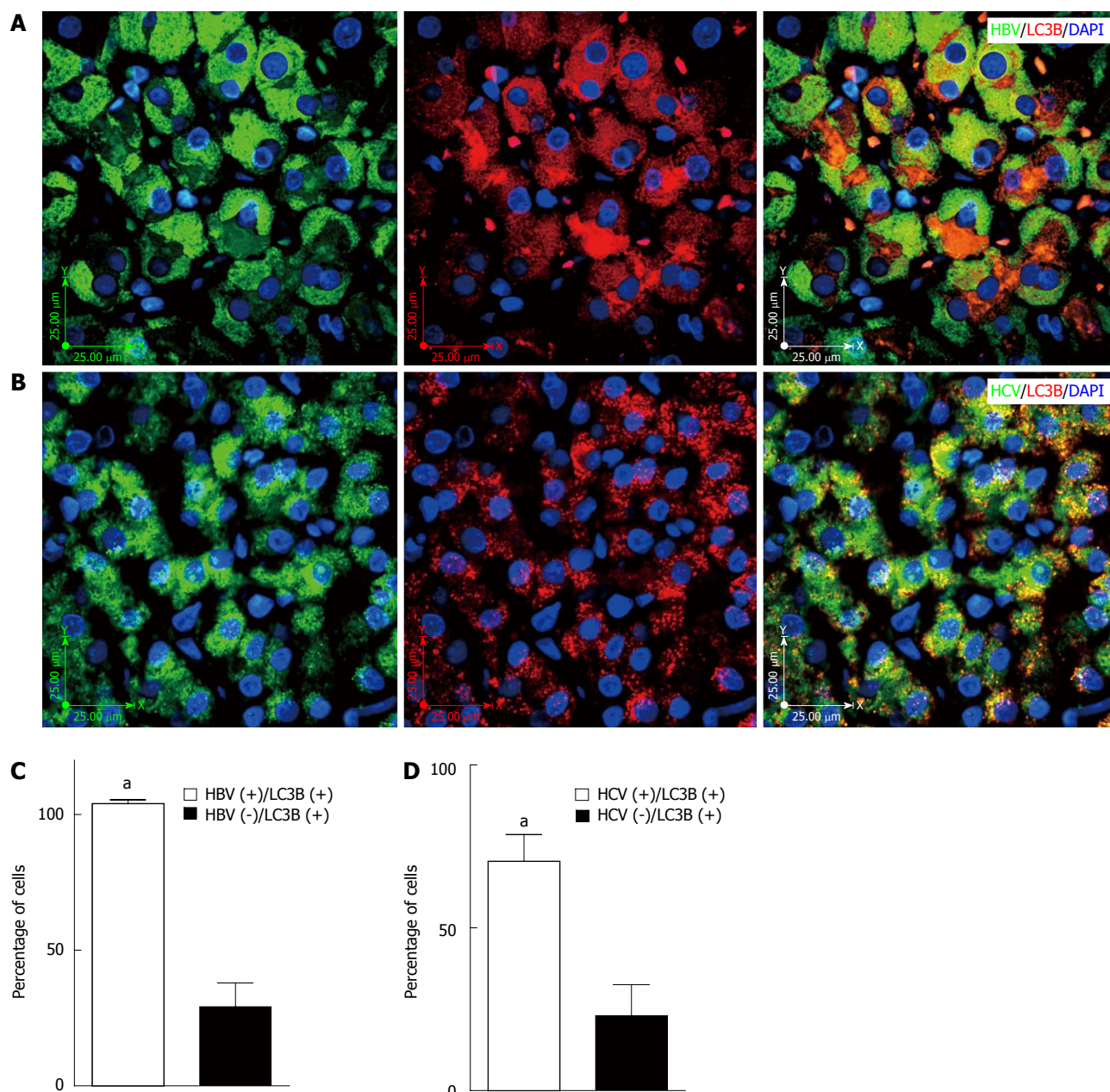


Figure 2 Hepatitis B and C virus infection induces autophagy in liver tissues. A: Fluorescent immunohistochemistry (IHC) confirms co-localization of hepatitis B virus (HBV) and punctate LC3 β in the HBV-infected liver tissue; the image is representative of fluorescence IHC for all patients; B: Fluorescent IHC confirms co-localization of hepatitis C virus (HCV) and punctate LC3 β in the HCV infected liver tissue; the image is representative of fluorescence IHC for all patients; C: HBV infection significantly ($^aP < 0.001$ vs HBV) induces autophagy in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event (HBV infection and punctuated LC3 β) in at least 50 cells counted in four different microscopic fields of view from each patient's sample. D: HCV infection significantly ($^aP < 0.001$ vs HCV) induces autophagy in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event (HCV infection and punctuated LC3 β) in at least 50 cells counted in four different microscopic fields of view from each patient's sample.

liver tissue. Normal liver cells, as well as adjacent non-infected cells in the HBV-infected samples, displayed weak LC3 β staining (Figure 3A). However, the majority of HBV-infected liver cells exhibit strong positive staining of LC3 β (Figure 3B). Evaluation of LC3 β protein expression by IHC using H-score showed a significant ($P < 0.001$) cytoplasmic staining of LC3 β compared with the normal liver samples (Figure 3C).

HBV and HCV infection induces apoptosis in human liver tissue

Previous reports have shown that caspase-3 cleavage is one of the most important hallmarks of apoptosis activation in different models^[6,32,33]. The results of immunofluorescence staining showed that both HBV and HCV infection induce caspase-3 cleavage in infected hepatocytes (Figure 4A and B). In addition,

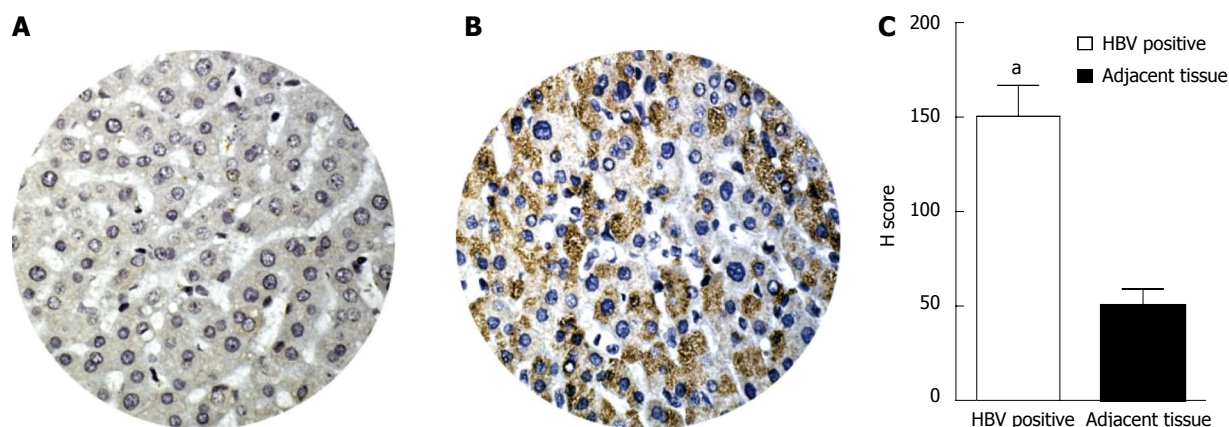


Figure 3 Comparison of LC3 β expression, determined by immunohistochemistry in HBV^{Pos} and HBV^{Neg} liver tissue microarrays. Immunohistochemistry was performed as described in Materials and Methods. H-scores were derived from semi-quantitative assessments of both staining intensity (scale 0-3) and the percentage of positive cells (0%-100%) and, when multiplied, generate a score ranging from 0 to 300. A: Representative image of tissue core from HBV^{Neg} liver showing low expression of LC3 β (H-score of 50); B: HBV^{Pos} liver tissue showing higher expression of LC3 β (H-score of 150); C: The bar graphs show expression levels (H-score) of LC3 β in HBV^{Pos} and HBV^{Neg} liver tissue microarrays. The bars represent the mean \pm SEM. ^a $P < 0.001$ vs the normal HBV^{Neg}. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

caspase-3 cleavage was significantly higher in HBV- and HCV-infected cells compared to non-infected adjacent cells (Figure 4C and D) ($P < 0.001$).

HBV and HCV infection induces UPR in human liver tissue

UPR induces expression of genes encoding proteins, such as BIP, to restore ER homeostasis^[29,34]. Therefore, higher expression of BIP is the major biochemical marker of the activation of UPR. Analysis of IHC staining showed significantly higher expression of BIP in HBV (Figure 5A and C) and HCV (Figure 5B and D) infected hepatocytes compared to non-infected adjacent cells ($P < 0.001$).

HBV infection induces XBP1 splicing in human liver tissue

IRE1 α is a bifunctional enzyme which possesses kinase and RNase activity. One of the primary targets of RNase activity of IRE1 α is XBP1, leading to production of spliced XBP1 (sXBP1) during the UPR^[13,34]. In our present investigation, we showed that both HBV and HCV infection induced XBP1 expression in infected hepatocytes (Figure 6A and B). Interestingly, HBV infection significantly induced XBP1 splicing (nuclear localized XBP1) compared to adjacent non-infected hepatocytes (Figure 6C) ($P < 0.001$) while HCV infection increased XBP splicing which is not statistically different compared to non-infected adjacent cells (Figure 6D) ($P > 0.05$).

Autophagy, apoptosis, and UPR do not correlate with the stage and the grade of liver disease in HBV and HCV patients

Our statistical analysis showed that autophagy, apoptosis, and UPR do not significantly correlate with the stage and the grade of the liver disease in both

HBV and HCV patient groups (Tables 1 and 2) ($P > 0.05$).

DISCUSSION

In the current study we showed that HBV or HCV infection can significantly induce apoptosis, autophagy, and UPR in infected human patient hepatocytes compared to non-infected adjacent cells. This study importantly represents real clinical sample evaluation of these events in the context of authentic HBV and HCV infection.

HCV infection has a striking tendency towards chronicity, often of significant liver diseases like chronic hepatitis, cirrhosis, and hepatocellular carcinoma^[35]. Apoptosis (programmed cell death) is a cellular process in which cells systematically kill themselves through activating intracellular death pathways in response to different kinds of stimuli^[36]. Although apoptosis has been observed as a crucial mechanism in viral clearance^[37], the exact mechanisms of HCV pathogenesis have not yet been fully delineated. There is an accumulating body of evidence that highlights the significant role of hepatocyte apoptosis regulation in HCV pathogenesis^[38] and a variety of apoptotic pathways were proposed that might be involved in this mechanism^[39]. The HCV core protein alone can greatly affect cellular functions. It can either induce or inhibit the apoptosis process. Apoptosis promotion by the core protein may be the reason for occurrence of hepatitis and liver damage and apoptosis inhibition. On the other hand, core might provide conditions for HCV to establish persistent infection^[40].

HBV is also a causative agent of chronic hepatitis and represents one of the major risk factors for development of HCC^[41-43]. The hepatitis B virus X protein (HBx) has recently garnered much attention

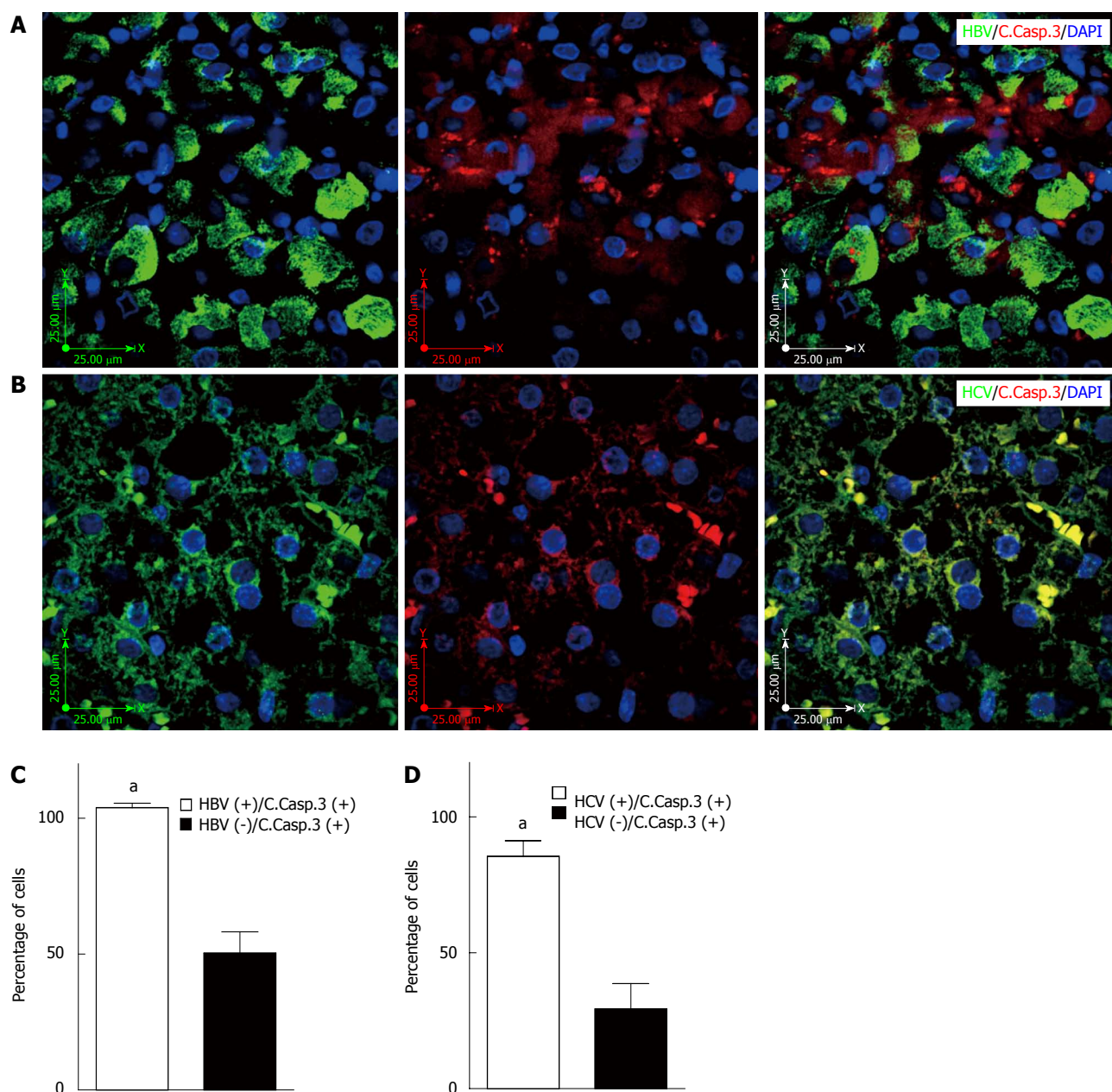


Figure 4 Hepatitis B and C virus infection induces apoptosis in the infected liver tissue. A: Immunofluorescence double-labeling shows co-localization of hepatitis B virus (HBV) and cleaved caspase-3 in the HBV infected liver tissue; the image is representative of fluorescence immunohistochemistry (IHC) for all patients; B: Immunofluorescence double-staining shows co-localization of hepatitis C virus (HCV) and cleaved caspase-3 in the HCV infected liver tissue; the image is representative of fluorescence IHC for all patients; C: HBV infection significantly ($P < 0.001$ vs HBV) induces apoptosis in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event (HBV infection and cleaved caspase-3) in at least 50 cells counted in four different microscopic fields from each patient's sample; D: HCV infection significantly ($P < 0.001$ vs HCV) induces apoptosis in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event (HCV infection and cleaved caspase-3) in at least 50 cells counted in four different microscopic fields from each patient's sample.

regarding its effect on cellular functions, especially apoptosis during HBV infection. Being the smallest protein encoded by HBV genome, HBx is expressed in 70% of patients with HBV-related HCC^[44,45], highly conserved in mammalian hepadnaviruses and essential for infection in mammals^[46]. HBx has various functions that may participate in HBV pathogenesis^[47] and different studies have investigated the role of HBx in multifaceted aspects of apoptosis process. One of the main mechanisms of HBx protein during HBV

infection and that contributes to HCC development is its influence on apoptosis. Since the pro-apoptotic functions of HBx were first described^[47], a wide range of different studies have evaluated the effect of HBx expression on apoptotic pathway regulation; however, results of such studies are variable. According to studies in multiple cellular contexts, HBx might induce^[19,20,47-53], inhibit^[54-57] or have no effect^[58] on apoptosis.

As previously mentioned, autophagy is often

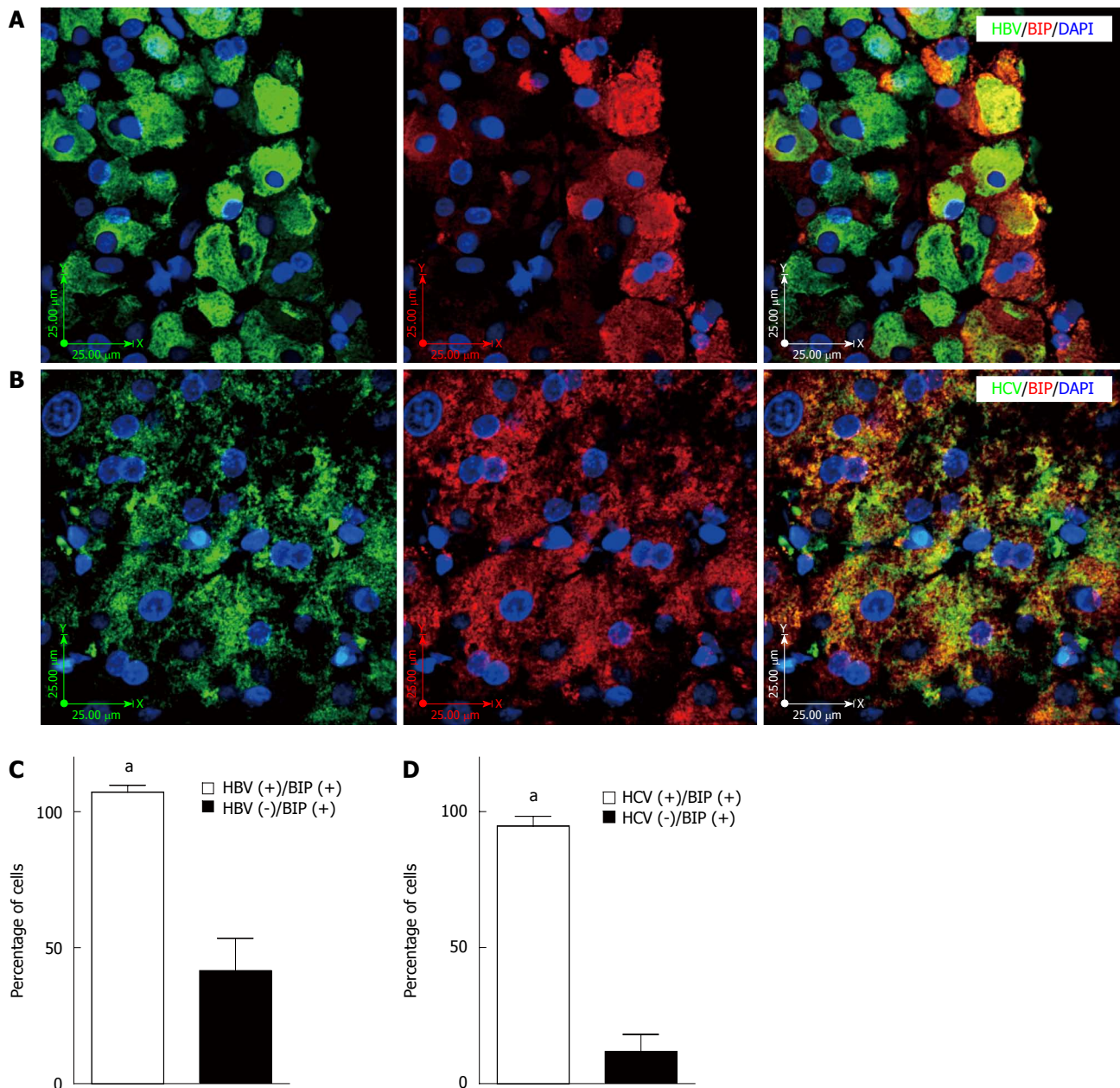


Figure 5 Hepatitis B and C virus infection induces unfolded protein response in the infected liver tissue. A: Fluorescence immunohistochemistry (IHC) confirms co-localization of hepatitis B virus (HBV) and BIP (GRP78) expression in the HBV-infected liver tissue; the image is representative of fluorescence IHC for all patients; B: Fluorescence IHC confirms co-localization of hepatitis C virus (HCV) and BIP (GRP78) expression in the HCV-infected liver tissue; the image is representative of fluorescence IHC for all patients; C: HBV infection significantly ($P < 0.001$ vs HBV) induces UPR in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event, HBV infection and BIP (GRP78) expression in at least 50 cells counted in four different views of fluorescence IHC in each patient's sample; D: HCV infection significantly ($P < 0.001$ vs HCV) induces UPR in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event (HCV infection and BIP (GRP78) expression) in at least 50 cells counted in four different views of fluorescence IHC in each patient's sample. UPR: Unfolded protein response.

defined as an evolutionarily conserved process that catabolizes intracellular components by delivering them into lysosomes^[59-61]. It has been shown that some viruses utilize and induce autophagy in favor of their own replication and survival using their proteins^[62-64]. To contribute to different steps of its life cycle like replication, translation, assembly, and lipo-viro-particle release, HCV takes advantage of the autophagic process. However, these effects are partially indirect^[65]. Similarly, several studies have been performed

aimed at clarifying the involvement of autophagy in establishment of chronic HCV infection^[26,66,67] and findings from these studies suggest that autophagy is involved in HCV infection. A subgenomic replicon, corresponding to the HCV NS3-NS5B coding region of HCV genotype 1b, can trigger autophagy^[68] which implies autophagy induction *via* viral nonstructural proteins. In addition, siRNA-targeting ATGs, that inhibit autophagy, abrogates HCV replication^[69]. Moreover, HCV infection activates various pattern recognition

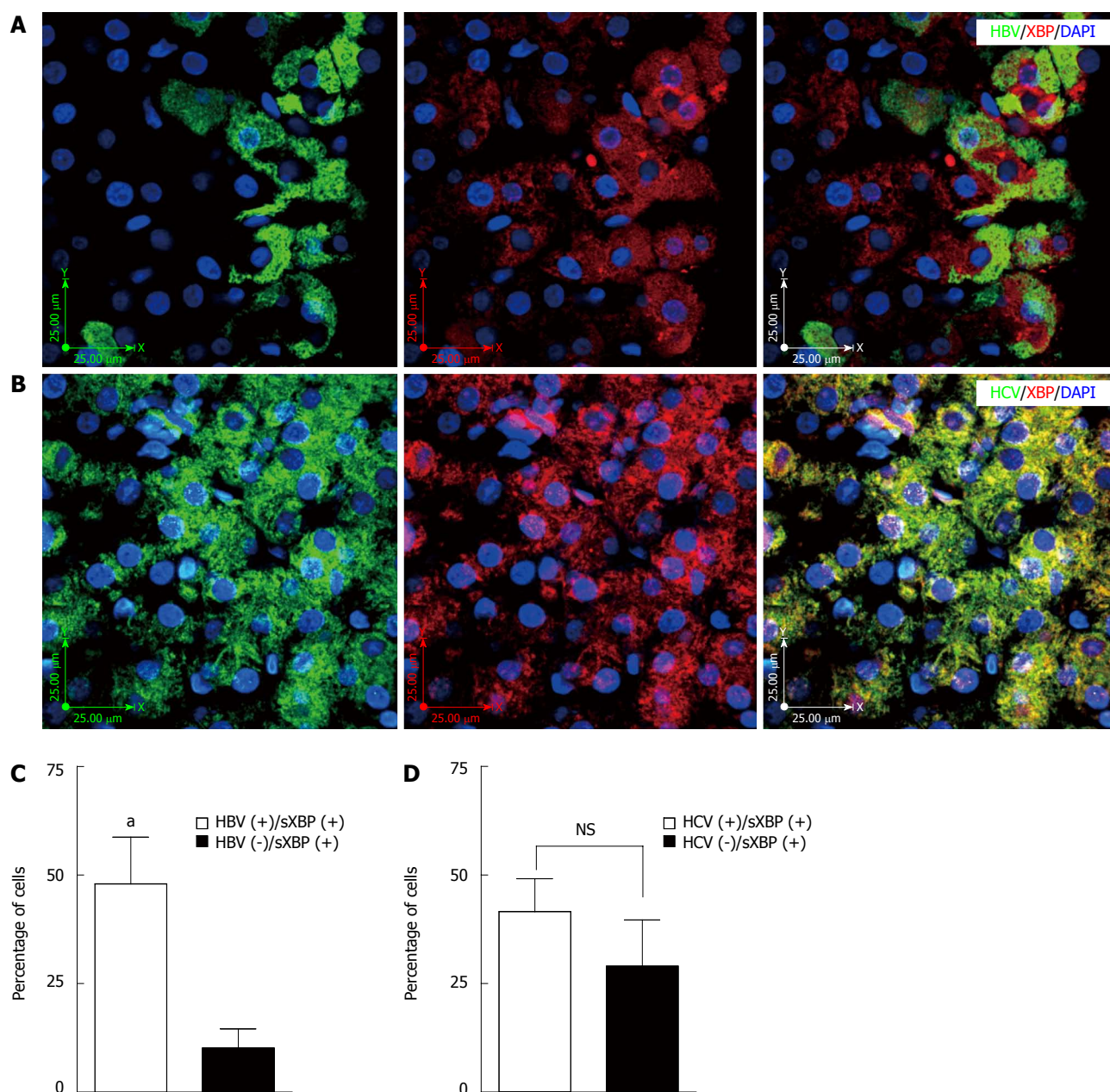


Figure 6 Hepatitis B virus infection induces XBP splicing in the infected liver tissue while hepatitis C virus infection does not induce XBP splicing. A: Fluorescence immunohistochemistry (IHC) confirms co-localization of hepatitis B virus (HBV) and spliced XBP (sXBP) (spliced XBP localized in the nucleus) in the HBV-infected liver tissue; the image is representative of fluorescence IHC for all patients; B: Fluorescence IHC shows that co-localization of hepatitis C virus (HCV) and spliced XBP (sXBP) (spliced XBP localized in the nucleus) is not a dominant event in the HCV-infected liver tissue; the image is representative of fluorescence IHC for all patients; C: HBV infection significantly ($P < 0.001$ vs HBV) induces XBP splicing (spliced XBP localized in the nucleus) in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event [HBV infection and spliced XBP (sXBP) (spliced XBP localized in the nucleus)] in at least 50 cells counted in four different views of fluorescence IHC in each patient's sample; D: HCV infection does not significantly ($P > 0.05$) induce XBP splicing in the infected hepatocytes compared to non-infected adjacent cells. The graph shows results of the event [HCV infection and spliced XBP (sXBP) (spliced XBP localized in the nucleus)] in at least 50 cells counted in four different views of fluorescence IHC in each patient's sample.

receptors^[70], being able to trigger autophagy^[71]. Nevertheless, their exact roles in autophagy induction during HCV infection remains poorly understood.

Some recent studies investigated the interaction between HBV chronic infection and autophagy^[72,73]. Autophagy is involved in different stages of HBV development and infection; however, its exact impacts are not fully known. Presumably, autophagy can either increase the replication of HBV DNA or contribute

to HBV envelopment^[22,74]. Results from electron microscopy, confocal microscopy, and biochemical assays have demonstrated that HBV can enhance autophagy during infection in cell cultures and mouse liver^[72]. There is compelling evidence that HBV acts on the early step of phagosome formation and that the degradation rate of autophagic protein is not increased while HBV triggers the formation of early phagosomes^[74,75]. Different HBV proteins are involved

in the autophagy process. HBx not only is involved in apoptosis induction^[19] but also in autophagy process in the course of HBV infection^[73]. HBV usurps cellular activities such as autophagy and proliferation in favor of virus replication^[22]. Nevertheless, the involved mechanisms for the induction of autophagy and the step of HBV replication affected by autophagy are not completely understood and open to interpretation^[72,74].

The UPR has recently been identified as a novel mechanism involved in a wide range of human diseases including viral infections^[76,77]. In fact, it has been revealed that a number of viruses utilize UPR to help attenuate anti-viral responses and establishment of infection^[78-82]. HCV uses the membranous compartment of the ER as the biogenesis site for its envelope protein and particle assembly^[83]. Therefore, there is general agreement on the induction of ER stress by HCV infection which can interfere with the ER function in host cells and upon sensing ER stress, cells activate the UPR signaling pathway^[18]. Moreover, there are some well documented data that HCV induces ER stress and UPR in both *in vitro* and *in vivo* experimental models^[27,84-86]. Virus-induced UPR is demonstrated to trigger apoptosis of the infected hepatocytes and also overwhelming evidence indicates the crucial role of UPR in the HCV replication^[68,84,85] and life cycle^[27,85-92]. HCV infection apparently activates all three UPR sensors^[27,60,84,93]. It was observed in a cell culture system that HCV-induced UPR plays a positive role in RNA replication and efficient propagation of HCV as HCV replication was suppressed when one of three UPR pathways was significantly abrogated^[68]. A study by Zheng *et al.*^[92] showed that HCV NS4B can activate UPR by induction of XBP1 mRNA splicing and ATF6 cleavage. Our finding shows that there is no significant difference in XBP splicing between HCV-infected and non-infected cells which does not correlate with previous reports that highlighted the role of XBP splicing in HCV infection in hepatocytes. We assume that this difference could be the result of the difference in genetic background of the population study or the lack of enough samples in our study.

Different studies have shown that HBx protein of HBV can trigger UPR. Meanwhile, a major drawback that has prevented us from broadening our in-depth knowledge in a natural infection system has been the lack of a strong and efficient *in vitro* infectivity model^[94]. Cho *et al.*^[24] observed that HBx downregulates the cellular ATP level and mitochondrial membrane potential; then ATP reduction induces ER stress. In addition, ATF4, which is a UPR marker, was demonstrated to up-regulate COX2 expression. This kind of UPR induction by HBx might trigger the development of HCC as well as liver inflammation^[24]. Moreover, HBx and S proteins both can activate the IRE1/XBP1 branch of the UPR. Li *et al.*^[23] demonstrated that the transiently-expressed HBx protein in Hep3B and HepG2 cells caused a high increase (up to 7-fold) in XBP1 promoter activity and also ATF6 cleavage in a dose-dependent manner. This

mechanism of HBx in inducing UPR was determined as a potential mechanism, contributing to the replication of HBV in liver cells^[23]. Similarly, in another study conducted by Li *et al.*^[74], they over-expressed the S protein in Huh7 hepatoma cells and observed the presence of the XBP1 mRNA (both precursor and spliced forms) which suggests the ability of S protein to trigger UPR by the IRE1/XBP1 pathway. XBP1, as a crucial sensor of UPR pathway, is a key protein in the growth and differentiation of hepatocytes as shown by XBP1 knockout mice^[95]. It is supposed that HBx might act as a kinase activator that increases the phosphorylation level of the ER stress sensor IRE1, thereby activating the IRE1-XBP1 pathway. On the other hand, HBx is able to interact with the bZIP class of transcription factors and both ATF6 and XBP1 belong to this family so that HBx can activate or co-activate them to enhance trans-activating activities^[95]. Furthermore, HBx might be involved in the expression and persistence of HBV since the ATF6 pathway associates with ER chaperone expression and helps the unfolded proteins to refold^[91,96]. It should be noted that studies on the UPR at early stages of HBV infection have not yet been addressed and this may be due to the difficulty in investigating HBV infection^[97].

In conclusion, our studies confirm previous *in vivo* and *in vitro* studies which showed HBV and HCV infection is associated with the induction of apoptosis, autophagy, and UPR in hepatocytes. In the present study, we showed, for the first time, simultaneous induction of apoptosis, autophagy, and UPR after HBV or HCV infection in human liver tissue. Our study highlights the co-incidence of all of these events in the human liver after natural HBV or HCV infection. As apoptosis, autophagy, and UPR are linked together *via* different regulatory proteins, it will be very important to address which events (apoptosis, autophagy, or UPR) are induced first after HBV or HCV induction. This understanding would be beneficial to design new strategies to control these pathways after HBV or HCV infection to ameliorate the process of liver injury after viral infection. As our study has been done in human samples, these results have great potential impact in HBV- and HCV-infection-initiated liver diseases. Further long term and follow up studies are in process to highlight the effect of these events in the progression of liver diseases.

ACKNOWLEDGMENTS

This study is a complementary and Side Project of the PhD thesis of Dr. Payam Peymani (Thesis Number: 92-6907).

COMMENTS

Background

Hepatitis caused by hepatitis B virus (HBV) and hepatitis C virus (HCV) infection has emerged as a major public health problem throughout the world.

Dysregulation of apoptosis, autophagy and the unfolded protein response (UPR) has been implicated in a wide spectrum of human diseases including viral infection. These pathways are tightly interconnected and their crosstalk is a key factor for cell-fate determination in response to different stimuli. However, their interplay during HBV and HCV infection remains unclear. In this study the authors investigated induction of autophagy, UPR, and apoptosis in HBV- and HCV-infected human liver tissues to examine the importance of these pathways in HBV- and HCV-induced liver damage. In addition, these studies will pave the way for the development and application of therapeutics that modulate these pathways to affect HBV and HCV replication and the progress of liver damage in patients.

Research frontiers

The authors investigate the importance of apoptosis, autophagy, and UPR in health and diseases. They modulate these pathways to provide new approaches in treatment of different diseases.

Innovations and breakthroughs

The current research showed the co-incidence of apoptosis, autophagy, and UPR in HBV and HCV infected hepatocytes.

Applications

The next step of this research would be to target the modulation of apoptosis, autophagy, UPR in the hepatocytes of HBV/HCV infected patients to prevent the progress of liver damage in these patients.

Peer-review

This is an interesting manuscript about cell fate in hepatocytes after hepatitis B and C virus infection. Also, the manuscript was clearly written and organized.

REFERENCES

- 1 El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007; **132**: 2557-2576 [PMID: 17570226 DOI: 10.1053/j.gastro.2007.04.061]
- 2 Beasley RP, Hwang LY, Lin CC, Chien CS. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22 707 men in Taiwan. *Lancet* 1981; **2**: 1129-1133 [PMID: 6118576]
- 3 Arzumanyan A, Reis HM, Feitelson MA. Pathogenic mechanisms in HBV- and HCV-associated hepatocellular carcinoma. *Nat Rev Cancer* 2013; **13**: 123-135 [PMID: 23344543 DOI: 10.1038/nrc3449]
- 4 Nguyen VT, Law MG, Dore GJ. Hepatitis B-related hepatocellular carcinoma: epidemiological characteristics and disease burden. *J Viral Hepat* 2009; **16**: 453-463 [PMID: 19302335 DOI: 10.1111/j.1365-2893.2009.01117.x]
- 5 Ghavami S, Cunningham RH, Gupta S, Yeganeh B, Filomeno KL, Freed DH, Chen S, Klonisch T, Halayko AJ, Ambrose E, Singal R, Dixon IM. Autophagy is a regulator of TGF- β 1-induced fibrogenesis in primary human atrial myofibroblasts. *Cell Death Dis* 2015; **6**: e1696 [PMID: 25789971 DOI: 10.1038/cddis.2015.36]
- 6 Marzban H, Del Bigio MR, Alizadeh J, Ghavami S, Zachariah RM, Rastegar M. Cellular commitment in the developing cerebellum. *Front Cell Neurosci* 2014; **8**: 450 [PMID: 25628535 DOI: 10.3389/fncel.2014.00450]
- 7 Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* 2004; **119**: 753-766 [PMID: 15607973 DOI: 10.1016/j.cell.2004.11.038]
- 8 Wasik AM, Grabarek J, Pantovic A, Cieřlar-Pobuda A, Asgari HR, Bundgaard-Nielsen C, Rafat M, Dixon IM, Ghavami S, Los MJ. Reprogramming and carcinogenesis--parallels and distinctions. *Int Rev Cell Mol Biol* 2014; **308**: 167-203 [PMID: 24411172 DOI: 10.1016/B978-0-12-800097-7.00005-1]
- 9 Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, Yokoyama M, Mishima K, Saito I, Okano H, Mizushima N. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* 2006; **441**: 885-889 [PMID: 16625204 DOI: 10.1038/nature04724]
- 10 Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, Ueno T, Koike M, Uchiyama Y, Kominami E, Tanaka K. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 2006; **441**: 880-884 [PMID: 16625205 DOI: 10.1038/nature04723]
- 11 Tanida I, Fukasawa M, Ueno T, Kominami E, Wakita T, Hanada K. Knockdown of autophagy-related gene decreases the production of infectious hepatitis C virus particles. *Autophagy* 2009; **5**: 937-945 [PMID: 19625776]
- 12 Alavian SM, Ande SR, Coombs KM, Yeganeh B, Davoodpour P, Hashemi M, Los M, Ghavami S. Virus-triggered autophagy in viral hepatitis - possible novel strategies for drug development. *J Viral Hepat* 2011; **18**: 821-830 [PMID: 22093031 DOI: 10.1111/j.1365-2893.2011.01530.x]
- 13 Ghavami S, Yeganeh B, Stelmack GL, Kashani HH, Sharma P, Cunningham R, Rattan S, Bathe K, Klonisch T, Dixon IM, Freed DH, Halayko AJ. Apoptosis, autophagy and ER stress in mevalonate cascade inhibition-induced cell death of human atrial fibroblasts. *Cell Death Dis* 2012; **3**: e330 [PMID: 22717585 DOI: 10.1038/cddis.2012.61]
- 14 Ghavami S, Cunningham RH, Yeganeh B, Davies JJ, Rattan SG, Bathe K, Kavosh M, Los MJ, Freed DH, Klonisch T, Pierce GN, Halayko AJ, Dixon IM. Autophagy regulates trans fatty acid-mediated apoptosis in primary cardiac myofibroblasts. *Biochim Biophys Acta* 2012; **1823**: 2274-2286 [PMID: 23026405 DOI: 10.1016/j.bbamcr.2012.09.008]
- 15 Qu X, Zou Z, Sun Q, Luby-Phelps K, Cheng P, Hogan RN, Gilpin C, Levine B. Autophagy gene-dependent clearance of apoptotic cells during embryonic development. *Cell* 2007; **128**: 931-946 [PMID: 17350577 DOI: 10.1016/j.cell.2006.12.044]
- 16 Malhi H, Kaufman RJ. Endoplasmic reticulum stress in liver disease. *J Hepatol* 2011; **54**: 795-809 [PMID: 21145844 DOI: 10.1016/j.jhep.2010.11.005]
- 17 Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, Murakami T, Taniguchi M, Tanii I, Yoshinaga K, Shiosaka S, Hammarback JA, Urano F, Imaizumi K. Autophagy is activated for cell survival after endoplasmic reticulum stress. *Mol Cell Biol* 2006; **26**: 9220-9231 [PMID: 17030611 DOI: 10.1128/MCB.01453-06]
- 18 Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* 2007; **8**: 519-529 [PMID: 17565364 DOI: 10.1038/nrm2199]
- 19 Chami M, Ferrari D, Nicotera P, Paterlini-Br  chet P, Rizzuto R. Caspase-dependent alterations of Ca²⁺ signaling in the induction of apoptosis by hepatitis B virus X protein. *J Biol Chem* 2003; **278**: 31745-31755 [PMID: 12799372 DOI: 10.1074/jbc.M304202200]
- 20 Liang X, Liu Y, Zhang Q, Gao L, Han L, Ma C, Zhang L, Chen YH, Sun W. Hepatitis B virus sensitizes hepatocytes to TRAIL-induced apoptosis through Bax. *J Immunol* 2007; **178**: 503-510 [PMID: 17182590 DOI: 10.4049/jimmunol.178.1.503]
- 21 Sir D, Kuo CF, Tian Y, Liu HM, Huang EJ, Jung JU, Machida K, Ou JH. Replication of hepatitis C virus RNA on autophagosomal membranes. *J Biol Chem* 2012; **287**: 18036-18043 [PMID: 22496373 DOI: 10.1074/jbc.M111.320085]
- 22 Tang SW, Ducroux A, Jeang KT, Neuveut C. Impact of cellular autophagy on viruses: Insights from hepatitis B virus and human retroviruses. *J Biomed Sci* 2012; **19**: 92 [PMID: 23110561 DOI: 10.1186/1423-0127-19-92]
- 23 Li B, Gao B, Ye L, Han X, Wang W, Kong L, Fang X, Zeng Y, Zheng H, Li S, Wu Z, Ye L. Hepatitis B virus X protein (HBx) activates ATF6 and IRE1-XBP1 pathways of unfolded protein response. *Virus Res* 2007; **124**: 44-49 [PMID: 17092596 DOI: 10.1016/j.virusres.2006.09.011]
- 24 Cho HK, Cheong KJ, Kim HY, Cheong J. Endoplasmic reticulum stress induced by hepatitis B virus X protein enhances cyclooxygenase 2 expression via activating transcription factor 4. *Biochem J* 2011; **435**: 431-439 [PMID: 21244365 DOI: 10.1042/

- BJ20102071]
- 25 **Honda M**, Kaneko S, Shimazaki T, Matsushita E, Kobayashi K, Ping LH, Zhang HC, Lemon SM. Hepatitis C virus core protein induces apoptosis and impairs cell-cycle regulation in stably transformed Chinese hamster ovary cells. *Hepatology* 2000; **31**: 1351-1359 [PMID: 10827163]
 - 26 **Mack HI**, Munger K. Modulation of autophagy-like processes by tumor viruses. *Cells* 2012; **1**: 204-247 [PMID: 24710474 DOI: 10.3390/cells1030204]
 - 27 **Merquiol E**, Uzi D, Mueller T, Goldenberg D, Nahmias Y, Xavier RJ, Tirosh B, Shibolet O. HCV causes chronic endoplasmic reticulum stress leading to adaptation and interference with the unfolded protein response. *PLoS One* 2011; **6**: e24660 [PMID: 21949742 DOI: 10.1371/journal.pone.0024660]
 - 28 **Yeganeh B**. Characterization of lung adenocarcinoma in transgenic mice overexpressing calreticulin under control of the Tie-2 promoter 2010, PhD Thesis, University of Manitoba. Available from: URL: <http://hdl.handle.net/1993/4239>
 - 29 **Ghavami S**, Sharma P, Yeganeh B, Ojo OO, Jha A, Mutawe MM, Kashani HH, Los MJ, Klonisch T, Unruh H, Halayko AJ. Airway mesenchymal cell death by mevalonate cascade inhibition: integration of autophagy, unfolded protein response and apoptosis focusing on Bcl2 family proteins. *Biochim Biophys Acta* 2014; **1843**: 1259-1271 [PMID: 24637330 DOI: 10.1016/j.bbamer.2014.03.006]
 - 30 **Jangamreddy JR**, Ghavami S, Grabarek J, Kratz G, Wiechec E, Fredriksson BA, Rao Pariti RK, Cieřlar-Pobuda A, Panigrahi S, Los MJ. Salinomycin induces activation of autophagy, mitophagy and affects mitochondrial polarity: differences between primary and cancer cells. *Biochim Biophys Acta* 2013; **1833**: 2057-2069 [PMID: 23639289 DOI: 10.1016/j.bbamer.2013.04.011]
 - 31 **Ghavami S**, Mutawe MM, Schaafsma D, Yeganeh B, Unruh H, Klonisch T, Halayko AJ. Geranylgeranyl transferase 1 modulates autophagy and apoptosis in human airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2012; **302**: L420-L428 [PMID: 22160308 DOI: 10.1152/ajplung.00312.2011]
 - 32 **Ghavami S**, Hashemi M, Ande SR, Yeganeh B, Xiao W, Eshraghi M, Bus CJ, Kadkhoda K, Wiechec E, Halayko AJ, Los M. Apoptosis and cancer: mutations within caspase genes. *J Med Genet* 2009; **46**: 497-510 [PMID: 19505876 DOI: 10.1136/jmg.2009.066944]
 - 33 **Chaabane W**, Cieřlar-Pobuda A, El-Gazzah M, Jain MV, Rzeszowska-Wolny J, Rafat M, Stetefeld J, Ghavami S, Los MJ. Human-gyrovirus-Apoptin triggers mitochondrial death pathway-Nur77 is required for apoptosis triggering. *Neoplasia* 2014; **16**: 679-693 [PMID: 25246270 DOI: 10.1016/j.neo.2014.08.001]
 - 34 **Chevet E**, Hetz C, Samali A. Endoplasmic reticulum stress-activated cell reprogramming in oncogenesis. *Cancer Discov* 2015; **5**: 586-597 [PMID: 25977222 DOI: 10.1158/2159-8290.CD-14-1490]
 - 35 **Villano SA**, Vlahov D, Nelson KE, Cohn S, Thomas DL. Persistence of viremia and the importance of long-term follow-up after acute hepatitis C infection. *Hepatology* 1999; **29**: 908-914 [PMID: 10051497 DOI: 10.1002/hep.510290311]
 - 36 **Schuchmann M**, Galle PR. Apoptosis in liver disease. *Eur J Gastroenterol Hepatol* 2001; **13**: 785-790 [PMID: 11474307 DOI: 10.1097/00042737-200107000-00005]
 - 37 **Zekri AR**, Bahnassy AA, Hafez MM, Hassan ZK, Kamel M, Loutfy SA, Sherif GM, El-Zayadi AR, Daoud SS. Characterization of chronic HCV infection-induced apoptosis. *Comp Hepatol* 2011; **10**: 4 [PMID: 21781333 DOI: 10.1186/1476-5926-10-4]
 - 38 **Mankouri J**, Dallas ML, Hughes ME, Griffin SD, Macdonald A, Peers C, Harris M. Suppression of a pro-apoptotic K⁺ channel as a mechanism for hepatitis C virus persistence. *Proc Natl Acad Sci USA* 2009; **106**: 15903-15908 [PMID: 19717445 DOI: 10.1073/pnas.0906798106]
 - 39 **Otsuka M**, Kato N, Taniguchi H, Yoshida H, Goto T, Shiratori Y, Omata M. Hepatitis C virus core protein inhibits apoptosis via enhanced Bcl-xL expression. *Virology* 2002; **296**: 84-93 [PMID: 12036320 DOI: 10.1006/viro.2002.1371]
 - 40 **Bantel H**, Schulze-Osthoff K. Apoptosis in hepatitis C virus infection. *Cell Death Differ* 2003; **10** Suppl 1: S48-S58 [PMID: 12655346 DOI: 10.1038/sj.cdd.4401119]
 - 41 **Cougot D**, Neuveut C, Buendia MA. HBV induced carcinogenesis. *J Clin Virol* 2005; **34** Suppl 1: S75-S78 [PMID: 16461228 DOI: 10.1016/S1386-6532(05)80014-9]
 - 42 **Waghay A**, Murali AR, Menon KN. Hepatocellular carcinoma: From diagnosis to treatment. *World J Hepatol* 2015; **7**: 1020-1029 [PMID: 26052391 DOI: 10.4254/wjh.v7.i8.1020]
 - 43 **Chan HLY**, Sung JY. Hepatocellular carcinoma and hepatitis B virus. Proceedings of the Seminars in liver disease. New York: Thieme Medical Publishers, 2006: 153-161
 - 44 **Fung J**, Lai CL, Yuen MF. Hepatitis B and C virus-related carcinogenesis. *Clin Microbiol Infect* 2009; **15**: 964-970 [PMID: 19874379 DOI: 10.1111/j.1469-0691.2009.03035.x]
 - 45 **Kanda T**, Yokosuka O, Imazeki F, Yamada Y, Imamura T, Fukai K, Nagao K, Saisho H. Hepatitis B virus X protein (HBx)-induced apoptosis in HuH-7 cells: influence of HBV genotype and basal core promoter mutations. *Scand J Gastroenterol* 2004; **39**: 478-485 [PMID: 15180187 DOI: 10.1080/00365520310008719]
 - 46 **Chen HS**, Kaneko S, Girones R, Anderson RW, Hornbuckle WE, Tennant BC, Cote PJ, Gerin JL, Purcell RH, Miller RH. The woodchuck hepatitis virus X gene is important for establishment of virus infection in woodchucks. *J Virol* 1993; **67**: 1218-1226 [PMID: 8437213]
 - 47 **Su F**, Schneider RJ. Hepatitis B virus HBx protein sensitizes cells to apoptotic killing by tumor necrosis factor alpha. *Proc Natl Acad Sci USA* 1997; **94**: 8744-8749 [PMID: 9238048 DOI: 10.1073/pnas.94.16.8744]
 - 48 **Chirillo P**, Pagano S, Natoli G, Puri PL, Burgio VL, Balsano C, Levvero M. The hepatitis B virus X gene induces p53-mediated programmed cell death. *Proc Natl Acad Sci USA* 1997; **94**: 8162-8167 [PMID: 9223332 DOI: 10.1073/pnas.94.15.8162]
 - 49 **Kim HJ**, Kim SY, Kim J, Lee H, Choi M, Kim JK, Ahn JK. Hepatitis B virus X protein induces apoptosis by enhancing translocation of Bax to mitochondria. *IUBMB Life* 2008; **60**: 473-480 [PMID: 18481805 DOI: 10.1002/iub.68]
 - 50 **Lee YI**, Hwang JM, Im JH, Lee YI, Kim NS, Kim DG, Yu DY, Moon HB, Park SK. Human hepatitis B virus-X protein alters mitochondrial function and physiology in human liver cells. *J Biol Chem* 2004; **279**: 15460-15471 [PMID: 14724286 DOI: 10.1074/jbc.M309280200]
 - 51 **Terradillos O**, Pollicino T, Lecoer H, Tripodi M, Gougeon ML, Tiollais P, Buendia MA. p53-independent apoptotic effects of the hepatitis B virus HBx protein in vivo and in vitro. *Oncogene* 1998; **17**: 2115-2123 [PMID: 9798683 DOI: 10.1038/sj.onc.1202432]
 - 52 **Kim KH**, Seong BL. Pro-apoptotic function of HBV X protein is mediated by interaction with c-FLIP and enhancement of death-inducing signal. *EMBO J* 2003; **22**: 2104-2116 [PMID: 12727877 DOI: 10.1093/emboj/cdg210]
 - 53 **Wang WH**, Grégori G, Hullinger RL, Andrisani OM. Sustained activation of p38 mitogen-activated protein kinase and c-Jun N-terminal kinase pathways by hepatitis B virus X protein mediates apoptosis via induction of Fas/FasL and tumor necrosis factor (TNF) receptor 1/TNF-alpha expression. *Mol Cell Biol* 2004; **24**: 10352-10365 [PMID: 15542843 DOI: 10.1128/MCB.24.23.10352-10365.2004]
 - 54 **Marusawa H**, Matsuzawa S, Welsh K, Zou H, Armstrong R, Tamm I, Reed JC. HBxIP functions as a cofactor of survivin in apoptosis suppression. *EMBO J* 2003; **22**: 2729-2740 [PMID: 12773388 DOI: 10.1093/emboj/cdg263]
 - 55 **Elmore LW**, Hancock AR, Chang SF, Wang XW, Chang S, Callahan CP, Geller DA, Will H, Harris CC. Hepatitis B virus X protein and p53 tumor suppressor interactions in the modulation of apoptosis. *Proc Natl Acad Sci USA* 1997; **94**: 14707-14712 [PMID: 9405677 DOI: 10.1073/pnas.94.26.14707]
 - 56 **Clippinger AJ**, Gearhart TL, Bouchard MJ. Hepatitis B virus X protein modulates apoptosis in primary rat hepatocytes by regulating both NF-kappaB and the mitochondrial permeability transition pore. *J Virol* 2009; **83**: 4718-4731 [PMID: 19279112]

- DOI: 10.1128/JVI.02590-08]
- 57 **Diao J**, Khine AA, Sarangi F, Hsu E, Iorio C, Tibbles LA, Woodgett JR, Penninger J, Richardson CD. X protein of hepatitis B virus inhibits Fas-mediated apoptosis and is associated with up-regulation of the SAPK/JNK pathway. *J Biol Chem* 2001; **276**: 8328-8340 [PMID: 11099494 DOI: 10.1074/jbc.M006026200]
 - 58 **Yun C**, Um HR, Jin YH, Wang JH, Lee MO, Park S, Lee JH, Cho H. NF-kappaB activation by hepatitis B virus X (HBx) protein shifts the cellular fate toward survival. *Cancer Lett* 2002; **184**: 97-104 [PMID: 12104053 DOI: 10.1016/S0304-3835(02)00187-8]
 - 59 **Degenhardt K**, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gélinas C, Fan Y, Nelson DA, Jin S, White E. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell* 2006; **10**: 51-64 [PMID: 16843265 DOI: 10.1016/j.ccr.2006.06.001]
 - 60 **Ke PY**, Chen SS. Autophagy: a novel guardian of HCV against innate immune response. *Autophagy* 2011; **7**: 533-535 [PMID: 21242722 DOI: 10.4161/auto.7.5.14732]
 - 61 **Mizushima N**, Yoshimori T, Ohsumi Y. The role of Atg proteins in autophagosome formation. *Annu Rev Cell Dev Biol* 2011; **27**: 107-132 [PMID: 21801009 DOI: 10.1146/annurev-cellbio-092910-154005]
 - 62 **Lee HK**, Iwasaki A. Autophagy and antiviral immunity. *Curr Opin Immunol* 2008; **20**: 23-29 [PMID: 18262399 DOI: 10.1016/j.coi.2008.01.001]
 - 63 **Lin LT**, Dawson PW, Richardson CD. Viral interactions with macroautophagy: a double-edged sword. *Virology* 2010; **402**: 1-10 [PMID: 20413139 DOI: 10.1016/j.virol.2010.03.026]
 - 64 **Kudchodkar SB**, Levine B. Viruses and autophagy. *Rev Med Virol* 2009; **19**: 359-378 [PMID: 19750559 DOI: 10.1002/rmv.630]
 - 65 **Vescovo T**, Refolo G, Romagnoli A, Ciccossanti F, Corazzari M, Alonzi T, Fimia GM. Autophagy in HCV infection: keeping fat and inflammation at bay. *Biomed Res Int* 2014; **2014**: 265353 [PMID: 25162004 DOI: 10.1155/2014/265353]
 - 66 **Chiramel AI**, Brady NR, Bartenschlager R. Divergent roles of autophagy in virus infection. *Cells* 2013; **2**: 83-104 [PMID: 24709646 DOI: 10.3390/cells2010083]
 - 67 **Dreux M**, Chisari FV. Impact of the autophagy machinery on hepatitis C virus infection. *Viruses* 2011; **3**: 1342-1357 [PMID: 21994783 DOI: 10.3390/v3081342]
 - 68 **Sir D**, Chen WL, Choi J, Wakita T, Yen TS, Ou JH. Induction of incomplete autophagic response by hepatitis C virus via the unfolded protein response. *Hepatology* 2008; **48**: 1054-1061 [PMID: 18688877 DOI: 10.1002/hep.22464]
 - 69 **Dreux M**, Gastaminza P, Wieland SF, Chisari FV. The autophagy machinery is required to initiate hepatitis C virus replication. *Proc Natl Acad Sci USA* 2009; **106**: 14046-14051 [PMID: 19666601 DOI: 10.1073/pnas.0907344106]
 - 70 **Imran M**, Waheed Y, Manzoor S, Bilal M, Ashraf W, Ali M, Ashraf M. Interaction of Hepatitis C virus proteins with pattern recognition receptors. *Virol J* 2012; **9**: 126 [PMID: 22726246 DOI: 10.1186/1743-422X-9-126]
 - 71 **Deretic V**, Saitoh T, Akira S. Autophagy in infection, inflammation and immunity. *Nat Rev Immunol* 2013; **13**: 722-737 [PMID: 24064518 DOI: 10.1038/nri3532]
 - 72 **Sir D**, Tian Y, Chen WL, Ann DK, Yen TS, Ou JH. The early autophagic pathway is activated by hepatitis B virus and required for viral DNA replication. *Proc Natl Acad Sci USA* 2010; **107**: 4383-4388 [PMID: 20142477 DOI: 10.1073/pnas.0911373107]
 - 73 **Tang H**, Da L, Mao Y, Li Y, Li D, Xu Z, Li F, Wang Y, Tiollais P, Li T, Zhao M. Hepatitis B virus X protein sensitizes cells to starvation-induced autophagy via up-regulation of beclin 1 expression. *Hepatology* 2009; **49**: 60-71 [PMID: 19065679 DOI: 10.1002/hep.22581]
 - 74 **Li J**, Liu Y, Wang Z, Liu K, Wang Y, Liu J, Ding H, Yuan Z. Subversion of cellular autophagy machinery by hepatitis B virus for viral envelopment. *J Virol* 2011; **85**: 6319-6333 [PMID: 21507968]
 - 75 **Tian Y**, Sir D, Kuo CF, Ann DK, Ou JH. Autophagy required for hepatitis B virus replication in transgenic mice. *J Virol* 2011; **85**: 13453-13456 [PMID: 21957292]
 - 76 **Favreau DJ**, Desforjes M, St-Jean JR, Talbot PJ. A human coronavirus OC43 variant harboring persistence-associated mutations in the S glycoprotein differentially induces the unfolded protein response in human neurons as compared to wild-type virus. *Virology* 2009; **395**: 255-267 [PMID: 19846189 DOI: 10.1016/j.virol.2009.09.026]
 - 77 **Wang S**, Kaufman RJ. The impact of the unfolded protein response on human disease. *J Cell Biol* 2012; **197**: 857-867 [PMID: 22733998 DOI: 10.1083/jcb.201110131]
 - 78 **Galindo I**, Hernández B, Muñoz-Moreno R, Cuesta-Geijo MA, Dalmau-Mena I, Alonso C. The ATF6 branch of unfolded protein response and apoptosis are activated to promote African swine fever virus infection. *Cell Death Dis* 2012; **3**: e341 [PMID: 22764100 DOI: 10.1038/cddis.2012.81]
 - 79 **Jheng JR**, Lau KS, Tang WF, Wu MS, Horng JT. Endoplasmic reticulum stress is induced and modulated by enterovirus 71. *Cell Microbiol* 2010; **12**: 796-813 [PMID: 20070307 DOI: 10.1111/j.1462-5822.2010.01434.x]
 - 80 **Qian Z**, Xuan B, Chapa TJ, Gualberto N, Yu D. Murine cytomegalovirus targets transcription factor ATF4 to exploit the unfolded-protein response. *J Virol* 2012; **86**: 6712-6723 [PMID: 22496230 DOI: 10.1128/jvi.00200-12]
 - 81 **Rathore AP**, Ng ML, Vasudevan SG. Differential unfolded protein response during Chikungunya and Sindbis virus infection: CHIKV nsP4 suppresses eIF2α phosphorylation. *Virol J* 2013; **10**: 36 [PMID: 23356742]
 - 82 **Stahl S**, Burkhardt JM, Hinte F, Tirosh B, Mohr H, Zahedi RP, Sickmann A, Ruzsics Z, Budt M, Brune W. Cytomegalovirus downregulates IRE1 to repress the unfolded protein response. *PLoS Pathog* 2013; **9**: e1003544 [PMID: 23950715 DOI: 10.1371/journal.ppat.1003544]
 - 83 **Jones DM**, McLauchlan J. Hepatitis C virus: assembly and release of virus particles. *J Biol Chem* 2010; **285**: 22733-22739 [PMID: 20457608 DOI: 10.1074/jbc.R110.133017]
 - 84 **Ke PY**, Chen SS. Activation of the unfolded protein response and autophagy after hepatitis C virus infection suppresses innate antiviral immunity in vitro. *J Clin Invest* 2011; **121**: 37-56 [PMID: 21135505 DOI: 10.1172/JCI41474]
 - 85 **Joyce MA**, Walters KA, Lamb SE, Yeh MM, Zhu LF, Kneteman N, Doyle JS, Katze MG, Tyrrell DL. HCV induces oxidative and ER stress, and sensitizes infected cells to apoptosis in SCID/Alb-uPA mice. *PLoS Pathog* 2009; **5**: e1000291 [PMID: 19242562 DOI: 10.1371/journal.ppat.1000291]
 - 86 **Tardif KD**, Mori K, Siddiqui A. Hepatitis C virus subgenomic replicons induce endoplasmic reticulum stress activating an intracellular signaling pathway. *J Virol* 2002; **76**: 7453-7459 [PMID: 12097557 DOI: 10.1128/JVI.76.15.7453-7459.2002]
 - 87 **Chan SW**, Egan PA. Hepatitis C virus envelope proteins regulate CHOP via induction of the unfolded protein response. *FASEB J* 2005; **19**: 1510-1512 [PMID: 16006626 DOI: 10.1096/fj.04-3455fje]
 - 88 **Ciccaglione AR**, Costantino A, Tritarelli E, Marcantonio C, Equestre M, Marziliano N, Rapicetta M. Activation of endoplasmic reticulum stress response by hepatitis C virus proteins. *Arch Virol* 2005; **150**: 1339-1356 [PMID: 15770357 DOI: 10.1007/s00705-004-0487-4]
 - 89 **Ciccaglione AR**, Marcantonio C, Tritarelli E, Equestre M, Vendittelli F, Costantino A, Geraci A, Rapicetta M. Activation of the ER stress gene gadd153 by hepatitis C virus sensitizes cells to oxidant injury. *Virus Res* 2007; **126**: 128-138 [PMID: 17368854 DOI: 10.1016/j.virusres.2007.02.006]
 - 90 **Top D**, Barry C, Racine T, Ellis CL, Duncan R. Enhanced fusion pore expansion mediated by the trans-acting Endodomain of the reovirus FAST proteins. *PLoS Pathog* 2009; **5**: e1000331 [PMID: 19266079 DOI: 10.1371/journal.ppat.1000331]
 - 91 **Tardif KD**, Mori K, Kaufman RJ, Siddiqui A. Hepatitis C virus suppresses the IRE1-XBP1 pathway of the unfolded protein response. *J Biol Chem* 2004; **279**: 17158-17164 [PMID: 14960590 DOI: 10.1074/jbc.M312144200]
 - 92 **Zheng Y**, Gao B, Ye L, Kong L, Jing W, Yang X, Wu Z, Ye L.

- Hepatitis C virus non-structural protein NS4B can modulate an unfolded protein response. *J Microbiol* 2005; **43**: 529-536 [PMID: 16410770]
- 93 **Kroemer G**, Mariño G, Levine B. Autophagy and the integrated stress response. *Mol Cell* 2010; **40**: 280-293 [PMID: 20965422 DOI: 10.1016/j.molcel.2010.09.023]
- 94 **Gripon P**, Rumin S, Urban S, Le Seyec J, Glaise D, Canine I, Guyomard C, Lucas J, Trepo C, Gugen-Guillouzo C. Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci USA* 2002; **99**: 15655-15660 [PMID: 12432097 DOI: 10.1073/pnas.232137699]
- 95 **Reimold AM**, Etkin A, Clauss I, Perkins A, Friend DS, Zhang J, Horton HF, Scott A, Orkin SH, Byrne MC, Grusby MJ, Glimcher LH. An essential role in liver development for transcription factor XBP-1. *Genes Dev* 2000; **14**: 152-157 [PMID: 10652269]
- 96 **Isler JA**, Skalet AH, Alwine JC. Human cytomegalovirus infection activates and regulates the unfolded protein response. *J Virol* 2005; **79**: 6890-6899 [PMID: 15890928 DOI: 10.1128/JVI.79.11.6890-6899.2005]
- 97 **Lazar C**, Uta M, Branza-Nichita N. Modulation of the unfolded protein response by the human hepatitis B virus. *Front Microbiol* 2014; **5**: 433 [PMID: 25191311 DOI: 10.3389/fmicb.2014.00433]

P- Reviewer: Kanda T **S- Editor:** Gong ZM **L- Editor:** A
E- Editor: Zhang DN



Basic Study

Histidine decarboxylase and urinary methylimidazoleacetic acid in gastric neuroendocrine cells and tumours

Apostolos V Tsolakis, Lars Grimelius, Göran Granerus, Mats Stridsberg, Sture E Falkmer, Eva T Janson

Apostolos V Tsolakis, Eva T Janson, Section of Endocrine Oncology, Department of Medical Sciences, Uppsala University, SE-75185 Uppsala, Sweden

Lars Grimelius, Section of Genetics and Pathology, Department of Immunology, Uppsala University, SE-75185 Uppsala, Sweden

Göran Granerus, Department of Medical and Health Sciences, Linköping University, SE-58183 Linköping, Sweden

Mats Stridsberg, Section of Biochemical Endocrinology, Department of Medical Sciences, Uppsala University, SE-75185 Uppsala, Sweden

Sture E Falkmer, Department of Pathology, Ryhov County Hospital, SE-55185 Jönköping, Sweden

Author contributions: Tsolakis AV, Grimelius L, Falkmer SE and Janson ET contributed to conception and design of the study; Tsolakis AV, Grimelius L, Granerus G, Stridsberg M, Falkmer SE and Janson ET contributed to acquisition, analysis and interpretation of data; Tsolakis AV, Grimelius L, Granerus G, Stridsberg M, Falkmer SE and Janson ET wrote and revised manuscript.

Supported by The Selander Foundation and the Foundation for Clinical Cancer Research in Jönköping.

Institutional review board statement: The research protocol was reviewed and approved by the local Research Ethics Board at Uppsala University Hospital.

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

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Apostolos V Tsolakis, MD, PhD, Section of Endocrine Oncology, Department of Medical Sciences, Uppsala University, SE-75185 Uppsala, Sweden. apobtsol@hotmail.com
Telephone: +46-18-6114913
Fax: +46-18-553943

Received: April 8, 2015
Peer-review started: April 9, 2015
First decision: June 19, 2015
Revised: August 27, 2015
Accepted: September 13, 2015
Article in press: September 14, 2015
Published online: December 21, 2015

Abstract

AIM: To study histidine decarboxylase (HDC) expression in normal and neoplastic gastric neuroendocrine cells in relationship to the main histamine metabolite.

METHODS: Control tissues from fundus ($n = 3$) and corpus ($n = 3$) mucosa of six patients undergoing operations for gastric adenocarcinoma, biopsy and/or gastric surgical specimens from 64 patients with primary gastric neuroendocrine tumours (GNETs), as well as metastases from 22 of these patients, were investigated using conventional immunohistochemistry and double immunofluorescence with commercial antibodies vs vesicular monoamine transporter 2 (VMAT-2), HDC and ghrelin. The urinary excretion of the main histamine metabolite methylimidazoleacetic acid (U-MeImAA) was determined using high-performance liquid chromatography in 27 of the 64 patients.

RESULTS: In the gastric mucosa of the control tissues, co-localization studies identified neuroendocrine cells that showed immunoreactivity only to VMAT-2 and others with reactivity only to HDC. A third cell

population co-expressed both antigens. There was no co-expression of HDC and ghrelin. Similar results were obtained in the foci of neuroendocrine cell hyperplasia associated with chronic atrophic gastritis type A and also in the tumours. The relative incidence of the three aforementioned markers varied in the tumours that were examined using conventional immunohistochemistry. All of these GNETs revealed both VMAT-2 and HDC immunoreactivity, and their metastases showed an immunohistochemical pattern and frequency similar to that of their primary tumours. In four patients, increased U-MeImAA excretion was detected, but only two of the patients exhibited related endocrine symptoms.

CONCLUSION: Human enterochromaffin-like cells appear to partially co-express VMAT-2 and HDC. Co-expression of VMAT-2 and HDC might be required for increased histamine production in patients with GNETs.

Key words: Enterochromaffin-like cells; High performance liquid chromatography; Gastric neuroendocrine tumours; Histidine decarboxylase; Immunohistochemistry; Urinary excretion of the main histamine metabolite methylimidazoleacetic acid; Vesicular monoamine transporter 2

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: It is suggested that only a fraction of vesicular monoamine transporter 2 (VMAT-2) immunoreactive neuroendocrine cells in human oxyntic mucosa co-express histidine decarboxylase (HDC), and *vice versa*, suggesting that the enterochromaffin-like (ECL) cells may not represent a homogeneous cell population when examined for HDC immunoreactivity. Co-expression of VMAT-2 and HDC might be important for giving rise to increased histamine production in patients with ECL cell neuroendocrine tumours. Furthermore, an increase of urinary excretion of the main histamine metabolite methylimidazoleacetic acid was not always associated with hormonal symptoms. This result could be attributed to the rate of histamine release. A sudden rapid release will cause a flush, whereas a slower release will not.

Tsolakis AV, Grimelius L, Granerus G, Stridsberg M, Falkmer SE, Janson ET. Histidine decarboxylase and urinary methylimidazoleacetic acid in gastric neuroendocrine cells and tumours. *World J Gastroenterol* 2015; 21(47): 13240-13249 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13240.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13240>

INTRODUCTION

The enterochromaffin-like (ECL) cell is the most abundant neuroendocrine cell type of the gastric oxyntic mucosa and is generally considered to produce, store and secrete histamine^[1]. This biogenic amine is formed by enzymatic decarboxylation of the amino acid histidine by histidine decarboxylase (HDC) and transported into secretory granules by proton-histamine counter transport *via* the vesicular monoamine transporter subtype 2 (VMAT-2)^[2-4].

Recent studies have shown that only some ghrelin immunoreactive (IR) cells in the gastric mucosa express VMAT-2^[5,6]. Thus, VMAT-2 does not seem specific for a homogeneous neuroendocrine cell type. However, VMAT-2 is suggested to be a specific marker for ECL cell neuroendocrine tumours (NETs) and is not expressed in ghrelinomas^[6-12].

At present, histamine cannot be detected immunohistochemically in routine formalin-fixed tissue specimens by any commercially available antibody because its preservation requires a specific fixation procedure^[13]. Because HDC is the specific enzyme for the production of histamine, its presence indicates synthesis of this amine and thus it can be used to visualize histamine-forming cells immunohistochemically^[14].

Two immunohistochemical studies have examined human ECL cell NETs by means of both VMAT-2 and HDC antibodies^[10,15]. In these tumours, some of the neoplastic parenchymal cells were IR to HDC, whereas the transporter had a wider distribution.

The production and release of histamine can be estimated by measuring the urinary excretion of the main and specific histamine metabolite methylimidazoleacetic acid (U-MeImAA)^[16]. Patients with various types of ECL cell NETs occasionally have an increased excretion of U-MeImAA^[17-21]. Some of these patients also suffer from the atypical carcinoid syndrome (ACS)^[17-20].

The purpose of this study was to characterize normal gastric mucosa, foci of neuroendocrine cell hyperplasia associated with ECL cell NETs, and different types of gastric NETs with respect to the occurrence of HDC expression in relation to VMAT-2- and ghrelin-IR cells. Furthermore, the immunohistochemical expression of HDC in gastric NETs was compared to U-MeImAA levels and clinical symptoms.

MATERIALS AND METHODS

Patients and tumours

Biopsy and/or gastric surgical specimens from 64 patients with primary gastric NETs, and metastases from 22 of these patients, were included in this study. Non-neoplastic oxyntic mucosa surrounding the

Table 1 Summary of clinical and tumour characteristics

Tumour type	n	TNM/stage	Age (yr), median (range)	M:F (ratio)	Tumour size (mm)	Localization
Type I ECL cell NETs	37		65 (29-80)	13:24	1-50	Fundus, corpus
	22	T1N0M0/ I				
	12	T2N0M0/ II a				
	2	T2N1M0/ III b				
	1	T2N1M1/IV				
Type II ECL cell NETs	3		48 (47-49)	1:2	0.5-25	Fundus, corpus, antrum
	2	T1N0M0/I				
	1	T2N1M0/ II a				
Type III ECL cell NETs	10		62 (23-77)	4:6	7-55	Fundus, Corpus, antrum
	1	T1N0M0/I				
	3	T2N1M0/ III b				
	2	T2N0M1/IV				
	2	T2N1M1/IV				
	2	T4N1M1/IV				
Non-ECL cell NET	1	T2N1M1/IV	71	0:1	20	Corpus, antrum
Ghrelinomas	2	T2N1M1/IV	55 (47-63)	2:0	35-40	Corpus
NECs	11		58 (39-77)	8:3	30-100	Fundus, corpus
Small cell	4					
	2	T2N0M0/ II a				
	1	T3N1M1/IV				
	1	T4N1M1/IV				
Large cell	7	T4N0M0/ III a				
	1	T2N1M1/IV				
	4	T3N1M1/IV				
	1	T4N1M1/IV				
	1					
Total number	64					

TNM/stage according to Rindi *et al*^[23]. ECL cell NETs: Enterochromaffin-like cell neuroendocrine tumours; NECs: Neuroendocrine carcinomas.

tumours was also included with a view to examine the possible existence of foci of neuroendocrine cell hyperplasia. Based on clinico-pathological criteria, the tumours were classified as type I ($n = 37$), type II ($n = 3$) or type III ($n = 10$) ECL cell NETs, as non-ECL cell NET ($n = 1$), as ghrelinomas ($n = 2$), and as neuroendocrine carcinomas (NECs) ($n = 11$)^[22]. The latter included four small-cell and seven large-cell type NECs. The cases of metastases that were examined included type I ($n = 3$), type II ($n = 1$) and type III ($n = 7$) ECL cell NETs, ghrelinomas ($n = 2$), and NECs ($n = 9$). The tumours were also classified according to the staging system based on TNM (Tables 1 and 2)^[23]. One patient with type II ECL cell NET complained of flushes and another with type III developed ACS.

Control tissue specimens

Control tissues were from the fundus ($n = 3$) and corpus ($n = 3$) mucosa of six patients undergoing operations for gastric adenocarcinoma. All of the specimens examined originated from macro- and microscopically normal gastric mucosa. The surgical specimens were located at least 3 cm from the neoplasm. In the tissue samples obtained from the gastric NETs, adjacent non-neoplastic mucosa was also used as an internal control.

Immunohistochemistry and cell density

All of the tissue samples were conventionally fixed in

10% buffered neutral formalin and routinely processed to paraffin wax. Consecutive sections, approx. 4 μ m thick, were attached to positively charged glass slides (Superfrost® Plus; Menzel Gläser, Braunschweig, Germany).

The primary antibodies used were rabbit polyclonal antibodies vs VMAT-2 (AB1767, Chemicon International, Temecula, CA, United States, 1:400), vs human ghrelin (H-031-30, Phoenix Pharmaceuticals Inc., Belmont, CA, United States, 1:4800) and vs recombinant HDC raised in *E. coli* (B 260-1, Euro-Diagnostica, Malmö, Sweden, diluted 1:5000). The tissues were immunostained using a polymer-detection system (DakoCytomation, EnVision® + System-HRP, K4010 for primary rabbit) with diaminobenzidine as the chromogen. Before immunostaining, the sections were microwave treated for 2 min \times 5 min at 750 W, using Tris buffer saline, pH 8.0, as a retrieval solution.

The density of the IR cells, expressed as the number of IR cells per one mm² of mucosal area, was calculated using a square grid in one of the oculars. In small lesions with a tumour area of < 1 mm², all of the neoplastic parenchymal cells were examined.

Immunofluorescence co-localization studies

For immunofluorescence staining, the following primary antibodies were used: polyclonal rabbit anti-HDC (B 260-1, Euro-Diagnostica, Malmö, diluted 1:100 when the conventional indirect method was used and 1:800

Table 2 Clinical and tumour characteristics in the subgroup of patients undergoing the U-MeImAA assay

Age/Gender	VMAT-2-IR tumour cells (primary)	HDC-IR tumour cells (primary)	HDC-IR tumour cells (metastases)	U-MeImAA (mmol/mol creatinine)	Diameter (mm)	TNM/stage
Type I ECL cell NETs (<i>n</i> = 15)						
47/F	> 90%	0%	-	1.6	2	T1m,N0,M0/I
52/M	> 90%	0%	-	1.6	10	T1m,N0,M0/I
55/F	> 90%	0% ^D	-	1.1	23	T2,N0,M0/ II a
61/F	> 90%	0%	-	1.3	2	T1,N0,M0/I
62/F	> 90%	0%	-	1.4	3	T1m,N0,M0/I
64/M	> 90%	0%	-	1.1	5	T1m,N0,M0/I
65/F	> 90%	0%	-	2.0	25	T2,N0,M0/ II a
72/F	> 90%	0%	-	1.1	5	T1,N0,M0/I
74/F	> 90%	0% ^D	-	1.7	2.2	T1m,N0,M0/I
78/F	> 90%	0%	-	1.8	4	T1m,N0,M0/I
79/F	> 90%	0%	-	2.4	1.5	T1m,N0,M0/I
80/M	> 90%	0%	-	1.5	7	T1m,N0,M0/I
54/F	> 90%	1%	-	1.5	5	T1m,N0,M0/I
71/F	> 90%	1%	-	1.8	15	T2,N0,M0/ II a
65/M	> 90%	3% ^D	-	1.6	12	T2m,N0,M0/ II a
Type II ECL cell NET (<i>n</i> = 1)						
49/F	> 90%	10% ^{D,L}	40%	9.0 ¹	3	T1m,N0,M0/I
Type III ECL cell NETs (<i>n</i> = 6)						
44/F	> 90%	0%	0%	1.6	22	T2,N1,M0/ III b
60/M	> 90%	0%	0%	1.4	11	T2,N1,M0/ III b
60/F	> 90%	0%	-	2.4	7	T1,N0,M0/I
77/F	> 90%	1%	40%	40.8	30	T2,N0,M1/IV
72/M	> 90%	10%	70%	1.1	11	T2,N1,M1/IV
62/M	> 90%	20%	40%	18.2 ²	45	T4,N1,M1/IV
Ghrelinoma (<i>n</i> = 1)						
47/M	0%	0%	0%	1.2	40	T2,N1,M1/IV
NECs (<i>n</i> = 4)						
76/M	0%	0%	0%	1.6	30	T2,N1,M1/IV
69/M	10%	0%	0%	1.8	100	T4,N1,M1/IV
61/F	60%	15%	15%	2.9	100	T4,N1,M1/IV
58/M	0%	60%	60%	1.2	90	T4,N0,M0/ III a

¹Flush; ²Atypical carcinoid syndrome. TNM/Stage according to Rindi *et al*^[23]. Diffuse (D) and Linear (L) pattern of neuroendocrine cell hyperplasia in the adjacent to the tumour mucosa; -: no metastases; U-MeImAA: Urine Methyl Imidazol Acetic Acid, reference range 0.4-2.4 mmol/mol creatinine; Diameter: The largest dimension of the tumour is given; m: Multiple tumours; VMAT-2: Vesicular monoamine transporter-2; HDC: Histidine decarboxylase; IR: Immunoreactive; ECL cell NETs: Enterochromaffin-like cell neuroendocrine tumours; NECs: Neuroendocrine carcinomas.

with the streptavidin), polyclonal goat anti-VMAT-2 (C-20, sc-7721, Santa Cruz Biotechnology®, Santa Cruz, CA, 1:200), and polyclonal chicken anti-ghrelin (a-a 17-28, Y-031-44, Phoenix Pharmaceuticals, Belmont, CA, 1:800).

The sections were microwave treated as described above and then incubated overnight with a cocktail of two primary antibodies at 4 °C. Before application of the antibody cocktail, the sections were incubated with a mixture of non-immune sera from the animal species producing the secondary antibodies, diluted 1:10. The following secondary antisera were used: (1) tetramethyl rhodamine isothiocyanate (TRITC)-conjugated AffiniPure donkey anti-rabbit (711-025-152, Jackson ImmunoResearch Laboratories, 1:100) and biotinylated horse anti-goat (BA-9500, Vector Laboratories, 1:100) for the co-localization studies of HDC and VMAT-2; and (2) biotinylated goat anti-rabbit (BA-1000, Vector Laboratories, 1:100) and fluorescein isothiocyanate (FITC)-conjugated AffiniPure donkey anti-chicken (703-095-155, Jackson ImmunoResearch Laboratories, 1:100) for HDC and

ghrelin. The incubation time for the secondary antisera was 30 min at room temperature. A further incubation was performed for 30 min at room temperature with a streptavidin-conjugated fluorophore. Streptavidin-FITC (SA-5001, Vector Laboratories) was used as a fluorophore for the first combination, and streptavidin-Texas Red (SA-5006, Vector Laboratories) was used for the second combination. Tests for inappropriate binding of the secondary antisera were performed as described previously^[11]. TRITC and Texas Red fluorophores give rise to red fluorescence, and FITC gives rise to green fluorescence.

The sections were examined using a Zeiss Axioplan2 fluorescence microscope and photographed with an AxioCam HRm camera employing Axiovision imaging software and a 63X plan-apochromat objective. In the co-localization studies, the tissue specimens from normal fundus (*n* = 3) and corpus (*n* = 3) and from types I (*n* = 5), II (*n* = 1), and III (*n* = 2) ECL cell NETs, including their respective metastases (one lymph node metastasis from each type of ECL cell NET, respectively), were examined.

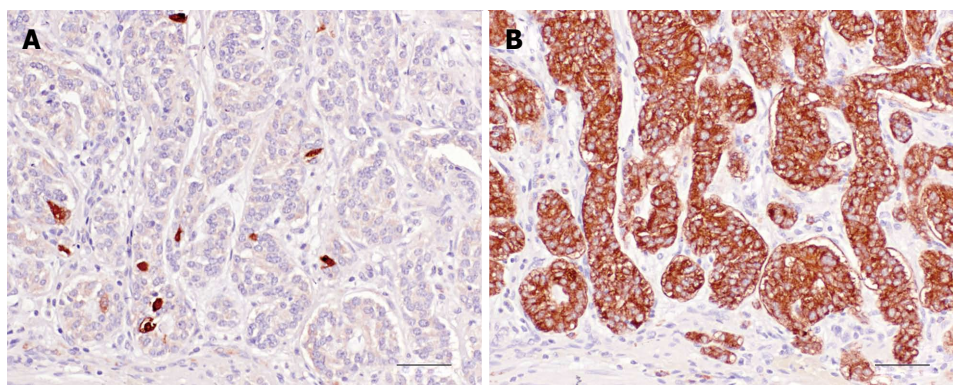


Figure 1 Histidine decarboxylase (A) and vesicular monoamine transporter 2 (B) immunostained type I enterochromaffin-like cell neuroendocrine tumour (consecutive sections). Only a few scattered histidine decarboxylase (HDC)-immunoreactive cells are present in the tumour, whereas virtually all neoplastic cells display VMAT-2 immunoreactivity. Bars = 50 µm (A, B).

When the gastric fundus ($n = 3$) and corpus ($n = 3$) mucosal specimens were examined, photos were taken from randomly identified neuroendocrine cells with a view to investigate a possible co-expression of HDC-with VMAT-2- or ghrelin-IR cells. A total of 1000 cells were examined, including 750 cells (125 randomly chosen cells/specimen) that were HDC- and/or VMAT-2-IR and 250 cells (approximately 40 cells/specimen) that were ghrelin-IR.

Control immunostainings

The immunostained controls included omission of the primary antisera and replacement of the primary antibody by non-immune serum at the same dilution as the primary antibody in question and in the same diluent.

U-MeImAA determination

U-MeImAA was measured in 27 patients with various types of gastric NETs before obtaining biopsy samples or operation and before specific treatment (Table 2). A 24-h urine sample, collected while patients were on a histamine-restricted diet, was analysed for U-MeImAA^[16]. All of the patients were instructed to avoid antihistamine medication and alcohol for at least 24 h prior to admission. U-MeImAA was determined using a HPLC assay with UV detection^[24]. The reference range was 0.4-2.4 mmol/mol creatinine.

Approval by an ethics committee

The research protocol was reviewed and approved by the local research ethics board at Uppsala University Hospital.

RESULTS

Conventional immunohistochemistry

Multiple foci of neuroendocrine cell hyperplasia were observed adjacent to types I and II ECL cell NETs where VMAT-2-IR cells predominated followed by ghrelin-IR cells. In these foci, HDC-IR cells were few

in number, but in occasional foci an inverse result occurred. The latter type of foci was observed in 14 cases of type I and two of type II ECL cell NETs (diffuse, linear and nodular hyperplasia patterns, in various combinations). Focally, diffuse HDC-IR cell hyperplasia was detected in the mucosa adjacent to the non-ECL cell NET. In the latter foci, no VMAT-2 cell hyperplasia was observed. No signs of neuroendocrine cell hyperplasia expressing HDC were observed in the peritumourous mucosa of the type III ECL cell NETs, the ghrelinomas and the NECs.

All the ECL cell NETs were VMAT-2-IR in more than 80% of the neoplastic parenchymal cells. In type I ECL cell NETs, ghrelin immunoreactivity varied from negative to the majority of the tumour cells. The two ghrelinomas examined expressed only ghrelin and did not display VMAT-2 immunoreactivity. The non-ECL cell NET examined contained a small fraction of VMAT-2- (approximately 3%) and ghrelin-IR cells (approximately 5%). Three of the NECs expressed VMAT-2 in more than half of the total number of the neoplastic cells. The remaining NECs were occasional- or non-IR for VMAT-2. None of the NECs examined expressed ghrelin.

Sixteen of the 37 type I ECL cell NETs contained HDC-IR cells (< 5% in 15 cases, approximately 50% in one case) (Figure 1). In the cases with metastases, a similar distribution pattern and frequency of HDC-IR cells was seen both in the primary tumour and in its respective metastases.

Two of the three type II ECL cell NETs contained HDC-IR cells in a frequency varying from 1% to 10%. In the lymph node metastases present in one case, the relative incidence of HDC-IR cells was greater (up to 40%).

Four of the ten type III ECL cell NETs contained HDC-IR cells with a relative incidence between 1% and 30%. Their metastases usually expressed HDC more abundantly than in the primary tumours.

The non-ECL cell NET and the two ghrelinomas did not contain HDC-IR cells.

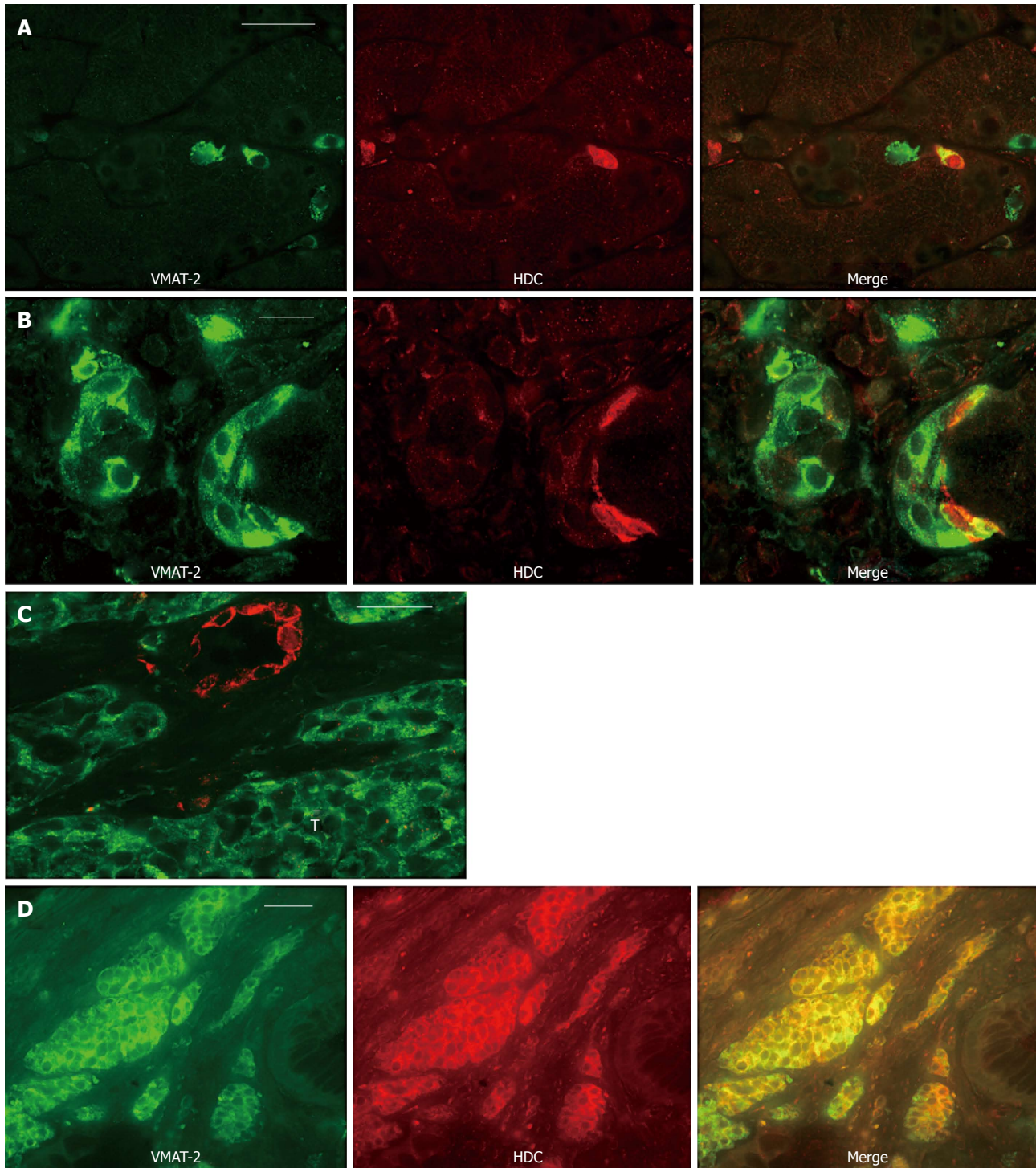


Figure 2 Co-localization studies. A: Normal human oxyntic mucosa double-immunostained for vesicular monoamine transporter 2 (VMAT-2) (green) and histidine decarboxylase (HDC) (red). Co-localization (yellow) depicts that VMAT-2 and HDC are co-expressed in the same neuroendocrine cell, whereas the remaining immunoreactive (IR) cells in the gastric glands do not display co-expression; B: Oxyntic mucosa adjacent to a type I enterochromaffin-like (ECL) cell neuroendocrine tumour (NET) double immunostained for VMAT-2 (green) and HDC (red). In one focus of neuroendocrine cell hyperplasia identified by VMAT-2 (linear pattern), only a fraction of cells co-express HDC (yellow), whereas another is non-IR for the enzyme; C: Oxyntic mucosa adjacent to a type I ECL cell NET double immunostained for VMAT-2 (green) and HDC (red). The HDC-IR cells display a linear pattern of neuroendocrine cell hyperplasia and do not co-express the transporter protein. In the foci of nodular cell hyperplasia identified by VMAT-2, no cell is HDC-IR. Adjacent tumour cells (T) reveal only VMAT-2 immunoreactivity; D: A malignant type III ECL cell NET accompanied by increased U-MeImAA excretion was double-immunostained for VMAT-2 (green) and HDC (red). Co-localization (yellow) shows that the tumour cells that are HDC-IR co-express the transporter protein. VMAT-2-IR cells slightly outnumber the HDC-IR cells. Bars = 30 μ m (A, C, D); 20 μ m (B).

Three NECs contained HDC-IR tumour cells in approximately 15%, 20% and 60% of the neoplastic cell population. The first two above mentioned NECs revealed VMAT-2 immunoreactivity in approx. 60% of the tumour cells, whereas the latter case lacked such cells. Their metastases revealed an immunohistochemical pattern and frequency similar to that of the primary tumours.

Co-localization studies

By using double immunofluorescence techniques, three patterns of immunoreactivity could be identified in the glands of the normal mucosa examined: (1) cells expressing only VMAT-2 (335/750 IR cells identified, approximately 45%); (2) only HDC (144/750 IR cells, approximately 19%); and (3) cells co-expressing both proteins (271/750 IR cells, approximately 36%) (Figure 2A). Ghrelin cells ($n = 250$) did not express HDC in the normal oxyntic mucosa.

In the foci of neuroendocrine cell hyperplasia in types I and II ECL cell NETs, VMAT-2-IR cells were predominant, but only a few of them co-expressed HDC (Figure 2B). In occasional foci of neuroendocrine cell hyperplasia, the HDC-IR cells were predominant, whereas VMAT-2-IR cells were few or absent (Figure 2C). In types I, II and III ECL cell NETs, most HDC-IR cells co-expressed VMAT-2 and occasional cells expressed only HDC (Figure 2D). However, the majority of the tumour cells were only VMAT-2-IR. The same finding was observed in the metastases of the type III ECL cell NETs examined. None of the neoplastic cells in types I, II and III ECL cell NETs or in the foci of neuroendocrine cell hyperplasia in the former two types of ECL cell NETs that showed ghrelin immunoreactivity were HDC-IR.

Control immunostainings

Immunoreactivity was not observed when the primary antibodies were omitted or replaced by non-immune serum. In double immunostaining, the omission of one of the primary antibodies, or its replacement by non-immune serum, gave an immunostaining pattern corresponding to that obtained with the remaining primary antibody. When both antibodies were omitted or replaced by the respective non-immune sera, the tissues were non-IR.

U-MeImAA excretion and clinical symptoms in relation to HDC and VMAT-2 immunoreactivity

Four of 27 patients in whom U-MeImAA was determined had an increased excretion of the histamine metabolite (results summarized in Table 2). None of the patients with type I ECL cell NETs had increased U-MeImAA or clinical symptoms that might be related to increased histamine production, irrespective of the presence of HDC-IR tumour cells.

The U-MeImAA excretion was increased moderately in one patient with metastatic disease of type II ECL

cell NET expressing VMAT-2 in virtually all neoplastic cells and HDC in 10% in the primary tumour and 40% in the respective lymph node metastasis. The patient suffered occasionally from flushes.

Two patients diagnosed with metastatic type III ECL cell NETs had increased and high levels of U-MeImAA. One of these patients developed ACS (the tumour cells were HDC-IR in 20% and 40% of the primary tumour and omentum metastasis, respectively), whereas the other patient did not report clinical symptoms related to increased histamine production (the primary tumour contained few HDC-IR cells, but the liver metastasis showed 40% HDC immunoreactivity). By contrast, one other patient with type III ECL cell NET, who had liver metastases and skin metastasis expressing HDC and VMAT-2, had U-MeImAA within the reference range.

One of the four investigated patients with NEC had a slightly elevated level of U-MeImAA without any clinical symptoms related to ACS (HDC immunoreactivity of approximately 15% of the neoplastic cells in both the primary tumour and the lymph node metastasis examined). The NEC patient with numerous HDC-IR cells in the primary tumour and in the adrenal metastasis (60%) had normal levels of the histamine metabolite in the urine. The tumour cells of that patient were non-IR for VMAT-2.

DISCUSSION

Human oxyntic mucosa contains four major types of neuroendocrine cells (ECL, ghrelin, serotonin and somatostatin cells), which have been described morphologically and functionally^[25]. ECL cells are the most abundant type, and they produce histamine. The histamine-synthesizing ability of ECL cells is based on cytosolic HDC. Histamine might be transported by VMAT-2 into the secretory vesicles. The ghrelin-producing cells are the second most frequent cell type and have been reported to express VMAT-2^[5]. However, these results differ from findings presented in a study where only approximately 2% of the ghrelin cell population expressed VMAT-2^[11]. This disparity may be due to the differing techniques used to compare the two cell types. Our results were based on double immunofluorescence stainings, which enable visualization of a large number of cells, whereas the other results were achieved by means of immunoelectron microscopy and using immunostaining consecutive sections with the respective antibodies.

The current study showed that approximately one-third of the VMAT-2-IR cells in normal human gastric mucosa co-express HDC. Additionally, numerous HDC-IR cells in the oxyntic mucosa were non-IR for VMAT-2, implying that HDC is expressed in only a fraction of VMAT-2-IR cells. Thus, we show that there is by no means a complete co-existence between histamine expression visualized by HDC immunoreactivity and VMAT-2. In animal models it has been shown that a

disappearance of HDC-IR cells occurs after 24 h of food deprivation^[26]. Although our specimens were obtained during the operation from patients fasting overnight, it is less likely that the disparity between VMAT-2- and HDC-IR cells can be entirely attributed to down regulation of HDC because of food deprivation. Although VMAT-2 is well characterized, it cannot be excluded that other proteins or forms of VMAT-2 exist that were not identified by the chosen antibody. As previously reported for the fundus, ghrelin did not co-localize with HDC in the corpus mucosa^[27].

In the foci of neuroendocrine cell hyperplasia, two different patterns appeared: a more common one identified primarily by VMAT-2 immunoreactivity and a less common one with predominantly HDC-IR cells. According to our co-localization studies in the former foci, only a few cells co-expressed HDC, whereas in the latter foci an inverse result occurred. It is well established that hypergastrinaemia causes ECL cell hyperplasia, which can transform into ECL cell NETs^[28,29]. Biochemical studies have shown that the total mucosa content of HDC increases in patients with chronic atrophic gastritis type A^[30]. However, remains unclear whether this increase in concentration depends on a higher frequency of HDC expressing cells or on an increase in HDC content in individual cells or both. It was expected that the increase in VMAT-2-IR cells would be accompanied by a similar increase in HDC-IR cells. However, only occasional foci with HDC-IR cell hyperplasia were identified in the atrophic mucosa. The HDC antibody used is well characterized, but we cannot exclude that HDC may undergo post-translational modifications and exist in forms not identified by the present antibody^[10].

In agreement with previous studies, our results showed that all types of ECL cell NETs, as well their respective metastases, can express HDC-IR tumour cells in varying relative incidences^[10,15]. Furthermore, according to our co-localization findings, occasional neoplastic cells displaying HDC immunoreactivity were non-IR for VMAT-2. This may be due to tumour de-differentiation or, more plausibly, because these neoplastic cells are derived from the HDC-IR cells that do not express VMAT-2.

Four of the 27 patients in whom U-MeImAA was determined had increased urinary excretion of this histamine metabolite. All of these patients had metastatic tumours that showed both HDC and VMAT-2 immunoreactivity. In other studies, it has been reported that even type I ECL cell NETs may be accompanied by increased U-MeImAA levels^[19,21]. Our results indicate that only disseminated disease can give rise to increased U-MeImAA excretion, although disseminated disease does not invariably lead to increased U-MeImAA levels. It is likely that the tumour load, often high in type III ECL cell NETs, is decisive; however, the tumour load also has to be accompanied

most likely by both HDC and VMAT-2 expression, as HDC expression alone does not invariably predict increased U-MeImAA excretion. Other factors involved in the production, transport and/or secretion of histamine that were not examined in the present study may also play an important role.

Furthermore, no apparent relationship was found between increased U-MeImAA excretion and the clinical symptoms of the respective patients. This could probably be attributed to the rate of histamine release. A sudden rapid release will cause a flush whereas a slower release will not. Additionally, an efficient local inactivation of histamine before it reaches the systemic circulation might explain the absence of symptoms even when large amounts of histamine are being released.

In conclusion, only a fraction of the VMAT-2-IR neuroendocrine cells in human oxyntic mucosa co-express HDC, and *vice versa*, suggesting that the ECL cells may not represent a homogeneous cell population when examined for HDC immunoreactivity. Co-expression of VMAT-2 and HDC might be substantial for giving rise to increased histamine production in patients with ECL cell NETs. Furthermore, an increase in U-MeImAA excretion is not always associated with hormonal symptoms.

COMMENTS

Background

Enterochromaffin-like (ECL) cells are the most abundant neuroendocrine cell type in the gastric mucosa, and they produce histamine. The histamine-synthesizing ability of ECL cells is based on cytosolic histidine decarboxylase (HDC). Histamine is transported by vesicular monoamine transporter subtype 2 (VMAT-2) into the secretory vesicles. The production and release of histamine can be estimated by measuring the urinary excretion of the main and specific histamine metabolite tele-methylimidazoleacetic acid (U-MelMAA). ECL cells can undergo malignant transformation to ECL cell neuroendocrine tumours (NETs)

Research frontiers

Previous immunohistochemical studies have examined human ECL cell NETs by means of both VMAT-2 and HDC antibodies. In these tumours, some of the neoplastic parenchymal cells were immunoreactive to HDC, whereas the transporter had a wider distribution.

Innovations and breakthroughs

This study shows that there is by no means a complete co-existence between histamine expression visualized by HDC immunoreactivity and VMAT-2, neither in the gastric mucosa nor in the foci of endocrine cell hyperplasia/ECL cell NETs. The results indicate that only disseminated disease can give rise to increased U-MelMAA excretion, although disseminated disease does not invariably lead to increased U-MelMAA levels. Furthermore, no apparent relationship was found between increased U-MelMAA excretion and the clinical symptoms of the respective patients.

Applications

The ECL cells may not represent a homogeneous cell population when examined for HDC immunoreactivity. Co-expression of VMAT-2 and HDC might be important for giving rise to increased histamine production in patients with ECL cell NETs. Furthermore, an increase in U-MelMAA excretion is not always associated with hormonal symptoms.

Terminology

ECL cells use the enzyme HDC to produce histamine, which is transported by VMAT-2 from the cytoplasm into the secretory vesicles. The production and release of histamine can be estimated by measurement of the urinary excretion of the main and specific histamine metabolite U-MeImAA.

Peer-review

The paper is well written but the topic is extremely specific and it may be of limited interest for the most of journal readers.

REFERENCES

- Bordi C, D'Adda T, Azzoni C, Ferraro G. Classification of gastric endocrine cells at the light and electron microscopical levels. *Microsc Res Tech* 2000; **48**: 258-271 [PMID: 10700043 DOI: 10.1002/(SICI)1097-0029(20000301)48]
- Viguera E, Trelles O, Urdiales JL, Matés JM, Sánchez-Jiménez F. Mammalian L-amino acid decarboxylases producing 1,4-diamines: analogies among differences. *Trends Biochem Sci* 1994; **19**: 318-319 [PMID: 7940675]
- Dimaline R, Struthers J. Expression and regulation of a vesicular monoamine transporter in rat stomach: a putative histamine transporter. *J Physiol* 1996; **490** (Pt 1): 249-256 [PMID: 8745292 DOI: 10.1113/jphysiol.1996.sp021140]
- Zhao CM, Jacobsson G, Chen D, Håkanson R, Meister B. Exocytotic proteins in enterochromaffin-like (ECL) cells of the rat stomach. *Cell Tissue Res* 1997; **290**: 539-551 [PMID: 9369530 DOI: 10.1007/s004410050960]
- Rindi G, Necchi V, Savio A, Torsello A, Zoli M, Locatelli V, Raimondo F, Cocchi D, Solcia E. Characterisation of gastric ghrelin cells in man and other mammals: studies in adult and fetal tissues. *Histochem Cell Biol* 2002; **117**: 511-519 [PMID: 12107501 DOI: 10.1007/s00418-002-0415-1]
- Tsolakis AV, Grimelius L, Stridsberg M, Falkmer SE, Waldum HL, Saras J, Janson ET. Obestatin/ghrelin cells in normal mucosa and endocrine tumours of the stomach. *Eur J Endocrinol* 2009; **160**: 941-949 [PMID: 19289536]
- Eissele R, Anlauf M, Schäfer MK, Eiden LE, Arnold R, Weihe E. Expression of vesicular monoamine transporters in endocrine hyperplasia and endocrine tumors of the oxyntic stomach. *Digestion* 1999; **60**: 428-439 [PMID: 10473967 DOI: 10.1159/000007688]
- Rindi G, Paolotti D, Fiocca R, Wiedenmann B, Henry JP, Solcia E. Vesicular monoamine transporter 2 as a marker of gastric enterochromaffin-like cell tumors. *Virchows Arch* 2000; **436**: 217-223 [PMID: 10782879 DOI: 10.1007/s004280050033]
- Jakobsen AM, Andersson P, Saglik G, Andersson E, Kölby L, Erickson JD, Forssell-Aronsson E, Wängberg B, Ahlman H, Nilsson O. Differential expression of vesicular monoamine transporter (VMAT) 1 and 2 in gastrointestinal endocrine tumours. *J Pathol* 2001; **195**: 463-472 [PMID: 11745679 DOI: 10.1002/path.973]
- Uccella S, Cerutti R, Vigetti D, Furlan D, Oldrini R, Carnevali I, Pelosi G, La Rosa S, Passi A, Capella C. Histidine decarboxylase, DOPA decarboxylase, and vesicular monoamine transporter 2 expression in neuroendocrine tumors: immunohistochemical study and gene expression analysis. *J Histochem Cytochem* 2006; **54**: 863-875 [PMID: 16517981]
- Tsolakis AV, Stridsberg M, Grimelius L, Portela-Gomes GM, Falkmer SE, Waldum HL, Janson ET. Ghrelin immunoreactive cells in gastric endocrine tumors and their relation to plasma ghrelin concentration. *J Clin Gastroenterol* 2008; **42**: 381-388 [PMID: 18277901 DOI: 10.1097/MCG.0b013e318032338c]
- Tsolakis AV, Portela-Gomes GM, Stridsberg M, Grimelius L, Sundin A, Eriksson BK, Oberg KE, Janson ET. Malignant gastric ghrelinoma with hyperghrelinemia. *J Clin Endocrinol Metab* 2004; **89**: 3739-3744 [PMID: 15292299 DOI: 10.1210/jc.2003-032118]
- Håkanson R, Böttcher G, Ekblad E, Panula P, Simonsson M, Dohlsten M, Hallberg T, Sundler F. Histamine in endocrine cells in the stomach. A survey of several species using a panel of histamine antibodies. *Histochemistry* 1986; **86**: 5-17 [PMID: 2878908]
- Dartsch C, Chen D, Håkanson R, Persson L. Histidine decarboxylase in rat stomach ECL cells: relationship between enzyme activity and different molecular forms. *Regul Pept* 1999; **81**: 41-48 [PMID: 10395406 DOI: 10.1016/S0167-0115(99)00016-6]
- Rindi G, Savio A, Torsello A, Zoli M, Locatelli V, Cocchi D, Paolotti D, Solcia E. Ghrelin expression in gut endocrine growths. *Histochem Cell Biol* 2002; **117**: 521-525 [PMID: 12107502 DOI: 10.1007/s00418-002-0416-0]
- Granerus G. Urinary excretion of histamine, methylhistamine and methylimidazoleacetic acids in man under standardized dietary conditions. *Scand J Clin Lab Invest Suppl* 1968; **104**: 59-68 [PMID: 4974103 DOI: 10.1080/00365516809168041]
- Granerus G, Lindell SE, Waldenström J, Westling H, White T. Histamine metabolism in carcinoidosis. *Lancet* 1966; **1**: 1267-1268 [PMID: 4161228 DOI: 10.1016/S0140-6736(66)90283-2]
- Ahlman H, Dahlström A, Enerbäck L, Granerus G, Nilsson O, Persson S, Tisell LE. Two cases of gastric carcinoids: diagnostic and therapeutic aspects. *World J Surg* 1988; **12**: 356-361 [PMID: 2456643 DOI: 10.1007/BF01655671]
- Ahlman H, Wängberg B, Nilsson O, Grimelius L, Granerus G, Modlin IM, Stenqvist O, Scherstén T. Aspects on diagnosis and treatment of the foregut carcinoid syndrome. *Scand J Gastroenterol* 1992; **27**: 459-471 [PMID: 1385890 DOI: 10.3109/00365529209000106]
- Ahlman H, Kölby L, Lundell L, Olbe L, Wängberg B, Granerus G, Grimelius L, Nilsson O. Clinical management of gastric carcinoid tumors. *Digestion* 1994; **55** Suppl 3: 77-85 [PMID: 7698542 DOI: 10.1159/000201206]
- Borch K, Ahrén B, Ahlman H, Falkmer S, Granerus G, Grimelius L. Gastric carcinoids: biologic behavior and prognosis after differentiated treatment in relation to type. *Ann Surg* 2005; **242**: 64-73 [PMID: 15973103 DOI: 10.1097/01.sla.0000167862.52309.7d]
- Solcia E, Arnold R, Capella C, Klimstra DS, Klöppel G, Komminoth P, Rindi G. Neuroendocrine neoplasms of the stomach. In: Bosman FT, Carneiro F, Hruban RH, Theise ND, editors. WHO Classification of Tumours of the Digestive System. 4th ed. Lyon: World Health Organization, 2010: 64-68
- Rindi G, Klöppel G, Ahlman H, Caplin M, Couvelard A, de Herder WW, Eriksson B, Falchetti A, Falconi M, Komminoth P, Körner M, Lopes JM, McNicol AM, Nilsson O, Perren A, Scarpa A, Scoazec JY, Wiedenmann B; all other Frascati Consensus Conference participants; European Neuroendocrine Tumor Society (ENETS). TNM staging of foregut (neuro)endocrine tumors: a consensus proposal including a grading system. *Virchows Arch* 2006; **449**: 395-401 [PMID: 16967267 DOI: 10.1007/s00428-006-0250-1]
- Granerus G, Lönnqvist B, Wass U. Determination of the histamine metabolite tele-methylimidazoleacetic acid and of creatinine in urine by the same HPLC system. *Inflamm Res* 1999; **48**: 75-80 [PMID: 10202992 DOI: 10.1007/s000110050413]
- Solcia E, Rindi G, Buffa R, Fiocca R, Capella C. Gastric endocrine cells: types, function and growth. *Regul Pept* 2000; **93**: 31-35 [PMID: 11033050 DOI: 10.1016/S0167-0115(00)00175-0]
- Torbergson K, Wiksén H, Johansen K, Rahimipoor S, Falkmer UG, Zhao CM. Immunoreactivity of gastric ECL and A-like cells in fasted and fed rats and mice. *Biotech Histochem* 2005; **80**: 21-30 [PMID: 15804823]
- Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 2000; **141**: 4255-4261 [PMID: 11089560 DOI: 10.1210/endo.141.11.7757]
- Bordi C, D'Adda T, Azzoni C, Pilato FP, Caruana P. Hypergastrinemia and gastric enterochromaffin-like cells. *Am J Surg Pathol* 1995; **19** Suppl 1: S8-S19 [PMID: 7762739]
- Thomas D, Tsolakis AV, Grozinsky-Glasberg S, Fraenkel M, Alexandraki K, Sougioultzis S, Gross DJ, Kaltsas G. Long-term follow-up of a large series of patients with type 1 gastric carcinoid

tumors: data from a multicenter study. *Eur J Endocrinol* 2013; **168**: 185-193 [PMID: 23132699]

30 Cattani D, Roucayrol AM, Launay JM, Callebort J, Charasz N,

Nurit Y, Belaiche J, Kalifat R. Circulating gastrin, endocrine cells, histamine content, and histidine decarboxylase activity in atrophic gastritis. *Gastroenterology* 1989; **97**: 586-596 [PMID: 2753321]

P- Reviewer: Cananzi FCM **S- Editor:** Yu J **L- Editor:** A
E- Editor: Ma S



Basic Study

Protective role of adiponectin in a rat model of intestinal ischemia reperfusion injury

Xu-Hui Liu, Yue-Wu Yang, Hai-Tao Dai, Song-Wang Cai, Rui-Han Chen, Zhi-Qiang Ye

Xu-Hui Liu, Hai-Tao Dai, Rui-Han Chen, Zhi-Qiang Ye, Department of Emergency Care, the Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510630, Guangdong Province, China

Yue-Wu Yang, Department of Traditional Chinese Medicine, the Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510630, Guangdong Province, China

Song-Wang Cai, Department of Cardiothoracic Surgery, the Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510630, Guangdong Province, China

Author contributions: Liu XH, Yang YW and Dai HT contributed equally to this work; Ye ZQ designed research; Liu XH and Yang YW performed research; Dai HT and Cai SW contributed new reagents or analytical tools; Chen RH analyzed data; Liu XH and Yang YW wrote the paper.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of the Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China. The animal protocol was designed to minimize pain or discomfort to the animals.

Conflict-of-interest statement: The authors declared no conflicts of interest in this study.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Dr. Zhi-Qiang Ye, Department of Emergency Care, the Third Affiliated Hospital of Sun Yat-Sen University, No. 600 Tianhe Street, Guangzhou 510630, Guangdong Province, China. yezhiqiang150211@163.com
Telephone: +86-20-85253010
Fax: +86-20-85252553

Received: March 3, 2015
Peer-review started: March 5, 2015
First decision: May 18, 2015
Revised: June 21, 2015
Accepted: September 2, 2015
Article in press: September 2, 2015
Published online: December 21, 2015

Abstract

AIM: To determine the potential protective role of adiponectin in intestinal ischemia reperfusion (I/R) injury.

METHODS: A rat model of intestinal I/R injury was established. The serum level of adiponectin in rats with intestinal I/R injury was determined by enzyme-linked immunosorbent assay (ELISA). The serum levels of interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α were also measured by ELISA. Apoptosis of intestinal cells was detected using the terminal deoxynucleotidyl transferase dUTP nick end labeling assay. The production of malondialdehyde (MDA) and superoxide dismutase (SOD) and villous injury scores were also measured.

RESULTS: Adiponectin was downregulated in the serum of rats with intestinal I/R injury compared with sham rats. No significant changes in the expression of adiponectin receptor 1 and adiponectin receptor 2 were found between sham and I/R rats. Pre-treatment with recombinant adiponectin attenuated intestinal I/R injury. The production of pro-inflammatory cytokines,

including IL-6, IL-1 β , and TNF- α , in rats with intestinal I/R injury was reduced by adiponectin pre-treatment. The production of MDA was inhibited, and the release of SOD was restored by adiponectin pre-treatment in rats with intestinal I/R injury. Adiponectin pre-treatment also inhibited cell apoptosis in these rats. Treatment with the AMP-activated protein kinase (AMPK) signaling pathway inhibitor, compound C, or the heme oxygenase 1 (HO-1) inhibitor, Snpp, attenuated the protective effects of adiponectin against intestinal I/R injury.

CONCLUSION: Adiponectin exhibits protective effects against intestinal I/R injury, which may involve the AMPK/HO-1 pathway.

Key words: Adiponectin; Ischemia reperfusion injury; Intestine

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Serum adiponectin was downregulated in the rat model of intestinal ischemia reperfusion (I/R) injury, and adiponectin pre-treatment attenuated intestinal I/R injury in rats. While the underlying mechanism of adiponectin-induced protection against intestinal I/R injury is not fully understood, the results of the present study suggest that the AMP-activated protein kinase (AMPK)/heme oxygenase 1 (HO-1) signaling pathway may be involved in this process. Therefore, adiponectin and components of the AMPK/HO-1 signaling pathway may be promising targets in the treatment of intestinal I/R injury.

Liu XH, Yang YW, Dai HT, Cai SW, Chen RH, Ye ZQ. Protective role of adiponectin in a rat model of intestinal ischemia reperfusion injury. *World J Gastroenterol* 2015; 21(47): 13250-13258 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13250.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13250>

INTRODUCTION

Intestinal ischemia reperfusion injury (I/R), a critical complication in patients with trauma and after liver and intestinal transplantation, is associated with high morbidity and mortality^[1-3]. Interruption of the blood supply may cause tissue damage. However, restoration of blood flow does not relieve tissue damage but leads to additional injury^[4]. Previous studies have shown that oxidative stress, the inflammatory response, and cell apoptosis are involved in I/R injury, which leads to multiple organ dysfunction syndrome (MODS) and acute respiratory distress syndrome^[5-9]. Extensive research has been conducted to develop effective treatments for I/R injury that regulate oxidative stress and the inflammatory reaction.

Adiponectin, also referred to as gelatin-binding

protein-28 (GBP28), ACRP30, AdipoQ, or apM1, is a hormone secreted mainly by adipocytes. As a serum protein similar to C1q, adiponectin is exclusively produced in adipocytes. Adiponectin exerts its function primarily via two membrane receptors, adiponectin receptor-1 and -2 (AdipoR1/2), which interact with AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)^[10]. In addition, previous studies have reported that adiponectin exhibited anti-inflammatory and anti-apoptosis effects and regulated the metabolism of glucose and lipid^[11-13]. Interestingly, a number of studies have also reported the role of adiponectin in protection against I/R injury in myocardial, cerebral, liver, and renal tissues^[14-17]. However, the effects of adiponectin in intestinal I/R have not been confirmed. The present study aimed to investigate the potential role of adiponectin in protecting against intestinal I/R injury based on a rat model of I/R injury.

MATERIALS AND METHODS

Preparation of recombinant adiponectin

Rat adiponectin was cloned into the pET30 vector (Novagen, Darmstadt, Germany) as described previously^[18]. The DNA constructs were then transfected into competent *Escherichia coli* BL21 (DE3) (Takara, Shiga, Japan). Isopropyl-1-thio- β -D-galactopyranoside was used to induce the expression of His-tagged adiponectin at 37 °C. Recombinant His-tagged fusion protein was isolated from the cytoplasm and purified using a His Bind resin column (Novagen).

Establishment of a rat model of intestinal I/R injury

All animal experiments were approved by the Medical Ethics Committee of the Third Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China. Female Wistar rats were randomly grouped and underwent surgery to induce intestinal I/R injury. There were five rats in each group weighing 180-230 g. The rats were deprived of food and received only water 12 h prior to laparotomy. The rats were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). A midline laparotomy was performed, as previously described, before equilibration for 30 min^[19]. The superior mesenteric artery was identified and isolated, and an atraumatic microvascular clamp (Roboz Surgical Instruments, Rockville, MD, United States) delivering 85 g of pressure was used to block blood flow in the mesenteric artery in rats in the intestinal I/R injury group. Intestinal ischemia was identified by the pale color of the intestine. After intestinal ischemia for 45 min, the clamp was removed to allow reperfusion for different time periods. When the color of the intestine returned to pink, the intestine was assumed to be reperfused. At the end of reperfusion, an approximately 10 cm segment of the small intestine 10 cm from the appendix was harvested for follow-up experiments. As a control, the sham group (sham)

was subjected to the same surgical intervention but without superior mesenteric artery occlusion. The body temperature of the animals was maintained throughout the surgical procedure using a water-circulating heating pad.

Rats in the adiponectin pre-treatment group (IR + adiponectin) received a tail vein injection of recombinant adiponectin at different concentrations (0.25, 0.5, or 1 mg/kg) 30 min before the establishment of intestinal I/R, as mentioned above.

To determine the possible pathway involved in the protective effects of adiponectin, the IR + adiponectin (1 mg/kg) group received an intraperitoneal injection of 20 mg/kg compound C (Millipore, Cambridge, MA, United States) or 50 μ mol/kg Snpp (Sigma, St. Louis, MO, United States).

Enzyme-linked immunosorbent assay

Serum adiponectin level in rats was determined using a rat enzyme-linked immunosorbent assay (ELISA) kit (R&D Corp., Minneapolis, MN, United States) according to the protocol described previously^[18]. Sections of the small intestine were homogenized and the supernatant was collected by centrifugation to measure the levels of cytokines, including interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α , in intestine tissues. The levels of IL-1 β , IL-6, and TNF- α in intestine tissues were determined using a commercial ELISA kit (USCN, Wuhan, China)^[20].

Malondialdehyde and superoxide dismutase determinations

Small intestine tissues were homogenized in 1.5% cold KCl solution at a ratio of 1:10 (weight:volume). The lipid peroxide level in the supernatant was measured according to the method described previously^[21]. Absorbance of the reaction was measured at 532 nm (Shimadzu UV-1700, Kyoto, Japan). The lipid peroxide level was expressed as nmol of malondialdehyde (MDA) per mg of tissue protein. Superoxide dismutase (SOD) activity was evaluated using a commercially available kit (Cayman Chemical, Ann Arbor, MI, United States) according to the protocol described in a previous report^[22]. SOD activity was expressed as unit (U) per mg protein. One U of SOD activity was defined as the amount of enzyme needed to inhibit 50% dismutation of the superoxide radical.

Western blot analysis

The small intestine tissues were homogenized and lysed in radio immunoprecipitation assay buffer [50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] incorporated with phenylmethylsulfonyl fluoride (Forevergen, Guangzhou, China). Isolated total proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were then

incubated with primary antibodies and then horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, Dallas, TX, United States). Bands were visualized using an enhanced chemiluminescence kit (Pierce, Rockford, IL, United States). The following antibodies were used in Western blot analysis: anti-caspase 3, anti-AMPK, anti-pAMPK, anti-heme oxygenase 1 (HO-1), anti-GAPDH antibodies (Cell Signaling, Beverly, MA, United States), anti-Adipo 1 (Abcam Inc., Cambridge, United Kingdom) and anti-Adipo 2 (Abcam Inc.).

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in the sections of jejunum which had undergone ischemia for 45 min and reperfusion for 3 h were detected by TUNEL assay using a Cell Death Detection kit (Roche Applied Science) according to the manufacturer's instructions.

Tissue damage evaluation

Intestinal mucosal lesions were graded as described previously^[21]. Briefly, grade 0 was defined as normal mucosal villi. Grade 1 was defined as the development of subepithelial Gruenhagen's space, usually at the apex of the villous, often with capillary congestion. Grade 2 was described as extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria. Grade 3 was defined as massive epithelial lifting with a few denuded villi. Grade 4 was defined as denuded villi with exposed dilated capillaries, and Grade 5 was defined as digestion and disintegration of the lamina propria, hemorrhage, and ulceration.

Statistical analysis

The data obtained from independent experiments were expressed as mean \pm SD. Differences among groups were analyzed by analysis of variance followed by the least significant difference method to determine statistical significance. A *P* value < 0.05 was defined as statistically significant.

RESULTS

Reduced serum adiponectin in rats with intestinal I/R

The serum levels of adiponectin in rats in the sham group and the intestinal I/R group were determined and compared by ELISA. A gradual decrease over time (1, 2, 3, 6, and 12 h after reperfusion) was observed in the serum level of adiponectin in the I/R rats compared with that in the sham group (Figure 1A). The lowest level of serum adiponectin was detected 3 h after reperfusion. At 12 h after reperfusion, the serum adiponectin level partially recovered. No significant decrease in the expression of AdipoR1 and AdipoR2 was detected in the intestinal tissues of rats with intestinal I/R based on western blot analysis

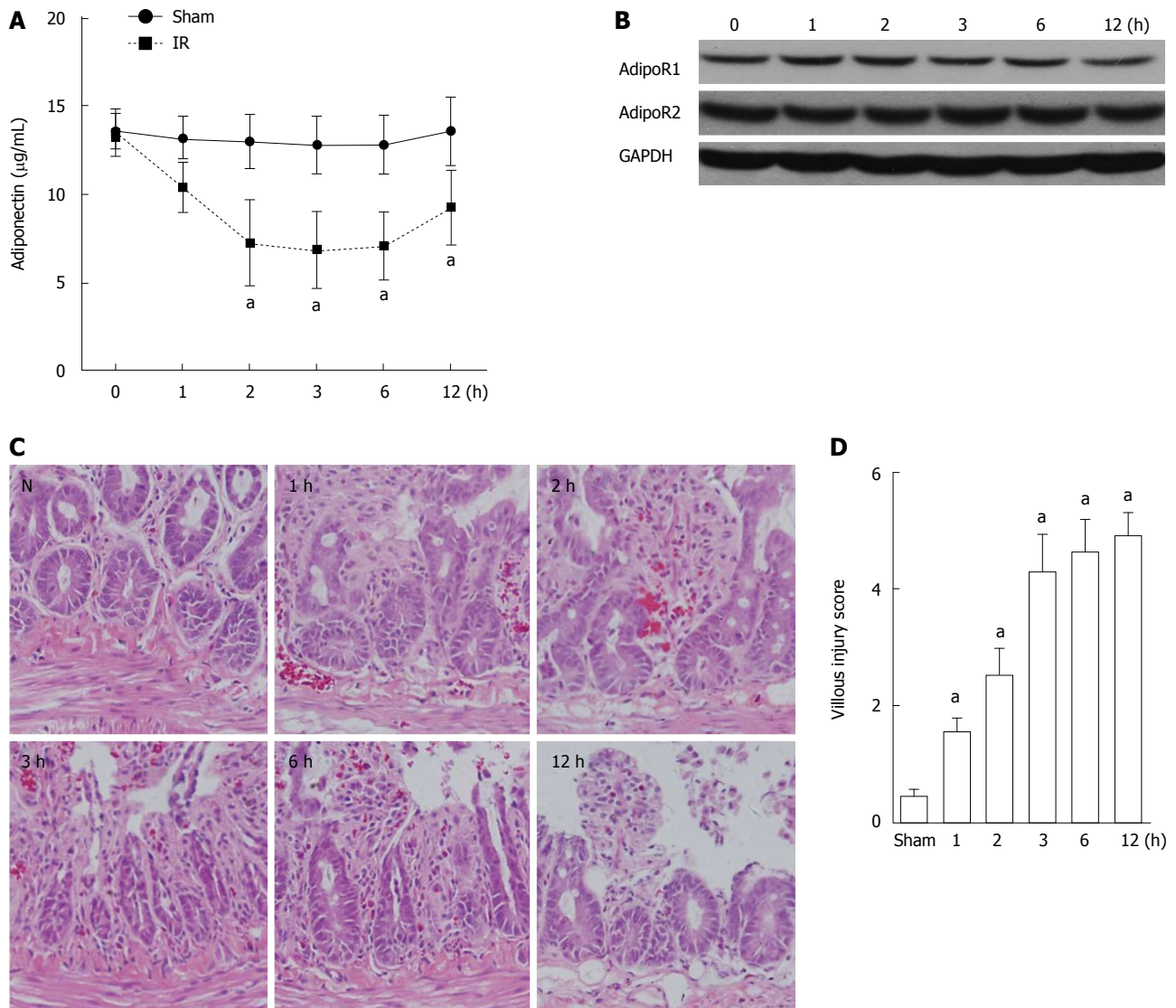


Figure 1 Adiponectin levels in rats in the sham and ischemia reperfusion injury groups. **A**: ELISA was used to determine the adiponectin serum levels in the sham and ischemia reperfusion (I/R) injury rats. Data are presented as mean \pm SD obtained from five independent experiments; **B**: Western blot analysis of the expression of adiponectin receptor 1 and adiponectin receptor 2 in the small intestine of rats with I/R injury; **C**: Hematoxylin-eosin staining of the small intestine at different time points after I/R injury; **D**: Villous injury scores of **C**. Necrosis was the major damage after intestinal I/R. N: Colon tissue in normal control rat. ^a $P < 0.05$ vs sham.

(Figure 1B). The evaluation of intestinal mucosal lesions showed significantly increased villous injury scores in rats with I/R injury (1, 2, 3, 6, and 12 h after reperfusion) compared with that in the sham group (Figure 1C and D).

Over-expression of adiponectin attenuated intestinal I/R injury

To investigate the effects of adiponectin on the production of pro-inflammatory cytokines, the rats were pre-treated with recombinant adiponectin 30 min before laparotomy. The rats were randomly divided into three subgroups, which were pre-treated with adiponectin at different concentrations (0.25, 0.5, and 1 mg/kg). Sham and I/R rats were used as controls. To assess the inflammatory response to I/R injury, the serum levels of IL-6, IL-1 β , and TNF- α were measured by ELISA. As shown in Figure 2A, the serum levels of

IL-6, IL-1 β , and TNF- α in the rats with I/R injury were significantly higher than those in the sham group. In addition, pre-treatment with recombinant adiponectin significantly reduced the serum levels of IL-6, IL-1 β , and TNF- α in a dose-dependent manner.

Consistent with the ELISA results, the level of MDA and villous injury scores in the I/R injury group were significantly higher than those in the sham group. Pre-treatment with recombinant adiponectin partially restored the level of MDA and villous injury scores. In addition, the SOD level was reduced in the I/R injury group and showed a decrease in the group pre-treated with adiponectin.

TUNEL staining was performed to investigate the effects of adiponectin on apoptosis in intestinal tissues undergoing I/R injury. As shown in Figure 2B, marked cell apoptosis was observed in intestinal tissues in the I/R injury group, but almost no apoptosis was found in

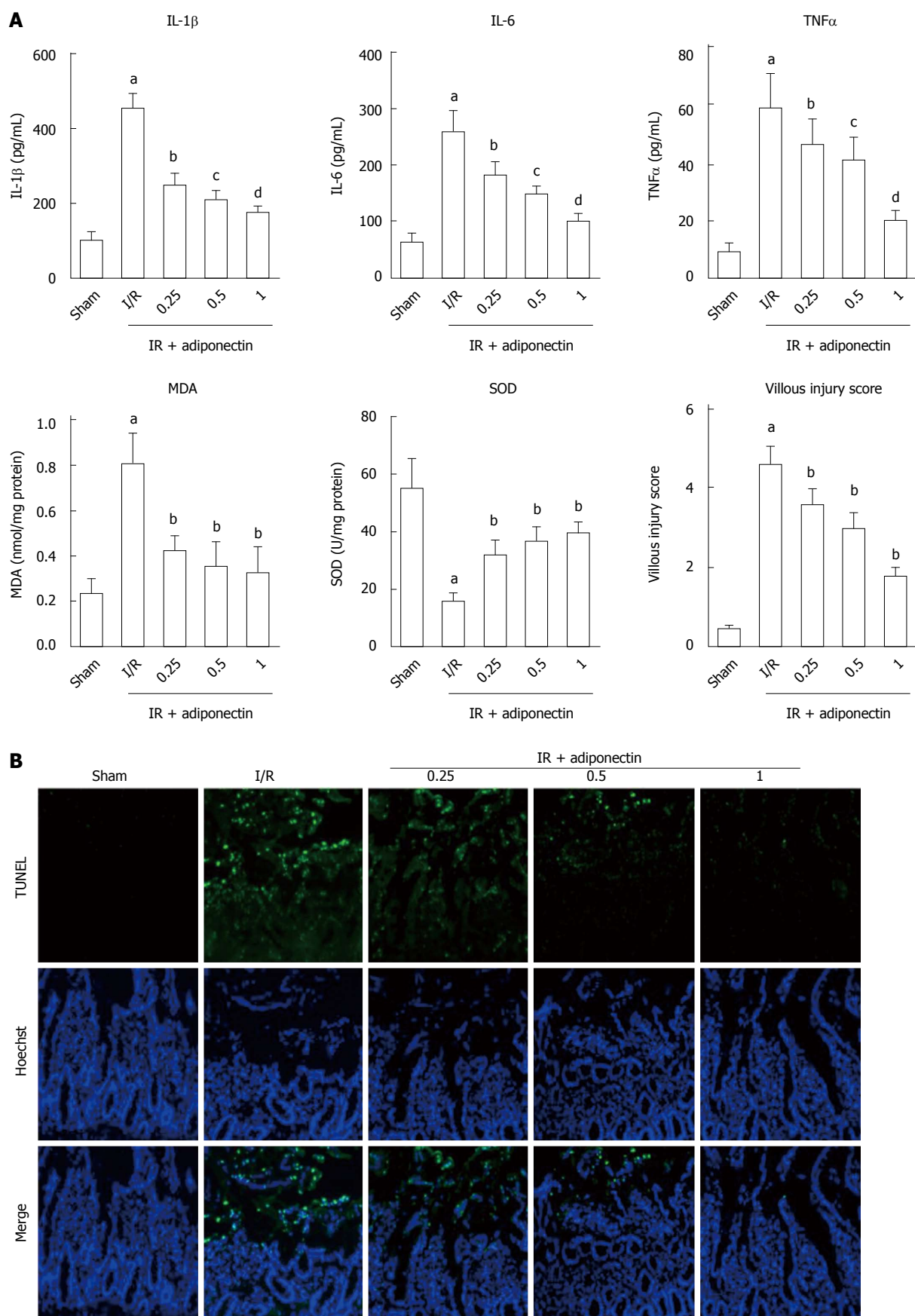


Figure 2 Adiponectin attenuated ischemia reperfusion injury in rats. A: The expression levels of IL-1 β , IL-6, TNF- α , MDA, and SOD, and the villous damage score. The data are expressed as mean \pm SD obtained from five independent experiments; B: TUNEL/Hoechst staining was performed to assess the effects of adiponectin treatment on cell apoptosis. Nuclei were stained with Hoechst (blue staining). Apoptotic nuclei were identified by TUNEL staining (green). ^a P < 0.05 vs sham, ^b P < 0.05 vs IR, ^c P < 0.05 vs 0.25 mg/kg group, ^d P < 0.05 vs 0.5 mg/kg group. SOD: Superoxide dismutase; MDA: Malondialdehyde.

the sham group. In addition, intestinal cell apoptosis in the I/R injury group was reduced by pre-treatment with adiponectin. The rats that received pre-treatment with recombinant adiponectin followed by intestinal I/R injury showed a statistically significant decrease in TUNEL-positive cells in intestinal tissues compared to rats with intestinal I/R injury without pre-treatment with recombinant adiponectin. The protective effect of adiponectin against apoptosis of intestinal cells in rats with intestinal I/R injury was dose-dependent (Figure 2B). Taken together, these results suggest that adiponectin was protective against intestinal I/R injury by inhibiting the apoptosis of intestinal cells and the production of pro-inflammatory cytokines.

AMPK/HO-1 is involved in the protective role of adiponectin in intestinal ischemia reperfusion

AMPK, AKT, and signal transducer and activator of transcription-3 have been reported to be associated with adiponectin-mediated effects^[23,24]. To determine the potential underlying mechanism responsible for the reduction in intestinal I/R injury, the expression levels of AMP, p-AMPK, and HO-1 were determined by western blot. As shown in Figure 3A, no significant difference in phosphorylated AMPK (p-AMPK) expression was observed between the sham and I/R groups. However, following adiponectin pre-treatment, AMPK was active and the level of p-AMPK was increased. No significant change in total AMPK level was found among the groups. Furthermore, the increased level of p-AMPK was partially abrogated by compound C, an AMPK inhibitor. Similar results were also found for the expression of HO-1, which was suggested to be activated by adiponectin in renal I/R injury^[25]. Rats administered adiponectin following I/R injury showed a higher level of HO-1 than the I/R group (Figure 3A), which was partially reversed in the compound C treatment group, indicating that the protective role of adiponectin in I/R may be *via* the AMPK/HO-1 pathway.

In addition, to confirm that the anti-apoptotic role of adiponectin is associated with the AMPK signaling pathway, the expression of cleaved caspase 3 was determined. As shown in Figure 3B, significant upregulation of cleaved caspase-3 was observed in the I/R rats compared with the sham rats. The upregulation induced by I/R was diminished by pre-treatment with adiponectin, compound C, and the HO-1 inhibitor (Snpp). Among these groups, the adiponectin group showed the lowest cleaved caspase 3 level.

Finally, as shown in Figure 3C, adiponectin-mediated I/R injury protection was partially abolished by compound C and Snpp. Increased levels of IL-6, IL-1 β , and TNF- α , increased production of MDA and SOD, and a higher villous injury score were seen in the IR + adiponectin + compound C and IR + adiponectin + Snpp groups compared with the IR + adiponectin group. These findings confirmed the involvement

of the AMPK/HO-1 pathway in the protective role of adiponectin in intestinal I/R injury.

DISCUSSION

Previous studies have demonstrated that adiponectin plays an important role in preventing I/R injury^[14-17]. However, whether adiponectin functions in the prevention of intestinal I/R injury is unknown. In the present study, we provide evidence that adiponectin is protective against intestinal I/R injury in rats.

IL-6, IL-1 β , and TNF- α are important pro-inflammatory cytokines and are regarded as molecular markers of inflammatory responses in human cells^[26]. In the present study, increased serum levels of IL-6, IL-1 β , and TNF- α were observed in rats with intestinal I/R injury. Interestingly, adiponectin reduced the serum levels of IL-6, IL-1 β , and TNF- α in rats with I/R injury, indicating that adiponectin has anti-inflammatory effects in intestinal I/R injury.

The production of free radicals and oxidants are dramatically increased during ischemia and reperfusion^[7,9,27]. Increased levels of oxidative stress markers such as MDA (an indicator of lipid peroxidation and free radical-induced cellular damage) were observed in I/R injury, accompanied by reduced scavenging ability against oxidative injury, such as low activity of SOD^[3,8]. Consistent with previous studies, increased MDA production and reduced activity of SOD were observed in the rat model of intestinal I/R injury. Reduced MDA and increased SOD levels were found in rats with I/R injury that received pre-treatment with adiponectin. These results suggest that adiponectin pre-treatment inhibited oxidant generation, maintaining a higher level of antioxidant activity in rats with I/R injury than in rats without adiponectin pre-treatment.

Intestinal villi have been reported to be susceptible to I/R injury and are involved in cellular necrosis^[3]. In the present study, we found that adiponectin pre-treatment attenuated villi damage in the rat model of I/R injury, indicating the therapeutic application of adiponectin in intestinal I/R injury. However, the underlying mechanisms should be investigated further.

AMPK plays an important role in mediating the production of adenosine triphosphate and fatty acid oxidation^[28]. In addition, it has been reported that adiponectin activated AMPK signaling in cat cardiomyocytes *in vitro*^[29] and protected against heart I/R injury in mice *via* an AMPK-dependent pathway^[16]. Based on previous studies, we hypothesized that adiponectin may also protect against intestinal I/R injury through the AMPK signaling pathway. In the present study, an increased level of phosphorylated AMPK was detected in rats with I/R injury that received adiponectin pre-treatment compared to the sham group, which supports our hypothesis.

HO-1, also known as heat shock protein 32, is a stress inducible protein involved in the protection

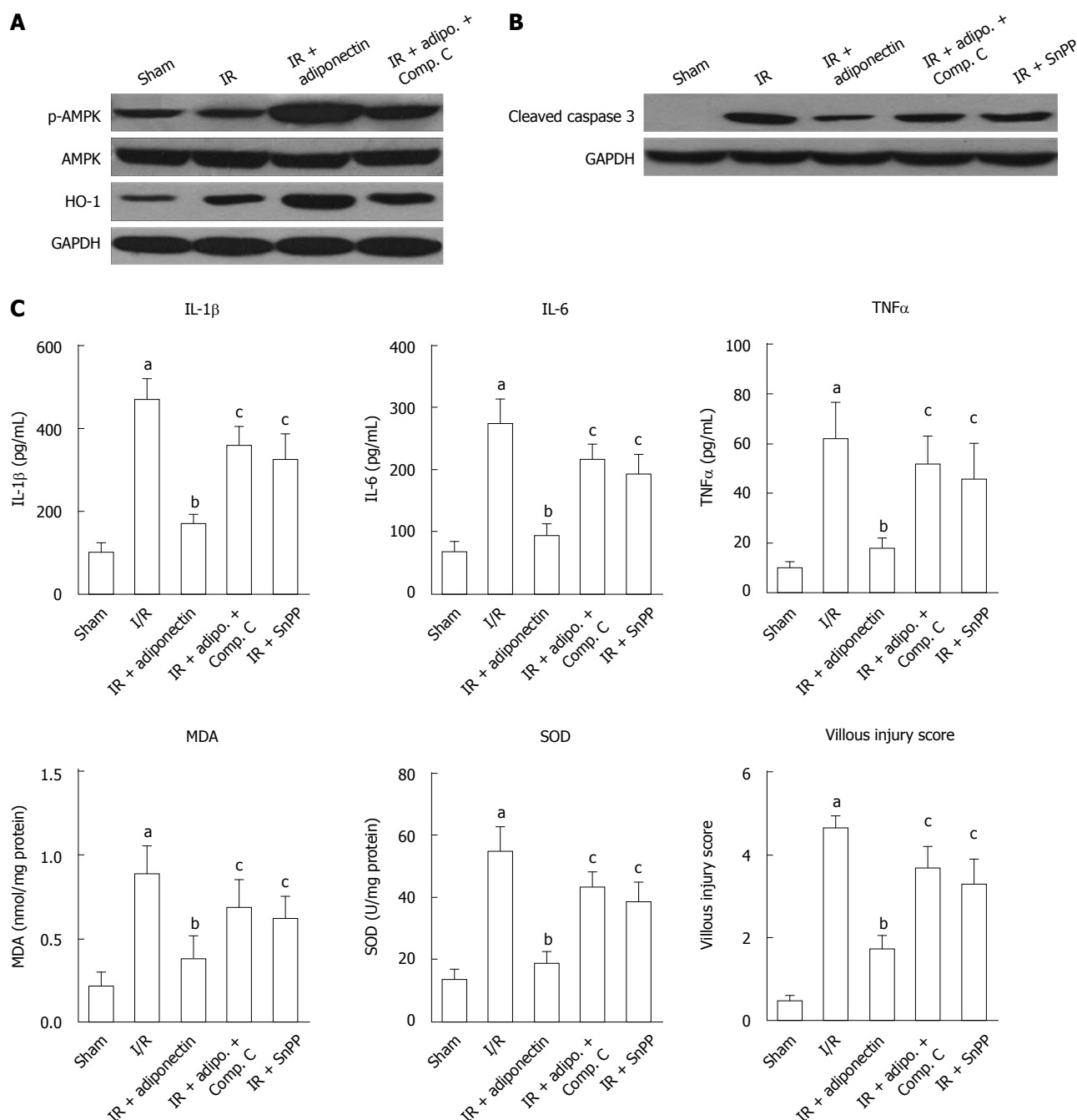


Figure 3 AMPK inhibition attenuated ischemia reperfusion injury. A: Western blot analysis of the expression of p-AMPK and HO-1; B: Western blot analysis of the expression of cleaved caspase 3; C: The serum levels of IL-1 β , IL-6, TNF- α , MDA, and SOD and the villus damage score. Compound C: AMPK signaling pathway inhibitor; Snpp, HO-1 inhibitor. Adipo: Adiponectin; Compo. C: Compound C; The data are expressed as mean \pm SD obtained from 5 independent experiments. ^a $P < 0.05$ vs sham, ^b $P < 0.05$ vs IR, ^c $P < 0.05$ vs IR + adiponectin. I/R: Ischemia reperfusion; HO-1: Heme oxygenase 1; SOD: Superoxide dismutase; MDA: Malondialdehyde.

against cellular injury^[30]. Previous studies have shown that adiponectin upregulated HO-1 expression and exerted beneficial effects in the protection of renal I/R injury^[25,31]. It has also been reported that increased HO-1 expression by adiponectin played an anti-apoptotic role in iron-induced liver injury^[32]. The present study showed that the expression of HO-1 was increased in rats with intestinal I/R injury and adiponectin pre-treatment. These results support the involvement of HO-1 in cellular injury protection. In addition, a previous study suggested that the anti-

apoptotic effect of AMPK mediated by HO-1 may provide an important adaptive response to preserve endothelial cell viability during metabolic stress^[33]. Here, we found that the expression of p-AMPK and HO-1 was inhibited by compound C, suggesting that the AMPK-HO-1 signaling pathway was involved in the protective effects of adiponectin against intestinal I/R injury.

AdipoR is involved in various biological effects of adiponectin, and is closely related to obesity, diabetes, and cardiovascular disease^[34]. In different

tissues, the expression levels and the regulating factors of AdipoR are different. Adiponectin receptor expression is elevated in colorectal carcinoma but not in gastrointestinal stromal tumors^[35]. The expression of placental adiponectin is increased during pregnancy in the rat, while Adipo R2 shows the opposite pattern. In this study, protein levels of Adipo R1 and Adipo R2 in the I/R and I/R + adiponectin groups did not change significantly, while p-AMPK and HO-1 increased in the I/R + adiponectin group compared to the I/R group which are consistent with the results of Chen *et al.*^[25] and Cheng *et al.*^[25]. However, it is unclear whether it is Adipo R1, AdipoR 2, or their combination that have an effect on the protein level of p-AMPK and HO-1. Perhaps the function of AdipoR is not dependent on the protein level in intestinal I/R injury. In the future, we intend to conduct experiments to understand further this mechanism.

In summary, we showed that serum adiponectin was downregulated in the rat model of intestinal I/R injury, and adiponectin pre-treatment attenuated intestinal I/R injury in rats. While the underlying mechanism of adiponectin in protection against intestinal I/R injury is not fully understood, the present results suggest that the AMPK/HO-1 signaling pathway may be involved in this process. Therefore, adiponectin and components of the AMPK/HO-1 signaling pathway may be promising targets in the treatment of intestinal I/R injury.

COMMENTS

Background

It has been reported that adiponectin protects against ischemia reperfusion (I/R) injury in several tissues. This study aimed to determine the potential protective role of adiponectin in intestinal I/R injury.

Research frontiers

Previous studies have shown that oxidative stress, the inflammatory response, and cell apoptosis are involved in I/R injury. Interestingly, a number of studies have also reported the role of adiponectin in protection against I/R injury in myocardial, cerebral, liver, and renal tissues. However, whether adiponectin functions in the prevention of intestinal I/R injury is unknown.

Innovations and breakthroughs

This study showed that serum adiponectin was downregulated in the rat model of intestinal I/R injury, and adiponectin pre-treatment attenuated intestinal I/R injury in rats. While the underlying mechanism of adiponectin in protection against intestinal I/R injury is not fully understood, the results of this study suggest that the AMP-activated protein kinase (AMPK)/heme oxygenase 1 (HO-1) signaling pathway may be involved in this process. Therefore, adiponectin and components of the AMPK/HO-1 signaling pathway may be promising targets in the treatment of intestinal I/R injury.

Applications

Adiponectin and components of the AMPK/HO-1 signaling pathway may be promising targets in the treatment of intestinal I/R injury.

Terminology

Intestinal I/R injury is a critical complication in patients with trauma and after liver and intestinal transplantation, and it is associated with high morbidity and mortality. Adiponectin, also referred to as gelatin-binding protein-28, ACRP30, AdipoQ, or apM1, is a hormone secreted mainly by adipocytes.

Peer-review

The title is short, conclusive, and reflects the content of the study. The abstract is clear with obvious delineation between the various parts.

REFERENCES

- 1 Farmer DG, Venick RS, Colangelo J, Esmailian Y, Yersiz H, Duffy JP, Cortina GR, Artavia K, Ngo K, McDiarmid SV, Busuttil RW. Pretransplant predictors of survival after intestinal transplantation: analysis of a single-center experience of more than 100 transplants. *Transplantation* 2010; **90**: 1574-1580 [PMID: 21107306 DOI: 10.1097/TP.0b013e31820000a1]
- 2 Koike K, Moore FA, Moore EE, Read RA, Carl VS, Banerjee A. Gut ischemia mediates lung injury by a xanthine oxidase-dependent neutrophil mechanism. *J Surg Res* 1993; **54**: 469-473 [PMID: 8395621]
- 3 Vincenti M, Behrends M, Dang K, Park YH, Hirose R, Blasi-Ibanez A, Liu T, Serkova NJ, Niemann CU. Induction of intestinal ischemia reperfusion injury by portal vein outflow occlusion in rats. *J Gastroenterol* 2010; **45**: 1103-1110 [PMID: 20549254 DOI: 10.1007/s00535-010-0262-0]
- 4 Mallick IH, Yang W, Winslet MC, Seifalian AM. Ischemia-reperfusion injury of the intestine and protective strategies against injury. *Dig Dis Sci* 2004; **49**: 1359-1377 [PMID: 15481305]
- 5 Köksoy C, Kuzu MA, Kuzu I, Ergün H, Gürhan I. Role of tumour necrosis factor in lung injury caused by intestinal ischaemia-reperfusion. *Br J Surg* 2001; **88**: 464-468 [PMID: 11260117 DOI: 10.1046/j.1365-2168.2001.01737.x]
- 6 Marshall JC. The gut as a potential trigger of exercise-induced inflammatory responses. *Can J Physiol Pharmacol* 1998; **76**: 479-484 [PMID: 9839072]
- 7 Robin E, Guzy RD, Loor G, Iwase H, Waypa GB, Marks JD, Hoek TL, Schumacker PT. Oxidant stress during simulated ischemia primes cardiomyocytes for cell death during reperfusion. *J Biol Chem* 2007; **282**: 19133-19143 [PMID: 17488710 DOI: 10.1074/jbc.M701917200]
- 8 Xiao F, Eppihimer MJ, Young JA, Nguyen K, Carden DL. Lung neutrophil retention and injury after intestinal ischemia/reperfusion. *Microcirculation* 1997; **4**: 359-367 [PMID: 9329012]
- 9 Zweier JL, Talukder MA. The role of oxidants and free radicals in reperfusion injury. *Cardiovasc Res* 2006; **70**: 181-190 [PMID: 16580655 DOI: 10.1016/j.cardiores.2006.02.025]
- 10 Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. *Endocr Rev* 2005; **26**: 439-451 [PMID: 15897298 DOI: 10.1210/er.2005-0005]
- 11 Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE, Lodish HF. Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci USA* 2001; **98**: 2005-2010 [PMID: 11172066 DOI: 10.1073/pnas.041591798]
- 12 Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, Kadowaki T. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 2002; **8**: 1288-1295 [PMID: 12368907 DOI: 10.1038/nm788]
- 13 Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipodystrophy and obesity. *Nat Med* 2001; **7**: 941-946 [PMID: 11479627 DOI: 10.1038/90984]
- 14 Chen B, Liao WQ, Xu N, Xu H, Wen JY, Yu CA, Liu XY, Li CL, Zhao SM, Campbell W. Adiponectin protects against cerebral ischemia-reperfusion injury through anti-inflammatory action. *Brain Res* 2009; **1273**: 129-137 [PMID: 19362080 DOI: 10.1016/j.brainres.2009.04.002]

- 15 **Massip-Salcedo M**, Zaouali MA, Padrisa-Altés S, Casillas-Ramirez A, Rodés J, Roselló-Catafau J, Peralta C. Activation of peroxisome proliferator-activated receptor- α inhibits the injurious effects of adiponectin in rat steatotic liver undergoing ischemia-reperfusion. *Hepatology* 2008; **47**: 461-472 [PMID: 18098300 DOI: 10.1002/hep.21935]
- 16 **Shibata R**, Sato K, Pimentel DR, Takemura Y, Kihara S, Ohashi K, Funahashi T, Ouchi N, Walsh K. Adiponectin protects against myocardial ischemia-reperfusion injury through AMPK- and COX-2-dependent mechanisms. *Nat Med* 2005; **11**: 1096-1103 [PMID: 16155579 DOI: 10.1038/nm1295]
- 17 **Tao L**, Gao E, Jiao X, Yuan Y, Li S, Christopher TA, Lopez BL, Koch W, Chan L, Goldstein BJ, Ma XL. Adiponectin cardioprotection after myocardial ischemia/reperfusion involves the reduction of oxidative/nitrative stress. *Circulation* 2007; **115**: 1408-1416 [PMID: 17339545 DOI: 10.1161/CIRCULATIONAHA.106.666941]
- 18 **Zhang C**, Liao Y, Li Q, Chen M, Zhao Q, Deng R, Wu C, Yang A, Guo Z, Wang D, He X. Recombinant adiponectin ameliorates liver ischemia reperfusion injury via activating the AMPK/eNOS pathway. *PLoS One* 2013; **8**: e66382 [PMID: 23762489 DOI: 10.1371/journal.pone.0066382]
- 19 **Yoshiya K**, Lapchak PH, Thai TH, Kannan L, Rani P, Dalle Lucca JJ, Tsokos GC. Depletion of gut commensal bacteria attenuates intestinal ischemia/reperfusion injury. *Am J Physiol Gastrointest Liver Physiol* 2011; **301**: G1020-G1030 [PMID: 21903760 DOI: 10.1152/ajpgi.00239.2011]
- 20 **Serafin A**, Roselló-Catafau J, Prats N, Gelpí E, Rodés J, Peralta C. Ischemic preconditioning affects interleukin release in fatty livers of rats undergoing ischemia/reperfusion. *Hepatology* 2004; **39**: 688-698 [PMID: 14999687 DOI: 10.1002/hep.20089]
- 21 **Chiu CJ**, McArdle AH, Brown R, Scott HJ, Gurd FN. Intestinal mucosal lesion in low-flow states. I. A morphological, hemodynamic, and metabolic reappraisal. *Arch Surg* 1970; **101**: 478-483 [PMID: 5457245]
- 22 **Mihara M**, Uchiyama M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem* 1978; **86**: 271-278 [PMID: 655387]
- 23 **Maruyama S**, Shibata R, Ohashi K, Ohashi T, Daida H, Walsh K, Murohara T, Ouchi N. Adiponectin ameliorates doxorubicin-induced cardiotoxicity through Akt protein-dependent mechanism. *J Biol Chem* 2011; **286**: 32790-32800 [PMID: 21784858 DOI: 10.1074/jbc.M111.245985]
- 24 **Shu RZ**, Zhang F, Wang F, Feng DC, Li XH, Ren WH, Wu XL, Yang X, Liao XD, Huang L, Wang ZG. Adiponectin deficiency impairs liver regeneration through attenuating STAT3 phosphorylation in mice. *Lab Invest* 2009; **89**: 1043-1052 [PMID: 19564844 DOI: 10.1038/labinvest.2009.63]
- 25 **Cheng CF**, Lian WS, Chen SH, Lai PF, Li HF, Lan YF, Cheng WT, Lin H. Protective effects of adiponectin against renal ischemia-reperfusion injury via prostacyclin-PPAR α -heme oxygenase-1 signaling pathway. *J Cell Physiol* 2012; **227**: 239-249 [PMID: 21412771 DOI: 10.1002/jcp.22726]
- 26 **Molmenti EP**, Ziambaras T, Perlmutter DH. Evidence for an acute phase response in human intestinal epithelial cells. *J Biol Chem* 1993; **268**: 14116-14124 [PMID: 7686149]
- 27 **Chien CT**, Lee PH, Chen CF, Ma MC, Lai MK, Hsu SM. De novo demonstration and co-localization of free-radical production and apoptosis formation in rat kidney subjected to ischemia/reperfusion. *J Am Soc Nephrol* 2001; **12**: 973-982 [PMID: 11316856]
- 28 **Young LH**, Li J, Baron SJ, Russell RR. AMP-activated protein kinase: a key stress signaling pathway in the heart. *Trends Cardiovasc Med* 2005; **15**: 110-118 [PMID: 16039971 DOI: 10.1016/j.tcm.2005.04.005]
- 29 **Guarnieri C**, Flamigni F, Calderara CM. Role of oxygen in the cellular damage induced by re-oxygenation of hypoxic heart. *J Mol Cell Cardiol* 1980; **12**: 797-808 [PMID: 7420425]
- 30 **Zweier JL**. Measurement of superoxide-derived free radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury. *J Biol Chem* 1988; **263**: 1353-1357 [PMID: 2826476]
- 31 **Tracz MJ**, Juncos JP, Croatt AJ, Ackerman AW, Grande JP, Knutson KL, Kane GC, Terzic A, Griffin MD, Nath KA. Deficiency of heme oxygenase-1 impairs renal hemodynamics and exaggerates systemic inflammatory responses to renal ischemia. *Kidney Int* 2007; **72**: 1073-1080 [PMID: 17728706 DOI: 10.1038/sj.ki.5002471]
- 32 **Lin H**, Yu CH, Jen CY, Cheng CF, Chou Y, Chang CC, Juan SH. Adiponectin-mediated heme oxygenase-1 induction protects against iron-induced liver injury via a PPAR α dependent mechanism. *Am J Pathol* 2010; **177**: 1697-1709 [PMID: 20709802 DOI: 10.2353/ajpath.2010.090789]
- 33 **Liu XM**, Peyton KJ, Shebib AR, Wang H, Korthuis RJ, Durante W. Activation of AMPK stimulates heme oxygenase-1 gene expression and human endothelial cell survival. *Am J Physiol Heart Circ Physiol* 2011; **300**: H84-H93 [PMID: 21037234]
- 34 **Sowers JR**. Endocrine functions of adipose tissue: focus on adiponectin. *Clin Cornerstone* 2008; **9**: 32-38; discussion 39-40 [PMID: 19046738]
- 35 **Williams CJ**, Mitsiades N, Sozopoulos E, Hsi A, Wolk A, Nifli AP, Tseleni-Balafouta S, Mantzoros CS. Adiponectin receptor expression is elevated in colorectal carcinomas but not in gastrointestinal stromal tumors. *Endocr Relat Cancer* 2008; **15**: 289-299 [PMID: 18310295 DOI: 10.1677/ERC-07-0197]
- 36 **Chen H**, Zhang L, Li X, Li X, Sun G, Yuan X, Lei L, Liu J, Yin L, Deng Q, Wang J, Liu Z, Yang W, Wang Z, Zhang H, Liu G. Adiponectin activates the AMPK signaling pathway to regulate lipid metabolism in bovine hepatocytes. *J Steroid Biochem Mol Biol* 2013; **138**: 445-454 [PMID: 23994141]

P- Reviewer: Hei ZQ, Shafik AN **S- Editor:** Yu J

L- Editor: Filipodia **E- Editor:** Zhang DN



Basic Study

Evaluation of epithelial-mesenchymal transitioned circulating tumor cells in patients with resectable gastric cancer: Relevance to therapy response

Ting-Ting Li, Hao Liu, Feng-Ping Li, Yan-Feng Hu, Ting-Yu Mou, Tian Lin, Jiang Yu, Lei Zheng, Guo-Xin Li

Ting-Ting Li, Hao Liu, Feng-Ping Li, Yan-Feng Hu, Ting-Yu Mou, Tian Lin, Jiang Yu, Guo-Xin Li, Department of General Surgery, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China

Lei Zheng, Department of Laboratory Medicine, Sino-UK Circulating Biomarkers' Exploration and Detection Centre, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China

Author contributions: Li TT, Liu H and Yu J contributed equally to this work and should be considered as co-first authors; Li TT, Liu H and Li GX designed the research; Lin T, Mou TY, Hu YF, Yu J and Li GX performed the surgeries and collected the data; Li TT, Li FP and Zheng L performed the experiment; Li TT and Liu H analyzed the data; Li TT, Liu H and Yu J wrote the paper.

Supported by Major Program of Science and Technology Program of Guangzhou, No. 201300000087; Research Fund of Public Welfare in Health Industry of National Health and Family Planning Commission of China, No. 201402015 and No. 201502039; National Key Technology R&D Program, No. 2013BAI05B05; and Key Clinical Specialty Discipline Construction Program.

Institutional review board statement: The study protocol was reviewed and approved by Institutional Review Board of Nanfang Hospital.

Institutional animal care and use committee statement: The present study did not involve animal experiments.

Conflict-of-interest statement: The authors do not have any conflict of interest to disclose.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on

different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Guo-Xin Li, MD, PhD, FRCS, Department of General Surgery, Nanfang Hospital, Southern Medical University, 1838 North Guangzhou Avenue, Guangzhou 510515, Guangdong Province, China. [gzlguoxin@163.com](mailto:gzglguoxin@163.com)
Telephone: +86-20-61641681
Fax: +86-20-62787626

Received: July 31, 2015
Peer-review started: August 1, 2015
First decision: August 26, 2015
Revised: August 31, 2015
Accepted: October 17, 2015
Article in press: October 20, 2015
Published online: December 21, 2015

Abstract

AIM: To evaluate the epithelial-to-mesenchymal transition (EMT) of circulating tumor cells (CTCs) in gastric cancer patients.

METHODS: We detected tumor cells for expression of four epithelial (E⁺) transcripts (keratins 8, 18, and 19 and epithelial cell adhesion molecule) and two mesenchymal (M⁺) transcripts (Vimentin and Twist) by a quantifiable, dual-colorimetric RNA-*in situ* hybridization assay. Between July 2014 and October 2014, 44 patients with gastric cancer were recruited for CTC evaluation. Blood samples were obtained from selected patients during the treatment course [before surgery, after surgery and at the 6th cycle of XELOX based chemotherapy (about 6 mo postoperatively)].

RESULTS: We found the EMT phenomenon in which there were a few biphenotypic E⁺/M⁺ cells in primary

human gastric cancer specimens. Of the 44 patients, the presence of CTCs was reported in 35 (79.5%) patients at baseline. Five types of cells including from exclusively E⁺ CTCs to intermediate CTCs and exclusively M⁺ CTCs were identified (4 patients with M⁺ CTCs and 10 patients with M⁺ or M⁺ > E⁺ CTCs). Further, a chemotherapy patient having progressive disease showed a proportional increase of mesenchymal CTCs in the post-treatment blood specimens. We used NCI-N87 cells to analyze the linearity and sensitivity of CanPatrol™ system and the correlation coefficient (R²) was 0.999.

CONCLUSION: The findings suggest that the EMT phenomenon was both in a few cells of primary tumors and abundantly in CTCs from the blood of gastric cancer patients, which might be used to monitor therapy response.

Key words: Gastric cancer; Epithelial-to-mesenchymal transition; Circulating tumor cells; Chemotherapy; Therapy response

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Epithelial-to-mesenchymal transition has been thought to play a critical role in tumor metastatic progression in preclinical models, however, characterizing the epithelial vs mesenchymal phenotypes of circulating tumor cells has been challenging. In this study, we aimed to evaluate epithelial-to-mesenchymal transition phenomenon in circulating gastric tumor cells by a combination of physical and biological methods. Our findings have provided evidence of the phenomenon both in rare cells within primary tumors and more abundantly in circulating tumor cells. Furthermore, we demonstrated that the evaluation of the mesenchymal circulating tumor cells in peripheral blood can be used to monitor therapy response in gastric cancer patients.

Li TT, Liu H, Li FP, Hu YF, Mou TY, Lin T, Yu J, Zheng L, Li GX. Evaluation of epithelial-mesenchymal transitioned circulating tumor cells in patients with resectable gastric cancer: Relevance to therapy response. *World J Gastroenterol* 2015; 21(47): 13259-13267 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13259.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13259>

INTRODUCTION

Gastric cancer is a serious public health concern in East Asia, South America and Eastern Europe, accounting for more than 950000 new cases per year (China alone accounts for 42% of new cases worldwide), and it is the third cause of cancer death all around the world (GLOBOCAN 2012)^[1]. Because mass

screening is rarely practiced worldwide except Japan and Korea, gastric cancer is often diagnosed at an advanced stage. Like common cancers, most gastric cancer-related deaths result from metastasis^[2], which is rarely predictable by standard imaging work-ups like positron emission tomography/computed tomography scans or tumor markers tests. Circulating tumor cells (CTCs) originating from solid tumors are related with the course of hematogenous metastatic spread to the distant sites^[3], exemplifying the switch from localized to systemic disease. Therefore, evaluating CTCs has clinical relevance in the monitoring and the outcomes of metastatic tumors. The recent discoveries on CTCs demonstrate how these cells are related with hematogenous metastasis, with an opinion on the epithelial-mesenchymal transition (EMT) phenomenon^[4]. The investigation by Yu *et al*^[5] found dynamic changes in the number of epithelial and mesenchymal CTCs in breast cancer patients as well as the potential of monitoring therapy response.

It was thought that EMT phenomenon played a critical role in tumor metastatic progression in preclinical models^[6,7], however, characterizing the epithelial vs mesenchymal phenotypes of CTCs has been challenging. Increasing evidence coming from clinical setting of CTCs supports the phenomenon of the EMT in human tumors. Accordingly, we are exploring the methods to identify the unique stem CTC subpopulation^[7], and its significance is further emphasized by recent findings suggesting the occurrence of mesenchymal markers in tumor tissues as a poor prognostic factor in many cancers^[5,8,9]. Furthermore, sequential analysis of CTCs, so called "liquid biopsy", may provide clinical significance on the effectiveness and progression of systemic therapies and consequently would facilitate "tailor-made" therapeutic strategies^[10,11].

To date, the CellSearch System is the only FDA-cleared CTC enumeration assay, which defines a CTC according to its size, positivity for epithelial cell adhesion molecule (EpCAM) and CK, and negativity of CD45 expression^[12]. The current techniques besides the CellSearch System are able to isolate CTCs by epithelial markers, however, these epithelial markers based methods most likely overlook a subpopulation of CTCs undergoing EMT^[13,14]. Thus, the new CTC capture systems should be essential to isolate the cell subpopulation with mesenchymal phenotype. To our knowledge, there have been few reports regarding the detecting methods and clinical significance of mesenchymal CTCs in cancer patients, specifically gastric cancer.

In the present study, we adopted two mesenchymal transcripts, Vimentin and Twist, to detect mesenchymal phenotypes of CTCs and tumor tissues in advanced gastric cancer, which have been reported as sensitive markers to detect them^[12]. Accordingly, the EMT phenomenon of CTCs in advanced gastric cancer and

its relationship with chemotherapy response were evaluated as well.

MATERIALS AND METHODS

Gastric cancer cell line

We used the NCI-N87 cell line for the analysis. NCI-N87 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 µg/mL of streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂.

Patients and study design

Between July 2014 and October 2014, 44 patients with newly diagnosed gastric cancer in our institution, without receiving neoadjuvant chemo- or radiotherapy, were prospectively enrolled in the study. The blood samples from our selected patients were acquired during the treatment course [before surgery, after surgery and at the 6th cycle of XELOX based chemotherapy (about 6 mo postoperatively)^[15]] and analyzed with CanPatrol™ System (Surexam Biotech, Guangzhou, China)^[16]. The blood samples from ten healthy volunteers were used as controls.

Isolation and enumeration of CTCs (CanPatrol™ system)

For the gastric cancer patients, 5 mL peripheral blood samples were collected in EDTA tubes by venipuncture and filtered with a 8-µm diameter pores calibrated membrane (Millipore, Billerica, MA, United States)^[16]. The required filtration system included a filtration tube containing the membrane (SurExam, Guangzhou, China), a manifold vacuum plate with valve settings (SurExam, Guangzhou, China), an E-Z 96 vacuum manifold (Omega, Norcross, GA, United States), and a vacuum pump (Auto Science, Tianjin, China). Before filtration, red blood cell lysis buffer was used to remove erythrocyte, then PBS with 4% formaldehyde (Sigma, St. Louis, MO, United States) was used to resuspend the remaining cells for 5 min. The pumping pressure is 0.08 MPa.

We established three groups of nucleic acid probes to identify and examine the expression levels of epithelial and mesenchymal genes in CTCs by a multiplex RNA-*in situ* hybridization (RNA-ISH) assay. Group 1 probes contained four pooled epithelial transcripts [keratins (KRT) 8, 18 and 19; EpCAM]. Group 2 probes had two mesenchymal transcripts (Vimentin and Twist). The last group only contained a CD45 transcript which was used to discriminate white blood cells and CTCs. The detail hybridization assay procedure followed the published literature^[16]. Briefly, the cells retained on the filter were permeabilized and digested with protease. And then, the cells were subjected to a serial of hybridization reactions with a cocktail probe specific to the intended examined genes described above. Finally, we used 4',6-diamidino-2-phenylindole (DAPI) to stain the cell nucleus.

The samples were analyzed with a fluorescent microscope. The red and green dots of fluorescent signal observed in the cells represented the epithelial and mesenchymal gene expression, respectively. The purple fluorescent dots represented the CD45 gene expression (the markers of white blood cells). The assays were applied in both selected primary human gastric cancer specimens and all blood specimens.

Statistical analysis

Data are presented as median and range (or mean ± SD) for continuous variables or as frequencies and proportions for categorical variables. Mann-Whitney *U* test and the Kruskal-Wallis *H* test were used to analyze the relationship between CTC count at baseline and tumor stage. The relationship between CTC count and the molecular classification of primary tumor according to Her2 status was examined using Spearman's rank correlation coefficient. All statistical calculations were performed with SPSS for Windows release 18.0. A *P* value < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

Totally 44 patients with gastric cancer (median age 56 years, range 25-87 years) were enrolled in this study. These patients consisted of 6 (13.6%) patients with gastric signet-ring cell carcinoma (SRCC) and 38 (86.4%) patients with gastric adenocarcinoma. Of these, 11 patients (7 men and 4 women) had early gastric cancer diagnosed pathologically as American Joint Committee on Cancer stage IA and IB. All 37 patients underwent laparoscopic gastrectomy with D2 lymphadenectomy; the remaining 7 patients with unresectable tumors were treated by laparoscopic exploration or palliative surgery. Thirty-six patients had no detectable overt metastasis (stage M0), and 5 patients presented with existing synchronous metastases in peritoneal carcinosis (Table 1). CTCs were detected in 35 (79.5%) patients from the gastric cancer group and the average count was 7.25 CTCs. No CTCs were detected in all of the samples from healthy volunteers (Figure 1). With regard to clinicopathological features, 11 (37.1%) patients had lymphatic emboli and elevated Her2 levels (+++ or ++) were detected in 9 (22%) gastric cancer patients.

Detection of EMT markers in primary tumors and draining lymph nodes

We first applied the RNA-ISH assays to primary human gastric cancer specimens. There were a small number of biphenotypic E⁺/M⁺ cells among the majority of E⁺ cancer cells with clear epithelial histology of primary tumors (Figure 2A). However, we only found E⁺ cancer cells in draining lymph nodes (Figure 2B).

Table 1 Characteristics of gastric cancer patients *n* (%)

	All	CanPatrol	
		Positive, <i>n</i> /total	<i>P</i> value
Median age, yr (range)	56 (25-87)		
Gender			0.780
Male	31	25/31 (80.6)	
Female	13	10/13 (76.9)	
Post-TNM stage			
Tumor			0.799
T1	11	9/11 (81.2)	
T2	4	3/4 (75)	
T3	5	4/5 (80)	
T4	21	19/21 (90.5)	
Node			0.710
N0	22	17/22 (77.3)	
N+	19	18/19 (94.7)	
Metastasis			0.115
M0	36	30/36 (83.3)	
M1	5	5/5 (100)	
Site of metastases			
Peritoneal carcinosis	5	5/5 (100)	
Histology			0.400
Adenocarcinoma	38	31/38 (81.6)	
Signet-ring cell carcinoma	6	4/6 (66.7)	
Lymphatic emboli			0.461
No	21	17/21 (81)	
Yes	11	10/11 (90.9)	
Her2			0.303
+++	2	2/2 (100)	
++	7	7/7 (100)	
+	12	11/12 (91.7)	
0	20	15/20 (75)	

T1: Tumor invades lamina propria or submucosa; T2: Tumor invades muscularis propria or subserosa; T3: Tumor penetrates serosa (visceral peritoneum) without invasion of adjacent structures; T4: Tumor invades adjacent structures; N0: No regional lymph node metastasis; N+: Lymph node metastasis; M0: No distant metastasis; M1: Distant metastasis.

Correlation of CTC detection with clinico-histopathologic risk factors

We also analyzed blood samples from 44 gastric cancer patients. Using the RNA-ISH assays, we defined five types of cells including from exclusively epithelial (E⁺) CTCs to intermediate (E⁺ > M⁺, E⁺ = M⁺, M⁺ > E⁺) CTCs and exclusively mesenchymal (M⁺) CTCs from blood samples (Figure 3A). Of 35 patients, four patients with M⁺ CTCs and ten patients with M⁺ or M > E CTCs were observed. CTCs had been captured in 35 (79.5%) patients, with EMT features varying according to histological subtype (SRCC and adenocarcinoma) and molecular classification (Her2⁻ and Her2⁺) (Figure 3B). Among them, nine (25.7%) patients were detected with Her2⁺ gastric cancer, and 26 (74.3%) patients with Her2⁻ gastric cancer. The CTCs from patients with both SRCC and adenocarcinoma were predominantly mesenchymal phenotypes. Furthermore, we compared the CTC counts from patients with Her2⁺ vs Her2⁻ in primary tumors but observed no statistically significant correlations with the number of E⁺/M⁺ cells.

EMT of CTCs and therapeutic response

To test the possibility of the ratio of mesenchymal

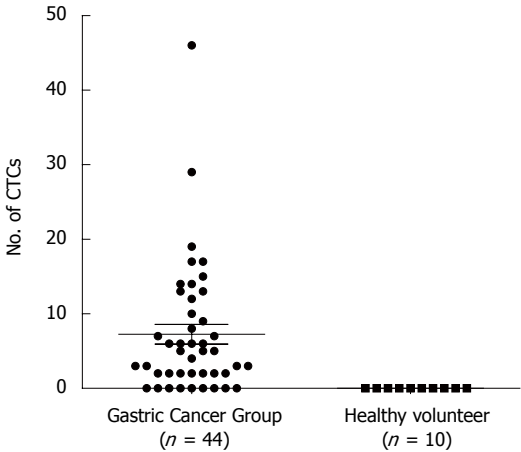


Figure 1 Circulating tumor cells were detected in 35 (79.5%) patients from gastric cancer group. The average number of circulating tumor cells (CTCs) was 7.25 in the group. CTCs were not observed in any samples from healthy volunteers.

(M) to epithelial (E) content serving as an indicator of therapeutic response, we combined both the CTC counts and mesenchymal features of CTCs (Figure 4). Sequential blood samples were obtained from four patients undergoing adjuvant chemotherapy (XELOX regimen, capecitabine plus oxaliplatin). Three patients showed a decrease in CTC counts and/or a proportional decrease in mesenchymal feature in post-treatment samples at the 6th cycle adjuvant chemotherapy, compared with those in the post-surgery samples (Figure 4A-C). In contrast, one patient who had progressive disease evaluated by a CT scan showed an increased number or proportion of mesenchymal CTCs in the post-treatment specimen (Figure 4D).

Sensitivity of the CanPatrol™ system

We used NCI-N87 cells to analyze the sensitivity and linearity of the CanPatrol™ system. We spiked 10, 50, 100 and 200 NCI-N87 cells into 5 mL of blood to get the recovery of the cells (Figure 5). The correlation coefficient (R²) was 0.999.

DISCUSSION

Because of the minimally invasive feature of obtaining sequential blood specimens from cancer patients as well as potential clinical implications of the CTCs, seeking for methods to isolate and analyze CTCs for diagnosis, prognosis evaluation, and monitoring of cancer patients has been highlighted in recent years^[17]. There are several underlying benefits for successful CTCs/circulating tumor DNA-based diagnostics, due to the ability to obtain sequential blood samples from cancer patients throughout the treatment course^[18]. That is, characterizing CTCs may potentially provide clinicians with: (1) biomarkers predictive of therapy resistance^[19,20]; (2) an independent biomarker of prognosis^[3,21-23]; and (3) an indicator for early relapse^[24], as well as materials for the evaluation of

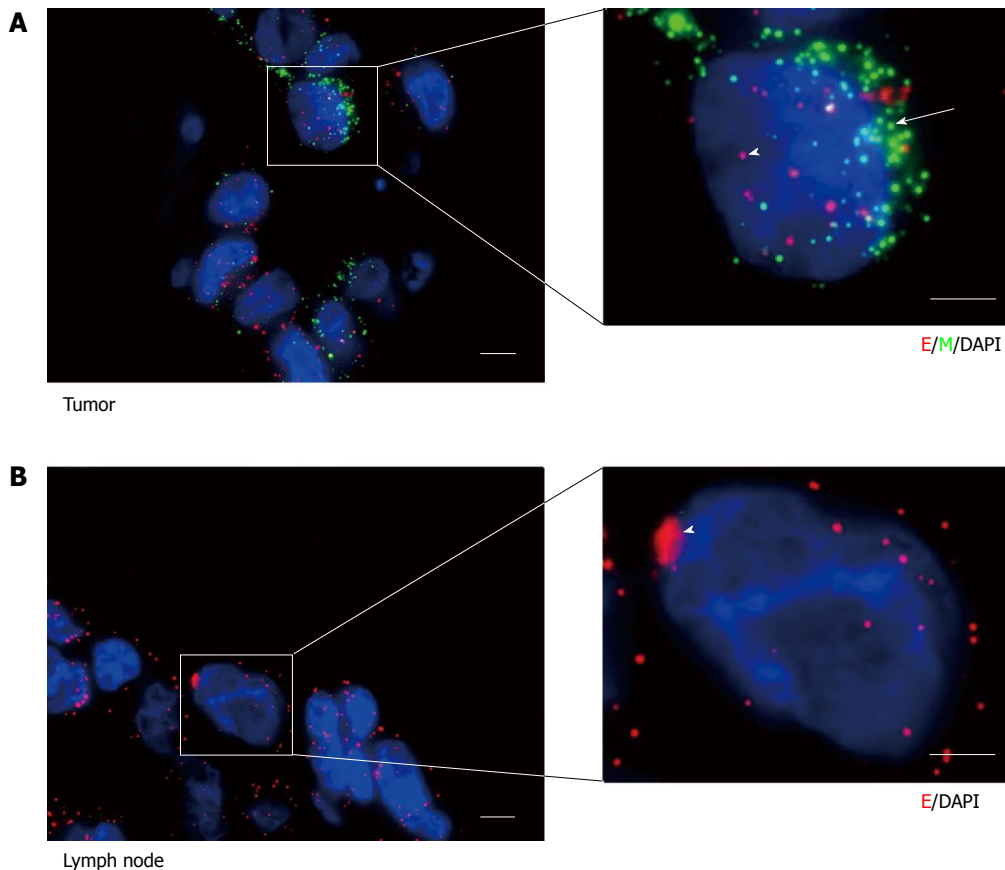


Figure 2 RNA-*in situ* hybridization analysis of epithelial-to-mesenchymal transition markers in human gastric tumors. The probes were constructed as described in the method section. Representative RNA-*in situ* hybridization analysis of pooled epithelial (E) (red dots, arrowheads) and mesenchymal (M) (green dots, arrows) markers in (A) primary tumor and (B) tumor-infiltrated lymph node of a patient with gastric cancer. Scale bars: (A) to (B), 20 mm; inserts, 10 mm.

therapy resistance^[17,25,26]. Currently, CTC enumerations have been widely admitted as an independent prognostic biomarker for a few tumors, however, their tentative roles as predictive biomarkers that may influence treatment decisions have been highly elusive and challenging to carry out, partially attributing to the extreme exiguity of CTCs in the peripheral circulation.

CTC enumeration

CellSearch System was used in the majority of published studies, which depends on epithelial tumor cell expression of KRT 8, 18, and 19 and EpCAM, presence of an intact nucleus (DAPI), and absence of the leukocyte marker CD45. However, because of the heterogeneity of CTCs, most current platforms overlooked mesenchymal-like CTCs, on which mesenchymal markers, such as Vimentin and N-cadherin, are upregulated^[4]. Like a recent published report^[5], a novel technology with a combination of epithelial and mesenchymal markers was used in our study, resulting in an increase of ten percent (4 patients with mesenchymal CTCs) in gastric CTC enumeration as well as identifying 10 more patients with intermediate phenotypes (biphenotypic E⁺/M⁺ cells). The approaches of CTC isolation or enrichment can be mainly categorized into two groups: physical

methods and biological methods^[14]. Perhaps using more than one technique, limited isolation may be improved by the inclusion of physical method-based isolation of CTCs, which may help to specifically target CTCs. Besides the biological method (immunological antibody-based capture of CTCs), we simultaneously used a filtration-based approach (a physical method, an 8 μ m filtration tube in the present study)^[16] for CTC isolation, further increasing sensitivity and specificity.

Mesenchymal CTC detection and the EMT phenomenon

The vital technical challenge for CTC research comes from the rarity of tumor cells in blood. Most CTC technologies rely on the expression of epithelial markers (EpCAM-positive and keratins-positive cells) by tumor cells for their capture. But considerable disparity exists between the numbers of "epithelial" CTCs detected in different cancer types, perhaps because of a subpopulation of CTCs undergoing EMT, linked to their stemness^[27]. Studies aiming to research the EMT phenomenon of CTCs have revealed high expression levels of mesenchymal markers such as AKT2, TWIST, PIK3 α , N-cadherin and Vimentin^[28]. Mesenchymal CTCs were correlated with cancer prognosis and therapy resistance in several cancer types^[5,9,29]. While there exist few studies on the EMT

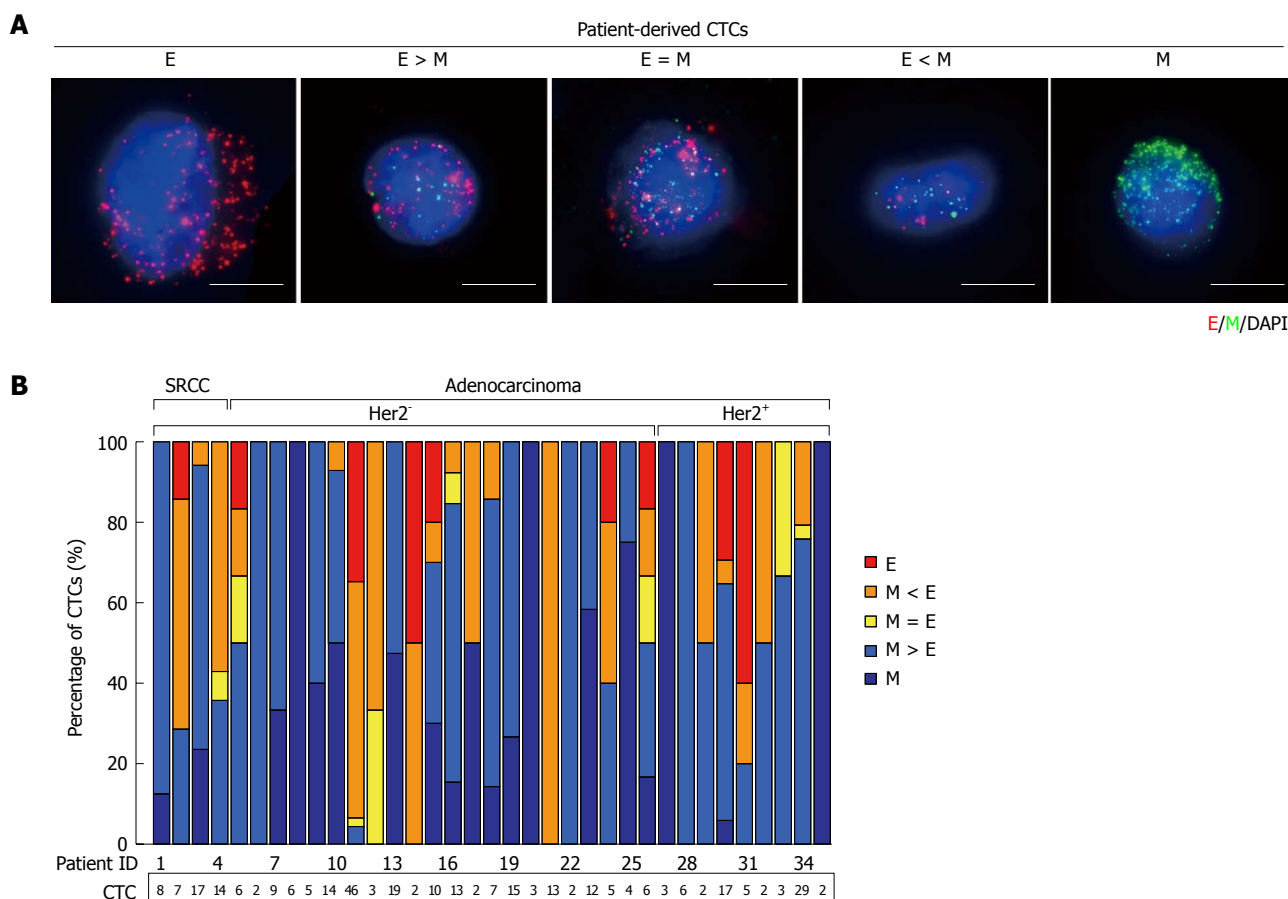


Figure 3 RNA-*in situ* hybridization analysis of epithelial-to-mesenchymal transition markers in circulating tumor cells from patients with gastric cancer. A: Representative images of five types of CTCs isolated from patients with gastric cancer, based on RNA-ISH staining of E (red dots) and M (green dots) markers. Scale bar, 5 μ m; B: Quantitation of EMT features in CTCs based on E and M RNA-ISH staining of histological subtypes of gastric cancer (SRCC and Adenocarcinoma), along with molecular classification of primary tumors (Her2⁻ and Her2⁺). CTC numbers per 10 mL of blood are listed below. RNA-ISH: RNA-*in situ* hybridization; CTCs: Circulating tumor cells; E: Epithelial; M: Mesenchymal; EMT: Epithelial-to-mesenchymal transition; DAPI: 4',6-diamidino-2-phenylindole.

phenomenon in CTCs specifically in gastric cancer, it is currently a distinct research focus. In the present study, we attempted at detecting and characterizing the EMT phenomenon in CTCs and gastric tumor tissues in clinical settings by using mesenchymal CTC markers (Vimentin and Twist). We found that human primary gastric tumor tissues contain scarce tumor cells that express epithelial and mesenchymal markers, but not in lymph node metastasis. In addition, the presence of CTCs bearing a mesenchymal phenotype has also been detected in the present study, which highlights the heterogeneity present in the circulating gastric tumor cells. Although there was an obvious increase in the number of mesenchymal CTCs in late-stage gastric cancer (data now shown), these data did not show a statistically significant difference in our analysis, which might be due to the relative small sample size. A large scale trial with higher statistic power is warranted.

Mesenchymal CTCs and monitoring chemotherapy

CTCs may be the promise of serving as "liquid biopsies" for tumors with the potential for providing information predictive of response and chemotherapy resistance.

Several reports have demonstrated the ratio of epithelial to mesenchymal markers on CTCs can be used to monitor the likeliness of therapy response. Yu and colleagues^[5] found a subpopulation of CTCs with a mixed epithelial-mesenchymal phenotype at baseline and the mesenchymal phenotype was observed at stages of disease progression (suppressed at stages of treatment response), further implicating mesenchymal CTCs in the metastatic progression. Additionally, Satelli *et al*^[9] suggested that CTC enumeration from a combination of EpCAM and Vimentin-based methods appeared to be a strong and reliable predictor for therapeutic outcomes in metastatic breast cancer with chemotherapy. Likewise, we compared CTC features in serial blood samples from four patients who underwent D2 gastrectomy (pre-operative, post-operative and post-adjuvant chemotherapy). One case who had progressive disease after 6 cycles of XELOX regimen showed the phenotypic changes in post-adjuvant chemotherapy specimen, compared with pre-treatment, showing an increased numbers of mesenchymal CTCs (Twist and Vimentin upregulated). The remaining three cases who responded to therapy showed a decrease in CTC counts and/or a

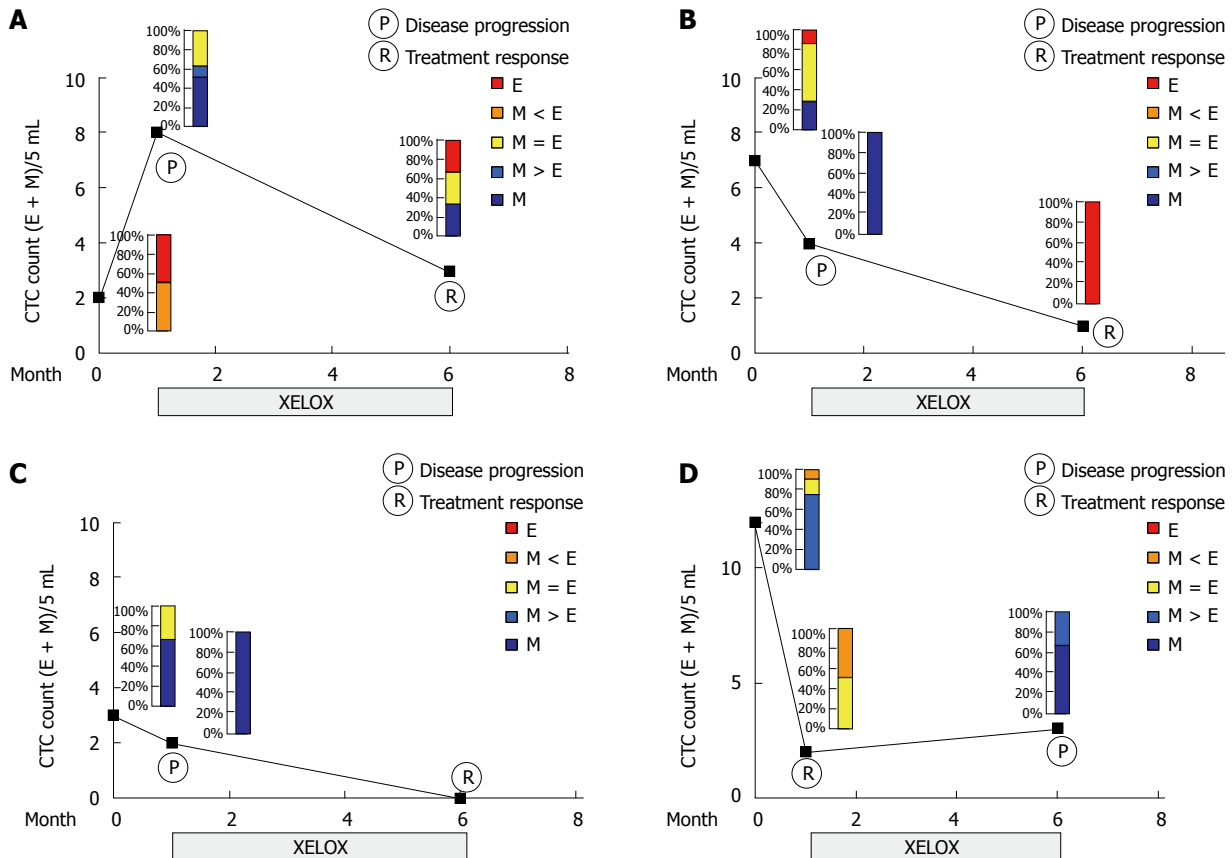


Figure 4 Longitudinal monitoring of epithelial-to-mesenchymal transition features in circulating tumor cells from a patient with resectable gastric cancer following surgery and adjuvant chemotherapy. CTC: Circulating tumor cell; E: Epithelial; M: Mesenchymal.

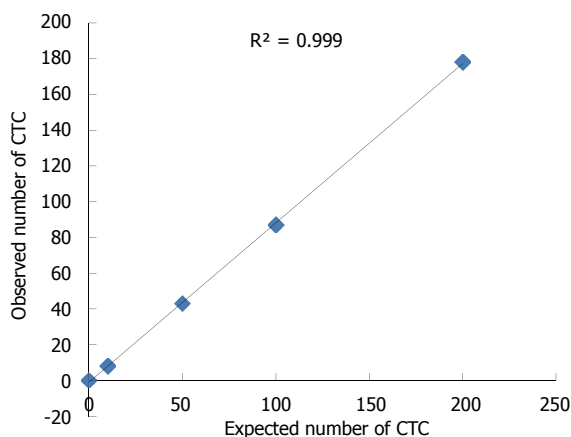


Figure 5 Regression analysis of the number of observed tumor cells vs the number of expected tumor cells produced a correlation coefficient (R^2) of 0.999. Even a single cell spiked into the samples was detected using this system. CTC: Circulating tumor cell.

proportional decrease in mesenchymal CTCs. Notably, one case who underwent curable resection surgery showed an increased number of mesenchymal CTCs in post-operative samples compared with pre-operative one, suggesting that surgical operation may play a critical role in the detachment of primary tumor cells to the peripheral circulation^[30]. Therefore, adjuvant therapy should be highlighted to reduce the risk of

hematogenous metastasis even after curable resection in selected patients.

Limitations

A caveat has to be noted for the present study as well as all other studies which do not confirm the tumor cell identity by genomic markers. Because markers for mesenchymal-like CTCs are mostly not tumor-specific^[31]. Furthermore, small sample size is another drawback when evaluating therapy response through obtaining sequential blood specimens.

In conclusion, our findings have provided evidence of the EMT phenomenon in human gastric cancer specimens, both in rare cells within primary tumors and more abundantly in CTCs by a combination of physical and biological methods. Furthermore, we demonstrated that the evaluation of the mesenchymal CTCs in peripheral blood can be used to monitor therapy response in gastric cancer patients. Clinical relevance of mesenchymal CTCs as a potential biomarker of therapeutic resistance and as a potential drug target in gastric cancer warrants further investigation.

ACKNOWLEDGMENTS

The authors would like to thank Surexam Biotech,

Guangzhou, China for the technical support.

COMMENTS

Background

Like common cancers, most gastric cancer-related deaths result from metastasis, which is rarely predictable by standard imaging work-ups like positron emission tomography/computed tomography scans or tumor marker tests. Circulating tumor cells (CTCs) originating from solid tumors are related with the course of hematogenous metastatic spread to the distant sites, exemplifying the switch from localized to systemic disease. Therefore, evaluating CTCs has clinical relevance in the monitoring and the outcomes of metastatic tumors.

Research frontiers

The recent discoveries on CTCs demonstrate how these cells are involved in hematogenous metastasis, with a focus on the epithelial-mesenchymal transition (EMT). The investigation by Yu and colleagues found that dynamic changes in the number of epithelial and mesenchymal CTCs in breast cancer patients as well as the potential of monitoring therapy response.

Innovations and breakthroughs

So far, there have been few reports regarding the detecting methods and clinical significance of mesenchymal CTCs in cancer patients, specifically gastric cancer. This is the first study evaluating the mesenchymal CTCs in peripheral blood and therapy response in gastric cancer patients.

Applications

The EMT phenomenon in human gastric cancer specimens was found both in rare cells within primary tumors and more abundantly in CTCs by a combination of physical and biological methods. Furthermore, the evaluation of the mesenchymal CTCs in peripheral blood can be used to monitor therapy response in gastric cancer patients. Mesenchymal CTCs maybe is a potential biomarker of therapeutic resistance or a potential drug target in gastric cancer.

Terminology

CTCs in peripheral circulation originating from solid tumors are involved in the process of hematogenous metastatic spreading to distant sites, exemplifying the switch from localized to systemic disease. Mesenchymal-to-epithelial transition (MET) is a crucial physiologic event that converts mesenchymal cells to epithelial cells. There is increasing evidence suggesting that MET maybe also regulate epithelial carcinogenesis.

Peer-review

The paper is a good contribution in investigating the role of mesenchymal-to-epithelial transition in circulating tumor cells of gastric cancer. The issue is not new but every new contribution confirming the feasibility and efficacy of a possible new marker is welcome.

REFERENCES

- 1 Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015; **136**: E359-E386 [PMID: 25220842 DOI: 10.1002/ijc.29210]
- 2 Nguyen DX, Bos PD, Massagué J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer* 2009; **9**: 274-284 [PMID: 19308067 DOI: 10.1038/nrc2622]
- 3 Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW, Hayes DF. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med* 2004; **351**: 781-791 [PMID: 15317891 DOI: 10.1056/NEJMoa040766]
- 4 Krebs MG, Metcalf RL, Carter L, Brady G, Blackhall FH, Dive C. Molecular analysis of circulating tumour cells-biology and biomarkers. *Nat Rev Clin Oncol* 2014; **11**: 129-144 [PMID: 24445517 DOI: 10.1038/nrclinonc.2013.253]
- 5 Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, Isakoff SJ, Ciciliano JC, Wells MN, Shah AM, Concannon KF, Donaldson MC, Sequist LV, Brachtel E, Sgroi D, Baselga J, Ramaswamy S, Toner M, Haber DA, Maheswaran S. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science* 2013; **339**: 580-584 [PMID: 23372014 DOI: 10.1126/science.1228522]
- 6 Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009; **139**: 871-890 [PMID: 19945376 DOI: 10.1016/j.cell.2009.11.007]
- 7 Kang Y, Pantel K. Tumor cell dissemination: emerging biological insights from animal models and cancer patients. *Cancer Cell* 2013; **23**: 573-581 [PMID: 23680145 DOI: 10.1016/j.ccr.2013.04.017]
- 8 Okabe H, Ishimoto T, Mima K, Nakagawa S, Hayashi H, Kuroki H, Imai K, Nitta H, Saito S, Hashimoto D, Chikamoto A, Ishiko T, Watanabe M, Nagano O, Beppu T, Saya H, Baba H. CD44s signals the acquisition of the mesenchymal phenotype required for anchorage-independent cell survival in hepatocellular carcinoma. *Br J Cancer* 2014; **110**: 958-966 [PMID: 24300972 DOI: 10.1038/bjc.2013.759]
- 9 Satelli A, Brownlee Z, Mitra A, Meng QH, Li S. Circulating tumor cell enumeration with a combination of epithelial cell adhesion molecule- and cell-surface vimentin-based methods for monitoring breast cancer therapeutic response. *Clin Chem* 2015; **61**: 259-266 [PMID: 25336717 DOI: 10.1373/clinchem.2014.228122]
- 10 Tsujiura M, Ichikawa D, Konishi H, Komatsu S, Shiozaki A, Otsuji E. Liquid biopsy of gastric cancer patients: circulating tumor cells and cell-free nucleic acids. *World J Gastroenterol* 2014; **20**: 3265-3286 [PMID: 24696609 DOI: 10.3748/wjg.v20.i12.3265]
- 11 Alix-Panabières C, Pantel K. Circulating tumor cells: liquid biopsy of cancer. *Clin Chem* 2013; **59**: 110-118 [PMID: 23014601 DOI: 10.1373/clinchem.2012.194258]
- 12 Esmaeilsabzali H, Beischlag TV, Cox ME, Parameswaran AM, Park EJ. Detection and isolation of circulating tumor cells: principles and methods. *Biotechnol Adv* 2013; **31**: 1063-1084 [PMID: 23999357 DOI: 10.1016/j.biotechadv.2013.08.016]
- 13 Friedlander TW, Premasekharan G, Paris PL. Looking back, to the future of circulating tumor cells. *Pharmacol Ther* 2014; **142**: 271-280 [PMID: 24362084 DOI: 10.1016/j.pharmthera.2013.12.011]
- 14 Harouaka R, Kang Z, Zheng SY, Cao L. Circulating tumor cells: advances in isolation and analysis, and challenges for clinical applications. *Pharmacol Ther* 2014; **141**: 209-221 [PMID: 24134902 DOI: 10.1016/j.pharmthera.2013.10.004]
- 15 Noh SH, Park SR, Yang HK, Chung HC, Chung IJ, Kim SW, Kim HH, Choi JH, Kim HK, Yu W, Lee JI, Shin DB, Ji J, Chen JS, Lim Y, Ha S, Bang YJ. Adjuvant capecitabine plus oxaliplatin for gastric cancer after D2 gastrectomy (CLASSIC): 5-year follow-up of an open-label, randomised phase 3 trial. *Lancet Oncol* 2014; **15**: 1389-1396 [PMID: 25439693 DOI: 10.1016/S1470-2045(14)70473-5]
- 16 Wu S, Liu Z, Liu S, Lin L, Yang W, Xu J. Enrichment and enumeration of circulating tumor cells by efficient depletion of leukocyte fractions. *Clin Chem Lab Med* 2014; **52**: 243-251 [PMID: 24021598 DOI: 10.1515/cclm-2013-0558]
- 17 Pavese JM, Bergan RC. Circulating tumor cells exhibit a biologically aggressive cancer phenotype accompanied by selective resistance to chemotherapy. *Cancer Lett* 2014; **352**: 179-186 [PMID: 25016063 DOI: 10.1016/j.canlet.2012.06.012]
- 18 Heitzer E, Ulz P, Geigl JB. Circulating tumor DNA as a liquid biopsy for cancer. *Clin Chem* 2015; **61**: 112-123 [PMID: 25388429 DOI: 10.1373/clinchem.2014.222679]
- 19 Maheswaran S, Sequist LV, Nagrath S, Ulkus L, Brannigan B, Collura CV, Inserra E, Diederichs S, Iafraite AJ, Bell DW, Digumarthy S, Muzikansky A, Irimia D, Suttleman J, Tompkins RG, Lynch TJ, Toner M, Haber DA. Detection of mutations in EGFR in circulating lung-cancer cells. *N Engl J Med* 2008; **359**: 366-377 [PMID: 18596266 DOI: 10.1056/NEJMoa0800668]
- 20 Miyamoto DT, Lee RJ, Stott SL, Ting DT, Wittner BS, Ulman M,

- Smas ME, Lord JB, Brannigan BW, Trautwein J, Bander NH, Wu CL, Sequist LV, Smith MR, Ramaswamy S, Toner M, Maheswaran S, Haber DA. Androgen receptor signaling in circulating tumor cells as a marker of hormonally responsive prostate cancer. *Cancer Discov* 2012; **2**: 995-1003 [PMID: 23093251 DOI: 10.1158/2159-8290.CD-12-0222]
- 21 **Hayes DF**, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, Matera J, Allard WJ, Doyle GV, Terstappen LW. Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. *Clin Cancer Res* 2006; **12**: 4218-4224 [PMID: 16857794 DOI: 10.1158/1078-0432.CCR-05-2821]
 - 22 **de Bono JS**, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, Doyle GV, Terstappen LW, Pienta KJ, Raghavan D. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin Cancer Res* 2008; **14**: 6302-6309 [PMID: 18829513 DOI: 10.1158/1078-0432.CCR-08-0872]
 - 23 **Muinelo-Romay L**, Vieito M, Abalo A, Nocelo MA, Barón F, Anido U, Brozos E, Vázquez F, Aguin S, Abal M, López RL. Evaluation of Circulating Tumor Cells and Related Events as Prognostic Factors and Surrogate Biomarkers in Advanced NSCLC Patients Receiving First-Line Systemic Treatment. *Cancers (Basel)* 2014; **6**: 153-165 [PMID: 24452143 DOI: 10.3390/cancers6010153]
 - 24 **Pachmann K**, Camara O, Kavallaris A, Krauspe S, Malarski N, Gajda M, Kroll T, Jörke C, Hammer U, Altendorf-Hofmann A, Rabenstein C, Pachmann U, Runnebaum I, Höflken K. Monitoring the response of circulating epithelial tumor cells to adjuvant chemotherapy in breast cancer allows detection of patients at risk of early relapse. *J Clin Oncol* 2008; **26**: 1208-1215 [PMID: 18323545 DOI: 10.1200/JCO.2007.13.6523]
 - 25 **Scher HI**, Jia X, de Bono JS, Fleisher M, Pienta KJ, Raghavan D, Heller G. Circulating tumour cells as prognostic markers in progressive, castration-resistant prostate cancer: a reanalysis of IMMC38 trial data. *Lancet Oncol* 2009; **10**: 233-239 [PMID: 19213602 DOI: 10.1016/S1470-2045(08)70340-1]
 - 26 **Friedlander TW**, Ngo VT, Dong H, Premasekharan G, Weinberg V, Doty S, Zhao Q, Gilbert EG, Ryan CJ, Chen WT, Paris PL. Detection and characterization of invasive circulating tumor cells derived from men with metastatic castration-resistant prostate cancer. *Int J Cancer* 2014; **134**: 2284-2293 [PMID: 24166007 DOI: 10.1002/ijc.28561]
 - 27 **Mani SA**, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Briskin C, Yang J, Weinberg RA. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 2008; **133**: 704-715 [PMID: 18485877 DOI: 10.1016/j.cell.2008.03.027]
 - 28 **Satelli A**, Mitra A, Brownlee Z, Xia X, Bellister S, Overman MJ, Kopetz S, Ellis LM, Meng QH, Li S. Epithelial-mesenchymal transitioned circulating tumor cells capture for detecting tumor progression. *Clin Cancer Res* 2015; **21**: 899-906 [PMID: 25516888 DOI: 10.1158/1078-0432.ccr-14-0894]
 - 29 **Mego M**, Mani SA, Lee BN, Li C, Evans KW, Cohen EN, Gao H, Jackson SA, Giordano A, Hortobagyi GN, Cristofanilli M, Lucci A, Reuben JM. Expression of epithelial-mesenchymal transition-inducing transcription factors in primary breast cancer: The effect of neoadjuvant therapy. *Int J Cancer* 2012; **130**: 808-816 [PMID: 21387303 DOI: 10.1002/ijc.26037]
 - 30 **Miyazono F**, Natsugoe S, Takao S, Tokuda K, Kijima F, Aridome K, Hokita S, Baba M, Eizuru Y, Aikou T. Surgical maneuvers enhance molecular detection of circulating tumor cells during gastric cancer surgery. *Ann Surg* 2001; **233**: 189-194 [PMID: 11176124 DOI: 10.1097/0000658-200102000-00007]
 - 31 **Bednarz-Knoll N**, Alix-Panabières C, Pantel K. Plasticity of disseminating cancer cells in patients with epithelial malignancies. *Cancer Metastasis Rev* 2012; **31**: 673-687 [PMID: 22733306 DOI: 10.1007/s10555-012-9370-z]

P- Reviewer: Di Vita M, Furka A **S- Editor:** Gong ZM
L- Editor: Wang TQ **E- Editor:** Zhang DN



Basic Study

Potential roles of EZH2, Bmi-1 and miR-203 in cell proliferation and invasion in hepatocellular carcinoma cell line Hep3B

Fang Yang, Li-Zhi Lv, Qiu-Cheng Cai, Yi Jiang

Fang Yang, Li-Zhi Lv, Qiu-Cheng Cai, Yi Jiang, Department of Hepatobiliary Surgery, Fuzong Clinical Medical College of Fujian Medical University, Fuzhou 350025, Fujian Province, China

Author contributions: Yang F contributed new reagents and analytic tools, and analyzed the data; Lv LZ conducted the experimental work; Cai QC participated in the manuscript drafting work; Jiang Y designed the research.

Institutional review board statement: All study protocols were approved by the Ethics Committee for Clinical Research of Fuzhou General Hospital.

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

Data sharing statement: No additional data are available

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Yi Jiang, PhD, Department of Hepatobiliary Surgery, Fuzong Clinical Medical College of Fujian Medical University, No. 156 Xierhuan North Road, Fuzhou 350025, Fujian Province, China. jiangyi8183@163.com
 Telephone: +86-591-22859377
 Fax: +86-591-22859377

Received: January 10, 2015
 Peer-review started: January 10, 2015
 First decision: March 10, 2015
 Revised: May 3, 2015
 Accepted: July 3, 2015
 Article in press: July 3, 2015
 Published online: December 21, 2015

Abstract

AIM: To investigate the potential roles of enhancer of zeste homolog2 (EZH2), Bmi-1 and miR-203 in cell proliferation and invasion in hepatocellular carcinoma (HCC) cell line Hep3B.

METHODS: A total of 73 patients who underwent surgical resection at Fuzong Clinical Medical College of Fujian Medical University were enrolled in this study. Hep3B cells were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 °C. Vectors that containing cDNA of the *EZH2* gene or miR-203 targeted shRNA plasmid were constructed, and then transfected into Hep3B cells. The mRNA expression of miR-203, EZH2, and Bmi-1 was analyzed using quantitative real-time polymerase chain reaction analysis, and the protein levels of EZH2 and Bmi-1 were detected by Western blot analysis. Effect of EZH2 or miR-203 on cell proliferation was observed by methyl thiazolyl tetrazolium assay, and cell apoptosis was assessed using flow cytometry. Besides, effect of EZH2 or miR-203 on tumor cell invasion was detected using Transwell assay.

RESULTS: The mRNA levels of EZH2 and Bmi-1 in HCC tissues and in Hep3B cells were significantly higher compared with those in normal samples ($P < 0.01$), while miR-203 level was significantly lower in HCC tissues ($P < 0.01$). Hep3B cells transfected with EZH2-shRNA or miR-203-shRNA showed lower expression levels of EZH2 and Bmi-1 ($P < 0.05$). Compared with controls, Hep3B cells transfected with EZH2-shRNA had relative slow cell proliferation, indicating that low expression of EZH2 and Bmi-1 and overexpression of miR-203 could inhibit Hep3B cell proliferation ($P < 0.05$). The average apoptosis rate of Hep3B cells transfected with EZH2-shRNA vector was about 18.631%, while that of Hep3B cells transfected with shRNA vector was about 5.33%, suggesting that EZH2 was down-

regulated by transfecting with EZH2-shRNA, and the down-regulated EZH2 contributed to the cell apoptosis. Low expression of EZH2 and Bmi-1 and overexpression of miR-203 could reduce Hep3B cell invasion ($P < 0.05$).

CONCLUSION: Our study suggests that EZH2 and Bmi-1 are up-regulated while miR-203 is down-regulated in Hep3B cells. MiR-203 may contribute to the metastasis and enhance apoptosis of HCC cells by regulating EZH2 and Bmi-1. Our study may provide a theoretical basis for metastasis of HCC and targeted therapy of HCC.

Key words: EZH2; Bmi-1; miR-203; Hepatocellular carcinoma; Hep3B cell line; Invasion; Proliferation

© **The Author(s) 2015.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: In this study, we analyzed the expression levels of Bmi-1, EZH2, and miR-203 in hepatocellular carcinoma (HCC) tissues and in Hep3B cell line. Comprehensive experimental methods were used to investigate the roles of Bmi-1, EZH2, and miR-203 in Hep3B cell proliferation, invasion and apoptosis. This study aimed to investigate the potential collaborate regulation mechanism of EZH2, Bmi-1, and miR-203 in metastasis and invasion of HCC.

Yang F, Lv LZ, Cai QC, Jiang Y. Potential roles of EZH2, Bmi-1 and miR-203 in cell proliferation and invasion in hepatocellular carcinoma cell line Hep3B. *World J Gastroenterol* 2015; 21(47): 13268-13276 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13268.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13268>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide, with increasing incidence in many countries^[1]. Due to the easy metastasis and easy recurrence of HCC, hepatic resection is the main treatment method for patients suffering HCC^[2]. However, liver transplantation provides the best chance for such patients and offers long-term survival^[3]. Therefore, to identify some reliable biomarkers for the prediction of metastasis and recurrence of HCC will be of great significance.

Polycomb group (PcG) is a group of proteins that control the transcriptional memory of cells by maintaining the stable silencing of specific sets of genes through chromatin modifications^[4]. The polycomb repressive complex 1 (PRC1) and PRC2 are two distinct PcG complexes for PcG protein^[5]. Increasing studies prove that enhancer of zeste homolog2 (EZH2) is a polycomb group protein and that EZH2 is overexpressed in HCC^[6,7]. Also, B cell-

specific moloney murine leukaemia virus insertion site 1 (Bmi-1) represses the transcription of their target genes *via* an epigenetic mechanism^[8]. Effendi *et al*^[9] proves that overexpression of Bmi-1 in early-stage HCC is correlated with ATP-binding cassette transporter B1 expression. Also, up-regulated Bmi-1 enhances the invasion and metastasis of HCC^[10]. Besides, Fu *et al*^[11] prove that Bmi-1 and EZH2 are associated with the progression and aggressive biological behavior of HCC.

MicroRNAs (miRNAs) are some endogenous non-coding small molecules that regulate the gene expression at the posttranscriptional level. Numerous miRNAs play crucial roles in HCC, and miR-203 has been suggested to be a predictor for HCC after liver transplantation^[12]. Also, miR-203 induces cell apoptosis and represses cell growth by targeting Bmi-1 in HCC^[13]. EZH2 could regulate the expression of some miRNAs, although the mechanism is still unclear^[14]. Although many studies have devoted to elucidating the roles of Bmi-1 and EZH2 in HCC progression, collaborate regulation mechanism of EZH2, Bmi-1, and miR-203 in proliferation and invasion of HCC remains incompletely described.

In this study, we analyzed the expression levels of Bmi-1, EZH2, and miR-203 in HCC tissues and in Hep3B cell line. Comprehensive experimental methods were used to investigate the roles of Bmi-1, EZH2, and miR-203 in Hep3B cell proliferation, invasion and apoptosis. This study aimed to investigate the potential collaborate regulation mechanism of EZH2, Bmi-1, and miR-203 in metastasis and invasion of HCC.

MATERIALS AND METHODS

HCC tissues

A total of 73 patients who underwent surgical resection at Department of Hepatobiliary Surgery, Fuzong Clinical Medical College of Fujian Medical University from January 2007 to January 2014 were enrolled in this study. Informed consent was obtained from all cases for research use of the specimens, and all study protocols were approved by the Ethics Committee for Clinical Research of Fuzhou General Hospital. All the patients have received no radiotherapy or chemotherapy before routine surgery.

HCC cell cultivation

The HCC cell line Hep3B was given as a gift by Shanghai Institute of Biochemistry and Cell Biology (China) and was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Hyclone, United States) in a humidified chamber with 95% air and 5% CO₂ at 37 °C.

ShRNA of EZH2/miR-203 lentiviral vector construction and cell transfection

The cDNA corresponding to the full-length human EZH2 gene was amplified by sequencing and

subcloned into the lentivirus vector pLKO.1-TRC cloning vector (10878). The specific primers for EZH2 were: sense, 5'-CCGGGCTAGGTTAATTGGGACCAAACTCGAG-TTTGGTCCCAATTAACCTAGCTTTTG-3' and antisense, 5'-AAT-TCAAAAAGCTAGGTTAATTGGGACCAAACTCGAG-TTTGGTCCCAATTAACCTAG-3'.

In addition, to construct the overexpression vector of miR-203, the cultivated Hep3B cells at logarithmic phase were digested using 0.25% trypsin to make the cell concentration to 5×10^6 /mL. The digested Hep3B cells (100 μ L) were injected into the cell incubator (10 cm²). After being incubated at 37 °C with 5% of CO₂ until cell confluence reached 50%-60%, shRNA (10 μ L) and opti-MEM (490 μ L) were mixed with the Hep3B cells. The total mixture was incubated in 10 mL medium with 10% FBS.

Finally, Hep3B cells transfected with EZH2-shRNA/miR-203-shRNA were seeded in 6-well plates using Deofect EU transfection reagent (Roche, Germany) according to the manufacturer's protocol. Clones with stable transfection of EZH2-shRNA/miR-203-shRNA or empty vector were selected using 3 μ g/mL puromycin dihydrochloride (Sigma, United States).

Quantitative real-time polymerase chain reaction

Total RNA from HCC tissues and Hep3B cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. Expression of miR-203 was determined using the Taqman microRNA assay kit (Taqman microRNA assay kit), and U6 snRNA was used for the normalization of relative abundance of miR-203. Total RNA was reverse-transcribed into cDNA using the primescript RT reagent kit (Takara, Japan), and 1 μ L of cDNA was used for each PCR reaction.

Western blot analysis

Hep3B cells transfected with the EZH2-shRNA or empty vector were lysed with RIPA and total proteins were separated by SDS-PAGE after the concentration was determined, and transferred to PVDF membranes. Primary antibodies against EZH2 and BMI-1 were added and incubated overnight in TBST with 5% bovine serum albumin. After washing with TBST, the membranes were further incubated for 1 h at room temperature with a secondary antibody conjugated with horseradish peroxidase. Finally, the immunoreactive protein bands were visualized with a chemiluminescence kit (NEN Life Science Products).

Cell proliferation assay

To investigate the role of miR-203 in HCC cell proliferation, cells (100 μ L, about 5000 cells) transfected with miR-203 were injected into 96-well plates and then incubated at 37 °C with 5% CO₂. Methyl thiazolyl tetrazolium (10 μ g/mL) was added into the cells to determine the cell viability and the cells were further incubated at 37 °C for 4 h. Cell

proliferation reagent WST-1 (10 μ L; Roche, United States) was added into the cultivated cells. The absorbance of cells was read at 450 nm using a microplate reader (Bio-Rad, 3550, United States).

Cell apoptosis assay

Hep3B cells were transfected with 50 μ L EZH2-shRNA/miR-203 shRNA or control vector, and cell apoptosis assay was performed using Annexin V FITC apoptosis Detection Kit I (BD Pharmingen, United States) at 72 h after transfection according to the manufacturer's protocol. Cell suspension (100 μ L) was incubated with 5 μ L of annexin-V and 1 μ L of propidium iodide at room temperature for 10 min. Total stained cells were analyzed using flow cytometry (BD-bio, San Diego, CA, United States).

Cell invasion assay in vitro

To determine the effect of EZH2 or miR-203 on HCC cell invasion, clones of Hep3B cells transfected with different vectors were injected into the 24-well Transwell cell culture chambers (8- μ m pores; Millipore, United States) and Matrigel (BD, United States). After serum starvation for 24 h, Hep3B cells in 350 μ L serum-free DMEM were transferred to the upper chamber. The lower chamber was added with DMEM with 15% FBS as a chemoattractant. The number of invasive cells that remained adherent to the outside of the membrane were fixed and stained.

Statistical analysis

All the data are expressed as the mean \pm SD. Correlations between clinicopathological variables and EZH2, BMI-1 and miR-203 expression were analyzed with Pearson's tests. Variance analysis between groups was performed by one-way ANOVA and the significance of differences between control and treatment groups was tested using Dummett's multiple comparisons test. All statistical analyses were performed using the SPSS software package (SPSS, Chicago, IL, United States). $P < 0.05$ was considered statistically significant.

RESULTS

mRNA levels of EZH2, Bmi-1, and miR-203 in HCC

The results of quantitative real-time PCR analysis are shown in Figure 1. The mRNA levels of both EZH2 and Bmi-1 were significantly higher in HCC tissues compared with normal tissues ($P < 0.01$; Figure 1A and B). Besides, miR-203 level was lower in HCC tissue samples than in normal tissues ($P < 0.01$; Figure 1C).

Western blot analysis of EZH2 and Bmi-1 expression

To further investigate the expression protein levels of EZH2 and Bmi-1, Western blot analysis was conducted (Figure 2). The results showed that the expression

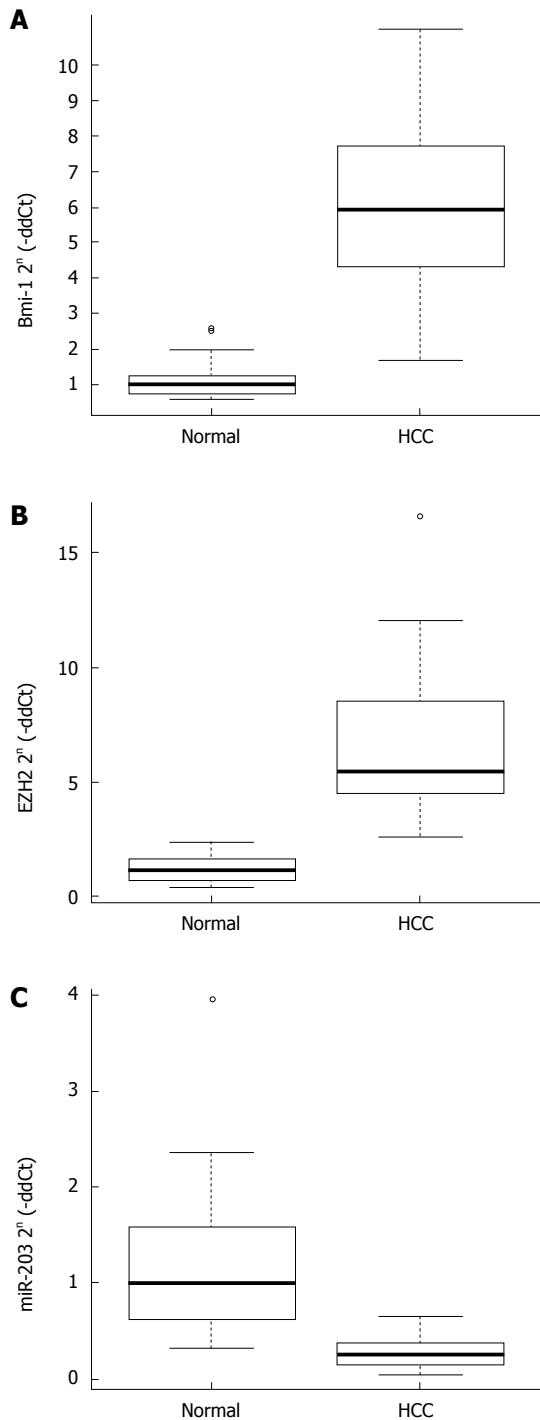


Figure 1 Quantitative real-time polymerase chain reaction detection of mRNA levels of Bmi-1, EZH2, and miR-203. The mRNA levels of Bmi-1 (A), EZH2 (B), and miR-203 (C) in HCC tissues.

levels of both EZH2 and Bmi-1 proteins were higher in HCC tissues compared with control tissues ($P < 0.05$; Figure 2A).

Besides, Western blot analysis showed that the expression levels of Bmi-1 and EZH2 in Hep3B cells transfected with EZH2-shRNA declined compared with control cells (Figure 2B). Meanwhile, expression levels of Bmi-1 and EZH2 in Hep3B cells overexpressing miR-203 were lower than those in Hep3B cells

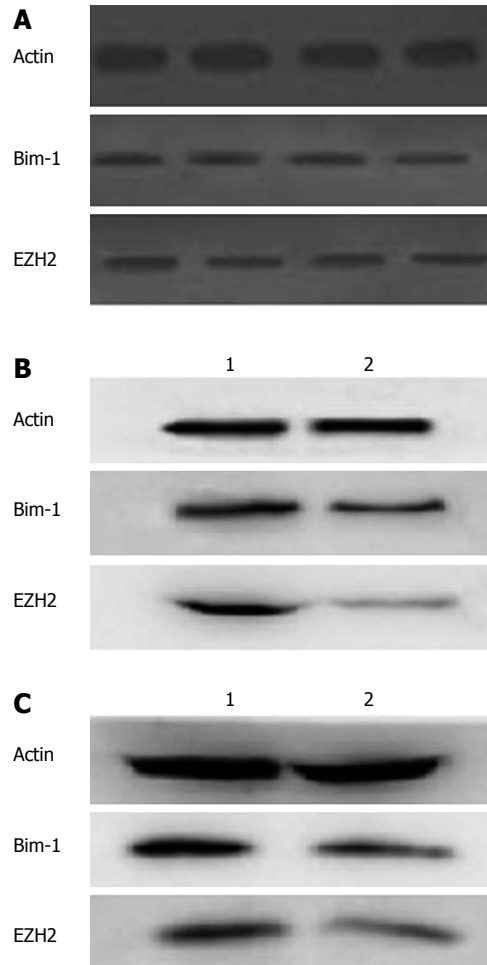


Figure 2 Western blot analysis of Bmi-1 and EZH2 protein levels. A: Expression levels of Bmi-1 and EZH2 in HCC tissues and in normal tissues; B: Expression levels of Bmi-1 and EZH2 in Hep3B cells transfected with EZH2-shRNA or control vector. Lane 1 stands for the protein levels in Hep3B cells, and lane 2 stands for the protein levels in Hep3B cells transfected with EZH2-shRNA; C: Expression levels of Bmi-1 and EZH2 in Hep3B cells transfected with miR-203-shRNA or control vector. Lane 1 stands for the protein levels in Hep3B cells, and lane 2 stands for the protein levels in Hep3B cells with miR-203 overexpression.

transfected with control vector (Figure 2C).

Cell proliferation analysis

After being cultivated for 4 d in the 96-well plates, the cell proliferation ability of Hep3B cells transfected with EZH2-shRNA/miR-203-shRNA or control vector was detected (Figure 3). Compared with the controls, Hep3B cells transfected with EZH2-shRNA had relative slow cell proliferation (Figure 3A), and the same tendency was observed in cells transfected with miR-203-shRNA (Figure 3B), suggesting that low expression of EZH2 and high expression of miR-203 both could inhibit Hep3B cell proliferation.

Cell apoptosis assay

Flow cytometry was used to investigate the role of EZH2 and miR-203 in apoptosis of HCC cells (Figure 4). The results showed that the average apoptosis

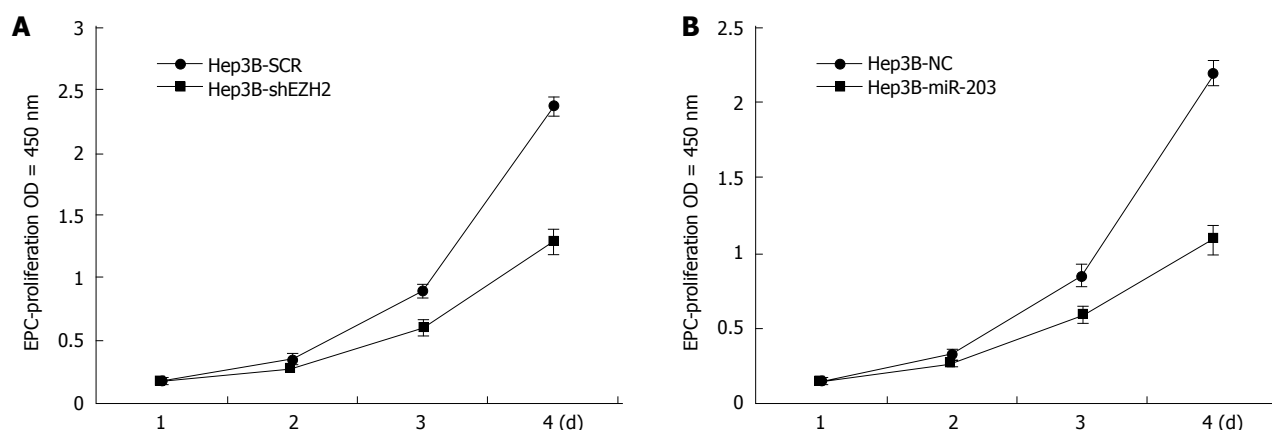


Figure 3 Methyl thiazolyl tetrazolium analysis of the effect of EZH2 and miR-203 on the proliferation of Hep3B cells. A: Proliferation of Hep3B cells transfected with control shRNA vector or EZH2-shRNA vector; B: Proliferation of Hep3B cells transfected with control shRNA vector or miR-203-shRNA vector.

rate of Hep3B cells transfected with EZH2-shRNA vector was about 18.631%, while that of Hep3B cells transfected with control shRNA vector was about 5.33%, suggesting that EZH2 was down-regulated by transfecting with EZH2-shRNA, and the down-regulated EZH2 contributed to the cell apoptosis (Figure 4A and B).

Cell invasion assay

The roles of EZH2 and miR-203 on HCC tumor cells invasion were shown in Figure 5. The results showed that violet crystals in Hep3B cells transfected with EZH2-shRNA were less than that in control group, indicating that the cell invasion ability of Hep3B cells transfected with EZH2-shRNA was obviously abated compared with that of Hep3B cells transfected with shRNA vector (Figure 5A and B).

Besides, Transwell invasion assay displayed that violet crystals in Hep3B cells with miR-203 overexpression were less than that in control group, suggesting that overexpression of miR-203 inhibiting the invasion of HCC cells (Figure 5C and D).

DISCUSSION

HCC is the third leading cause of cancer-related deaths worldwide, with increasing incidence in many countries^[1]. Due to the easy metastasis and recurrence of HCC, hepatic resection is the main treatment method for patients with HCC^[2]. High recurrence rate of intra-hepatic and distant metastases is the major obstacle to improving the survival of patients with HCC. Clinical data showed that PcG proteins such as EZH2 and Bmi-1, and miR-203 play key roles in HCC metastasis and recurrence. Clarifying the mechanisms and identifying key factors underlying invasion and metastasis that could reflect the metastasis and recurrence of HCC will be necessary. In the present study, we found that PcG proteins Bmi-1 and EZH2, and miRNA-203 coordinately regulated HCC, which might play an important role in HCC recurrence and

metastasis.

Our data showed that the two PcG proteins EZH2 and Bmi-1 had high expression levels both in HCC tissues and in Hep3B cells, suggesting their crucial roles in HCC development. PcG proteins are important in maintaining cell identity and regulation of the cell cycle^[15]. EZH2 was found to be associated with adverse pathological characteristics by some investigators^[16]. Overexpression of EZH2 and Bmi-1 is associated with a poor prognosis in several types of human tumors^[17,18,19]. Sasaki *et al*^[20] proved that high levels of EZH2 and Bmi-1 were related to HCC progression. Besides, qRT-PCR analysis showed that miR-203 is down-regulated in HCC tissues and in Hep3B cells. It has been demonstrated that miR-203 plays key roles in controlling proliferation, migration, and invasion of cancer cells, such as prostate cancer cells^[21]. Recent studies show that miR-203 is epigenetically silenced in HCC^[22,23]. Therefore, our results are consistent with those of the former studies.

Our study showed that proliferation ability of Hep3B cells transfected with EZH2-shRNA or miR-203-shRNA was lower compared with Hep3B cells transfected with control vector. The polycomb group protein EZH2 was up-regulated in proliferating HCC cells^[24] and EZH2 could be inhibited by the tumor suppressor miR-124 in HCC^[25]. Besides, Wang *et al*^[26] proved that down-regulation of Bmi-1 could retard the cell proliferation in HCC, and Chiba *et al*^[27] proved that Bmi-1 enhanced the proliferation of HCC cells. Thus, high levels of Bmi-1 and EZH2 may contribute to cell proliferation of HCC. Meanwhile, bioinformatics analysis showed that miR-203 was related to the cell growth of HCC^[28]. Our results are consistent with those of Sasaki *et al*^[20], who found that the expression of Bmi-1 and EZH2 was heterogeneous and associated with vascular infiltration, histological grade and cell proliferation in HCC. Based on our results, we speculate that high expression of Bmi-1 and EZH2 or low expression of miR-203 could contribute to Hep3B cell proliferation.

On the other hand, our data showed that cell

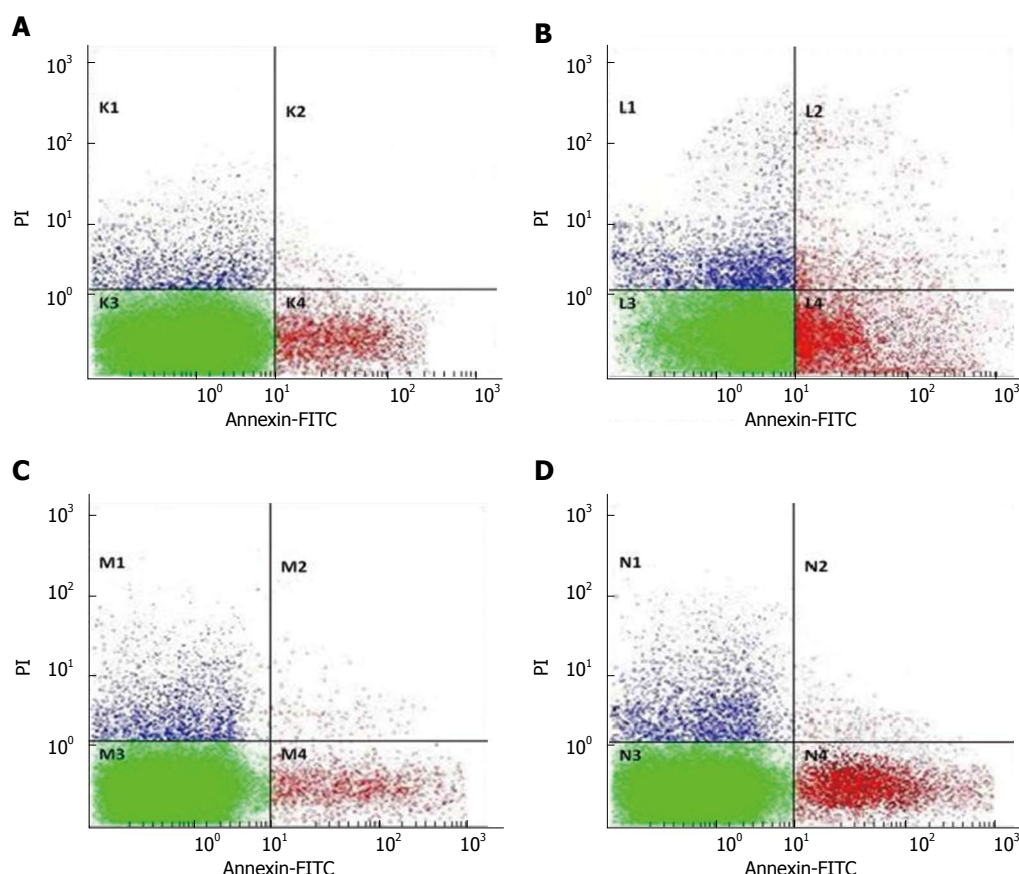


Figure 4 Flow cytometry analysis of apoptosis of Hep3B cells. A: Hep3B cells transfected with control shRNA vector; B: Hep3B cells transfected with EZH2-shRNA vector; C: Hep3B cells transfected with control shRNA vector; D: Hep3B cells transfected with miR-203-shRNA vector.

apoptosis of Hep3B cells transfected with EZH2-shRNA was lower than that in Hep3B cells transfected with control vector, and the same tendency was observed in Hep3B cells transfected with miR-203 shRNA. This finding indicates the crucial role of EZH2 and Bmi-1 in regulating HCC cell apoptosis and invasion. Su *et al.*^[29] proved that EZH2 could be silenced by the down-regulated miR-101, which contributed to HCC cell apoptosis. Also, overexpression of Bmi-1 leads to increased self-renewal and tumorigenesis of liver stem cells in mice^[30], and high expression level of Bmi-1 in HCC was significantly correlated with recurrence of HCC *via* increasing the cell apoptosis^[31]. Yonemitsu *et al.*^[17] proved that cell proliferation was apparently inhibited in HepG2 cells with EZH2 and Bmi-1 knockdown. Thus, high levels of EZH2 and Bmi-1 may be the contributors to HCC metastasis and recurrence. Based on our results, we speculate that EZH2 may collaborate with Bmi-1 in HCC development and progression by regulating Hep3B cell apoptosis and invasion. Meanwhile, miR-203 is the first skin-specific miRNA discovered in recent years^[12]. It is involved not only in the regulation of embryonic epidermal differentiation, building a protective layer of skin for psoriasis and other skin diseases, but also in proliferation, differentiation, invasion, metastasis and apoptosis of tumor cells as a tumor suppressor

or oncogenic factor^[32]. The abnormal expression of miR-203 can alter the expression of many targeted genes, leading to breast, prostate, liver and other tumors^[22]. Abnormal expression of miR-203 caused by promotor methylation resulted in aberrant expression of many target genes including Bmi-1, thus leading to the occurrence of breast cancer and liver cancer^[33,34]. Knockdown of EZH2 both in breast cancer and in breast cancer cell line resulted in the down-regulation of specific miRNAs such as miR-203 and miR-200 to enhance the expression of Bmi-1^[35], indicating that miR-203 may function by regulating many target genes.

In conclusion, our study suggests that EZH2 and Bmi-1, together with miR-203, may form a regulatory axis (EZH2-Bmi-1-miR-203) to regulate Hep3B cell proliferation and invasion. Down-regulating EZH2 causes the down-regulation of Bmi-1 and the up-regulation of miR-203. The overexpression of miR-203 and down-regulation of EZH2 and Bmi-1 may decrease the invasion and proliferation but increase the apoptosis of Hep3B cells. miR-203 might play an important role in the link of EZH2 and Bmi-1. Our study may provide evidence of coordinated regulation of PcG proteins EZH2 and Bmi-1 through miR-203, which regulates the invasion and proliferation of Hep3B cells. However, further studies that focus on the role of

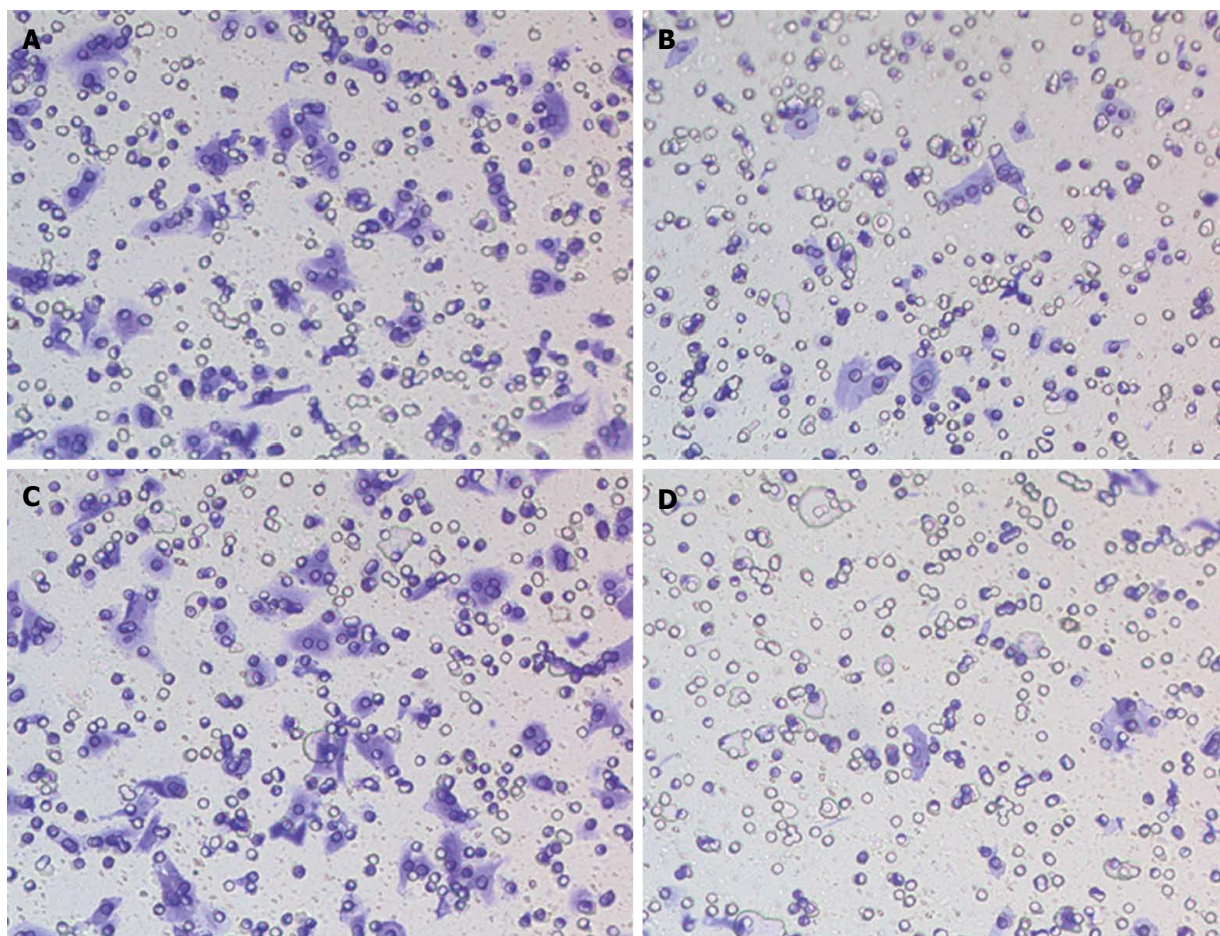


Figure 5 Analysis of Hep3B cell invasion *in vitro*. A: Invasion of Hep3B cells transfected with control shRNA vector; B: Invasion of Hep3B cells transfected with EZH2-shRNA; C: Invasion of Hep3B cells transfected with control shRNA vector; D: Invasion of Hep3B cells transfected with miR-203-shRNA.

miR-203 are still needed.

COMMENTS

Background

To clarify the mechanisms and identify the key factors of Bmi-1/EZH2/miR-203 underlying invasion and metastasis that could reflect the metastasis and recurrence of hepatocellular carcinoma.

Research frontiers

This study found that PcG proteins of Bmi-1 and EZH2, and miR-203 coordinately regulated hepatocellular carcinoma, which may play crucial roles in hepatocellular carcinoma recurrence and metastasis.

Innovations and breakthroughs

This study found that two PcG proteins EZH2 and Bmi-1 had high expression levels in hepatocellular carcinoma tissues and in Hep3B cells, suggesting their crucial roles in hepatocellular carcinoma development. Low expression of miR-203 or high expression of EZH2 and Bmi-1 contribute to Hep3B cell proliferation. The present study suggests that EZH2 and Bmi-1, together with miR-203, may form a regulatory axis to regulate Hep3B cell proliferation and invasion.

Applications

This study may provide evidence of coordinated regulation of PcG proteins EZH2, Bmi-1 through miR-203, which regulated invasion and proliferation of Hep3B cells.

Terminology

The authors detected the expression of PcG proteins EZH2 and Bmi-1 both in hepatocellular carcinoma tissues and in Hep3B cells. SiRNA of miR-203 was constructed and transfected into Hep3B cells.

Peer-review

This study investigated the coordinated role between PcG proteins EZH2 and Bmi-1, and miR-203 in regulating hepatocellular carcinoma Hep3B cell proliferation, invasion, and cell apoptosis, and the study suggested that EZH2-Bmi-1-miR-203 may be a regulatory axis to regulate Hep3B cell proliferation and invasion.

REFERENCES

- 1 **Tung-Ping Poon R**, Fan ST, Wong J. Risk factors, prevention, and management of postoperative recurrence after resection of hepatocellular carcinoma. *Ann Surg* 2000; **232**: 10-24 [PMID: 10862190 DOI: 10.3748/wjg.v17.i27.3213]
- 2 **Zhou Y**, Zhao Y, Li B, Xu D, Yin Z, Xie F, Yang J. Meta-analysis of radiofrequency ablation versus hepatic resection for small hepatocellular carcinoma. *BMC Gastroenterol* 2010; **10**: 78 [PMID: 20618937 DOI: 10.1186/1471-230X-10-78]
- 3 **Cheng YC**, Chen TW, Fan HL, Yu CY, Chang HC, Hsieh CB. Transarterial chemoembolization for intrahepatic multiple recurrent HCC after liver resection or transplantation. *Ann Transplant* 2014; **19**: 309-316 [PMID: 24975583 DOI: 10.12659/AOT.890505]
- 4 **Delers A**, Stroud H, Bernatavichute Y, Johnson E, Klein G, Schubert D, Jacobsen SE. Loss of the DNA methyltransferase

- MET1 Induces H3K9 hypermethylation at PcG target genes and redistribution of H3K27 trimethylation to transposons in *Arabidopsis thaliana*. *PLoS Genet* 2012; **8**: e1003062 [PMID: 23209430 DOI: 10.1371/journal.pgen.1003062]
- 5 **Raaphorst FM**, van Kemenade FJ, Blokzijl T, Fieret E, Hamer KM, Satijn DP, Otte AP, Meijer CJ. Coexpression of BMI-1 and EZH2 polycomb group genes in Reed-Sternberg cells of Hodgkin's disease. *Am J Pathol* 2000; **157**: 709-715 [PMID: 10980109 DOI: 10.1016/S0002-9440(10)64583-X]
 - 6 **Gong Y**, Wang X, Liu J, Shi L, Yin B, Peng X, Qiang B, Yuan J. NSPc1, a mainly nuclear localized protein of novel PcG family members, has a transcription repression activity related to its PKC phosphorylation site at S183. *FEBS Lett* 2005; **579**: 115-121 [PMID: 15620699 DOI: 10.1016/j.febslet.2004.11.056]
 - 7 **Ciarapica R**, De Salvo M, Carcarino E, Bracaglia G, Adesso L, Leoncini PP, Dall'Agnese A, Walters ZS, Verginelli F, De Sio L, Boldrini R, Inserra A, Bisogno G, Rosolen A, Alaggio R, Ferrari A, Collini P, Locatelli M, Stifani S, Screpanti I, Rutella S, Yu Q, Marquez VE, Shipley J, Valente S, Mai A, Miele L, Puri PL, Locatelli F, Palacios D, Rota R. The Polycomb group (PcG) protein EZH2 supports the survival of PAX3-FOXO1 alveolar rhabdomyosarcoma by repressing FBXO32 (Atrogin1/MAFbx). *Oncogene* 2014; **33**: 4173-4184 [PMID: 24213577 DOI: 10.1038/onc.2013.471]
 - 8 **Kim HG**, Kim DJ, Li S, Lee KY, Li X, Bode AM, Dong Z. Polycomb (PcG) proteins, BMI1 and SUZ12, regulate arsenic-induced cell transformation. *J Biol Chem* 2012; **287**: 31920-31928 [PMID: 22843710 DOI: 10.1074/jbc.M112.360362]
 - 9 **Effendi K**, Mori T, Komuta M, Masugi Y, Du W, Sakamoto M. Bmi-1 gene is upregulated in early-stage hepatocellular carcinoma and correlates with ATP-binding cassette transporter B1 expression. *Cancer Sci* 2010; **101**: 666-672 [PMID: 20085590 DOI: 10.1111/j.1349-7006.2009.01431.x]
 - 10 **Li X**, Yang Z, Song W, Zhou L, Li Q, Tao K, Zhou J, Wang X, Zheng Z, You N, Dou K, Li H. Overexpression of Bmi-1 contributes to the invasion and metastasis of hepatocellular carcinoma by increasing the expression of matrix metalloproteinase (MMP)-2, MMP-9 and vascular endothelial growth factor via the PTEN/PI3K/Akt pathway. *Int J Oncol* 2013; **43**: 793-802 [PMID: 23807724 DOI: 10.3892/ijo.2013.1992]
 - 11 **Fu WM**, Tang LP, Zhu X, Lu YF, Zhang YL, Lee WY, Wang H, Yu Y, Liang WC, Ko CH, Xu HX, Kung HF, Zhang JF. MiR-218-targeting-Bmi-1 mediates the suppressive effect of 1,6,7-trihydroxyxanthone on liver cancer cells. *Apoptosis* 2015; **20**: 75-82 [PMID: 25416134 DOI: 10.1007/s10495-014-1047-3]
 - 12 **Chen HY**, Han ZB, Fan JW, Xia J, Wu JY, Qiu GQ, Tang HM, Peng ZH. miR-203 expression predicts outcome after liver transplantation for hepatocellular carcinoma in cirrhotic liver. *Med Oncol* 2012; **29**: 1859-1865 [PMID: 21786180 DOI: 10.1007/s12032-011-0031-9]
 - 13 **Sato F**, Tsuchiya S, Meltzer SJ, Shimizu K. MicroRNAs and epigenetics. *FEBS J* 2011; **278**: 1598-1609 [PMID: 21395977 DOI: 10.1111/j.1742-4658.2011.08089.x]
 - 14 **Thomas MB**, Zhu AX. Hepatocellular carcinoma: the need for progress. *J Clin Oncol* 2005; **23**: 2892-2899 [PMID: 15860847 DOI: 10.1200/JCO.2005.03.196]
 - 15 **Furuyama T**, Tie F, Harte PJ. Polycomb group proteins ESC and E(Z) are present in multiple distinct complexes that undergo dynamic changes during development. *Genesis* 2003; **35**: 114-124 [PMID: 12533794 DOI: 10.1002/gene.10173]
 - 16 **Mihic-Probst D**, Kuster A, Kilgus S, Bode-Lesniewska B, Ingold-Heppner B, Leung C, Storz M, Seifert B, Marino S, Schraml P, Dummer R, Moch H. Consistent expression of the stem cell renewal factor BMI-1 in primary and metastatic melanoma. *Int J Cancer* 2007; **121**: 1764-1770 [PMID: 17597110 DOI: 10.1002/ijc.22891]
 - 17 **Yonemitsu Y**, Imazeki F, Chiba T, Fukai K, Nagai Y, Miyagi S, Arai M, Aoki R, Miyazaki M, Nakatani Y, Iwama A, Yokosuka O. Distinct expression of polycomb group proteins EZH2 and BMI1 in hepatocellular carcinoma. *Hum Pathol* 2009; **40**: 1304-1311 [PMID: 19386347 DOI: 10.1016/j.humpath.2009.01.017]
 - 18 **Breuer RH**, Snijders PJ, Smit EF, Sutedja TG, Sewalt RG, Otte AP, van Kemenade FJ, Postmus PE, Meijer CJ, Raaphorst FM. Increased expression of the EZH2 polycomb group gene in BMI-1-positive neoplastic cells during bronchial carcinogenesis. *Neoplasia* 2004; **6**: 736-743 [PMID: 15720799 DOI: 10.1593/neo.04160]
 - 19 **Liu S**, Dontu G, Mantle ID, Patel S, Ahn NS, Jackson KW, Suri P, Wicha MS. Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res* 2006; **66**: 6063-6071 [PMID: 16778178]
 - 20 **Sasaki M**, Ikeda H, Itatsu K, Yamaguchi J, Sawada S, Minato H, Ohta T, Nakanuma Y. The overexpression of polycomb group proteins Bmi1 and EZH2 is associated with the progression and aggressive biological behavior of hepatocellular carcinoma. *Lab Invest* 2008; **88**: 873-882 [PMID: 18591938 DOI: 10.1038/labinvest.2008.52]
 - 21 **Viticchiè G**, Lena AM, Latina A, Formosa A, Gregersen LH, Lund AH, Bernardini S, Mauriello A, Miano R, Spagnoli LG, Knight RA, Candi E, Melino G. MiR-203 controls proliferation, migration and invasive potential of prostate cancer cell lines. *Cell Cycle* 2011; **10**: 1121-1131 [PMID: 21368580 DOI: 10.4161/cc.10.7.15180]
 - 22 **Saini S**, Majid S, Yamamura S, Tabatabai L, Suh SO, Shahryari V, Chen Y, Deng G, Tanaka Y, Dahiya R. Regulatory Role of mir-203 in Prostate Cancer Progression and Metastasis. *Clin Cancer Res* 2011; **17**: 5287-5298 [PMID: 21159887 DOI: 10.1158/1078-0432.CCR-10-2619]
 - 23 **Furuta M**, Kozaki KI, Tanaka S, Arai S, Imoto I, Inazawa J. miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. *Carcinogenesis* 2010; **31**: 766-776 [PMID: 19843643 DOI: 10.1093/carcin/bgp250]
 - 24 **Sudo T**, Utsunomiya T, Mimori K, Nagahara H, Ogawa K, Inoue H, Wakiyama S, Fujita H, Shirouzu K, Mori M. Clinicopathological significance of EZH2 mRNA expression in patients with hepatocellular carcinoma. *Br J Cancer* 2005; **92**: 1754-1758 [PMID: 15856046 DOI: 10.1038/sj.bjc.6602531]
 - 25 **Zheng F**, Liao YJ, Cai MY, Liu YH, Liu TH, Chen SP, Bian XW, Guan XY, Lin MC, Zeng YX, Kung HF, Xie D. The putative tumour suppressor microRNA-124 modulates hepatocellular carcinoma cell aggressiveness by repressing ROCK2 and EZH2. *Gut* 2012; **61**: 278-289 [PMID: 21672940 DOI: 10.1136/gut.2011.239145]
 - 26 **Wang H**, Pan K, Zhang HK, Weng DS, Zhou J, Li JJ, Huang W, Song HF, Chen MS, Xia JC. Increased polycomb-group oncogene Bmi-1 expression correlates with poor prognosis in hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2008; **134**: 535-541 [PMID: 17917742 DOI: 10.1007/s00432-007-0316-8]
 - 27 **Chiba T**, Miyagi S, Saraya A, Aoki R, Seki A, Morita Y, Yonemitsu Y, Yokosuka O, Taniguchi H, Nakauchi H, Iwama A. The polycomb gene product BMI1 contributes to the maintenance of tumor-initiating side population cells in hepatocellular carcinoma. *Cancer Res* 2008; **68**: 7742-7749 [PMID: 18829528 DOI: 10.1158/0008-5472]
 - 28 **Ladeiro Y**, Couchy G, Balabaud C, Bioulac-Sage P, Pelletier L, Rebouissou S, Zucman-Rossi J. MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. *Hepatology* 2008; **47**: 1955-1963 [PMID: 18433021 DOI: 10.1002/hep.22256]
 - 29 **Su H**, Yang JR, Xu T, Huang J, Xu L, Yuan Y, Zhuang SM. MicroRNA-101, down-regulated in hepatocellular carcinoma, promotes apoptosis and suppresses tumorigenicity. *Cancer Res* 2009; **69**: 1135-1142 [PMID: 19155302]
 - 30 **Lessard J**, Sauvageau G. Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature* 2003; **423**: 255-260 [PMID: 12714970 DOI: 10.1038/nature01572]
 - 31 **Lee TK**, Cheung VC, Ng IO. Liver tumor-initiating cells as a therapeutic target for hepatocellular carcinoma. *Cancer Lett* 2013; **338**: 101-109 [PMID: 22579789 DOI: 10.1016/j.canlet.2012.05.001]
 - 32 **Wei W**, Wan Jun L, Hui S, Dongyue C, Xinjun Y, Jisheng Z. miR-203 inhibits proliferation of HCC cells by targeting survivin.

- Cell Biochem Funct* 2013; **31**: 82-85 [PMID: 22886454 DOI: 10.1002/cbf.2863]
- 33 **Karakatsanis A**, Papaconstantinou I, Gazouli M, Lyberopoulou A, Polymeneas G, Voros D. Expression of microRNAs, miR-21, miR-31, miR-122, miR-145, miR-146a, miR-200c, miR-221, miR-222, and miR-223 in patients with hepatocellular carcinoma or intrahepatic cholangiocarcinoma and its prognostic significance. *Mol Carcinog* 2013; **52**: 297-303 [PMID: 22213236 DOI: 10.1002/mc.21864]
 - 34 **Jin J**, Deng J, Wang F, Xia X, Qiu T, Lu W, Li X, Zhang H, Gu X, Liu Y, Cao W, Shao W. The expression and function of microRNA-203 in lung cancer. *Tumour Biol* 2013; **34**: 349-357 [PMID: 23073851 DOI: 10.1007/s13277-012-0556-3]
 - 35 **Varambally S**, Cao Q, Mani RS, Shankar S, Wang X, Ateeq B, Laxman B, Cao X, Jing X, Ramnarayanan K, Brenner JC, Yu J, Kim JH, Han B, Tan P, Kumar-Sinha C, Lonigro RJ, Palanisamy N, Maher CA, Chinnaiyan AM. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. *Science* 2008; **322**: 1695-1699 [PMID: 19008416 DOI: 10.1126/science.1165395]

P- Reviewer: Deepak P, Siddiqui I **S- Editor:** Yu J
L- Editor: Wang TQ **E- Editor:** Zhang DN





Basic Study

Guggulsterone induces apoptosis of human hepatocellular carcinoma cells through intrinsic mitochondrial pathway

Juan-Juan Shi, Xiao-Li Jia, Mei Li, Ning Yang, Ya-Ping Li, Xin Zhang, Ning Gao, Shuang-Suo Dang

Juan-Juan Shi, Xiao-Li Jia, Mei Li, Ning Yang, Ya-Ping Li, Xin Zhang, Ning Gao, Shuang-Suo Dang, Department of Infectious Diseases, the Second Affiliated Hospital of Medical School of Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China

Author contributions: Shi JJ, Jia XL and Dang SS designed the research; Shi JJ, Li M, Yang N, Li YP, Zhang X and Gao N performed the research; Shi JJ, Yang N and Li YP analyzed the data; and Shi JJ wrote the paper.

Supported by Science and Technology Foundation of Shaanxi Province, China, No. 2007K16-07(9).

Institutional review board statement: No human or animal subjects in the study.

Institutional animal care and use committee statement: Animals were not used in the study.

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Xiao-Li Jia, PhD, Department of Infectious Diseases, the Second Affiliated Hospital of Medical School of Xi'an Jiaotong University, No. 157 Xi'wu Road, Xi'an 710004, Shaanxi Province, China. drjxl@163.com
Telephone: +86-29-87679688
Fax: +86-29-87679688

Received: April 29, 2015
Peer-review started: May 8, 2015
First decision: July 19, 2015

Revised: August 24, 2015
Accepted: October 17, 2015
Article in press: October 20, 2015
Published online: December 21, 2015

Abstract

AIM: To investigate the effects of guggulsterone on the proliferation and apoptosis of human hepatoma HepG2 cells *in vitro* and relevant mechanisms.

METHODS: Human hepatocellular carcinoma HepG2 cells and normal human liver L-02 cells were treated with different concentrations of guggulsterone (5-100 $\mu\text{mol/L}$) for 24-72 h. Cell proliferation was tested by MTT assay. Cell cycle and apoptosis were investigated using flow cytometry (FACS). Bcl-2 and Bax mRNA and protein expression was detected by real-time PCR and Western blot, respectively. TGF- β 1, TNF- α , and VEGF contents were determined by ELISA.

RESULTS: Guggulsterone significantly inhibited HepG2 cell proliferation in a dose- and time-dependent manner. FACS showed that guggulsterone arrested HepG2 cell cycle at G0/G1 phase. Guggulsterone induced apoptosis was also observed in HepG2 cells, with 24.91% \pm 2.41% and 53.03% \pm 2.28% of apoptotic cells in response to the treatment with 50 $\mu\text{mol/L}$ and 75 $\mu\text{mol/L}$ guggulsterone, respectively. Bax mRNA and protein expression was significantly increased and Bcl-2 mRNA and protein expression was decreased. ELISA analysis showed that the concentrations of TGF- β 1 and VEGF were significantly decreased and TNF- α concentration was increased.

CONCLUSION: Guggulsterone exerts its anticancer effects by inhibiting cell proliferation and inducing apoptosis in HepG2 cells. Guggulsterone induces apoptosis by activation of the intrinsic mitochondrial

pathway.

Key words: Guggulsterone; Hepatocellular carcinoma cells; Apoptosis; Cell cycle; Mitochondrial pathway

© **The Author(s)** 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer and the second leading cause of tumor mortality worldwide. Guggulsterone (GS) is a phytosterol extracted from the gum resin of guggul plants, and its pro-apoptotic effect has been found to play a pivotal role in its anti-carcinogenic mechanisms. In this study, we investigated the anticancer effects of GS-induced apoptosis in human HCC cells and the underlying molecular mechanisms. Our results demonstrated that GS induced HepG2 cell apoptosis through regulating Bcl-2 and Bax expression levels.

Shi JJ, Jia XL, Li M, Yang N, Li YP, Zhang X, Gao N, Dang SS. Guggulsterone induces apoptosis of human hepatocellular carcinoma cells through intrinsic mitochondrial pathway. *World J Gastroenterol* 2015; 21(47): 13277-13287 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13277.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13277>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer and the second leading cause of tumor mortality worldwide^[1]. The number of new cases of HCC is increasing year by year and about 748000 new cases are being diagnosed annually, accounting for 9.2% of all new global tumor cases^[2,3]. HCC has a low resectability rate, high recurrence rate, insidious onset, rapid progression, grave prognosis and high mortality^[1], therefore development of anticancer drugs with explicit efficacy on HCC has now become a challenge worldwide.

Many phytochemicals derived from edible plants have shown cancer therapeutic potential^[4,5]. Guggulsterone (GS) is a phytosterol extracted from the gum resin of guggul plants that shows strong antioxidant, anti-inflammatory, hypolipidemic and hypocholesteremic properties^[6-9], and has been used for treatment of several malignant diseases^[10-13]. Pro-apoptotic effect of GS has been found to play a pivotal role in its anti-carcinogenic mechanisms^[10,13].

Apoptosis is known as programmed cell death, accompanying specific morphological and biochemical changes such as cell shrinkage, nuclear and DNA fragmentation, and apoptotic body formation^[14,15]. These changes are associated with a distinct set of signaling pathways including the intrinsic mitochondrial pathway and the extrinsic death receptor pathway^[16]. A common step is the caspase activation in both

pathways^[17]. The mitochondrial pathway is activated by a variety of stimuli such as heat shock, DNA damage and reactive oxygen to increase permeability of mitochondrial outer membrane and release cytochrome C into the cytoplasm. In the presence of ATP, the cytochrome C is combined with apoptosis protease-activating factor (Apaf-1) that is combined with caspase recruitment domain and caspase-9 precursor to activate caspase-9, leading to activation of caspase-3 and caspase-7^[14]. Bcl-2 family, which plays an important role in the mitochondrial pathway, contains anti-apoptotic proteins including Bcl-2, Bcl-XL and Mcl-1, and pro-apoptotic proteins such as Bax, Bad and Bak, in which Bcl-2 and Bax are more important proteins^[16].

Previous studies have shown that GS can induce human colon cancer HT-29 cells apoptosis through decreasing expression of anti-apoptotic proteins including Bcl-2, cIAP-1, cIAP-2 and increasing the Bid protein expression^[10]. Moreover, GS has been found to activate JNK signaling pathway in human prostate cancer cells, which subsequently upregulates the Bcl-2 family members including Bax and Bak, leading to the apoptosis of cancer cells^[18,19]. Several studies have indicated that GS enhanced the sensitivity of HCC cell lines Hep3B and HepG2 to TRAIL-induced apoptosis *via* ROS-dependent ER stress induction^[12,20,21]. However, it has not been determined whether GS has anti-HCC effects through other signaling pathways, such as the intrinsic mitochondrial pathway.

In this study, we investigated the anticancer effects of GS-induced apoptosis in human HCC cells and the underlying molecular mechanisms. Our results demonstrated that GS induced HepG2 cell apoptosis through regulating Bcl-2 and Bax expression levels.

MATERIALS AND METHODS

Reagents and antibodies

Z-guggulsterone (Z-GS) was purchased from ENZO (United States) and was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO) as a 16 mmol/L stock solution and stored at -20 °C. Various concentrations of Z-GS (0-100 μmol/L) were diluted in serum free RPMI1640 medium (HyClone, Utah, United States) with 0.5% (v/v) DMSO used as a vehicle control. 0.25% (w/v) trypsin was obtained from HyClone (Utah, United States). The rabbit monoclonal antibodies against Bcl-2, Bax and β-actin were purchased from Santa Cruz Biotechnology (CA, United States). Horseradish peroxidase conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from ABGENT Biotechnology (SD, United States).

Cell lines and cell culture

Human HCC cell line HepG2 and the normal human hepatic cell line L-02 were obtained from the

Table 1 Primers used for real-time PCR analysis

Name	Forward primer (5'-3')	Reverse primer (5'-3')
Bcl-2 (168 bp)	TGTGTGGAGAGCGTCAAC	GGAGAAATCAAACAGAGGC
Bax (164 bp)	ATGCGTCCACCAAGAAGC	CCAGTTGAAGTTGCCGTC
GAPDH (138 bp)	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

Experimental Center of Xi'an Jiaotong University. Cells were cultured in RPMI1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS, HyClone, Utah, United States), 100 U/mL penicillin (Sigma-Aldrich, St Louis, United States) and 100 µg/mL streptomycin (Sigma-Aldrich) in a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO₂ at 37 °C. Culture medium was changed every other day. When cells covered 80%-90% of the bottom of culture flasks, cell were washed twice with phosphate buffered saline (PBS, 137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na₂HPO₄, 1.4 mmol/L KH₂PO₄, pH 7.4) and then were digested with 0.25% (w/v) trypsin. Cells were harvested using RPMI1640 medium followed by centrifugation at 1000 rpm for 10 min. Cells were re-suspended in RPMI1640 medium and were plated in appropriate plates at appropriate density and serum-starved for 24 h using serum free RPMI1640. Then the cells were treated with RPMI1640 medium containing various concentrations of Z-GS. After 24, 48 or 72 h of culture, cells were harvested as usual.

MTT assay

Cell viability was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St Louis, MO) assay as described previously^[22]. Briefly, cells were plated in 96-well plates at a density of 6×10^3 cells/well followed by starvation for 24 h using serum free RPMI1640 culture medium. The culture medium was then replaced with RPMI1640 medium containing various concentrations of Z-GS (0-100 µmol/L). After 24, 48, and 72 h of culture, 20 µL of MTT solution (5 mg/mL) was added to each well and cells were continuously cultured for 4 h. Culture medium was then removed and 150 µL of DMSO was added to each well. After shaking the culture plates for 5 min, the solution was collected and the optical density (OD) was measured using a spectrophotometer (ND-1000, Thermo Fisher, United States) at a wavelength of 570 nm. The cell viability rate (%) was calculated as $(OD_{\text{treated}}/OD_{\text{control}}) \times 100\%$.

Cell cycle analysis

The logarithmic phase HepG2 cells and L-02 cells were plated in 6-well plates at a density of 6×10^5 cells/well and incubated in a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO₂ at 37 °C for 24 h. Cells were then treated with 50 µmol/L and 75 µmol/L Z-GS in RPMI1640 medium for 24 h. After washing with cold PBS twice, cells were fixed in ice-cold 70% (v/v) ethanol overnight at -20 °C. Cells were treated

with Tris-HCl buffer (10 mmol/L Tris-HCl, pH 7.5) containing 1% (w/v) RNase A (Sigma-Aldrich) for 15 min, followed by incubation with propidium iodide (PI, Sigma-Aldrich) for 15 min. Cell cycles were then analyzed using a flow cytometer (CALIBUR, BD, United States), and the outputs were processed using ModFit LT2.0 software (Verity Software House, United States).

Apoptosis assay

Cell apoptosis was determined using the Annexin V-FITC and PI double staining (Kaiji, Nanjing, China) as previously described^[22]. Briefly, HepG2 cells and L-02 cells were plated in 6-well plates at 6×10^5 cells/well and treated with various concentrations of Z-GS (0 µmol/L, 50 µmol/L and 75 µmol/L) for 24 h. Cells were then harvested and stained with Annexin V-FITC and PI in the cold binding buffer (50 mmol/L HEPES, 700 mmol/L NaCl, 12.5 mmol/L CaCl₂, pH 7.4) for 15 min at room temperature in the dark. After washing, cell apoptosis was analyzed using a flow cytometer (CALIBUR, BD, United States).

Real-time PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen, United States) as described previously^[23]. RNA (1 µg) was then used to synthesize complementary DNA (cDNA) with AMV Reverse Transcriptase (RT) kit (TAKARA, Japan) according to the manufacturer's protocols. The relative expression of Bcl-2 and Bax was analyzed by quantitative real-time PCR with SYBR Premix Ex Taq II kit (TAKARA, Japan) with GAPDH as an internal control. The reaction system contained 12.5 µL $2 \times$ SYBR Ex Taq II, 2 µL RNA reaction, 1 µL each of forward and reverse primers and 8.5 µL sterile distilled water in a final volume of 25 µL. The reaction was performed at 95 °C for one cycle for 30 s, 95 °C for 5 s and 60 °C for 60 s for 40 cycles, and 72 °C for 60 s. Table 1 shows all the primer sequences. PCR products were run on 2% (w/v) agarose gels (Sigma-Aldrich) and stained with ethidium bromide (Sigma-Aldrich). RT-PCR outputs were detected using Bio-Rad iQ5 software (CA, United States).

Western blot analysis

Bcl-2 and Bax protein expression was investigated using Western blot analysis as described previously^[23]. Briefly, cells were harvested and re-suspended in RIPA lysis buffer [20 mmol/L Tris, 150 mmol/L NaCl, 1% (v/v) Triton X-100, 1% (w/v) digestive phosphatase inhibitors, 1% (w/v) protease inhibitors, 1% (w/v) phenylmethyl sulfonyl fluoride (PMSF), pH 7.5] (Sigma-

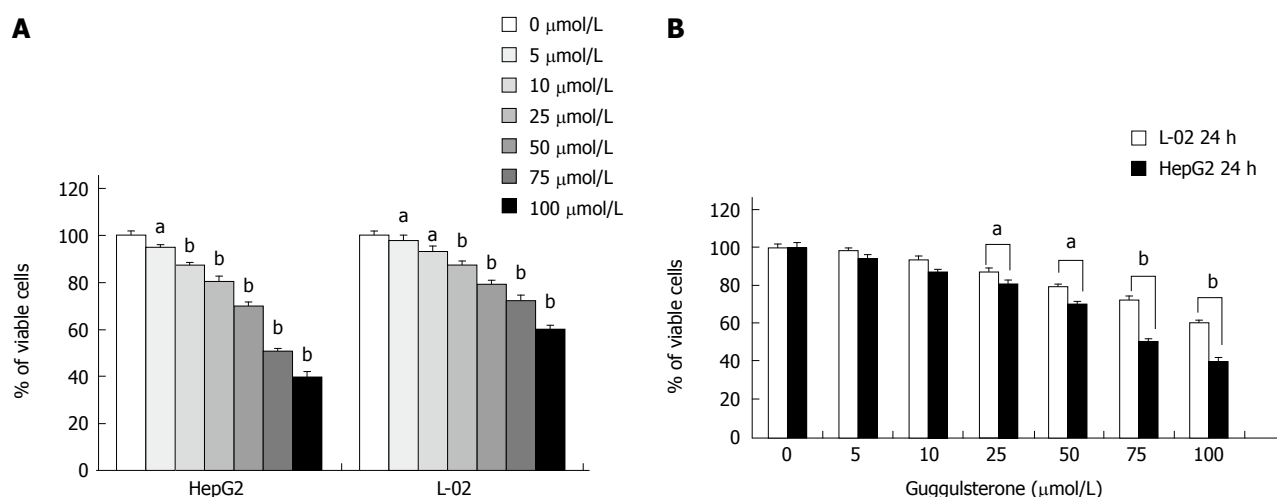


Figure 1 Human L-02 and HepG2 cells show different cell viabilities in response to guggulsterone treatment. Cell viability in L-02 and HepG2 cells was determined using MTT assay after incubation with 5, 10, 25, 50, 75 and 100 μmol/L guggulsterone, respectively, for 24 h. Guggulsterone significantly reduced cell viability in both L-02 and HepG2 cells in a dose- and time-dependent manner (A). Same concentration of guggulsterone differently reduced cytotoxicity between L-02 and HepG2 cells (B). ^a $P < 0.05$ or ^b $P < 0.01$, vs the control group.

Aldrich). Protein concentration was determined using BCA assay (Kangweishiji, Beijing, China) according to the manufacturer's protocols. Equal amounts of protein (30 μg/lane) were separated in a 10% (w/v) sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel (Sigma-Aldrich), and were then electrotransferred onto polyvinylidenedifluoride (PVDF) membranes (Sigma-Aldrich). After blocking with 5% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) in Tris-buffered saline (TBS, 0.1 mol/L, pH 7.4), membranes were incubated with primary antibodies (1:1000 dilution) overnight at 4 °C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution) for 1 h at room temperature with agitation. All membranes were detected using the ECL Western Blotting Kit Reagent (Thermo Fisher, United States).

Cytokine detection

Cell supernatants were collected from Z-GS treated and untreated control cells and filtered through a 0.22 μm filter (Millipore, United States). The levels of TGF-β1, TNF-α and VEGF in the culture medium were determined using enzyme-linked immunosorbent assay kit (ELISA, eBioscience, United States) according to the manufacturer's protocols.

Statistical analysis

Data are presented as mean ± SD, and were tested for normality and equal variance. Student's *t*-test or one-way analysis of variance (ANOVA) plus Bonferroni's post-test was carried out using SPSS 15.0 software (SPSS Inc., United States). *P*-values less than 0.05 were considered statistically significant.

RESULTS

GS inhibits the viability of human HCC cells

Compared with the control group, the viability of HepG2 and L-02 cells was significantly decreased in a dose- and time-dependent manner in response to 5, 10, 25, 50, 75 and 100 μmol/L of GS for 24 h (Figure 1A). Interestingly, 25, 50, 75 and 100 μmol/L of GS significantly reduced the HepG2 cell viability when compared with that in the L-02 cells after 24 h of treatment (Figure 1B), suggesting a less sensitivity to GS in L-02 cells relative to HepG2 cells. Similar results were revealed after 48 and 72 h of treatment (data not shown). The half maximal inhibitory concentration (IC₅₀) of GS in HepG2 cells for 24 h was 75 μmol/L. Therefore, 50 μmol/L and 75 μmol/L GS was used for all subsequent experiments in HepG2 cells.

Effect of GS on cell cycle in human HepG2 and L-02 cells

After treatment with 75 μmol/L of GS for 24 h, there was an increase in G0/G1 fraction (62.88% ± 2.67% vs 44.02% ± 1.07%) but decrease in G2/M fraction of HepG2 cells (13.33% ± 1.84% vs 28.33% ± 1.25%) in comparison with the untreated control cells ($P < 0.05$; Figure 2A). However, 50 μmol/L GS did not induce a significant difference in cell cycle fractions in HepG2 cells when compared with the control group ($P > 0.05$; Figure 2A). Differently, neither 75 μmol/L nor 50 μmol/L of GS induced a significant alteration in L-02 cell cycle ($P > 0.05$; Figure 2B).

GS induces apoptosis of human HepG2 cells

After treatment with 50 μmol/L and 75 μmol/L

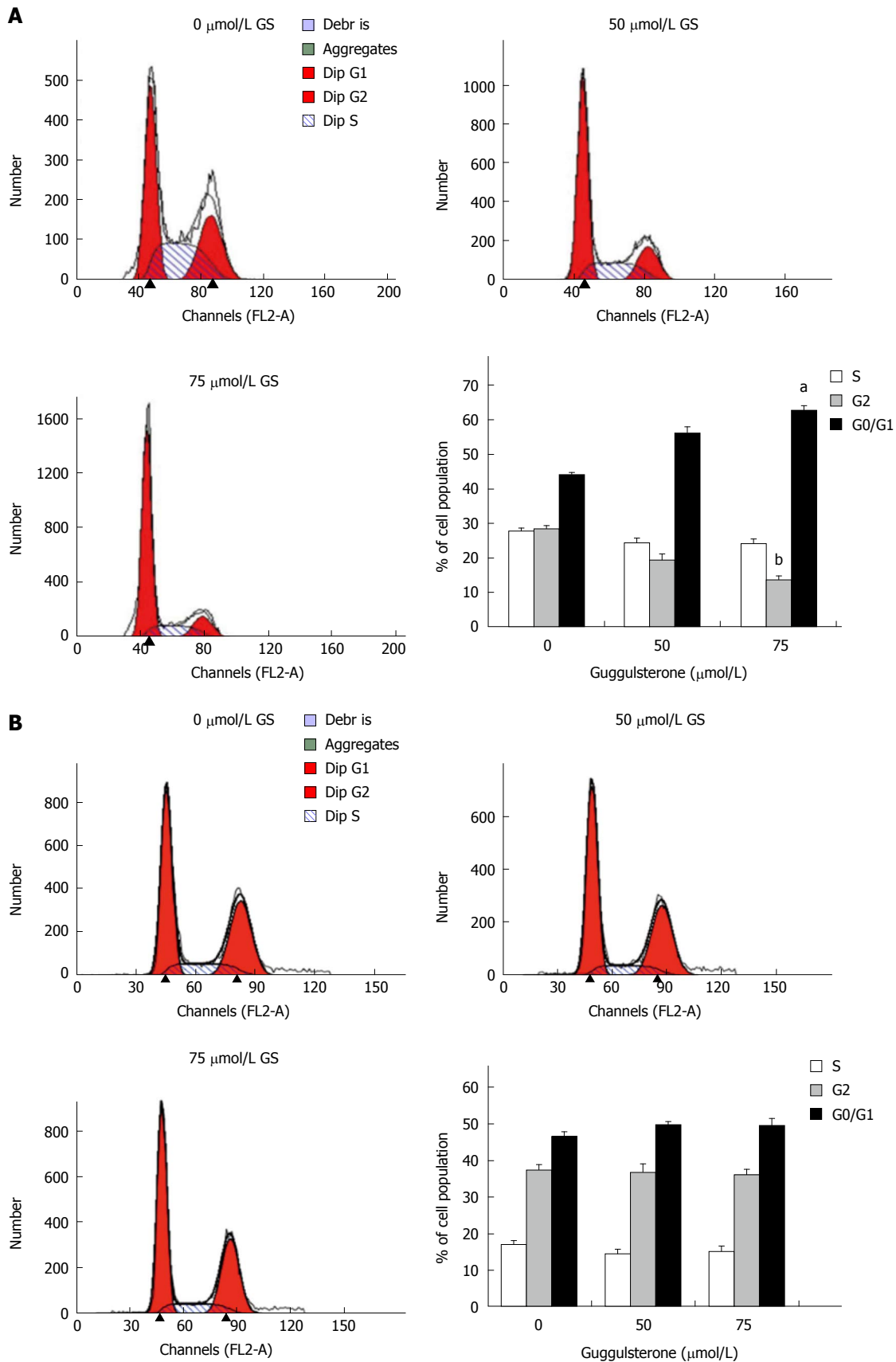


Figure 2 Guggulsterone alters cell cycle in human HepG2 and L-02 cells. Human HepG2 (A) and L-02 cells (B) were treated with 0 $\mu\text{mol/L}$, 50 $\mu\text{mol/L}$ and 75 $\mu\text{mol/L}$ of GS for 24 h and the cell cycle was analyzed using flow cytometry, respectively. The histogram showed mean % of cell population in each phase of cell cycle. ^a $P < 0.05$ or ^b $P < 0.01$, vs the untreated control group.

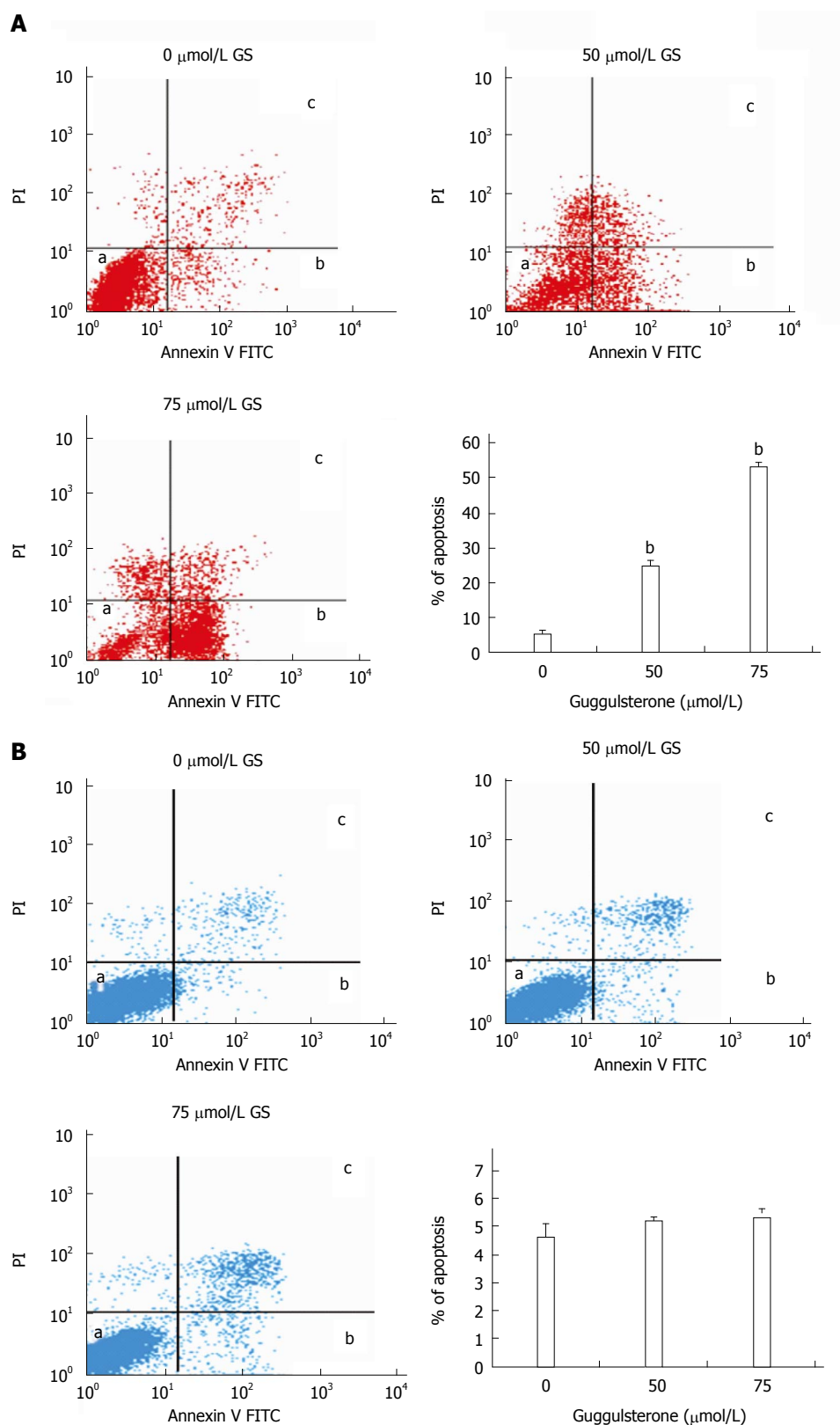


Figure 3 Guggulsterone induces apoptosis in human HepG2 and L-02 cells. Human HepG2 (A) and L-02 cells (B) were treated with 0 $\mu\text{mol/L}$, 50 $\mu\text{mol/L}$ and 75 $\mu\text{mol/L}$ of GS for 24 h and cell apoptosis was investigated using annexin V-FITC and PI. GS significantly increased HepG2 cell apoptosis in a dose-dependent manner. $^bP < 0.01$, vs the control group.

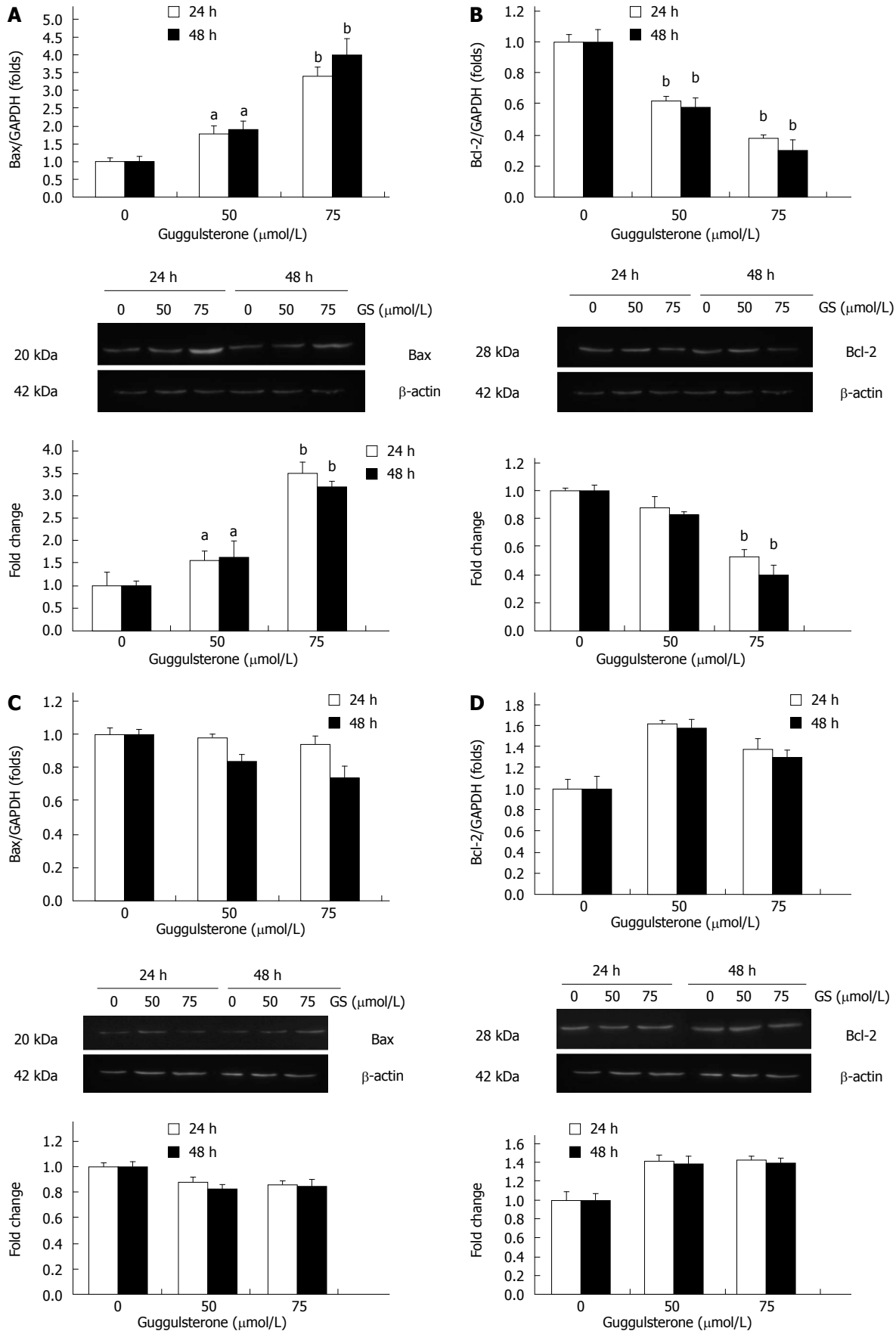


Figure 4 Guggulsterone differentially alters Bax and Bcl-2 expression in HepG2 cell. HepG2 (A and B) and L-02 cells (C and D) were treated with 0 μmol/L, 50 μmol/L and 75 μmol/L of GS for 24 and 48 h, respectively. Bax and Bcl-2 mRNA and protein levels were investigated using real-time PCR and Western blot, respectively. * $P < 0.05$ or ** $P < 0.01$, vs the control group.

GS for 24 h, the percentages of apoptotic cells in HepG2 cells were $24.91\% \pm 2.41\%$ and $53.03\% \pm 2.28\%$, respectively, significantly higher than that in the untreated control cells ($5.18\% \pm 1.74$, $P < 0.01$) (Figure 3A). However there was no significant difference in L-02 cell apoptosis in response to 50 $\mu\text{mol/L}$ and 75 $\mu\text{mol/L}$ GS treatment ($P > 0.05$; Figure 3B).

GS induces apoptosis through activation of intrinsic mitochondrial signaling pathway

There was a significant increase in Bax mRNA and protein levels in HepG2 cells treated with 50 $\mu\text{mol/L}$ and 75 $\mu\text{mol/L}$ GS for 24 and 48 h, respectively (Figure 4A). Differently, Bcl-2 mRNA and protein contents in HepG2 cells were significantly reduced in response to 50 $\mu\text{mol/L}$ and 75 $\mu\text{mol/L}$ GS (Figure 4B). However, neither 50 $\mu\text{mol/L}$ nor 75 $\mu\text{mol/L}$ induced significant alterations in Bax (Figure 4C) or Bcl-2 expression (Figure 4D) in L-02 cells ($P > 0.05$).

Effect of GS on TGF- β 1, TNF- α and VEGF cytokines

After treatment with 35, 50 and 75 $\mu\text{mol/L}$ of GS for 24 h, TGF- β 1 and VEGF levels in HepG2 cells were significantly decreased when compared with the control group ($P < 0.01$; Figure 5A and B). Differently, TNF- α contents in HepG2 cells were significantly increased in a dose-dependent manner in response to the treatment with 35, 50 and 75 $\mu\text{mol/L}$ of GS when compared with the untreated cells (Figure 5C). Interestingly, there was no significant difference in TGF- β 1 (Figure 5D), VEGF (Figure 5E) or TNF- α levels (Figure 5F) in L-02 cells after treatment with 35, 50 and 75 $\mu\text{mol/L}$ of GS for 24 h.

DISCUSSION

GS has been used as an anticancer agent due to its cell proliferation inhibition and pro-apoptosis effects in tumor cells^[10,18]. However, whether GS can be used in HCC treatment is still largely unknown. In this study, the proliferation of HepG2 cells was significantly inhibited by GS in a dose- and time-dependent manner. More importantly, cell proliferation in normal hepatic cell line L-02 was less affected by GS when compared with HepG2 cells, suggesting that normal hepatocytes are significantly more resistant to growth inhibition by GS compared with HCC cells. Therefore, we investigated whether GS treatment has a selective activity to human HCC cells and normal liver cells by conducting experiments on the effects of GS on cell cycle and apoptosis of L-02, a normal human hepatic cell line.

G0/G1 fractions of cell cycles were increased but G2/M fractions were decreased in HepG2 cells in response to treatment with GS, indicating that GS arrested HepG2 cell cycle in G0/G1 phase, and thereby inhibited HCC cell proliferation. The precise mechanism

is not clear, however, previous studies performed in other tumor cell types have illustrated that GS regulates cell cycle *via* downregulated expression level of cell cycle regulatory protein cyclin D1 and induced expression of cyclin dependent kinase inhibitor P21^{WAF1/CIP1} and P27^[24,25].

Furthermore, we found that GS induced HepG2 cell apoptosis in a dose-dependent way, which is also consistent with previous studies^[10,24,26]. Our further investigation indicated that GS increased Bax but decreased Bcl-2 gene and protein expression in HepG2 cells, illustrating that the intrinsic mitochondrial pathway was involved in the pro-apoptosis effect of GS. A variety of stress stimuli including growth factor withdrawal, heat shock, and oxidative damage have been shown to activate the apoptosis intrinsic or mitochondrial pathway^[27-29]. TGF- β is a multifunctional cytokine involved in the regulation of apoptosis of many cell types and is implicated in the pathogenesis of human diseases, including carcinogenesis. The VEGF family plays a pivotal role in tumor angiogenesis and is responsible for solid tumor growth and metastasis^[29-31]. We therefore investigated the effects of GS on the levels of TGF and VEGF which have been confirmed to be involved in the HCC occurrence and development. As expected, TGF- β 1 and VEGF concentrations were significantly decreased in response to the treatment with GS in a dose-dependent manner, indicating that these two growth factors may be negatively involved in the HCC cell apoptosis. The underlying mechanism is still unknown, however, previous studies have shown that the low expression of their receptors on the cell surface induced by GS may be involved^[32,33].

Accumulating evidence has suggested that GS has chemopreventive and chemotherapeutic potential for cancer treatment. Although the underlying mechanisms are not fully understood, these studies including ours clearly indicated that anticancer activity of GS is associated with apoptosis induction. Specifically, GS treatment of cancer cells induces the alteration and regulation of Bcl-2 gene family proteins, NF- κ B signaling, MAPK pathways, farnesoid X receptor, EGFR-STAT3 signaling, *etc.* However, these results are largely from other cancer cell types. It is still largely unknown whether GS has similar effects on HCC cells. In the present study, for the first time, we showed that GS treatment significantly inhibited cell growth, induced cell cycle arrest and caused apoptotic cell death in human HCC cells. Our results revealed a novel anti-HCC mechanism of GS, in which the mitochondrial pathway is involved in the GS induced apoptosis in human hepatocarcinoma.

Interestingly, GS treatment did not induce a significant cell cycle arrest and apoptosis in L-02 cells, which may explain the reason why a normal human hepatocyte cell line is more resistant to the growth inhibition by GS as described above. Unsurprisingly, our further investigation showed less alterations in

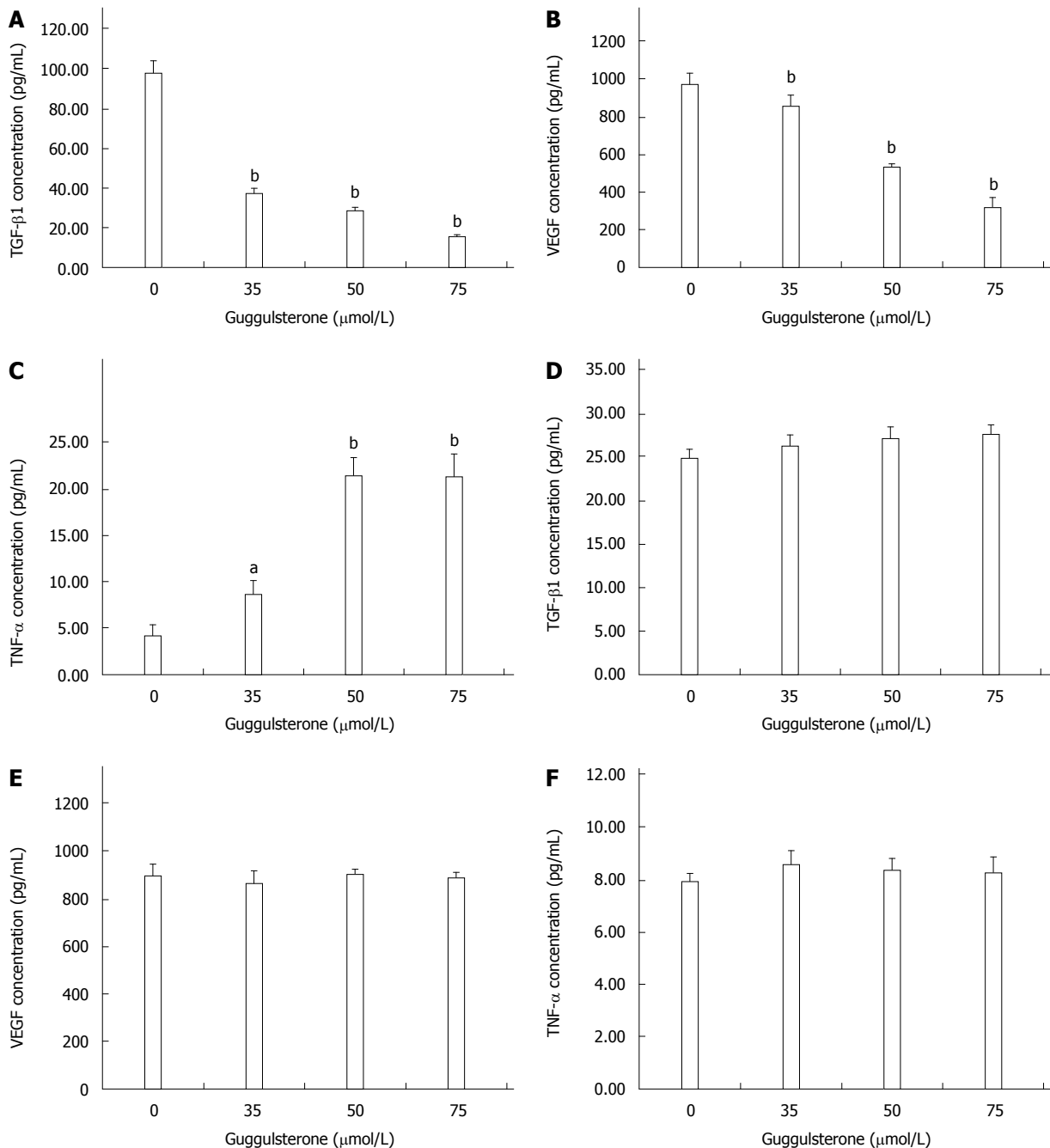


Figure 5 Guggulsterone alters TGF- β 1, TNF- α and VEGF levels in HepG2 and L-02 cells. HepG2 (A, B, and C) and L-02 cells (D, E, and F) were treated with 0, 35, 50 or 75 $\mu\text{mol/L}$ of GS for 24 h, respectively. TGF- β 1, TNF- α and VEGF levels in the culture medium were investigated using ELISA. ^a $P < 0.05$ or ^b $P < 0.01$, vs the control group.

mitochondrial pathway protein bax and Bcl-2, as well as growth factors/cytokines including TGF- β 1, VEGF and TNF- α levels in L-02 cells in response to the treatment with GS. These results indicated that normal hepatocytes are more resistant to the growth inhibition by GS when compared with the HCC cells, which is consistent to previous results in other cancer cell types^[34]. The mechanism is largely unknown, however, uncharacterized constituent(s) of GS may interact additively or synergistically to inhibit the viability of the

cancer cells.

In conclusion, we demonstrated that GS inhibited the viability of human HCC cells by regulated cell cycle, induced tumor cell apoptosis by activation of the mitochondrial pathway, and decreased TGF- β 1 and VEGF but increased TNF- α levels. These results suggest that GS influences HCC phenotypes by inhibiting cell proliferation, promoting cell death and regulating carcinogenesis-related growth factors, and therefore is a potential anticancer agent for the

treatment of human HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer and the second leading cause of tumor mortality worldwide. Guggulsterone (GS) is a phytosterol extracted from the gum resin of guggul plants that shows strong antioxidant, anti-inflammatory, hypolipidemic and hypocholesteremic properties, and has been used for treatment of several malignant diseases. Apoptosis is known as programmed cell death, which is associated with a distinct set of signaling pathways including the intrinsic mitochondrial pathway and the extrinsic death receptor pathway. Previous studies have shown that GS can induce cancer cell apoptosis through activation of the extrinsic death receptor pathway. However, it has not been determined whether GS has anti-HCC effects through other signaling pathways, such as the intrinsic mitochondrial pathway.

Research frontiers

Due to the strong antioxidant, anti-inflammatory, hypolipidemic and hypocholesteremic properties of GS, it has been used for treatment of several malignant diseases including HCC. Pro-apoptotic effect of GS has been found to play a pivotal role in its anti-carcinogenic mechanisms, however, its precise mechanism is not fully understood. Therefore, the relationship between GS and cell apoptosis becomes an important research area in this field.

Innovations and breakthroughs

In this study, the authors demonstrated that GS inhibited the viability of human HCC cells by regulated cell cycle, induced tumor cell apoptosis by activation of the mitochondrial pathway, and decreased TGF- β 1 and VEGF but increased TNF- α levels. These results suggest that GS influences HCC phenotypes by inhibiting cell proliferation, promoting cell death and regulating carcinogenesis-related growth factors, and therefore is a potential anticancer agent for the treatment of human HCC. This is the first systematical study demonstrating that GS induces HCC cell apoptosis through activation of the mitochondrial pathway, and evidence shown here expands our knowledge of the precise mechanism how GS inhibits HCC development.

Applications

The study results suggest that GS induces HCC apoptosis and could be a potential treatment drug for this commonly prevalent tumor in humans in the future.

Peer-review

Other reports have shown that GS is pro-apoptotic and modulates various genes. These authors show that GS arrested HepG2 cycle at G0/G1 phase. Bax mRNA was increased together with other changes, suggesting that the intrinsic mitochondrial pathway is involved in GS effects. The study indicates that GS is a potential anticancer therapy for HCC.

REFERENCES

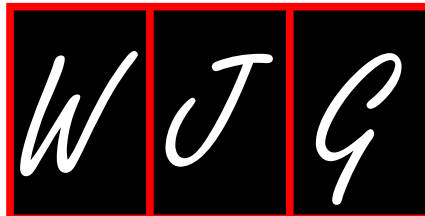
- 1 Kew MC. Hepatocellular carcinoma: epidemiology and risk factors. Available from: URL: <http://www.dovepress.com/hepatocellular-carcinoma-epidemiology-and-risk-factors-peer-reviewed-article-JHC#>
- 2 Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; **127**: 2893-2917 [PMID: 21351269 DOI: 10.1002/ijc.25516]
- 3 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]
- 4 Surh YJ. Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* 2003; **3**: 768-780 [PMID: 14570043 DOI: 10.1038/nrc1189]
- 5 Aggarwal BB, Takada Y, Oommen OV. From chemoprevention to chemotherapy: common targets and common goals. *Expert Opin Investig Drugs* 2004; **13**: 1327-1338 [PMID: 15461561 DOI: 10.1517/13543784.13.10.1327]
- 6 Lee JY, Lee KT, Lee JK, Lee KH, Jang KT, Heo JS, Choi SH, Kim Y, Rhee JC. Farnesoid X receptor, overexpressed in pancreatic cancer with lymph node metastasis promotes cell migration and invasion. *Br J Cancer* 2011; **104**: 1027-1037 [PMID: 21364590 DOI: 10.1038/bjc.2011.37]
- 7 Niranjana R, Kamat PK, Nath C, Shukla R. Evaluation of guggulipid and nimesulide on production of inflammatory mediators and GFAP expression in LPS stimulated rat astrocytoma, cell line (C6). *J Ethnopharmacol* 2010; **127**: 625-630 [PMID: 20018235 DOI: 10.1016/j.jep.2009.12.012]
- 8 Yang D, Yang J, Shi D, Xiao D, Chen YT, Black C, Deng R, Yan B. Hypolipidemic agent Z-guggulsterone: metabolism interplays with induction of carboxylesterase and bile salt export pump. *J Lipid Res* 2012; **53**: 529-539 [PMID: 22246918 DOI: 10.1194/jlr.M014688]
- 9 Almazari I, Park JM, Park SA, Suh JY, Na HK, Cha YN, Surh YJ. Guggulsterone induces heme oxygenase-1 expression through activation of Nrf2 in human mammary epithelial cells: PTEN as a putative target. *Carcinogenesis* 2012; **33**: 368-376 [PMID: 22095074 DOI: 10.1093/carcin/bgr259]
- 10 An MJ, Cheon JH, Kim SW, Kim ES, Kim TI, Kim WH. Guggulsterone induces apoptosis in colon cancer cells and inhibits tumor growth in murine colorectal cancer xenografts. *Cancer Lett* 2009; **279**: 93-100 [PMID: 19232820 DOI: 10.1016/j.canlet.2009.01.026]
- 11 Yamada T, Osawa S, Hamaya Y, Furuta T, Hishida A, Kajimura M, Ikuma M. Guggulsterone suppresses bile acid-induced and constitutive caudal-related homeobox 2 expression in gut-derived adenocarcinoma cells. *Anticancer Res* 2010; **30**: 1953-1960 [PMID: 20651339]
- 12 Chen KF, Chen HL, Liu CY, Tai WT, Ichikawa K, Chen PJ, Cheng AL. Dovitinib sensitizes hepatocellular carcinoma cells to TRAIL and tigatuzumab, a novel anti-DR5 antibody, through SHP-1-dependent inhibition of STAT3. *Biochem Pharmacol* 2012; **83**: 769-777 [PMID: 22230479 DOI: 10.1016/j.bcp.2011.12.035]
- 13 Shishodia S, Sethi G, Ahn KS, Aggarwal BB. Guggulsterone inhibits tumor cell proliferation, induces S-phase arrest, and promotes apoptosis through activation of c-Jun N-terminal kinase, suppression of Akt pathway, and downregulation of antiapoptotic gene products. *Biochem Pharmacol* 2007; **74**: 118-130 [PMID: 17475222 DOI: 10.1016/j.bcp.2007.03.026]
- 14 Debatin KM. Apoptosis pathways in cancer and cancer therapy. *Cancer Immunol Immunother* 2004; **53**: 153-159 [PMID: 14749900 DOI: 10.1007/s00262-003-0474-8]
- 15 Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell* 2004; **116**: 205-219 [PMID: 14744432 DOI: 10.1016/S0092-8674(04)00046-7]
- 16 Sprick MR, Walczak H. The interplay between the Bcl-2 family and death receptor-mediated apoptosis. *Biochim Biophys Acta* 2004; **1644**: 125-132 [PMID: 14996497 DOI: 10.1016/j.bbamer.2003.11.002]
- 17 Ricci MS, Zong WX. Chemotherapeutic approaches for targeting cell death pathways. *Oncologist* 2006; **11**: 342-357 [PMID: 16614230 DOI: 10.1634/theoncologist.11-4-342]
- 18 Singh SV, Choi S, Zeng Y, Hahm ER, Xiao D. Guggulsterone-induced apoptosis in human prostate cancer cells is caused by reactive oxygen intermediate dependent activation of c-Jun NH2-terminal kinase. *Cancer Res* 2007; **67**: 7439-7449 [PMID: 17671214 DOI: 10.1158/0008-5472.CAN-07-0120]
- 19 Singh SV, Zeng Y, Xiao D, Vogel VG, Nelson JB, Dhir R, Tripathi YB. Caspase-dependent apoptosis induction by guggulsterone, a constituent of Ayurvedic medicinal plant Commiphora mukul, in PC-3 human prostate cancer cells is mediated by Bax and Bak. *Mol Cancer Ther* 2005; **4**: 1747-1754 [PMID: 16275996 DOI: 10.1158/1535-7163.MCT-05-0223]
- 20 Anan A, Gores GJ. A new TRAIL to therapy of hepatocellular carcinoma: blocking the proteasome. *Hepatology* 2005; **42**:

- 527-529 [PMID: 16116625 DOI: 10.1002/hep.20869]
- 21 **Moon DO**, Park SY, Choi YH, Ahn JS, Kim GY. Guggulsterone sensitizes hepatoma cells to TRAIL-induced apoptosis through the induction of CHOP-dependent DR5: involvement of ROS-dependent ER-stress. *Biochem Pharmacol* 2011; **82**: 1641-1650 [PMID: 21903093 DOI: 10.1016/j.bcp.2011.08.019]
 - 22 **Sun MZ**, Dang SS, Wang WJ, Jia XL, Zhai S, Zhang X, Li M, Li YP, Xun M. Cytokeratin 8 is increased in hepatitis C virus cells and its ectopic expression induces apoptosis of SMMC7721 cells. *World J Gastroenterol* 2013; **19**: 6178-6187 [PMID: 24115814 DOI: 10.3748/wjg.v19.i37.6178]
 - 23 **Dang SS**, Sun MZ, Yang E, Xun M, Ma L, Jia ZS, Wang WJ, Jia XL. Prohibitin is overexpressed in Huh-7-HCV and Huh-7.5-HCV cells harboring in vitro transcribed full-length hepatitis C virus RNA. *Virol J* 2011; **8**: 424 [PMID: 21896168 DOI: 10.1186/1743-422X-8-424]
 - 24 **Macha MA**, Matta A, Chauhan S, Siu KM, Ralhan R. 14-3-3 zeta is a molecular target in guggulsterone induced apoptosis in head and neck cancer cells. *BMC Cancer* 2010; **10**: 655 [PMID: 21118500 DOI: 10.1186/1471-2407-10-655]
 - 25 **Leeman-Neill RJ**, Wheeler SE, Singh SV, Thomas SM, Seethala RR, Neill DB, Panahandeh MC, Hahm ER, Joyce SC, Sen M, Cai Q, Freilino ML, Li C, Johnson DE, Grandis JR. Guggulsterone enhances head and neck cancer therapies via inhibition of signal transducer and activator of transcription-3. *Carcinogenesis* 2009; **30**: 1848-1856 [PMID: 19762335 DOI: 10.1093/carcin/bgp211]
 - 26 **Patel MP**, Masood A, Patel PS, Chanan-Khan AA. Targeting the Bcl-2. *Curr Opin Oncol* 2009; **21**: 516-523 [PMID: 19730103 DOI: 10.1097/CCO.0b013e328331a7a4]
 - 27 **Sinha K**, Das J, Pal PB, Sil PC. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. *Arch Toxicol* 2013; **87**: 1157-1180 [PMID: 23543009 DOI: 10.1007/s00204-013-1034-4]
 - 28 **Schulz R**, Moll UM. Targeting the heat shock protein 90: a rational way to inhibit macrophage migration inhibitory factor function in cancer. *Curr Opin Oncol* 2014; **26**: 108-113 [PMID: 24225413 DOI: 10.1097/CCO.0000000000000036]
 - 29 **Principe DR**, Doll JA, Bauer J, Jung B, Munshi HG, Bartholin L, Pasche B, Lee C, Grippo PJ. TGF- β : duality of function between tumor prevention and carcinogenesis. *J Natl Cancer Inst* 2014; **106**: djt369 [PMID: 24511106 DOI: 10.1093/jnci/djt369]
 - 30 **Jakowlew SB**. Transforming growth factor-beta in cancer and metastasis. *Cancer Metastasis Rev* 2006; **25**: 435-457 [PMID: 16951986 DOI: 10.1007/s10555-006-9006-2]
 - 31 **Shahneh FZ**, Baradaran B, Zamani F, Aghebati-Maleki L. Tumor angiogenesis and anti-angiogenic therapies. *Hum Antibodies* 2013; **22**: 15-19 [PMID: 24284305 DOI: 10.3233/HAB-130267]
 - 32 **Kim ES**, Hong SY, Lee HK, Kim SW, An MJ, Kim TI, Lee KR, Kim WH, Cheon JH. Guggulsterone inhibits angiogenesis by blocking STAT3 and VEGF expression in colon cancer cells. *Oncol Rep* 2008; **20**: 1321-1327 [PMID: 19020709 DOI: 10.3892/or.00000147]
 - 33 **Xiao D**, Singh SV. z-Guggulsterone, a constituent of Ayurvedic medicinal plant Commiphora mukul, inhibits angiogenesis in vitro and in vivo. *Mol Cancer Ther* 2008; **7**: 171-180 [PMID: 18202020 DOI: 10.1158/1535-7163.MCT-07-0491]
 - 34 **Jiang G**, Xiao X, Zeng Y, Nagabhushanam K, Majeed M, Xiao D. Targeting beta-catenin signaling to induce apoptosis in human breast cancer cells by z-guggulsterone and Gugulipid extract of Ayurvedic medicine plant Commiphora mukul. *BMC Complement Altern Med* 2013; **13**: 203 [PMID: 23914993 DOI: 10.1186/1472-6882-13-203]

P- Reviewer: Shukla SD **S- Editor:** Ma YJ

L- Editor: Wang TQ **E- Editor:** Liu XM





Basic Study

Mast cell tryptase and carboxypeptidase A expression in body fluid and gastrointestinal tract associated with drug-related fatal anaphylaxis

Xiang-Jie Guo, Ying-Yuan Wang, Hao-Yue Zhang, Qian-Qian Jin, Cai-Rong Gao

Xiang-Jie Guo, Ying-Yuan Wang, Hao-Yue Zhang, Qian-Qian Jin, Cai-Rong Gao, Department of Forensic Medicine, Shanxi Medical University, Taiyuan 030001, Shanxi Province, China

Author contributions: Gao CR designed the research; Guo XJ, Wang YY, Zhang HY and Jin QQ performed the research; Guo XJ and Gao CR wrote the paper.

Supported by the National Natural Science Foundation of China, No. 81172905; and Shanxi Province Science Foundation for Youths, No. 2012021032-2.

Institutional review board statement: All experiments were approved by the ethics committee of Shanxi Medical University.

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

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at 258187101@qq.com.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Dr. Cai-Rong Gao, Department of Forensic Medicine, Shanxi Medical University, No. 56 South Xinjian Road, Taiyuan 030001, Shanxi Province, China. 258187101@qq.com
Telephone: +86-351-4135175
Fax: +86-351-4135175

Received: May 24, 2015
Peer-review started: May 25, 2015
First decision: June 25, 2015

Revised: July 9, 2015
Accepted: September 30, 2015
Article in press: September 30, 2015
Published online: December 21, 2015

Abstract

AIM: To investigate the expression of mast cell tryptase and carboxypeptidase A in drug-related fatal anaphylaxis.

METHODS: The expression of mast cell tryptase and carboxypeptidase A in 15 autopsy cases of drug-related fatal anaphylaxis and 20 normal autopsy cases were detected. First, the expression of mast cell tryptase was determined in stomach, jejunum, lung, heart, and larynx by immunofluorescence. Different tissues were removed and fixed in paraformaldehyde solution, then paraffin sections were prepared for immunofluorescence. Using specific mast cell tryptase and carboxypeptidase A antibodies, the expression of tryptase and carboxypeptidase A in gastroenterology tract and other tissues were observed using fluorescent microscopy. The postmortem serum and pericardial fluid were collected from drug-related fatal anaphylaxis and normal autopsy cases. The level of mast cell tryptase and carboxypeptidase A in postmortem serum and pericardial fluid were measured using fluor enzyme linked immunosorbent assay (FEIA) and enzyme linked immunosorbent assay (ELISA) assay. The expression of mast cell tryptase and carboxypeptidase A was analyzed in drug-related fatal anaphylaxis cases and compared to normal autopsy cases.

RESULTS: The expression of carboxypeptidase A was less in the gastroenterology tract and other tissues from anaphylaxis-related death cadavers than

normal controls. Immunofluorescence revealed that tryptase expression was significantly increased in multiple organs, especially the gastrointestinal tract, from anaphylaxis-related death cadavers compared to normal autopsy cases (46.67 ± 11.11 vs 4.88 ± 1.56 in stomach, 48.89 ± 11.02 vs 5.21 ± 1.34 in jejunum, 33.72 ± 5.76 vs 1.30 ± 1.02 in lung, 40.08 ± 7.56 vs 1.67 ± 1.03 in larynx, 7.11 ± 5.67 vs 1.10 ± 0.77 in heart, $P < 0.05$). Tryptase levels, as measured with FEIA, were significantly increased in both sera (43.50 ± 0.48 $\mu\text{g/L}$ vs 5.40 ± 0.36 $\mu\text{g/L}$, $P < 0.05$) and pericardial fluid (28.64 ± 0.32 $\mu\text{g/L}$ vs 4.60 ± 0.48 $\mu\text{g/L}$, $P < 0.05$) from the anaphylaxis group in comparison with the control group. As measured by ELISA, the concentration of carboxypeptidase A was also increased more than 2-fold in the anaphylaxis group compared to control (8.99 ± 3.91 ng/mL vs 3.25 ± 2.30 ng/mL in serum, 4.34 ± 2.41 ng/mL vs 1.43 ± 0.58 ng/mL in pericardial fluid, $P < 0.05$).

CONCLUSION: Detection of both mast cell tryptase and carboxypeptidase A could improve the forensic identification of drug-related fatal anaphylaxis.

Key words: Gastrointestinal tract; Drug-related fatal anaphylaxis; Forensic Pathology; Mast cell carboxypeptidase A; Mast cell tryptase

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Drug-related fatal anaphylaxis is occasionally encountered in forensic pathology routine. However, markers in the identification of drug-related fatal anaphylaxis still need further exploration. This study identified two important markers in drug-related fatal anaphylaxis, tryptase and carboxypeptidase A, which may improve postmortem diagnosis of anaphylaxis in medicolegal expertise.

Guo XJ, Wang YY, Zhang HY, Jin QQ, Gao CR. Mast cell tryptase and carboxypeptidase A expression in body fluid and gastrointestinal tract associated with drug-related fatal anaphylaxis. *World J Gastroenterol* 2015; 21(47): 13288-13293 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13288.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13288>

INTRODUCTION

Drug-induced anaphylaxis, also called allergic shock, is an immunologically mediated event that occurs after drug exposure in sensitized individuals and could lead to death^[1-3]. However, postmortem diagnosis of anaphylaxis is difficult in medicolegal expertise. There was less different clinical symptom and pathological morphologic change between fatal anaphylaxis and general sudden death^[4,5]. In current autopsy cases,

general disease, intoxication, and violent death should be excluded first, and then exposure to allergen and clinical symptoms are evaluated in combination to identify anaphylaxis^[2,6]. Therefore, exploration of novel, precise methods for anaphylaxis identification could be important in routine forensic pathology.

Drug-induced anaphylaxis can be initiated by binding of foreign drugs to specific immunoglobulin E (IgE) on mast cells^[7,8]. Subsequently, various kinds of mediators are secreted from the mast cells, thereby inducing anaphylaxis^[7,9,10]. Tryptase is a serine protease mainly stored in the granules of mast cells that is released at the onset of anaphylaxis^[11]. Several studies have reported that serum tryptase levels may be a reliable indicator of anaphylaxis because of its long serum half-life compared to other secreted mediators^[11-13]. However, the normal value of tryptase varies in different countries. Thus, more a more precise standard should be determined. Another chemical mediator, mast cell carboxypeptidase A, has been the focus of postmortem diagnosis of anaphylaxis. Carboxypeptidase A is a secreted protease that may be released following activation of mast cells to mediate acute anaphylaxis^[14,15].

Therefore, we determined whether the level of carboxypeptidase A or a combination of carboxypeptidase A and tryptase could be meaningful in the postmortem diagnosis of anaphylaxis. In this study, the expression of tryptase and carboxypeptidase A in multiple organs of cadavers was detected by immunofluorescence. Fluor enzyme linked immunosorbent assay (FEIA) and enzyme linked immunosorbent assay (ELISA) were used to measure the level of tryptase and carboxypeptidase A in postmortem serum and pericardial fluid, respectively.

MATERIALS AND METHODS

Immunofluorescence of tryptase in different tissues

During autopsy, the stomach, jejunum, lung, heart, and larynx were removed, fixed, and embedded in paraffin for preparation of sections. Immunofluorescence was performed as previously described with minor alterations^[16]. Briefly, mouse anti-human mast cell tryptase, mouse anti-human carboxypeptidase A, and rabbit anti-mouse IgG-TRITC (Santa Cruz, Dallas, TX, United States) were used to detect tryptase in different organs. The sections were observed using fluorescence microscopy (BX61, Olympus, Tokyo, Japan). Ten random visual fields were imaged per section and the number of tryptase-positive cells was counted. All experiments were approved by the Ethics Committee of Shanxi Medical University.

Quantification of tryptase and carboxypeptidase A levels in serum and pericardial fluid

Blood was collected from the right cardiac cavity and

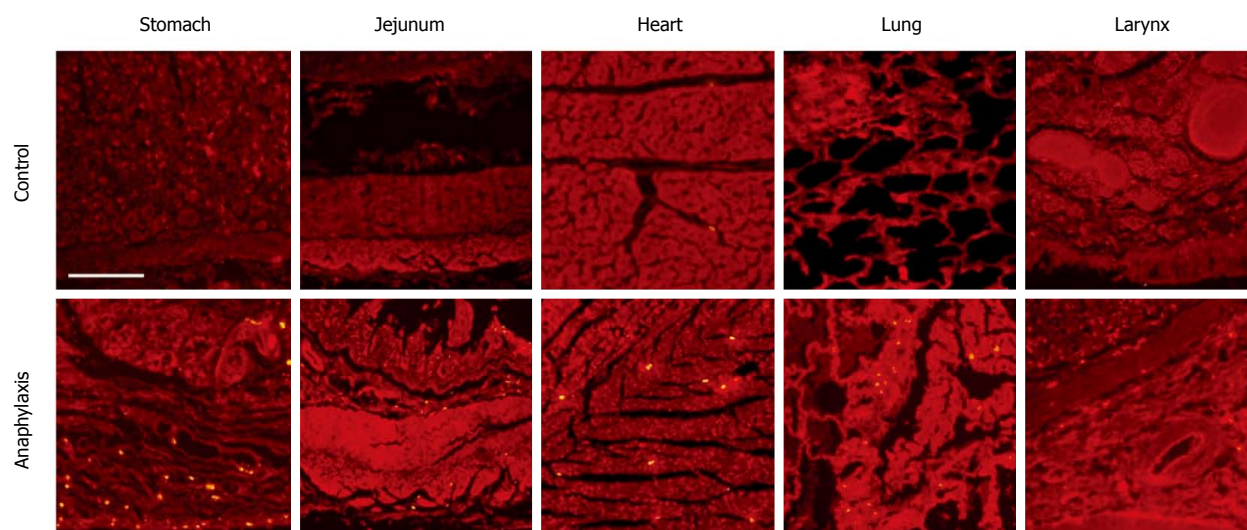


Figure 1 Immunofluorescence staining of tryptase in different organs. Scale bar = 200 μ m.

Table 1 Number of tryptase-positive particles in the anaphylaxis and control groups

	Control ($\times 100$)	Anaphylaxis ($\times 100$)
Stomach	4.88 \pm 1.56	46.67 \pm 11.11 ¹
Jejunum	5.21 \pm 1.34	48.89 \pm 11.02 ¹
Lung	1.30 \pm 1.02	33.72 \pm 5.76 ¹
Larynx	1.67 \pm 1.03	40.08 \pm 7.56 ¹
Heart	1.10 \pm 0.77	7.11 \pm 5.67 ¹

¹Denotes significant difference *vs* control, $P < 0.05$, $n = 10$.

centrifuged. The serum and pericardial fluid were stored at -80°C until use. Samples from 35 autopsy cases were measured. The causes of death in the anaphylaxis group (15 cases, 10 male, five female) included three of penicillin, three of ceftriaxone, three of levofloxacin, five of lomefloxacin *via* intravenous drip, and one of ibuprofen *via* oral administration. Anaphylaxis was diagnosed by clinical features, where the anaphylaxis symptoms occurred in all cases within 30 min. All postmortem autopsies were performed within 72 h. For the control group, 20 cases without allergic reaction, craniocerebral injury, coronary heart disease, and recreational drug use were selected. The level of tryptase in serum and pericardial fluid was measured by a commercial FEIA kit (Pharmacia Diagnostics, Uppsala, Sweden). Carboxypeptidase A levels were determined using an ELISA kit (Huamei Bio, Wuhan, China) according to the manufacturer's instructions.

Statistical analysis

Data were expressed as mean \pm SE, and a student's *t* test was used to compare differences between groups. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS version

17.0 software (Palo Alto, California, United States).

RESULTS

The expression of tryptase in different organs of anaphylaxis cadaver

Immunofluorescence was performed to detect the expression of carboxypeptidase A and tryptase in different organs. Less carboxypeptidase A was expressed in tissues from anaphylaxis cadaver than control (data not shown). Next, the expression of tryptase was detected in different organs. As shown in Figure 1, multiple tryptase-positive particles were observed in the mucous layer, with less in the muscular layer of the stomach and jejunum, from anaphylaxis cadaver. In contrast, the expression of tryptase was less in tissues from the control group. We also detected the expression of tryptase in some other tissues. Tryptase was observed in the bronchia wall and the small vessel wall in the lung, the small vessel wall in the submucosa of the larynx, and the periphery mesenchyme of the small vessels in the heart (Figure 1 and Table 1).

Determination of tryptase and carboxypeptidase A in postmortem serum and pericardial fluid

We examined tryptase in the sera and pericardial fluid from 15 autopsy cases who died of anaphylaxis and 20 control cases. The levels of tryptase were significantly increased in both sera and pericardial fluid from the anaphylaxis group in comparison with control group (Table 2). The concentrations of carboxypeptidase A were increased more than 2-fold in the anaphylaxis group compared to the control group (Table 3). Taken together, our results suggested that both tryptase and carboxypeptidase A were increased in drug-related fatal anaphylaxis.

Table 2 Expression of tryptase in serum and pericardial fluid

	<i>n</i>	Serum (μg/L)	Pericardial fluid (μg/L)
Control	20	5.40 ± 0.36	4.60 ± 0.48
Anaphylaxis	15	43.50 ± 0.48 ¹	28.64 ± 0.32 ¹

¹Denotes significant difference *vs* control, *P* < 0.05.**Table 3 Expression of carboxypeptidase A in serum and pericardial fluid**

	<i>n</i>	Serum (ng/mL)	Pericardial fluid (ng/mL)
Control	20	3.25 ± 2.30	1.43 ± 0.58
Anaphylaxis	15	8.99 ± 3.91 ¹	4.34 ± 2.41 ¹

¹Denotes significant difference *vs* control, *P* < 0.05.

DISCUSSION

Drug-induced fatal anaphylaxis is frequently encountered in medicolegal expertise. Some current indicators of anaphylaxis, including IgE and histamine, lack specificity or stability^[17-19]. Compared to other secreted mediators, tryptase and carboxypeptidase A have a long half-life *in vivo*, which led to the speculation that these two proteases may be superior indicators for the postmortem diagnosis of anaphylaxis^[20,21]. In the present study, we measured the levels of mast cell tryptase and carboxypeptidase A in postmortem serum and pericardial fluid. Schwartz *et al.*^[22] had reported that the concentration of tryptase increased rapidly after allergic shock and that it could be detected up to 4 d in autopsy. Moreover, the severity of the allergic reaction was shown to be highly related to tryptase level^[13,23,24]. Although the standard of serum tryptase is different in normal adults among countries, a tryptase value greater than 10 μg/L can be considered abnormal^[25,26]. We found that the level of tryptase in the serum from the anaphylaxis group was 8-fold higher than control. Meanwhile, this value in the pericardial fluid was 6-fold greater in the anaphylaxis group than control. These results were consistent with previous findings suggesting that tryptase may be a specific marker in the postmortem diagnosis of anaphylaxis. However, it has also been reported that serum tryptase levels are increased in patients with coronary heart disease, mastocytosis patients, and some drug abusers^[27-30]. Therefore, these causes of mortality should be excluded before making a diagnosis of anaphylaxis.

Another chemical mediator secreted from mast cells, carboxypeptidase A (also known as carboxypeptide A3, CPA3), was increased in allergic reactions, which was positively correlated to chymases^[31,32]. As shown for tryptase, carboxypeptide was also highly expressed in the epithelium of asthma patients^[33,34]. We confirmed that the level of carboxypeptidase A increased significantly in both postmortem serum and pericardial

fluid from anaphylaxis cadavers compared with control. Although there was less in-depth investigation of carboxypeptide levels in the postmortem serum from anaphylaxis cases, we speculate that the alteration of carboxypeptide was also meaningful. Measuring both carboxypeptide and tryptase could improve the postmortem diagnosis of anaphylaxis. Furthermore, determining levels of these mediators from the pericardial fluid in the closed serous cavity would help to avoid possible contamination after death.

During medicolegal expertise, the detection of indicators often occurs long after death, making it increasingly difficult to obtain the serum or pericardial fluid samples. Therefore, determining the expression of chemical markers in different organs from the cadavers is important. Although carboxypeptidase A was expressed less in tissues from both normal and anaphylaxis cadaver, the expression of tryptase in stomach, jejunum, lung, heart, and larynx from the drug-induced anaphylaxis group was significantly greater than the control group.

In conclusion, the expression of mast cell tryptase and carboxypeptidase A in body fluid and postmortem organs, especially gastrointestinal tract, could be meaningful in the identification of drug-related fatal anaphylaxis. Taken together with immunofluorescent identification, measurement of serum mast cell-specific tryptase and carboxypeptidase A levels might be a novel precise method that could improve postmortem diagnosis of anaphylaxis in medicolegal expertise. In addition, the detection of tryptase level in postmortem organs could also be meaningful in cases where it is difficult to collect serum/pericardial fluid due to the advanced state of decay during medicolegal expertise.

COMMENTS

Background

Drug-related fatal anaphylaxis could be occasionally encountered in routine forensic pathology. However, additional markers for the identification of drug-related fatal anaphylaxis are still needed.

Research frontiers

The exploration of novel markers and methods for the identification of drug-related fatal anaphylaxis could be important in the forensic identification of anaphylaxis.

Innovations and breakthroughs

This article provides new evidence for the use of mast cell tryptase and carboxypeptidase A as biomarkers to identify drug-related fatal anaphylaxis. It is suggested that the expression of tryptase and carboxypeptidase A in the gastroenterology tract and other tissues might be important markers in the case that it is difficult to collect serum/pericardial fluid because of the advanced state of decay during medicolegal expertise.

Applications

Combination of mast cell tryptase and carboxypeptidase A detection could improve the forensic identification of drug-related fatal anaphylaxis.

Peer-review

This is an interesting study about drug-related fatal anaphylaxis. The expression

of mast cell tryptase and carboxypeptidase A in drug-related fatal anaphylaxis are investigated. And the expression of mast cell tryptase and carboxypeptidase A in 15 autopsy cases of drug-related fatal anaphylaxis and 20 normal autopsy cases were detected. The authors concluded that combination of mast cell tryptase and carboxypeptidase A detection could improve the forensic identification of drug-related fatal anaphylaxis. And the detection of tryptase level in postmortem organs could also be meaningful in the case that hard to collect serum/pericardial fluid and advanced state of decay during medicolegal expertise.

REFERENCES

- Aun MV, Blanca M, Garro LS, Ribeiro MR, Kalil J, Motta AA, Castells M, Giavina-Bianchi P. Nonsteroidal anti-inflammatory drugs are major causes of drug-induced anaphylaxis. *J Allergy Clin Immunol Pract* 2014; **2**: 414-420 [PMID: 25017529 DOI: 10.1016/j.jaip.2014.03.014]
- Da Broi U, Moreschi C. Post-mortem diagnosis of anaphylaxis: A difficult task in forensic medicine. *Forensic Sci Int* 2011; **204**: 1-5 [PMID: 20684869 DOI: 10.1016/j.forsciint.2010.04.039]
- Kuruvilla M, Khan DA. Anaphylaxis to drugs. *Immunol Allergy Clin North Am* 2015; **35**: 303-319 [PMID: 25841553 DOI: 10.1016/j.iac.2015.01.008]
- Dworzynski K, Ardern-Jones M, Nasser S. Diagnosis and management of drug allergy in adults, children and young people: summary of NICE guidance. *BMJ* 2014; **349**: g4852 [PMID: 25186447 DOI: 10.1136/bmj.g4852]
- Jerschow E, Lin RY, Scaperotti MM, McGinn AP. Fatal anaphylaxis in the United States, 1999-2010: temporal patterns and demographic associations. *J Allergy Clin Immunol* 2014; **134**: 1318-1328.e7 [PMID: 25280385 DOI: 10.1016/j.jaci.2014.08.018]
- Kannan JA, Bernstein JA. Perioperative anaphylaxis: diagnosis, evaluation, and management. *Immunol Allergy Clin North Am* 2015; **35**: 321-334 [PMID: 25841554 DOI: 10.1016/j.iac.2015.01.002]
- Drain KL, Volcheck GW. Preventing and managing drug-induced anaphylaxis. *Drug Saf* 2001; **24**: 843-853 [PMID: 11665871]
- Burton OT, Noval Rivas M, Zhou JS, Logsdon SL, Darling AR, Koleoglou KJ, Roers A, Houshyar H, Crackower MA, Chatila TA, Oettgen HC. Immunoglobulin E signal inhibition during allergen ingestion leads to reversal of established food allergy and induction of regulatory T cells. *Immunity* 2014; **41**: 141-151 [PMID: 25017467 DOI: 10.1016/j.immuni.2014.05.017]
- Gruchalla RS. Clinical assessment of drug-induced disease. *Lancet* 2000; **356**: 1505-1511 [PMID: 11081549 DOI: 10.1016/S0140-6736(00)02885-3]
- Akin C. Mast cell activation syndromes presenting as anaphylaxis. *Immunol Allergy Clin North Am* 2015; **35**: 277-285 [PMID: 25841551 DOI: 10.1016/j.iac.2015.01.010]
- Matsson P, Enander I, Andersson AS, Nystrand J, Schwartz L, Watkins J. Evaluation of mast cell activation (tryptase) in two patients suffering from drug-induced hypotension reactions. *Agents Actions* 1991; **33**: 218-220 [PMID: 1897443]
- Cianferoni A, Novembre E, Mugnaini L, Lombardi E, Bernardini R, Pucci N, Vierucci A. Clinical features of acute anaphylaxis in patients admitted to a university hospital: an 11-year retrospective review (1985-1996). *Ann Allergy Asthma Immunol* 2001; **87**: 27-32 [PMID: 11476457 DOI: 10.1016/S1081-1206(10)62318-6]
- Sprung J, Weingarten TN, Schwartz LB. Presence or absence of elevated acute total serum tryptase by itself is not a definitive marker for an allergic reaction. *Anesthesiology* 2015; **122**: 713-714 [PMID: 25689761 DOI: 10.1097/ALN.0000000000000584]
- Lützelshwab C, Pejler G, Aveskogh M, Hellman L. Secretory granule proteases in rat mast cells. Cloning of 10 different serine proteases and a carboxypeptidase A from various rat mast cell populations. *J Exp Med* 1997; **185**: 13-29 [PMID: 8996238]
- Xing D, Zhang R, Li S, Huang P, Luo C, Hei Z, Xia Z, Gan X. Pivotal role of mast cell carboxypeptidase A in mediating protection against small intestinal ischemia-reperfusion injury in rats after ischemic preconditioning. *J Surg Res* 2014; **192**: 177-186 [PMID: 24953986 DOI: 10.1016/j.jss.2014.05.050]
- Chen M, Sun P, Liu XY, Dong D, Du J, Gu L, Ge YB. α -fetoprotein involvement during glucocorticoid-induced precocious maturation in rat colon. *World J Gastroenterol* 2011; **17**: 2933-2940 [PMID: 21734804 DOI: 10.3748/wjg.v17.i24.2933]
- Malo JL, Cartier A. Occupational reactions in the seafood industry. *Clin Rev Allergy* 1993; **11**: 223-240 [PMID: 8221510]
- Tyler D. Disability and medical management of natural latex sensitivity claims. *J Allergy Clin Immunol* 2002; **110**: S129-S136 [PMID: 12170254]
- Sakatani A, Doi Y, Matsuda T, Sasai Y, Nishida N, Sakamoto M, Uenoyama N, Matsumoto Y, Kinoshita K. Protracted anaphylaxis developed after peginterferon α -2a administration for chronic hepatitis C. *World J Gastroenterol* 2015; **21**: 2826-2829 [PMID: 25759556 DOI: 10.3748/wjg.v21.i9.2826]
- Nishio H, Takai S, Miyazaki M, Horiuchi H, Osawa M, Uemura K, Yoshida K, Mukaida M, Ueno Y, Suzuki K. Usefulness of serum mast cell-specific chymase levels for postmortem diagnosis of anaphylaxis. *Int J Legal Med* 2005; **119**: 331-334 [PMID: 15735956 DOI: 10.1007/s00414-005-0524-1]
- Cicarelli A, Calabrò C, Imperatore C, Scala G. Prick by prick induced anaphylaxis in a patient with peanuts and lupine allergy: awareness of risks and role of component resolved diagnosis. *Case Rep Med* 2014; **2014**: 892394 [PMID: 25477973 DOI: 10.1155/2014/892394]
- Schwartz LB, Yunginger JW, Miller J, Bokhari R, Dull D. Time course of appearance and disappearance of human mast cell tryptase in the circulation after anaphylaxis. *J Clin Invest* 1989; **83**: 1551-1555 [PMID: 2468689 DOI: 10.1172/JCI114051]
- Haeblerli G, Brönnimann M, Hunziker T, Müller U. Elevated basal serum tryptase and hymenoptera venom allergy: relation to severity of sting reactions and to safety and efficacy of venom immunotherapy. *Clin Exp Allergy* 2003; **33**: 1216-1220 [PMID: 12956741]
- Sprung J, Larson KJ, Divekar RD, Butterfield JH, Schwartz LB, Weingarten TN. Refractory intraoperative hypotension with elevated serum tryptase. *Asia Pac Allergy* 2015; **5**: 47-50 [PMID: 25653920 DOI: 10.5415/apallergy.2015.5.1.47]
- Vadas P, Perelman B, Liss G. Platelet-activating factor, histamine, and tryptase levels in human anaphylaxis. *J Allergy Clin Immunol* 2013; **131**: 144-149 [PMID: 23040367 DOI: 10.1016/j.jaci.2012.08.016]
- Edston E, van Hage-Hamsten M. Mast cell tryptase and hemolysis after trauma. *Forensic Sci Int* 2003; **131**: 8-13 [PMID: 12505465]
- Deligargyris EN, Upadhyay B, Sane DC, Dehmer GJ, Pye J, Smith SC, Boucher WS, Theoharides TC. Mast cell tryptase: a new biomarker in patients with stable coronary artery disease. *Atherosclerosis* 2005; **178**: 381-386 [PMID: 15694948 DOI: 10.1016/j.atherosclerosis.2004.09.008]
- Sperr WR, Jordan JH, Fiegl M, Escobedo L, Bellas C, Dirnhofer S, Semper H, Simonitsch-Klupp I, Horny HP, Valent P. Serum tryptase levels in patients with mastocytosis: correlation with mast cell burden and implication for defining the category of disease. *Int Arch Allergy Immunol* 2002; **128**: 136-141 [PMID: 12065914]
- Edston E, van Hage-Hamsten M. Anaphylactoid shock--a common cause of death in heroin addicts? *Allergy* 1997; **52**: 950-954 [PMID: 9298181]
- Maurer U, Kager C, Feller C, Loader D, Pollesböck A, Spitzer B, Jarisch R. Risk of anaphylaxis in opioid dependent persons: effects of heroin versus substitution substance. *Subst Abuse Treat Prev Policy* 2014; **9**: 12 [PMID: 24576327 DOI: 10.1186/1747-597X-9-12]
- Mayorga C, Sanz ML, Gamboa PM, García BE, Caballero MT, García JM, Labrador M, Lahoz C, Longo Aresó N, López Hoyos M, Martínez Quesada J, Monteseirín FJ. In vitro diagnosis of immediate allergic reactions to drugs: an update. *J Invest Allergol Clin Immunol* 2010; **20**: 103-109 [PMID: 20461964]
- Arias Á, Lucendo AJ, Martínez-Fernández P, González-Castro AM, Fortea M, González-Cervera J, Yagüe-Compadre JL, Mota-Huertas T, Vicario M. Dietary Treatment Modulates Mast Cell Phenotype, Density, and Activity in Adult Eosinophilic Esophagitis. *Clin Exp Allergy* 2015; Epub ahead of print [PMID: 25640519 DOI: 10.1111/cea.12504]

- 33 **Christ-Crain M**, Müller B. Biomarkers in respiratory tract infections: diagnostic guides to antibiotic prescription, prognostic markers and mediators. *Eur Respir J* 2007; **30**: 556-573 [PMID: 17766633 DOI: 10.1183/09031936.00166106]
- 34 **Kemona-Chetnik I**, Kowal K, Kucharewicz I, Pampuch A, Bodzenta-Lukaszyk A. [Thrombin activatable fibrinolysis inhibitor (TAFI) in allergic asthma patients]. *Przegl Lek* 2006; **63**: 1281-1285 [PMID: 17642140]

P- Reviewer: Ruffion A, Simpson ND **S- Editor:** Yu J
L- Editor: Filipodia **E- Editor:** Liu XM



Retrospective Study

Surgical care quality and oncologic outcome after D2 gastrectomy for gastric cancer

Johanna Mrena, Anne Mattila, Jan Böhm, Ismo Jantunen, Ilmo Kellokumpu

Johanna Mrena, Anne Mattila, Ilmo Kellokumpu, Department of Surgery, Central Hospital of Central Finland, 40620 Jyväskylä, Finland

Jan Böhm, Department of Pathology, Central Hospital of Central Finland, 40620 Jyväskylä, Finland

Ismo Jantunen, Department of Oncology, Central Hospital of Central Finland, 40620 Jyväskylä, Finland

Author contributions: Mrena J, Mattila A, Böhm J, Jantunen I and Kellokumpu I participated in the design of the study, acquisition of data, and article revisions; Kellokumpu I and Mrena J analyzed the data and wrote the article; all authors have approved the final version of the article.

Supported by the Central Hospital of Central Finland.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Central Hospital of Central Finland.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by verbal consent. Individuals can't be identified according to the data presented.

Conflict-of-interest statement: This work was presented as a poster at the ISW Congress in Helsinki, Finland, August 25-29, 2013. We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Johanna Mrena, MD, PhD, Department of Surgery, Central Hospital of Central Finland, Keskus-sairaalantie 19, 40620 Jyväskylä, Finland. johanna.mrena@ksshp.fi
Telephone: +358-14-2695048
Fax: +358-14-2692929

Received: May 29, 2015
Peer-review started: June 3, 2015
First decision: July 19, 2015
Revised: August 17, 2015
Accepted: October 23, 2015
Article in press: October 26, 2015
Published online: December 21, 2015

Abstract

AIM: To examine the quality of surgical care and long-term oncologic outcome after D2 gastrectomy for gastric cancer.

METHODS: From 1999 to 2008, a total of 109 consecutive patients underwent D2 gastrectomy without routine pancreateosplenectomy in a multimodal setting at our institution. Oncologic outcomes together with clinical and histopathologic data were analyzed in relation to the type of surgery performed. Staging was carried out according to the Union for International Cancer Control criteria of 2002. Patients were followed-up for five years at the outpatient clinic. The primary measure of outcome was long-term survival with the quality of surgery as a secondary outcome measure. Clinical data were retrospectively collected from the patient records, and causes of death were obtained from national registries.

RESULTS: A total of 109 patients (58 men) with a mean age of 67.4 ± 11.2 years underwent total

gastrectomy or gastric resection with D2 lymph node dissection. The tumor stage distribution was as follows: stage I, (27/109) 24.8%; stage II, (31/109) 28.4%; stage III, (41/109) 37.6%; and stage IV, (10/109) 9.2%. Forty patients (36.7%) received chemotherapy or chemoradiotherapy. The five-year overall survival rate for all 109 patients was 45.0%, and was 47.1% for the 104 patients treated with curative R0 resection. The five-year disease-specific survival rates were 53.0% and 55.8%, respectively. In a multivariate analysis, body mass index and tumor stage were independent prognostic factors for overall survival (both $P < 0.01$), whereas body mass index, tumor stage, tumor site, Lauren classification, and lymph node invasion were prognostic factors for cancer-specific survival (all $P < 0.05$). Postoperative 30-d mortality was 1.8% and 30-d, surgical (including three anastomotic leaks, two of which were treated conservatively), and general morbidities were 26.6%, 12.8%, and 14.7%, respectively.

CONCLUSION: D2 dissection is a safe surgical option for gastric cancer, providing quality surgical care and long-term oncologic outcomes that are in line with current Western standards.

Key words: Gastric cancer; Quality of care; Survival; Gastric surgery; Clinical practice

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Gastric cancer remains one of the most lethal malignancies worldwide. Although radical surgery with adequate lymphadenectomy is the cornerstone of curative treatment, whether D2 lymphadenectomy is applicable in Western hospitals is not clear, despite the low reported morbidity and mortality rates and survival benefit. This single-center study of 109 patients demonstrates that D2 lymphadenectomy can be performed with relatively low mortality (1.8%) and morbidity (26.6%). Five-year survival rates were 45.0% (overall) and 53.0% (disease-specific). Therefore, D2 gastrectomy can be considered as a safe surgical option for gastric cancer.

Mrena J, Mattila A, Böhm J, Jantunen I, Kellokumpu I. Surgical care quality and oncologic outcome after D2 gastrectomy for gastric cancer. *World J Gastroenterol* 2015; 21(47): 13294-13301 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13294.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13294>

INTRODUCTION

Gastric cancer is the sixth most common cancer and the third leading cause of cancer-related death worldwide^[1,2]. The cornerstone of treatment involves adequate surgery, though it is not curative, as most

patients present with advanced disease. As nodal involvement is indicative of a poor prognosis, more aggressive surgical approaches with lymph node removal have gained popularity^[3]. D2 lymph node dissection is currently the gold standard in gastric cancer surgery in Asia with regard to long-term survival and local recurrence^[4]. In the Western world, however, the role of D2 lymphadenectomy for the treatment of gastric cancer is controversial^[5,6].

Large European randomized studies report higher mortality and morbidity with no survival benefit from D2 dissection with splenic and/or pancreatic resection compared to D1 dissection^[7,8]. However, an Italian study found similar rates of postoperative complications (18% vs 12%) and mortality (2% vs 3%) when comparing D2 (with preservation of the spleen and pancreas) and D1 dissections^[9]. Another small single-center randomized trial comparing D1 and D3 (current D2) dissections showed a better overall survival after D2/3 dissection, but no significant difference in disease-specific survival^[10]. Finally, a Japanese randomized trial showed that extended D2 dissection with removal of para-aortic lymph nodes did not improve survival compared with standard D2 dissection^[11].

D2 lymphadenectomy with preservation of the spleen and pancreas is now regarded as the standard surgical technique for locally advanced gastric cancer. Patient outcome has been further improved by the centralization of procedures to experienced high-volume units and the use multimodal treatments^[12], such as chemotherapy and/or radiotherapy. An American study using postoperative chemoradiation^[13] and European studies using pre- and postoperative chemotherapy^[14,15] have shown improved survival with adjuvant therapies. Furthermore, a recent meta-analysis showed that adjuvant chemotherapy based on 5-fluorouracil (FU) regimens was associated with improved survival compared to surgery alone^[16]. However, whether to use pre- and postoperative chemotherapy, postoperative chemoradiotherapy, or adjuvant chemotherapy remains unclear.

Due to the availability of multiple individually tailored treatment options, it is important to monitor the quality of care, which also affects outcomes. In Finland, for example, there is no national quality audit for gastric cancer. Therefore, the aim of this study was to assess the long-term oncologic outcome and quality of surgical care for patients who underwent D2 gastrectomy at our institution between 1998 and 2008.

MATERIALS AND METHODS

Patients

This study examined 109 consecutive patients with histologically proven gastric adenocarcinoma that underwent D2 gastrectomy with curative intent at the Central Hospital of Central Finland in Jyväskylä

Table 1 Baseline characteristics (*n* = 109)

Characteristic	<i>n</i> (%)
Male gender	51 (46.8)
Body mass index (kg/m ²), mean (SD)	25.6 (4.9)
ASA score III-IV	67 (61.5)
Tumor site	
Upper	21 (19.3)
Middle	43 (39.4)
Lower	38 (34.9)
All levels	7 (6.4)
UICC tumor stage	
I	27 (24.8)
II	31 (28.4)
III	41 (37.6)
IV	10 (9.2)
Tumor type	
Intestinal	50 (45.9)
Diffuse	56 (51.4)
Mixed	3 (2.8)
Lymph node invasion	
No	45 (41.2)
Yes	64 (58.7)
Type of surgery	
Total gastrectomy	103 (94.5)
Subtotal gastric resection	6 (5.5)
D2-lymph node dissection	109 (100)
Splenectomy	48 (44.0)
Cholecystectomy	19 (17.4)
Additional organ resection due to cancer invasion	10 (9.2)
Radicality	
R0	104 (95.4)
Neoadjuvant/adjuvant treatment	
No	69 (63.3)
Neoadjuvant chemoradiotherapy	2 (1.8)
Adjuvant chemotherapy	26 (23.9)
Adjuvant chemoradiotherapy	12 (11.0)

ASA: American Society of Anesthesiologists; UICC: Union for International Cancer Control.

between January 1998 and December 2008. Pre-operative diagnosis and staging was performed *via* endoscopy and thoracoabdominal CT; laparoscopic staging was not routinely conducted during the study period. Clinical and follow-up patient data were collected retrospectively. Patients who underwent other surgical procedures (D1 dissection or palliative resections) were excluded. The study was approved by the ethics committee of the Central Hospital of Central Finland.

Surgical treatment

D2 lymph node dissections were conducted by senior upper gastrointestinal surgeons who also performed hepatobiliary and pancreatic surgeries. Resection of the pancreas was performed only when invasion by the gastric cancer was suspected. Indications for splenectomy were gastric carcinomas of the greater curvature in the upper and middle part of the stomach. Cholecystectomy was performed routinely, and additional organ resections were performed when deemed appropriate. The nodal dissection of the removed surgical specimen was performed by

surgeons on the bench in the operating room in a standardized fashion following the JRSGC classification system^[17]. R0 gastric resection was defined by the following parameters: negative resection margins, en bloc resection of adherent organs, and en bloc resection of the greater and lesser omentum.

Perioperative care during the study period included the assessment and optimization of medical risk factors, thromboprophylaxis with low-molecular-weight heparin and elastic stockings, prophylactic antibiotics, standard anesthesia with epidural analgesia, avoidance of hypothermia, and increased oxygen concentrations. Nasogastric tubes were removed in the operation theatre.

Pathology

Tumors were staged by pathologists according to the 2002 Union for International Cancer Control/Tumor-node-metastasis categories^[18]. Lymph node ratios were defined as the proportion of metastatic lymph nodes from the total number studied, and classified as follows: ratio, 0%, 1%-9%, 10%-25%, and > 25%^[19]. The histopathologic tumor type was evaluated according to Lauren classification^[20].

Neoadjuvant treatment and adjuvant chemotherapy

A selective approach for neoadjuvant and adjuvant oncologic treatments was used. Adjuvant postoperative chemotherapy consisted of FU-based regimens combined with epirubicin and cisplatin or oxaliplatin. Chemoradiotherapy (40-45 Gy and FU or capecitabine) was generally used in the postoperative setting, but two patients received preoperative chemoradiation (Table 1). None of the patients had perioperative chemotherapy^[14,15].

Follow-up

Patients were followed at the surgical outpatient clinic every six months for two years, and then once a year for up to five years from the operation. Tumor recurrence was defined as a recurrent tumor in the tumor bed or distant organs and diagnosed by CT, magnetic resonance imaging, or endoscopy. Histopathologic confirmation was not mandatory. The end of follow-up was June 30, 2014. The causes of death were obtained from the National Cause of Death Registry.

Statistical analysis

Statistical analyses were conducted using SPSS (version 15.0 for Windows; SPSS Inc., Chicago, IL, United States) and STATA (version 11; StatCorp LP, College Station, TX, United States) software. Results are given as mean ± SD or median (interquartile range). Pearson's χ^2 or Fisher's exact tests were used to compare frequencies, and Student's *t* and Mann-Whitney *U* tests were used for continuous variables. The Kaplan-Meier method was used to calculate the

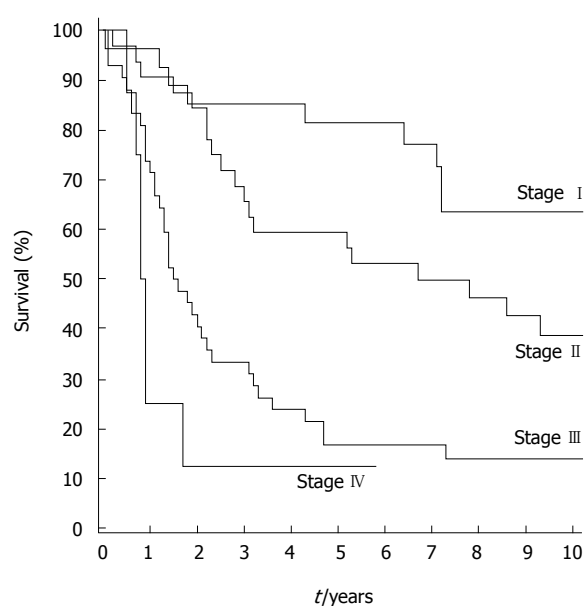


Figure 1 Overall survival rates by tumor stage.

survival, and the differences between groups were compared with the log-rank test. Survival times were calculated from the date of surgery until the time of death or the end of follow-up. Factors affecting survival were analyzed with univariate and multivariate Cox proportional hazards regression models; only variables with $P < 0.20$ were entered in the multivariate analysis. All statistical tests were two-sided, with a $P < 0.05$ considered as statistically significant.

RESULTS

Baseline characteristics

Baseline patient and tumor characteristics are shown in Table 1. A total of 109 patients with a mean age of 67.4 ± 11.2 years (median, 69 years; range: 59–77 years) underwent total gastrectomy or subtotal gastric resection with D2 lymph node dissection; 5/109 (4.6%) patients had residual microscopic disease upon histopathologic examination. Ten patients received multivisceral resections for locally invasive or metastatic disease, including resection of the colon ($n = 2$), pancreas ($n = 1$), liver ($n = 1$), esophagus ($n = 2$), kidney ($n = 2$), and ovaries ($n = 2$).

Survival

The overall median follow-up time was 3.3 (1.5–5.1) years, and 9.1 (7.9–12.3) years for the 37 surviving patients. No patients were lost to follow-up. The five- and 10-year overall survival rates were 45.0% [95%CI: 35.5%–54.0%] and 32.9% (95%CI: 23.9%–42.2%), respectively. The five- and 10-year disease-specific survival rates were 53.0% (95%CI: 42.7%–62.3%) and 47.8% (95%CI: 37.4%–57.5%). For the 104 patients with R0 resection, the five- and 10-year overall survival rates were 47.1% (95%CI:

37.3%–56.3%) and 34.5% (95%CI: 25.1%–44.0%) and disease-specific survival rates were 55.8% (95%CI: 45.2%–65.2%) and 50.3% (95%CI: 39.5%–60.2%), respectively. For tumor stages I, II, and III, the five-year overall survival rates were 81.5% (95%CI: 61.1%–91.8%), 61.3% (95%CI: 40.5%–74.0%), and 17.1% (95%CI: 7.3%–29.3%) (Figure 1), and disease-specific survival rates were 95.8% (95%CI: 73.9%–99.4%), 63.8% (95%CI: 44.2%–78.1%), and 22.6% (95%CI: 10.4%–37.7%), respectively.

Factors influencing survival

Univariate and multivariate analyses were performed to identify prognostic factors of overall and cancer-specific survival. The results of the univariate analysis are shown in Table 2. In the multivariate analysis, body mass index (BMI) and tumor stage were the independent prognostic factors of overall survival (both $P < 0.01$), and BMI, tumor stage, tumor site, lymph node invasion, and Lauren's classification (intestinal vs diffuse) were independent prognostic factors of cancer-specific survival (all $P < 0.05$). Of note, significantly more patients with BMI < 25 had metastatic lymph nodes than those with BMI > 25 (67.9% vs 49.1%, $P < 0.05$).

Quality of surgical care

The parameters reflecting the quality of care are shown in Table 3. Overall 30-d mortality was 1.8%, including one death from cerebral stroke and one from severe pneumonia. The overall complication rate was 26.6% (29/109) with 12.8% surgical and 14.7% general morbidity rates. The 30-d reoperation rate was 3.7% due to postoperative hemorrhage ($n = 1$), pleural empyema ($n = 1$), esophagojejunal anastomotic leak in a cirrhotic patient ($n = 1$), and colon obstruction ($n = 1$). The median postoperative hospital stay was 10 (8–12) d. Seven patients had a late reoperation because of bowel obstruction caused by locoregional recurrence or intra-abdominal carcinosis.

DISCUSSION

The treatment of gastric cancer is complex and has evolved from surgical management to a multi-disciplinary model^[12]. Consequently, overall five-year survival rates across all tumor stages range from 33%–47% as reported in European randomized trials^[7,8]. In the present cohort of patients undergoing D2 gastrectomy for gastric cancer without routine pancreaticosplenectomy and multimodal treatment, the five-year overall and cancer-specific survival rates were 45% and 53%, and slightly higher, at 47% and 56%, respectively, for those having R0 resection. Asian studies have reported higher survival rates ranging from 59% up to 70% depending on the extent of lymphadenectomy, with minor survival benefit gained

Table 2 Univariate analyses of prognostic factors for five-year survival (*n* = 109)

Variables	OS			DSS		
	Events/patients	HR (95%CI)	P value	Events/patients	HR (95%CI)	P value
Age (yr)			0.15 ¹			0.72 ¹
< 60	16/29	1		13/29	1	
60-75	31/47	1.33 (0.73-2.44)		23/47	1.22 (0.62-2.42)	
> 75	25/33	1.59 (0.85-2.97)		15/33	1.23 (0.54-2.79)	
Sex			0.83			0.67
Male	34/51	1		26/51	1	
Female	38/58	1.05 (0.66-1.67)		25/58	0.89 (0.51-1.54)	
BMI (kg/m ²)			0.02			0.01
< 25	43/56	1		33/56	1	
> 25	29/53	0.56 (0.35-0.90)		18/53	0.47 (0.27-0.84)	
ASA			0.61			0.43
I - II	25/42	1		22/42	1	
III-IV	47/67	1.13 (0.70-1.85)		29/67	0.80 (0.46-1.39)	
Operation type			0.05			0.04 ¹
D2	27/51	1		20/51	1	
D2 + splenectomy	38/48	1.59 (0.97-2.61)		24/48	1.44 (0.81-2.56)	
D2 + adjacent organ resection	7/10	1.80 (0.78-4.16)		7/10	3.67 (1.25-10.80)	
Radicality			0.02			< 0.01
R0	67/104	1		46/104	1	
R1	5/5	3.18 (1.24-8.14)		5/5	4.43 (1.69-11.59)	
Neoadjuvant/ adjuvant therapy			0.02 ¹			< 0.01 ¹
No	41/69	1		20/69	1	
Neoadjuvant	1/2	0.75 (0.10-5.48)		1/2	1.53 (0.21-11.40)	
Adjuvant	30/38	1.82 (1.13-2.93)		30/38	3.55 (2.01-6.28)	
Tumor site			0.07 ¹			< 0.01 ¹
Upper third	14/21	1		8/21	1	
Middle third	26/43	0.91 (0.48-1.75)		15/43	0.95 (0.40-2.25)	
Lower third	25/38	1.04 (0.54-2.01)		21/38	1.54 (0.68-3.48)	
All levels	7/7	2.87 (1.14-7.21)		7/7	4.74 (1.70-13.23)	
Tumor stage			< 0.01 ¹			0.03 ¹
I	13/38	1		4/38	1	
II	19/27	2.98 (1.47-6.05)		12/27	5.96 (1.92-18.50)	
III	31/34	5.99 (3.09-11.62)		26/34	15.34 (5.30-44.38)	
IV	9/10	10.15 (4.18-24.66)		9/10	30.67 (9.17-102.65)	
Lauren classification			0.66			0.03
Intestinal	34/50	1		18/50	1	
Diffuse ²	38/59	1.11 (0.66-1.77)		33/59	1.87 (1.05-3.33)	
Lymph node invasion						< 0.01
No	20/45	1		9/45	1	
Yes	52/64	2.90 (1.72-4.89)		42/64	4.95 (2.40-10.23)	
Lymph node ratio (%)			< 0.01 ¹			< 0.01
0	20/45	1		9/45	1	
1-9	9/15	1.88 (0.85-4.14)		7/15	3.03 (1.12-8.16)	
10-25	16/20	2.69 (1.37-5.28)		12/20	4.11 (1.70-9.95)	
> 25	27/29	3.74 (2.09-6.70)		23/29	7.00 (3.22-15.20)	

¹P for linearity; ²Three mixed type tumors included. ASA: American Society of Anesthesiologists; BMI: Body mass index; DSS: Disease-specific survival; HR: Hazard ratio; OS: Overall survival.

by D3 or D4 lymphadenectomy compared with less radical dissection^[10,11,21].

Variability in survival rates among studies may, in part, depend upon tumor stage distribution, use of neoadjuvant and adjuvant treatments, and differences in the quality of the surgical care. The reported five-year survival rates for curative surgical resection ranges from 60%-90% for patients with stage I, 30%-50% for patients with stage II disease, and 10%-25% for patients with stage III disease^[22], which is in line with what was observed in the present study. The results indicate that tumor stage and a BMI < 25 are predictive of overall survival, with additional

factors of tumor site, Lauren classification, and lymph node invasion predictive of cancer-free survival. Although the lymph node ratio is a prognostic tool that can be used to stratify patients with gastric cancer undergoing limited lymph node dissection and to reduce stage migration^[19], this was only a significant prognostic factor for survival in the univariate, but not multivariate, analysis in the present study.

Data from Asian studies show that D2 dissection provides better survival rates compared with D1 dissection^[10,11,21], and that a more extended lymphadenectomy does not improve survival. However, European randomized studies failed to demonstrate

Table 3 Parameters reflecting the quality of care (*n* = 109)

Parameter	Value
Duration of surgery (min)	230 (200-255)
Operative blood loss (mL)	500 (300-900)
Hospital stay (d)	10 (8-12)
Number of lymph nodes studied	19 (11-25)
30-d mortality	2 (1.8)
30-d morbidity ¹	29 (26.6)
General morbidity	
Cardiac	1 (0.9)
Pleural effusion	8 (7.3)
Pneumonia	3 (1.8)
Pleural empyema	1 (0.9)
Urinary tract infection	2 (1.8)
Thromboembolism	1 (0.9)
Cerebral infarction	1 (0.9)
Surgical morbidity	
Bleeding	1 (0.9)
Anastomotic leak ²	3 (2.8)
Abdominal abscess	7 (6.4)
Wound infection	1 (0.9)
Common bile duct injury	1 (0.9)
Bowel obstruction	1 (0.9)
Dindo-Clavien classification ^[32] , severe morbidity	9 (8.3)
Grade III	3 (2.8)
Grade IV	3 (2.8)
Grade V	3 (2.8)

¹Figures are not additive because some patients had > 1 complication;

²Two of these were treated conservatively. Data are presented as median (IQR) or *n* (%).

any survival benefit from D2 over D1 dissection. Five-year survival rates were 35% (D1) and 33% (D2) in the MRC trial^[7], and 45% and 47%, respectively, in the Dutch trial^[8]. This, in combination with high morbidity and mortality rates, has led to a reserved attitude against D2 surgery in Western centers. However, results from a 15-year follow-up of the Dutch trial showed a significant survival benefit for D2 without pancreaticosplenectomy compared to D1 (35% vs 22%)^[8]. Moreover, a recent meta-analysis including eight randomized controlled trials with 2044 patients confirmed that there is no overall survival benefit for D2 lymphadenectomy, but found a benefit among patients who had resection without a splenectomy and/or pancreatectomy^[6]. Importantly, some previous randomized trials have been scrutinized based on the inclusion of a large number of operating surgeons and heterogeneous surgical techniques. Thus, universal standardization and surgical quality control of D2 dissection is needed to improve evaluation of the oncologic outcome^[23,24]. Currently, radical D2 gastrectomy is indicated for resectable stage I B-III disease, provided that patients are medically fit and the procedure is carried out in specialized, high-volume centers with appropriate surgical expertise and postoperative care^[12].

Despite radical surgery, a substantial proportion of patients relapse, which indicates that there is a need for additional therapeutic modalities, such as radiation and chemotherapy, though more evidence

is needed with regard to the (neo)adjuvant setting^[25]. A randomized phase III study performed in the United States (Intergroup 0116) demonstrated a survival benefit with postoperative chemoradiotherapy compared with surgery alone based primarily on patients undergoing D1 gastrectomy^[26]. The European UK MRC MAGIC trial demonstrated a survival benefit when patients with resectable stage II and III gastric cancers undergoing either D1 or D2 surgery were treated with three cycles of preoperative chemotherapy (epirubicin, cisplatin, and FU) followed by an additional three cycles of postoperative chemotherapy compared with surgery alone^[15]. As a result, perioperative chemotherapy has been widely adopted as the standard of care throughout the United Kingdom and Europe. However, additional real-life data is needed, as a recent Norwegian study showed that perioperative chemotherapy was completed in less than half of the patients in line with the MAGIC trial, and the tumor response did not afford any long-term survival benefit^[27]. Nevertheless, a recent meta-analysis containing data from 17 randomized trials confirmed an overall survival benefit for FU-based chemotherapy for gastric cancer compared with surgery alone (55.3% vs 49.6%, HR = 0.82, 95%CI: 0.76-0.90)^[28]. Particularly, data from the Japanese ACTS-GC^[29] and the Korean CLASSIC^[30] trials showed that adjuvant chemotherapy improves overall survival after D2 gastrectomy. Recent European guidelines recommend either chemoradiotherapy or chemotherapy delivered in the adjuvant setting for patients with \geq stage I B gastric cancer who undergo surgery without preoperative chemotherapy^[12]. Although neoadjuvant and adjuvant treatments were used in our cohort of patients, the protocol was not uniform during the study period due to the lack of an international consensus regarding various treatment strategies.

Additional parameters can be used to assess the quality of surgical care, including postoperative mortality, reoperation rate, and 30-d readmission rate. The mortality rate within 30 d after a surgical procedure for gastric cancer has been reduced substantially over the last decades. Indeed, the postoperative mortality rate in this patient series (1.8%) is within the reported range of 0%-2%^[9-11], and is similar to earlier European multicenter randomized trials^[7,8]. Furthermore, the 30-d morbidity (26.6%) is in line with other reports: 20.9%-28.1% in the JCOG trial^[11] and 17.9% by Degiuli *et al.*^[9]. Early postoperative complications typically include bleeding, ileus, cholecystitis, pancreatitis, pulmonary infections, thromboembolism, and anastomotic leakage, which is the most significant complication after total gastrectomy. In our report, the rate of symptomatic leakage was 2.8%, within the commonly reported incidence range of 1.9%-4.5%. The reoperation rate (3.7%) also compares well with the figures reported from other centers^[10,11]. Asian studies show that, even in high-volume centers, there is a certain amount

of morbidity associated with major gastric surgery. Overall, the current international standards of care were well met in this series of patients treated with D2 gastrectomy and combined modality therapy for gastric cancer.

This single-center audit should be interpreted with some caution. The number of patients is relatively small due to a declining incidence of gastric cancer in Western Europe. In addition, some changes in the management were made during the study period, including the addition of multidisciplinary team meetings and some modifications in chemotherapy regimens. However, the mortality, reoperation, and local recurrence rates are low. Currently, endoscopic ultrasound can be used to determine the proximal and distal extent of the tumor and provide further assessment of the T and N stages. Moreover, laparoscopy with or without peritoneal washings for malignant cells is now recommended to exclude occult metastatic disease in all stage I B–III stomach cancers considered potentially resectable^[31].

In conclusion, the present audit indicates that D2 lymphadenectomy with pancreatic preservation and selective splenectomy is a safe surgical strategy for patients with locally advanced gastric cancer. The oncologic outcome is largely determined by the stage of the disease at presentation, which can be improved through the use of neoadjuvant and adjuvant chemotherapy regimens.

COMMENTS

Background

Morbidity and mortality rates after D2 gastrectomy are relatively high in European randomized studies, and evidence of a long-term survival benefit has been controversial. Nevertheless, D2 lymphadenectomy has been adopted for treatment protocols for gastric cancer, despite the lack of quality audits in some countries.

Research frontiers

Asian studies show that D2–3 lymphadenectomy improves survival and can be performed with acceptable complication rates. The present study evaluates the long-term oncologic outcomes of 109 consecutive D2 gastrectomy patients with gastric adenocarcinoma at the authors' institution. Clinicopathologic factors affecting survival were assessed, and the quality of surgery was a secondary outcome measure.

Innovations and breakthroughs

The overall five-year and disease-specific survivals for the 109 patients in this cohort were 45.0% and 53.0%, respectively, which are in line with European studies. The mortality associated with D2 lymphadenectomy without routine pancreatectomy and/or splenectomy was only 1.8%, and morbidity was 26.6%. Low body mass index, advanced stage, tumor site, Lauren classification, and lymph node invasion were independent prognostic factors for poor survival.

Applications

Large randomized studies are essential for defining clinical guidelines. However, it is important to report how these guidelines are implemented in clinical practice. In this series, the Western standards for long-term oncologic outcome and quality of surgical care were well met.

Peer-review

The authors examined long-term oncologic outcome and quality of surgical care in gastric cancer patients treated with D2 lymphadenectomy without routine pancreatectomy. Clinicopathologic factors affecting survival were assessed. Their results show that in this consecutive series, survival and morbidity rates were comparable with European studies. Mortality was low. To this end, D2 gastrectomy is a safe surgical option for gastric cancer, and can be well implemented in clinical practice.

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 Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015; **136**: E359–E386 [PMID: 25220842 DOI: 10.1002/ijc.29210]
- 3 Sasako M. Principles of surgical treatment for curable gastric cancer. *J Clin Oncol* 2003; **21**: 274s–275s [PMID: 14645409]
- 4 Shimada Y. JGCA (The Japan Gastric Cancer Association). Gastric cancer treatment guidelines. *Jpn J Clin Oncol* 2004; **34**: 58 [PMID: 15061149]
- 5 Verlato G, Giacomuzzi S, Bencivenga M, Morgagni P, De Manzoni G. Problems faced by evidence-based medicine in evaluating lymphadenectomy for gastric cancer. *World J Gastroenterol* 2014; **20**: 12883–12891 [PMID: 25278685 DOI: 10.3748/wjg.v20.i36.12883]
- 6 Jiang L, Yang KH, Chen Y, Guan QL, Zhao P, Tian JH, Wang Q. Systematic review and meta-analysis of the effectiveness and safety of extended lymphadenectomy in patients with resectable gastric cancer. *Br J Surg* 2014; **101**: 595–604 [PMID: 24668465 DOI: 10.1002/bjs.9497]
- 7 Cuschieri A, Weeden S, Fielding J, Bancewicz J, Craven J, Joypaul V, Sydes M, Fayers P. Patient survival after D1 and D2 resections for gastric cancer: long-term results of the MRC randomized surgical trial. Surgical Co-operative Group. *Br J Cancer* 1999; **79**: 1522–1530 [PMID: 10188901]
- 8 Songun I, Putter H, Kranenbarg EM, Sasako M, van de Velde CJ. Surgical treatment of gastric cancer: 15-year follow-up results of the randomised nationwide Dutch D1D2 trial. *Lancet Oncol* 2010; **11**: 439–449 [PMID: 20409751 DOI: 10.1016/S1470-2045(10)70070-X]
- 9 Degiuli M, Sasako M, Ponti A. Morbidity and mortality in the Italian Gastric Cancer Study Group randomized clinical trial of D1 versus D2 resection for gastric cancer. *Br J Surg* 2010; **97**: 643–649 [PMID: 20186890 DOI: 10.1002/bjs.6936]
- 10 Wu CW, Hsiung CA, Lo SS, Hsieh MC, Chen JH, Li AF, Lui WY, Whang-Peng J. Nodal dissection for patients with gastric cancer: a randomised controlled trial. *Lancet Oncol* 2006; **7**: 309–315 [PMID: 16574546]
- 11 Sasako M, Sano T, Yamamoto S, Kurokawa Y, Nashimoto A, Kurita A, Hiratsuka M, Tsujinaka T, Kinoshita T, Arai K, Yamamura Y, Okajima K. D2 lymphadenectomy alone or with para-aortic nodal dissection for gastric cancer. *N Engl J Med* 2008; **359**: 453–462 [PMID: 18669424]
- 12 Waddell T, Verheij M, Allum W, Cunningham D, Cervantes A, Arnold D. Gastric cancer: ESMO-ESSO-ESTRO clinical practice guidelines for diagnosis, treatment and follow-up. *Eur J Surg Oncol* 2014; **40**: 584–591 [PMID: 24685156 DOI: 10.1016/j.ejso.2013.09.020]
- 13 Macdonald JS, Smalley SR, Benedetti J, Hundahl SA, Estes NC, Stemmermann GN, Haller DG, Ajani JA, Gunderson LL, Jessup JM, Martenson JA. Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. *N Engl J Med* 2001; **345**: 725–730

- [PMID: 11547741]
- 14 **Ychou M**, Boige V, Pignon JP, Conroy T, Bouché O, Lebreton G, Ducourtieux M, Bedenne L, Fabre JM, Saint-Aubert B, Genève J, Lasser P, Rougier P. Perioperative chemotherapy compared with surgery alone for resectable gastroesophageal adenocarcinoma: an FNCLCC and FFCD multicenter phase III trial. *J Clin Oncol* 2011; **29**: 1715-1721 [PMID: 21444866 DOI: 10.1200/JCO.2010.33.0597]
 - 15 **Cunningham D**, Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, Scarffe JH, Lofts FJ, Falk SJ, Iveson TJ, Smith DB, Langley RE, Verma M, Weeden S, Chua YJ. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. *N Engl J Med* 2006; **355**: 11-20 [PMID: 16822992]
 - 16 **Ge L**, Wang HJ, Yin D, Lei C, Zhu JF, Cai XH, Zhang GQ. Effectiveness of 5-fluorouracil-based neoadjuvant chemotherapy in locally-advanced gastric/gastroesophageal cancer: a meta-analysis. *World J Gastroenterol* 2012; **18**: 7384-7393 [PMID: 23326149 DOI: 10.3748/wjg.v18.i48.7384]
 - 17 **Sayegh ME**, Sano T, Dexter S, Katai H, Fukagawa T, Sasako M. TNM and Japanese staging systems for gastric cancer: how do they coexist? *Gastric Cancer* 2004; **7**: 140-148 [PMID: 15449201]
 - 18 **Sobin LH**, Wittekind C. TNM classification of malignant tumours. 6th edition. New York: Wiley-Liss, 2002
 - 19 **Marchet A**, Mocellin S, Ambrosi A, Morgagni P, Garcea D, Marrelli D, Roviello F, de Manzoni G, Minicozzi A, Natalini G, De Santis F, Baiocchi L, Coniglio A, Nitti D. The ratio between metastatic and examined lymph nodes (N ratio) is an independent prognostic factor in gastric cancer regardless of the type of lymphadenectomy: results from an Italian multicentric study in 1853 patients. *Ann Surg* 2007; **245**: 543-552 [PMID: 17414602 DOI: 10.1097/01.sla.0000250423.43436.e1]
 - 20 **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]
 - 21 **Yonemura Y**, Wu CC, Fukushima N, Honda I, Bandou E, Kawamura T, Kamata T, Kim BS, Matsuki N, Sawa T, Noh SH. Randomized clinical trial of D2 and extended paraaortic lymphadenectomy in patients with gastric cancer. *Int J Clin Oncol* 2008; **13**: 132-137 [PMID: 18463957 DOI: 10.1007/s10147-007-0727-1]
 - 22 **Seevaratnam R**, Bocicariu A, Cardoso R, Mahar A, Kiss A, Helyer L, Law C, Coburn N. A meta-analysis of D1 versus D2 lymph node dissection. *Gastric Cancer* 2012; **15** Suppl 1: S60-S69 [PMID: 22138927 DOI: 10.1007/s10120-011-0110-9]
 - 23 **Dikken JL**, van Sandick JW, Allum WH, Johansson J, Jensen LS, Putter H, Coupland VH, Wouters MW, Lemmens VE, van de Velde CJ, van der Geest LG, Larsson HJ, Cats A, Verheij M. Differences in outcomes of oesophageal and gastric cancer surgery across Europe. *Br J Surg* 2013; **100**: 83-94 [PMID: 23180474 DOI: 10.1002/bjs.8966]
 - 24 **Kim HI**, Hur H, Kim YN, Lee HJ, Kim MC, Han SU, Hyung WJ. Standardization of D2 lymphadenectomy and surgical quality control (KLASS-02-QC): a prospective, observational, multicenter study [NCT01283893]. *BMC Cancer* 2014; **14**: 209 [PMID: 24646327 DOI: 10.1186/1471-2407-14-209]
 - 25 **Dikken JL**, van Sandick JW, Maurits Swellengrebel HA, Lind PA, Putter H, Jansen EP, Boot H, van Grieken NC, van de Velde CJ, Verheij M, Cats A. Neo-adjuvant chemotherapy followed by surgery and chemotherapy or by surgery and chemoradiotherapy for patients with resectable gastric cancer (CRITICS). *BMC Cancer* 2011; **11**: 329 [PMID: 21810227 DOI: 10.1186/1471-2407-11-329]
 - 26 **Smalley SR**, Benedetti JK, Haller DG, Hundahl SA, Estes NC, Ajani JA, Gunderson LL, Goldman B, Martenson JA, Jessup JM, Stemmermann GN, Blanke CD, Macdonald JS. Updated analysis of SWOG-directed intergroup study 0116: a phase III trial of adjuvant radiochemotherapy versus observation after curative gastric cancer resection. *J Clin Oncol* 2012; **30**: 2327-2333 [PMID: 22585691 DOI: 10.1200/JCO.2011.36.7136]
 - 27 **Bringeland EA**, Wasmuth HH, Fougner R, Mjones P, Grønbech JE. Impact of perioperative chemotherapy on oncological outcomes after gastric cancer surgery. *Br J Surg* 2014; **101**: 1712-1720 [PMID: 25312592 DOI: 10.1002/bjs.9650]
 - 28 **Paoletti X**, Oba K, Burzykowski T, Michiels S, Ohashi Y, Pignon JP, Rougier P, Sakamoto J, Sargent D, Sasako M, Van Cutsem E, Buyse M. Benefit of adjuvant chemotherapy for resectable gastric cancer: a meta-analysis. *JAMA* 2010; **303**: 1729-1737 [PMID: 20442389 DOI: 10.1001/jama.2010.534]
 - 29 **Sasako M**, Sakuramoto S, Katai H, Kinoshita T, Furukawa H, Yamaguchi T, Nashimoto A, Fujii M, Nakajima T, Ohashi Y. Five-year outcomes of a randomized phase III trial comparing adjuvant chemotherapy with S-1 versus surgery alone in stage II or III gastric cancer. *J Clin Oncol* 2011; **29**: 4387-4393 [PMID: 22010012 DOI: 10.1200/JCO.2011.36.5908]
 - 30 **Bang YJ**, Kim YW, Yang HK, Chung HC, Park YK, Lee KH, Lee KW, Kim YH, Noh SI, Cho JY, Mok YJ, Kim YH, Ji J, Yeh TS, Button P, Sirzén F, Noh SH. Adjuvant capecitabine and oxaliplatin for gastric cancer after D2 gastrectomy (CLASSIC): a phase 3 open-label, randomised controlled trial. *Lancet* 2012; **379**: 315-321 [PMID: 22226517 DOI: 10.1016/S0140-6736(11)61873-4]
 - 31 **Yoon H**, Lee DH. New approaches to gastric cancer staging: beyond endoscopic ultrasound, computed tomography and positron emission tomography. *World J Gastroenterol* 2014; **20**: 13783-13790 [PMID: 25320516 DOI: 10.3748/wjg.v20.i38.13783]
 - 32 **Clavien PA**, Strasberg SM. Severity grading of surgical complications. *Ann Surg* 2009; **250**: 197-198 [PMID: 19638901 DOI: 10.1097/SLA.0b013e3181b6dcab]

P- Reviewer: Abdel-Wahab M S- Editor: Yu J

L- Editor: A E- Editor: Liu XM



Retrospective Study

Clinical impact of atypical endoscopic features in rectal neuroendocrine tumors

Jong Hee Hyun, Seong Dae Lee, Eui Gon Youk, Jae Bum Lee, Enu-Jung Lee, Hee Jin Chang, Dae Kyung Sohn

Jong Hee Hyun, Hee Jin Chang, Dae Kyung Sohn, Center for Colorectal Cancer, National Cancer Center, Gyeonggi-do 10408, South Korea

Seong Dae Lee, Eui Gon Youk, Jae Bum Lee, Enu-Jung Lee, Department of Surgery, Daehang Hospital, Seoul 06699, South Korea

Author contributions: Sohn DK conceived the study, participated in study design and patient accrual, and provided final approval for publication of the manuscript; Hyun JH contributed to data collection, performed statistical analyses and drafted the manuscript; Chang HJ contributed to the design and coordination of the study and critical revision of the manuscript; Lee SD, Youk EG, Lee JB and Lee EJ contributed to patient accrual; all authors read and approved the final manuscript.

Supported by National Cancer Center Grant, No. NCC-1510150-1.

Institutional review board statement: The study was reviewed and approved by the National Cancer Center Institutional Review Board (NCC-2014-0104).

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: Statistical analysis and dataset are available from the corresponding author at gsgsbal@ncc.re.kr.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Dae Kyung Sohn, MD, Center for Colorectal Cancer, National Cancer Center, 323 Ilsan-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do 10408, South Korea. gsgsbal@ncc.re.kr
 Telephone: +82-31-9201636

Fax: +82-31-9201289

Received: August 4, 2015
 Peer-review started: August 5, 2015
 First decision: September 9, 2015
 Revised: September 20, 2015
 Accepted: November 13, 2015
 Article in press: November 13, 2015
 Published online: December 21, 2015

Abstract

AIM: To validate the association between atypical endoscopic features and lymph node metastasis (LNM).

METHODS: A total of 247 patients with rectal neuroendocrine tumors (NETs) were analyzed. Endoscopic images were reviewed independently by two endoscopists, each of whom classified tumors by sized and endoscopic features, such as shape, color, and surface change (kappa coefficient 0.76 for inter-observer agreement). All of patients underwent computed tomography scans of abdomen and pelvis for evaluation of LNM. Univariate and multivariate analyses were performed to identify the factors associated with LNM. Additionally, the association between endoscopic atypical features and immunohistochemical staining of tumors was analyzed.

RESULTS: Of 247 patients, 156 (63.2%) were male and 15 (6.1%) were showed positive for LNM. On univariate analysis, tumor size ($P < 0.001$), shape ($P < 0.001$), color ($P < 0.001$) and surface changes ($P < 0.001$) were significantly associated with LNM. On multivariate analysis, tumor size (OR = 11.53, 95%CI: 2.51-52.93, $P = 0.002$) and atypical surface (OR = 27.44, 95%CI: 5.96-126.34, $P < 0.001$) changes were independent risk factors for LNM. The likelihood of atypical endoscopic features increased

as tumor size increased. Atypical endoscopic features were associated with LNM in rectal NETs < 10 mm ($P = 0.005$) and 10-19 mm ($P = 0.041$) in diameter. Immunohistochemical staining showed that the rate of atypical endoscopic features was higher in non L-cell tumors.

CONCLUSION: Atypical endoscopic features as well as tumor size are predictive factors of LNM in patients with rectal NETs.

Key words: Rectal neuroendocrine tumor; Colonoscopy; Lymph node metastasis

© **The Author(s) 2015.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: We were studied about association between endoscopic atypical features in rectal neuroendocrine tumor and metastasis in 2008. Thus, our study was designed to validate the association between atypical endoscopic features and lymph node metastasis (LNM). Our study showed that the atypical endoscopic features, such as size > 10 mm, surface changes, were risk factors for LNM. Additionally, rectal neuroendocrine tumors which showed atypical endoscopic features were associated with non L-cell tumors. When we examined rectal neuroendocrine tumor using colonoscopy, atypical endoscopic features help to predict the treatment plan.

Hyun JH, Lee SD, Youk EG, Lee JB, Lee EJ, Chang HJ, Sohn DK. Clinical impact of atypical endoscopic features in rectal neuroendocrine tumors. *World J Gastroenterol* 2015; 21(47): 13302-13308 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13302.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13302>

INTRODUCTION

Increases in rate of screening colonoscopy have resulted in increases in incidence and prevalence of rectal neuroendocrine tumors (NETs). Most rectal NETs are slow growing tumors that originate from enterochromaffin cells and rarely metastasize^[1,2]. A recent consensus guideline suggests that tumor size is the most powerful predictor of lymph node metastasis (LNM) of rectal NETs^[3,4]. Guideline of the National Comprehensive Cancer Network recommend that NETs ≤ 2 cm in diameter be excised transanally or endoscopically excision and that NETs > 2 cm in diameter undergo radical resection^[5]. However, LNMs have been reported in patients with NETs < 6 mm in diameter^[6], suggesting that tumor size alone is not predictive of LNM.

Colonoscopy is the most useful method of diagnosing and treating rectal NETs. Although typical NETs appear as yellowish, sessile, submucosal tumors^[7,8], some are morphologically unusual, having irregular surfaces

or being pedunculated or hyperemic. These unusual features have been associated with LNM, suggesting an association between endoscopic findings and LNM^[9].

Study was designed to validate the association between endoscopically atypical features and LNM. In addition, the association between endoscopically atypical features and the immunohistochemistry of these tumors was analyzed^[10].

MATERIALS AND METHODS

Patients

Data from 287 patients with rectal NETs diagnosed and treated at the National Cancer Center (Goyang, South Korea) and Daehang Hospital (Seoul, South Korea) between January 2008 and December 2010 were retrospectively reviewed^[10]. Eight patients with synchronous colorectal cancer, eight who underwent multiple biopsies before visiting our institutions and 24 whose endoscopic images were unavailable were excluded from this study. Finally, 247 patients with rectal NETs were analyzed (Figure 1). Of these 247 lesions, 208 were endoscopically resected, 22 were removed transanally, and 16 were treated with radical surgery. One patient received only palliative chemotherapy, because he had multiple unresectable liver and peritoneal metastases. Clinicopathologic variables were retrospectively collected from the patients' medical records. This study was approved by the Institutional Review Board of the National Cancer Center of Korea (NCC2014-0104).

Evaluation

All patients underwent endoscopic examination with video colonoscopes (Olympus CF-Q240, CF-Q260 or CF-H260; Olympus, Tokyo, Japan) for diagnosis and treatment. Endoscopic images were reviewed independently by two endoscopists, resulting in kappa coefficient of 0.76 for interobserver agreement. Any disagreements between the two endoscopists were resolved by open discussions with all expert endoscopists.

All patients underwent computed tomography (CT) scans of the abdomen and pelvis for evaluation of LNM. Patients were considered positive for LNM if CT scans revealed nodes > 3 mm in diameter in the perirectal area or nodes > 1 cm in diameter in the pelvis^[10-12]. Tumor sizes were confirmed by pathology reports, except for the one patient who did not undergo curative resection because of extensive liver metastases. All tumors were classified by size (longest diameter), and then by endoscopic features such as shape, color, and surface changes, including depression, erosion and ulceration. Of the 247 lesions, 217 were also assessed immunohistochemically.

Statistical analysis

Interobserver agreement on endoscopic findings

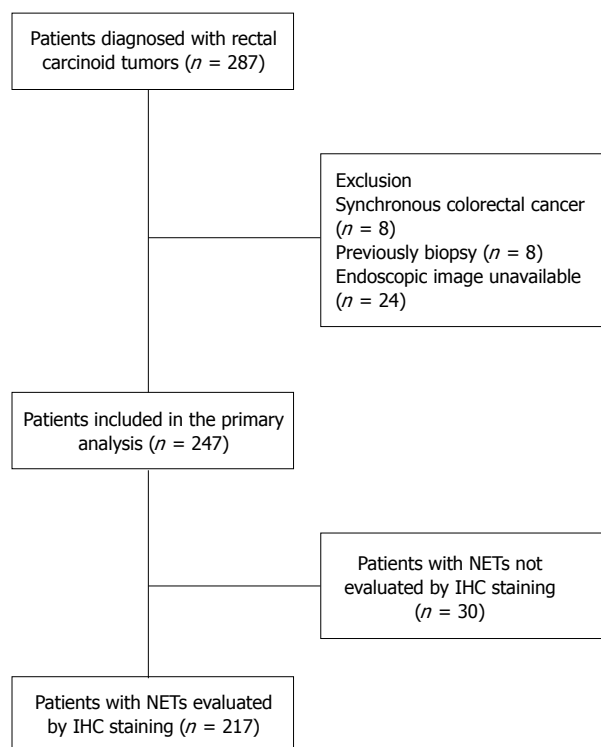


Figure 1 Study flow chart. NETs: Neuroendocrine tumors; IHC: immunohistochemistry.

was analyzed by calculating the kappa coefficient. The associations between endoscopic findings and LNM were analyzed by χ^2 or Fisher's exact tests. Multivariate analysis using a logistic regression model was performed to identify associations between all potential parameters and LNM. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 14.0 (SPSS Inc., Chicago, IL). A two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Table 1 shows the baseline clinicopathological characteristics of the 247 patients with rectal NETs. Of these patients, 91 (36.8%) were male and 156 (63.2%) were female. Mean age at diagnosis was 51.6 ± 10.7 years and mean tumor size was 5.76 ± 2.65 mm. Two patients had liver metastases at diagnosis, with one also having peritoneal seeding and 15 (6.1%) were diagnosed with LNM.

Fifty-five patients (22.3%) had rectal NETs with one or more atypical features (Figure 2), whereas the other 192 patients (77.7%) had rectal NETs with endoscopically typical features such as being sessile and having a smooth surface covered with normal or yellowish mucosa (Figure 3). On univariate analysis, tumor size, tumor shape, surface changes, and color were significantly associated with LNM. On multivariate analysis, tumor size (OR = 11.53, 95%CI: 2.51-52.93), atypical surface changes (OR = 27.44, 95%CI:

Table 1 Characteristics of the 247 patients with rectal neuroendocrine tumor *n* (%)

	Value
Age, mean \pm SD (range), yr	51.56 \pm 10.69 (27-76)
Sex	
Male	91 (36.8)
Female	156 (63.2)
Tumor size, mean \pm SD (mm)	5.76 \pm 2.76
Resection type	
Endoscopic resection	208 (84.2)
TEM or TAE	22 (8.9)
Radical resection	16 (6.5)
None	1 (0.4)
Distant organ metastasis at diagnosis	
Negative	245 (99.2)
Positive	2 (0.8)
LN metastasis	
Negative	232 (93.9)
Positive	15 (6.1)

TEM: Transanal endoscopic microsurgery; TAE: Transanal excision; LN: Lymph node.

5.96-126.34), and any type of atypical feature (OR = 4.38, 95%CI: 0.92-20.80) were independent risk factors for LNM (Table 2). Moreover, atypical features correlated with increased tumor size (Table 3).

Table 4 shows the association between endoscopic features and metastasis in rectal NETs < 10 mm and 10-19 mm in diameter were evaluated in Table 4, respectively. Tumor shape and color were not associated with LNM for either size range of rectal NETs. However, tumor surface changes were associated with LNM in patients with NETs < 10 mm ($P = 0.005$) and 10-19 mm ($P = 0.041$) in diameter. Ulceration was not observed in any tumor < 20 mm in diameter.

Table 5 shows the association between atypical features and the results of immunohistochemical staining results. L-cell phenotype and GLP1 were associated with atypical features, whereas non-L cell phenotype was associated with surface changes and color of NETs (Table 6).

DISCUSSION

Risk factors predictive of LNM of rectal NETs were assessed by univariate and multivariate analyses, with the latter showing that tumor size and atypical surface changes were significant independent predictors of LNM. The ability to predict the likelihood of LNM is important for managing patients requiring radical surgery to prevent tumor progression. Recent studies have reported that risk factors for LNM of rectal NETs include tumor size > 10 mm; atypical features; pathologic T stage; and muscular, perineural or lymphovascular invasion^[9,13-16]. Two studies recommended radical lymph node dissection for patients with rectal NETs > 10 mm and lymphatic invasion^[16,17]. Lymphatic invasion, however, cannot be evaluated

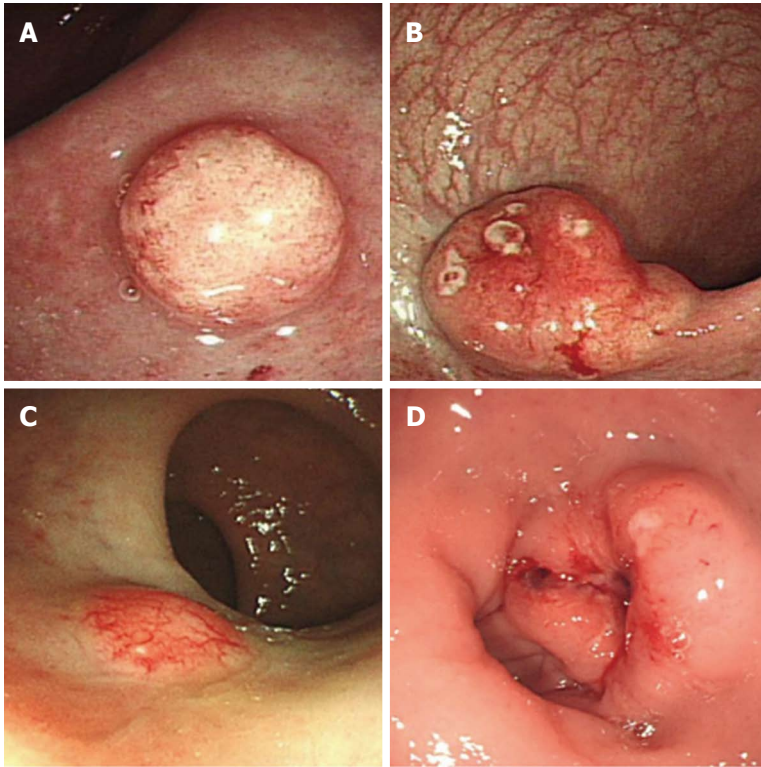


Figure 2 Endoscopic findings of atypical carcinoids. A: Semipedunculated type with hyperemia; B: Semipedunculated type with erosion and hyperemia; C: Sessile type with hyperemia; D: An ulcerofungating types mimicking rectal cancer.



Figure 3 Endoscopic image of a typical carcinoid, which was a sessile tumor with a yellow, smooth surface.

before resection of rectal NETs. On colonoscopy, the size of rectal NETs was the only predictor of LNM. We previously reported an association between atypical features of rectal NETs and LNM^[9]. Moreover, the incidence of atypical features was found to be associated with increased tumor size, suggesting that atypical features may be useful in determining treatment for tumors 11–19 mm in diameter. This study was performed to validate the predictive value of atypical features of NETs in a separate patient cohort.

The cutoff value for carcinoid tumor size that can determine the treatment plan and assess patient prognosis has not been definitively established. Tumors ≤ 10 mm in diameter are locally resected,

by methods such as endoscopic resection, transanal excision or transanal endoscopic microsurgery. The American Joint Committee on Cancer staging system has recommended that patients with tumors ≥ 20 mm undergo radical resection with lymph node dissection^[3,18]. However, the proper method of removing rectal NETs 11–19 mm in size remains undetermined, and no controlled prospective trials have assessed treatment plans for these patients. We found that all three patients with tumors ≥ 20 mm in diameter, 6 (27.3%) of 22 with tumors 10–19 mm, and 3 (1.4%) of 222 with tumors < 10 mm presented with LNM. Although, surprisingly, 3 patients with tumors < 10 mm in diameter had LNM, two studies observed metastases to lymph nodes and distant organs in patients with rectal NETs ≤ 10 mm in size^[6,19]. Thus, size of rectal NETs alone is insufficient to predict LNM and determine treatment plans.

The Surveillance, Epidemiology, and End Results registry database has shown that the incidence of rectal NETs has increased over the last 35 years^[20]. Most rectal NETs are diagnosed incidentally, with the increase in incidence likely due to increases in screening sigmoidoscopy and colonoscopy^[1]. Although size of rectal NETs incidentally diagnosed during lower endoscopy was the only factor associated with LNM, this study found that atypical features, especially surface changes, were strongly predictive of LNM. One of 3 patients with rectal NETs ≤ 10 mm and LNM had a semipedunculated lesion with surface erosion,

Table 2 Univariate and multivariate analyses of clinical factors associated with lymph node metastasis in patients with rectal neuroendocrine tumors *n* (%)

	Univariate analysis			Multivariate analysis	
	Metastasis (-)	Metastasis (+)	<i>P</i> value	OR (95%CI)	<i>P</i> value
Gender			0.183	-	-
Male	144 (92.3)	12 (7.7)			
Female	88 (96.7)	3 (3.3)			
Age (yr)			1.000	-	-
≤ 50	113 (94.2)	7 (5.8)			
> 50	119 (93.7)	8 (6.3)			
Size (mm)			< 0.001	11.53 (2.51-52.93)	0.002
< 10	219 (98.6)	3 (1.4)			
≥ 10, < 20	13 (59.1)	9 (40.9)			
≥ 20	0 (0)	3 (100)			
Tumor shape			< 0.001	-	-
Sessile	205 (97.6)	5 (2.4)			
Semipedunculated	27 (77.1)	8 (22.9)			
Ulcerofungating	0 (0)	2 (100)			
Surface change			< 0.001	27.44 (5.96-126.34)	< 0.001
Smooth	222 (97.8)	5 (2.2)			
Depressed/eroded	10 (55.6)	8 (44.4)			
Ulcerated	0 (0)	2 (100)			
Color			< 0.001	-	-
Normal or yellow	210 (96.8)	7 (3.2)			
Hyperemia	22 (73.3)	8 (26.7)			
Atypical features, any			< 0.001	4.38 (0.92-20.80)	0.064
Typical features	189 (98.4)	3 (1.6)			
Atypical features	43 (78.2)	12 (21.8)			

Table 3 Atypical features of rectal neuroendocrine tumors according to tumor size *n* (%)

	Typical (<i>n</i> = 192)	Atypical (<i>n</i> = 55)	<i>P</i> value
Tumor size (mm)			< 0.001
< 10	186 (83.8)	36 (16.2)	
≥ 10, < 20	6 (27.3)	16 (72.7)	
≥ 20	0 (0)	3 (100)	

while all 9 patients with tumors > 10 mm in size and LNM had lesions with one or more atypical features. The presence of atypical features can help determine treatment plans for patients with rectal NETs 11-19 mm in diameter. We suggest that rectal NTEs 11-19mm in diameter, which showed atypical features in endoscopic findings, should be performed the CT or EUS to evaluate the LNM.

In 2010, the World Health Organization (WHO) classified rectal NETs as malignant^[21], with L-cell, glucagon-like peptide producing and pancreatic polypeptide/peptide YY (PPY/PYY) producing NETs defined as borderline malignant or of uncertain malignant potential. Although most rectal NETs are L-cell tumors, the L-cell phenotype was not associated with biologically favorable characteristics^[10]. That study, with a population overlapping our study, recommended that clinical management of rectal NETs should depend on tumor size. Our analysis of the association between atypical features and immunohistochemical staining results found that L-cell phenotype and GLP1 were associated with atypical features. These

Table 4 Association between endoscopic features and metastasis in rectal neuroendocrine tumors < 10 mm and 10-19 mm in diameter *n* (%)

	Metastasis (-)	Metastasis (+)	<i>P</i> value
< 10 mm in diameter			
Shape			0.155
Sessile	199 (99.0)	2 (1.0)	
Semipedunculated	20 (95.2)	1 (4.8)	
Surface change			0.005
Smooth	212 (99.1)	2 (0.9)	
Depression/erosion	7 (87.5)	1 (12.5)	
Color			0.627
Yellow	203 (98.5)	3 (1.5)	
Hyperemia	16 (100)	0 (0)	
10-19 mm in diameter			
Shape			0.548
Sessile	6 (66.7)	3 (33.3)	
Semipedunculated	7 (53.8)	6 (46.2)	
Surface change			0.041
Smooth	10 (76.9)	3 (23.1)	
Depression/erosion	3 (33.3)	6 (66.7)	
Color			0.665
Yellow	7 (63.6)	4 (36.4)	
Hyperemia	6 (54.5)	5 (45.5)	

findings suggested that increased tumor size may be associated with atypical features as well as non L-cell type. Prospective observational studies in large cohorts of patients are required to clarify these associations.

Although we analyzed a relatively large patient cohort, our study had the inherent limitations of a retrospective study. To minimize such biases, we did not include and analyze consecutive patients with

Table 5 Association between atypical endoscopic features of rectal neuroendocrine tumors and immunohistochemical staining results *n* (%)

	Typical feature	Atypical feature	<i>P</i> value
L-cell			0.008
(-)	27 (62.8)	16 (37.2)	
(+)	142 (81.6)	32 (18.4)	
EC-cell			0.464
(-)	162 (78.3)	45 (21.7)	
(+)	7 (70.0)	3 (30.0)	
Serotonin			0.306
(-)	161 (78.5)	44 (21.5)	
(+)	8 (66.7)	4 (33.3)	
Somatostatin			1.000
(-)	161 (77.8)	46 (22.2)	
(+)	8 (80.0)	2 (20.0)	
GLP1			0.021
(-)	57 (69.5)	25 (30.5)	
(+)	112 (83.0)	23 (17.0)	
PYY1			0.513
(-)	72 (75.8)	23 (24.2)	
(+)	97 (79.5)	25 (20.5)	
PPY1			0.443
(-)	88 (75.9)	28 (24.1)	
(+)	81 (80.2)	20 (19.8)	

rectal NETs. Second, we investigated LNM by radiologic imaging or pathologic reports. Most patients with rectal NETs underwent local excision, such as transanal excision, transanal endoscopic microsurgery, and endoscopic procedures, rather than radical resection. Although the LNM status of patients who underwent local excision was evaluated by abdominopelvic CT, CT was used only to evaluate lymph node status. To evaluate the lymph node status, we were using criteria that distinguished positive node which showed > 3 mm in diameter in perirectal area or > 1 cm in diameter in the pelvis^[11,12]. These criteria showed about a sensitivity of 73% and a specificity of 58%. Thus, we have to consider a difference between CT finding and pathology. Third, we did not perform survival analysis. Median follow-up time of our study patients was 44 mo (range 0-78 mo), which, while longer than in other studies, was too short to assess distant metastases or tumor recurrence. Prospective long term follow-up studies are needed for survival analyses.

In conclusion, the present study, along with a previous study performed at our institution, suggests that rectal NETs ≤ 10 mm in diameter can be treated by local excision, whereas tumors ≥ 20 mm in diameter should be treated by radical resection with lymph node dissection. Atypical endoscopic features may help select the optimal treatment plans for patients with rectal NETs 11-19 mm in diameter.

COMMENTS

Background

Increases in rate of screening colonoscopy have resulted in increases in incidence and prevalence of rectal neuroendocrine tumors (NETs). The treatment plan of rectal NETs have been decided depends on tumor size.

Table 6 Association between atypical features and L-cell type in rectal neuroendocrine tumors *n* (%)

	L-cell (-)	L-cell (+)	<i>P</i> value
Tumor shape			0.107
Sessile	34 (18.4)	151 (81.6)	
Semipedunculated	8 (25.8)	23 (74.2)	
Ulcerofungating	1 (100)	0 (0)	
Surface change			0.022
Smooth	36 (18.0)	164 (82.0)	
Depression/erosion	6 (37.5)	10 (62.5)	
Ulceration	1 (100)	0 (0)	
Color			< 0.001
Normal or yellow	31 (16.1)	162 (83.9)	
Hyperemia	12 (50.0)	12 (50.0)	

Previously, the authors had proposed the possible association between endoscopic atypical features and lymph node metastasis (LNM) in rectal NETs.

Research frontiers

Tumor size, tumor shape, color and surface change were significantly related to LNM in univariate analysis. In multivariate analysis, tumor size and atypical surface change were independent risk factors for LNM [odds ratios with 95% confidence interval were 11.5 (2.5-52.9) and 27.4 (5.9-126.3), respectively]. The rate of atypical endoscopic feature was increased as tumor size increase (16.2% in less than 10 mm, 72.7% in 10-19 mm, 100% in 20 mm or over in diameter, *P* < 0.001).

Innovations and breakthroughs

In this study, the authors found atypical endoscopic features as well as tumor size were predictive factors for LNM in rectal NETs.

Applications

This study suggested that NETs which showed atypical endoscopic feature helpful for determining proper treatment.

Peer-review

The paper is an interesting analysis of the association between atypical features and LNM in rectal NETs. The atypical features of rectal NETs are important findings to determine the treatment plan during colonoscopy.

REFERENCES

- 1 **Taghavi S**, Jayarajan SN, Powers BD, Davey A, Willis AI. Examining rectal carcinoids in the era of screening colonoscopy: a surveillance, epidemiology, and end results analysis. *Dis Colon Rectum* 2013; **56**: 952-959 [PMID: 23838863 DOI: 10.1097/DCR.0b013e318291f512]
- 2 **Scherübl H**. Rectal carcinoids are on the rise: early detection by screening endoscopy. *Endoscopy* 2009; **41**: 162-165 [PMID: 19214898 DOI: 10.1055/s-0028-1119456]
- 3 **Modlin IM**, Kidd M, Latich I, Zikusoka MN, Shapiro MD. Current status of gastrointestinal carcinoids. *Gastroenterology* 2005; **128**: 1717-1751 [PMID: 15887161]
- 4 **Ramage JK**, Ahmed A, Ardill J, Bax N, Breen DJ, Caplin ME, Corrie P, Davar J, Davies AH, Lewington V, Meyer T, Newell-Price J, Poston G, Reed N, Rockall A, Steward W, Thakker RV, Toubanakis C, Valle J, Verbeke C, Grossman AB. Guidelines for the management of gastroenteropancreatic neuroendocrine (including carcinoid) tumours (NETs). *Gut* 2012; **61**: 6-32 [PMID: 22052063 DOI: 10.1136/gutjnl-2011-300831]
- 5 **Kulke MH**, Benson AB, Bergsland E, Berlin JD, Blaszkowsky LS, Choti MA, Clark OH, Doherty GM, Eason J, Emerson L, Engstrom PF, Goldner WS, Heslin MJ, Kandeel F, Kunz PL, Kuvshinov BW, Moley JF, Pillarisetty VG, Saltz L, Schteingart DE, Shah MH, Shibata S, Strosberg JR, Vauthey JN, White R, Yao JC, Freedman-Cass DA, Dwyer MA. Neuroendocrine tumors. *J Natl Compr Canc*

- Netw 2012; **10**: 724-764 [PMID: 22679117]
- 6 **Shinohara T**, Hotta K, Oyama T. Rectal carcinoid tumor, 6 mm in diameter, with lymph node metastases. *Endoscopy* 2008; **40** Suppl 2: E40-E41 [PMID: 18302079 DOI: 10.1055/s-2007-966849]
- 7 **Jetmore AB**, Ray JE, Gathright JB, McMullen KM, Hicks TC, Timmcke AE. Rectal carcinoids: the most frequent carcinoid tumor. *Dis Colon Rectum* 1992; **35**: 717-725 [PMID: 1643994]
- 8 **Matsui K**, Iwase T, Kitagawa M. Small, polypoid-appearing carcinoid tumors of the rectum: clinicopathologic study of 16 cases and effectiveness of endoscopic treatment. *Am J Gastroenterol* 1993; **88**: 1949-1953 [PMID: 8237948]
- 9 **Kim BN**, Sohn DK, Hong CW, Han KS, Chang HJ, Jung KH, Lim SB, Choi HS, Jeong SY, Park JG. Atypical endoscopic features can be associated with metastasis in rectal carcinoid tumors. *Surg Endosc* 2008; **22**: 1992-1996 [PMID: 18568372 DOI: 10.1007/s00464-008-0006-x]
- 10 **Lee SH**, Kim BC, Chang HJ, Sohn DK, Han KS, Hong CW, Lee EJ, Lee JB, Lee DS, Lee IT, Youk EG. Rectal neuroendocrine and L-cell tumors: diagnostic dilemma and therapeutic strategy. *Am J Surg Pathol* 2013; **37**: 1044-1052 [PMID: 23648459 DOI: 10.1097/PAS.0b013e3182819f0f]
- 11 **Rifkin MD**, Ehrlich SM, Marks G. Staging of rectal carcinoma: prospective comparison of endorectal US and CT. *Radiology* 1989; **170**: 319-322 [PMID: 2643135 DOI: 10.1148/radiology.170.2.2643135]
- 12 **Balthazar EJ**, Megibow AJ, Hulnick D, Naidich DP. Carcinoma of the colon: detection and preoperative staging by CT. *AJR Am J Roentgenol* 1988; **150**: 301-306 [PMID: 3257314 DOI: 10.2214/ajr.150.2.301]
- 13 **Kasuga A**, Chino A, Urugami N, Kishihara T, Igarashi M, Fujita R, Yamamoto N, Ueno M, Oya M, Muto T. Treatment strategy for rectal carcinoids: a clinicopathological analysis of 229 cases at a single cancer institution. *J Gastroenterol Hepatol* 2012; **27**: 1801-1807 [PMID: 22743039 DOI: 10.1111/j.1440-1746.2012.07218.x]
- 14 **Kim MS**, Hur H, Min BS, Baik SH, Lee KY, Kim NK. Clinical outcomes for rectal carcinoid tumors according to a new (AJCC 7th edition) TNM staging system: a single institutional analysis of 122 patients. *J Surg Oncol* 2013; **107**: 835-841 [PMID: 23505038 DOI: 10.1002/jso.23327]
- 15 **Li AF**, Hsu CY, Li A, Tai LC, Liang WY, Li WY, Tsay SH, Chen JY. A 35-year retrospective study of carcinoid tumors in Taiwan: differences in distribution with a high probability of associated second primary malignancies. *Cancer* 2008; **112**: 274-283 [PMID: 18008361 DOI: 10.1002/cncr.23159]
- 16 **Shields CJ**, Tiet E, Winter DC. Carcinoid tumors of the rectum: a multi-institutional international collaboration. *Ann Surg* 2010; **252**: 750-755 [PMID: 21037430 DOI: 10.1097/SLA.0b013e3181fb8df6]
- 17 **Konishi T**, Watanabe T, Kishimoto J, Kotake K, Muto T, Nagawa H. Prognosis and risk factors of metastasis in colorectal carcinoids: results of a nationwide registry over 15 years. *Gut* 2007; **56**: 863-868 [PMID: 17213340 DOI: 10.1136/gut.2006.109157]
- 18 **Ramage JK**, Davies AH, Ardill J, Bax N, Caplin M, Grossman A, Hawkins R, McNicol AM, Reed N, Sutton R, Thakker R, Aylwin S, Breen D, Britton K, Buchanan K, Corrie P, Gillams A, Lewington V, McCance D, Meeran K, Watkinson A. Guidelines for the management of gastroenteropancreatic neuroendocrine (including carcinoid) tumours. *Gut* 2005; **54** Suppl 4: iv1-i16 [PMID: 15888809 DOI: 10.1136/gut.2004.053314]
- 19 **Yoon SN**, Yu CS, Shin US, Kim CW, Lim SB, Kim JC. Clinicopathological characteristics of rectal carcinoids. *Int J Colorectal Dis* 2010; **25**: 1087-1092 [PMID: 20397020 DOI: 10.1007/s00384-010-0949-y]
- 20 **Modlin IM**, Oberg K, Chung DC, Jensen RT, de Herder WW, Thakker RV, Caplin M, Delle Fave G, Kaltsas GA, Krenning EP, Moss SF, Nilsson O, Rindi G, Salazar R, Ruszniewski P, Sundin A. Gastroenteropancreatic neuroendocrine tumours. *Lancet Oncol* 2008; **9**: 61-72 [PMID: 18177818 DOI: 10.1016/s1470-2045]
- 21 **Bosman F**. World Health Organization International Agency for Research on Cancer. WHO classification of tumours of the digestive system 2010. Available from: URL: http://www.who.int/dg/lee/speeches/2004/iarc_governingcouncil/en/

P- Reviewer: Boulay B, Osawa S **S- Editor:** Ma YJ **L- Editor:** A
E- Editor: Zhang DN





Retrospective Study

Features associated with progression of small pancreatic cystic lesions: A retrospective study

Hong-Ming Tsai, Chiao-Hsiung Chuang, Yan-Shen Shan, Yi-Sheng Liu, Chiung-Yu Chen

Hong-Ming Tsai, Yi-Sheng Liu, Department of Radiology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan

Chiao-Hsiung Chuang, Chiung-Yu Chen, Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan

Yan-Shen Shan, Department of Surgery, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan

Author contributions: Chen CY was in charge of drafting and manuscript composition; Tsai HM and Liu YS contributed to the imaging review; Shan YS provided the original concept and study design; Chuang CH provided his expertise for data interpretation.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the National Cheng Kung University Hospital.

Informed consent statement: The IRB allowed us to waive the requirement for obtaining informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to have imaging study by written consent.

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

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Chiung-Yu Chen, MD, Associate Professor, Department of Internal Medicine, National Cheng Kung University Hospital, No. 138 Sheng-Li Road, Tainan 704, Taiwan. chiungyu@mail.ncku.edu.tw
Telephone: +886-6-2353535-2689
Fax: +886-6-2347270

Received: May 13, 2015
Peer-review started: May 15, 2015
First decision: July 10, 2015
Revised: July 21, 2015
Accepted: October 12, 2015
Article in press: October 13, 2015
Published online: December 21, 2015

Abstract

AIM: To investigate the progression rate of small pancreatic cystic lesions and identify characteristics associated with their progression.

METHODS: Patients with pancreatic cystic lesions with at least 1-year of follow-up were evaluated retrospectively. We excluded patients with cysts larger than 3 cm or with features that were a concern for malignancy. In total, 135 patients were evaluated. The interval progression of the cysts was examined. Characteristics were compared between patients with and without progression.

RESULTS: The pancreatic cysts ranged from 3 to 29 mm. The mean follow-up period was 4.5 ± 2.3 years and the mean progression rate was 1.0 ± 1.3 mm/year. Ninety patients showed interval progression and were divided into two groups; the minimal-change group ($n = 41$), who had cyst progression at less than 1 mm/year, and the progression group ($n = 49$), who had a progression rate of more than 1 mm/year. Compared

with the cysts without progression, the lesions of the progression group were more frequently associated with tubular cyst, septation or a prominent pancreatic duct ($P < 0.05$). The odds ratio for progression was 5.318 for septation and 4.582 for tubular cysts.

CONCLUSION: Small pancreatic cysts progress slowly. Lesions with tubular shape, septa, or prominent pancreatic duct were more likely to progress, and required further diagnostic intervention or shorter surveillance interval.

Key words: Pancreas; Cystic lesion; Progression; Imaging features; Observation

© **The Author(s) 2015.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Observation is advised for small pancreatic cyst without features that are a concern for malignancy. Our study determined that small pancreatic cysts with borderline pancreatic duct dilation, tubular shape, or septa were associated with risk of progression. Our findings may be helpful to stratify patients for different management planning according to their risk of progression.

Tsai HM, Chuang CH, Shan YS, Liu YS, Chen CY. Features associated with progression of small pancreatic cystic lesions: A retrospective study. *World J Gastroenterol* 2015; 21(47): 13309-13315 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13309.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13309>

INTRODUCTION

The increased utilization of cross-sectional imaging, such as multi-detector computed tomography (MDCT) and magnetic resonance (MR) imaging, has resulted in the increased detection of pancreatic cystic lesions, generally for unrelated reasons^[1,2]. Pancreatic cystic lesions are encountered in up to 3% of computed tomography (CT) examinations and 20% of MR imaging studies among individuals with no prior history of pancreatic disease^[3,4]. It is estimated that 10% of individuals have a pancreatic cystic lesions by the age of 70^[4].

Pancreatic cystic lesions not only have diverse histologies and imaging features, but also differ in terms of clinical presentation, biological behavior and risk of malignancy^[5]. According to the risk of malignancy, lesions can be categorized into: (1) those with no malignant potential (pseudocysts and serous cystadenomas); and (2) those that are precancerous or cancerous (mucinous cystic neoplasms and intra-ductal papillary mucinous neoplasms)^[6]. Patients with imaging features that are considered to be associated

with increased risk of malignancy, including cystic lesions larger than 3 cm, a dilated pancreatic duct and the presence of solid mural nodules^[7-10], are advised to undergo further pathology diagnosis or surgical resection. Conversely, emerging data support observation as the preferred approach for patients who have small cystic lesions detected incidentally^[11-13]. Nevertheless, approximately 3.3% of patients with small pancreatic cystic lesions were found to have occult malignancy after surgical resection^[10]. Subjecting these patients to unnecessary testing and treatment can result in a potentially harmful and expensive cascade of tests and procedures. However, lesions left for observation can cause anxiety for both patients and clinicians because of the potential specter of a lethal malignancy.

The shape and number of cysts have been used to differentiate low or no malignant potential for serous oligocystic adenoma from lesions with malignant potential, including mucinous cystic neoplasm and IPMN^[14]. By reviewing the images of our patients with small pancreatic cystic lesions, we aimed to determine the progression rate of these small lesions and to find the characteristic features that could differentiate progressive from non-progressive lesions. Hopefully, our findings will help in risk stratification and decision-making for the appropriate management and follow-up strategy in patients with small pancreatic cystic lesions.

MATERIALS AND METHODS

Patient selection

Institutional review board approval was obtained for this retrospective study; informed patient consent was not obtained. From January 2004 through December 2011, we identified 523 patients with cystic lesions of the pancreas that had been detected by sonography, CT or MR imaging. One hundred and sixty seven patients with prior clinical and laboratory evidence of pancreatitis were excluded. Eight patients with co-existing pancreatic malignancies, including four cases of adenocarcinoma and four cases of lymphoma, were excluded. Of the remaining 348 patients, 41 (11.8%) underwent surgical resection because of presence of symptoms, high-risk of stigmata (such as dilated main pancreatic duct or mural nodule), or at the patient's request. To focus on the progression of small pancreatic cystic lesions without features that are a concern for malignancy, we further excluded 172 patients with lesions larger than 3 cm ($n = 12$), a dilated pancreatic duct greater than 5 mm ($n = 5$), less than 1-year of follow-up ($n = 123$), or those who had only been evaluated by ultrasonography ($n = 32$). Finally, data from the remaining 135 patients with incidentally detected small pancreatic cystic lesions (94 men, 41 women; age range, 20-92 years; mean age, 67 years) were evaluated. Of these patients, 90 had

Table 1 Clinical characteristics of patients

	Non-progression group (<i>n</i> = 45)	Minimal-change group (<i>n</i> = 41)	Progression group (<i>n</i> = 49)
Follow-up (yr)	3.6 ± 2.0 ¹	5.6 ± 2.0 ^{2,3}	4.3 ± 2.5 ¹
Age (yr)	67.9 ± 10.3	63.2 ± 12.9 ³	70.0 ± 13.0 ¹
Sex (M:F)	29:16	33:8	32:17

¹Significant difference *vs* minimal-change group; ²Significant difference *vs* non-progression group; ³Significant difference *vs* progression group. By ANOVA and χ^2 test, data are expressed as the mean ± SD.

undergone CT only, 22 had undergone MR imaging only and 23 had undergone CT and MR imaging. The interval progression was examined by using the initial and the last imaging of the cyst, not necessarily using a the same imaging modality. None of our patients were associated with features that were a concern malignancy; therefore, none of them had been studied by endoscopic ultrasonography.

Imaging protocol

Most of our patients underwent the imaging examination to study other abdominal disease; therefore, the protocols we used were not specific to pancreatic disease but were those that we routinely used for abdomen examination.

Helical CT with two different multi-detector helical CT scanners (Sensation-16, Siemens Medical Systems, Forchheim, Germany or Definition Flash, Siemens Medical Systems, Forchheim, Germany) were performed in all patients. A Routine abdominal CT beginning 60–70 s after intravenous injection of contrast material was the most commonly performed examination. For routine CT scanning, 120–150 mL of nonionic contrast material (300–350 mg/mL) was injected at a rate of 2.5–3.0 mL/s, and images were acquired at a 5-mm section thickness after a 70 s delay. The field of view was adjusted according to the size of the patient.

MR imaging was performed with a 1.5-T system (Achieva, Philips Medical Systems, Netherland B.V.) using a phased-array torso coil. T2 weighted axial (TR/TE/FA 558/150/90) and coronal fat suppression images (TR/TE/FA 686/150/90), as well as two-dimensional (TR/TE/FA 8000/800/90) and three-dimensional MR cholangiopancreatography (TR/TE/FA 2000/700/90) were obtained. T1-weighted in-phase (TR/TE/FA 175/4.6/80) and opposed-phase (TR/TE/FA 175/2.3/80) images were acquired. Subsequently, axial breath hold three-dimensional T1-weighted high resolution isotropic volume examinations (TR/TE/FA 4.3/2.1/14; reconstructed slice thickness, 2.5 mm) were taken before and after intravenous contrast injection during the arterial (15 and 50 s), portal venous (90 s), and equilibrium (150 and 300 s) phases.

Image analysis

The CT and MR images were reviewed retrospectively by two radiologists (Tsai HM and Liu YS, with 20 and 10 years of experience, respectively). The shape (spherical or tubular), number, size and location of the cysts, the existence of a prominent pancreatic duct (3–4 mm in diameter), and the morphological features of the cysts, such as presence or absence of calcifications, and/or septa on CT and MR images, were recorded. When multiple cysts were present within the pancreas, the diameter and morphological features of the largest lesion were recorded. When the shape was not spherical, the longest diameter was recorded.

Grouping

The sizes of the cysts on the patient's first and last follow-up images were measured. The cyst progression rate was determined by dividing the change in size by the follow-up time in years. Accordingly, patients were first divided into those without progression of cystic lesions and those with interval progression. Patients with interval progression of cysts were further divided into the minimal-change and the progression groups, determined by a progression rate that was lower or higher than the mean cyst progression rate. Accordingly, the patients were divided into three groups: non-progression, minimal-change, and the progression.

Statistical analysis

The demographic data and imaging features were compared among the three groups. Comparisons were made using a Fisher's exact test or χ^2 test for nominal variables and a Student's *t*-test for continuous variables. A univariate analysis was performed to identify risk factors for progression, using logistic regression analysis. To evaluate independent predictors of malignancy, a multivariate analysis was performed with a model using factors identified as being significant in the univariate analysis or those with a *P*-value less than 0.20. A *P*-value less than 0.05 was considered statistically significant. Results are presented as means ± standard deviation.

RESULTS

Clinical features

The mean cyst progression rate was 1 mm per year (1.0 ± 1.3 mm/year). The patients were divided into three groups, the non-progression (*n* = 45), the minimal change (*n* = 41), and the progression groups (*n* = 49). Although the age of our patients ranged from 20 to 92 years, most of them were elderly (mean age, 67.3 ± 12.4 years). The mean age of the progression group was the highest among the three groups,

Table 2 Imaging characteristics of cysts *n* (%)

	Non-progression group (<i>n</i> = 45)	Minimal-change group (<i>n</i> = 41)	Progression group (<i>n</i> = 49)
Lesion characteristics			
Initial size (mm)	12.0 ± 5.4	11.6 ± 6.5	13.8 ± 7.4
Multiple cystic lesions	3 (6.7)	5 (12.2)	9 (18.4)
Location			
Uncinate	1 (0.2)	1 (2.4)	4 (8.2)
Head	9 (20.0)	9 (22.0)	13 (26.5)
Neck	17 (37.8)	7 (17.1)	9 (18.4)
Body	10 (22.2)	14 (34.1)	12 (24.5)
Tail	8 (17.8)	10 (24.4)	11 (22.4)
Imaging features			
Septation	4 (8.9) ¹	5 (12.2)	13 (26.5) ²
Tubular cyst	5 (11.1) ¹	8 (19.5)	14 (28.6) ²
Calcification	2 (4.5)	1 (2.5)	1 (2.0)
Prominent pancreatic duct	0 (0) ¹	3 (7.3)	9 (18.4) ²

¹Significant difference *vs* the progression group; ²Significant difference *vs* the non-progression group, by ANOVA and χ^2 test. Data are expressed as the mean ± SD.

but it was not significantly different from that of the non-progression group (Table 1). Male gender was predominant in all three groups. The follow-up period of our patients ranged from 1.1 to 10.1 years, with a mean of 4.5 ± 2.3 years. Although the progression group had a longer follow-up time than the non-progression group, it was not statistically significant (Table 1).

Imaging characteristics

The pancreatic cystic lesions ranged from 3 to 29 mm (mean, 12.5 ± 6.5 mm), based on the longest diameter. The initial size was not related to future progression and was similar among the three groups (Table 2). Although a trend for increasing multiple-cystic lesions was noted from the non-progression group to the progression group, the difference was not statistically significant (Table 2). Few cystic lesions were detected over the uncinate process. The remaining cystic lesions were evenly distributed across all the other sites of the pancreas and there was no predilection site for progressive pancreatic cystic lesions (Table 2).

As shown in Table 2, more patients in the progression group had a septated (Figure 1) or a tubular (Figure 2) cyst than those in the non-progression group (septated: 26.5% *vs* 8.9%, $P < 0.05$, tubular: 28.6% *vs* 11.1%, $P < 0.05$). Nine patients (18.4%) in the progression group had a prominent pancreatic duct (Figure 3), which was in marked contrast to none observed in the non-progression group ($P < 0.01$). E could not check the calcification of pancreatic cysts in 22 of our patients because they underwent MR imaging only. In the other 113 patients who had a CT scan examined during the follow-up, only four patients had a calcified pancreatic cystic lesions and all calcifications

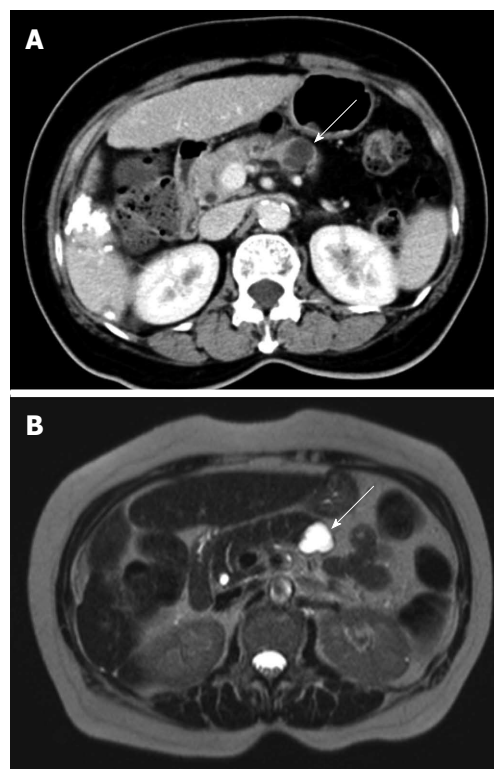


Figure 1 Contrast-enhanced computed tomography scans and T2-weighted magnetic resonance images. Contrast-enhanced computed tomography scans obtained during the portal venous phase showing a septated cystic lesion in the body of the pancreas (A; arrow). An axial T2-weighted magnetic resonance image showing a T2 hyperintense lesion with septa (B; arrow) in the body of the pancreas.

were located in the periphery.

Regression analysis

Cystic lesions with septa or a tubular shape were included for the regression analysis; whereas, cysts with a prominent pancreatic duct were not analyzed, because this feature was exclusively found in lesions with interval progression. By multivariate regression analysis, lesions with septa had 5.318-fold increased odds of interval progression than lesions without septa; the odds ratio of progression was 4.582 for the tubular shape lesion (Table 3).

Follow-up

The interval progression diameter ranged from 0 to 54 mm (average, 4.1 ± 7.2 mm) with a progression rate ranging from 0 to 6.2 mm per year (average 1.0 ± 1.3 mm per year). No mural nodules were found during the follow-up period. Twelve patients had cysts that progressed to more than 3 cm in diameter (range from 6.4 cm to 3.0 cm) within a mean follow-up period of 5.9 ± 2.8 years (range from 1.5 to 10.0 years). Two patients underwent surgery because of concern of malignant potential based on the growth characteristics. The pathologies of these two lesions were serous adenoma and serous oligocystic adenoma,

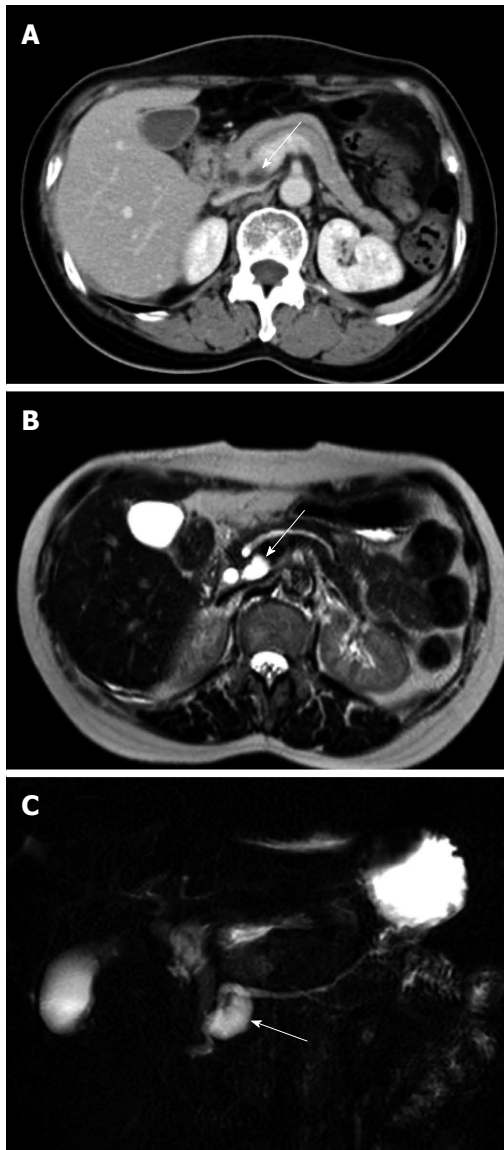


Figure 2 Contrast-enhanced computed tomography scans and T2-weighted magnetic resonance images. Contrast-enhanced computed tomography scans obtained during the portal venous phase showing a tubular cyst in the neck of the pancreas (A; white arrow). Axial T2-weighted magnetic resonance image showing a T2 hyperintense tubular lesion (B; white arrow) in the neck of the pancreas. The tubular lesion (C; white arrow) was more clearly appreciated on two dimensional magnetic resonance cholangiopancreatography.

respectively. The remaining patients were kept under observation according to the patients' preference.

DISCUSSION

The decision to proceed with surgical resection for a small pancreatic cystic lesion is difficult because of the significant morbidity and mortality associated with pancreatic surgery, especially in the elderly. Accordingly, the Sendai guidelines recommend surveillance only for cysts less than 3 cm without "worrisome features" or "high-risk stigmata"^[15]. Despite the good performance of the Sendai surveillance guidelines, which have been

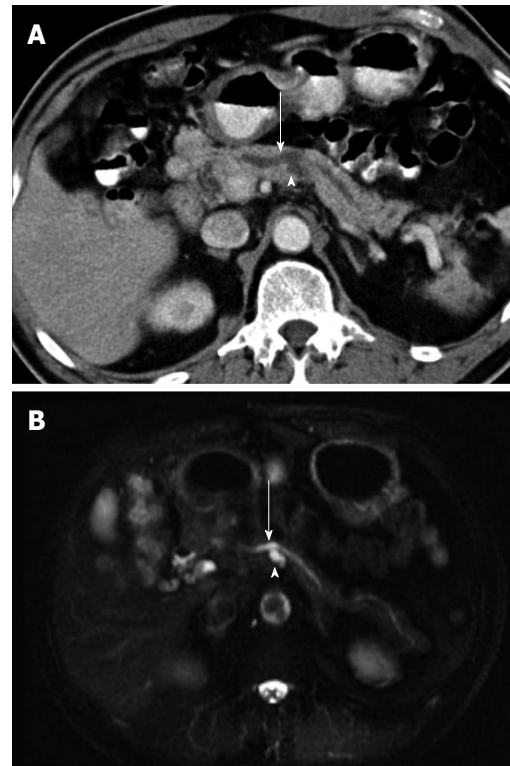


Figure 3 Contrast-enhanced computed tomography scans and T2-weighted magnetic resonance images. Contrast-enhanced computed tomography scans obtained during the portal venous phase showing a tubular cyst (A; arrowhead) in the body of the pancreas, with a prominent pancreatic duct (A; arrow). Axial T2-weighted magnetic resonance image showing a T2 hyperintense tubular cyst (B; arrowhead) in the body of the pancreas, with a prominent pancreatic duct (B; arrow).

validated recently in a report showing that patients who met the criteria had a 97% probability of benign follow-up for up to 7 years and 8 mo^[16], they are not perfect, because some small cysts were still found to have malignant outcomes on long-term follow-up. Surveillance, therefore, is associated with concern, anxiety and fear about the uncertainty of the diagnosis and the natural history of these cysts. Our study revealed the slow progression rate of small pancreatic cystic lesions and indicated that tubular cysts or cysts associated with prominent pancreatic ducts or septa tended to be progressive. Our study may provide more information to ease the uncertainty associated with the follow-up strategy chosen for patients with a small pancreatic cystic lesions.

Septa can be associated both with a malignant or potentially malignant mucinous cystic neoplasm and a benign serous cystadenoma^[17,18]. Sahani *et al.*^[12] reported that small pancreatic cysts without septa or solid components are almost always benign; however, since 20% of their septated cystic lesions were associated with malignancies or borderline malignancies, detecting septa within small cysts should raise a concern for malignancy. Our study found that small septated cysts had an increased risk of interval progression, further supporting the observation that

Table 3 Regression analysis between non-progression and progression groups

	Univariate		Multivariate	
	OR (95%CI)	P value	OR (95%CI)	P value
Septation	3.701 (1.107-2.372)	0.034	5.318 (1.539-18.374)	0.008
Tubular content	3.200 (1.047-9.782)	0.041	4.582 (1.450-14.480)	0.010

By binary logistic regression analysis.

septa in a small cyst increase the level of suspicion of malignancy.

IPMN has a malignant potential and tends to be progressive. As the tumor produces mucin content, dilatation of the main pancreatic duct is frequently found with main duct IPMN. A dilated main pancreatic duct of 5-9 mm is considered a "worrisome feature", while a diameter of more than 10 mm is one of the "high-risk stigmata"^[15] that warrant further diagnosis or surgery. In our study, cysts with these features were initially excluded; however, we found that cysts with a prominent pancreatic duct of 3 to 4 mm in diameter still showed a tendency for interval progression. In addition, cysts with a tubular shape could be caused by the dilation of the pancreatic duct branch and are frequently associated with branch duct IPMN^[14]. As both features are characteristics of IPMN, which possesses malignant potential, the more tubular cysts or cysts with prominent pancreatic ducts found in our progression group may indicate the presence of more IPMN cases in this group.

Peripheral calcification was prevalent in mucinous cystic neoplasms, while head location or multiple cysts were more frequently associated with branch duct IPMN^[5]; therefore, cysts with these features would be expected to be progressive. However, calcification was rarely found in our cases. While the number of cases with multi-cystic lesions showed an increasing trend in the progression group, it did not reach statistical significance. The above-mentioned imaging features, although also characteristic features of mucinous cystic lesions, were less valuable to distinguish small pancreatic cystic lesions with different progression rates in our study.

Male gender was predominant across all three groups in this study, which was contradictory to previous studies reporting that female gender was predominant in serous and mucinous cystic neoplasms^[19]. All our cystic lesions were asymptomatic and were found coincidentally during imaging examinations for abdominal organs other than the pancreas; therefore, this finding might simply reflect the male predominance of liver diseases in this liver diseases endemic country.

This was a retrospective study; therefore, the follow-up intervals of imaging were not the same for all patients. It may be argued that the progressive lesions observed in our patients could simply result from a

longer follow-up period. Undeniably, the longest follow-up period seen in our minimal-change group may actually reflect this possibility. However, the similar follow-up periods between the non-progression and progression groups indicated there were factors other than "time" that determined the risk of progression in pancreatic cystic lesions.

We suspected that the progression rate of cyst might not be constant and could be accelerated when the cyst becomes larger, which challenged the progression rate of 1 mm/year that was determined by observing cyst progression at different follow-up intervals instead of year-by-year. Such suspicion might be reasonable in those lesions larger than 3 cm, but it should be trivial in our study, because all our lesions were smaller than 3 cm and the mean initial size was similar among groups with different interval changes.

As observed from the benign pathology in our two patients who had cysts growing greater than 3 cm and who underwent surgery, the increase in the size of the cyst was not necessarily associated with malignancy. However, cyst progression remains a key point to be followed on surveillance images^[15,20]. Our study determined the mean growth rate as 1 mm per year after a mean follow-up period of about 6 years, which supports the low incidence of malignant transformation; *i.e.*, of 0.4% per year during surveillance^[20], and the current proposed annual surveillance strategy for small cysts without high risk stigmata or worrisome features^[15]. The addition of molecular profiling, cytology and chemistry of pancreatic cystic fluid to imaging studies, has defined an integrated approach to molecular pathology testing that has increased the accuracy of assessing the malignant potential of pancreatic cysts^[16]. Our study explored imaging features associated with the risk of progression in small pancreatic cystic lesions that might be helpful to stratify patients into those who require cystic fluid testing and those who merely require observation.

COMMENTS

Background

Pancreatic cystic lesions are encountered in up to 3% of computed tomography examinations and 20% of magnetic resonance imaging studies among individuals with no prior history of pancreatic disease. Pancreatic cystic lesions not only have diverse histologies and imaging features, but also differ in terms of clinical presentation, biological behavior and risk of malignancy. Emerging data supports observation as the preferred approach for patients who have small cystic lesions without features that are a concern for malignancy. Nevertheless, approximately 3.3% of patients with small pancreatic cystic lesions were found to have occult malignancy after surgical resection. The authors aimed to determine the progression rate of these small lesions and to identify the characteristic features that can differentiate progressive from non-progressive lesions.

Research frontiers

There was little data dealing with the progression and characteristic findings of small pancreatic cysts. The results of this study contributed to the identification of the progression rate of small pancreatic cysts and the characteristics

associated with such progressive cysts.

Innovations and breakthroughs

This study determined the mean growth rate of small pancreatic cyst as 1 mm per year after a mean follow-up period of about 6 years. This study indicated that tubular cysts or cysts with prominent pancreatic ducts or septa were imaging features associated with risk of progression in small pancreatic cystic lesions.

Applications

This study provides more information to ease the uncertainty associated with the follow-up strategy chosen for patients with a small pancreatic cyst and to select patients at risk of progressive disease to undergo EUS-FNA for cystic fluid analysis.

Terminology

High-risk stigmata: pancreatic cyst with enhanced solid components and a main pancreatic duct dilation ≥ 10 mm. Worrisome features: pancreatic cyst ≥ 3 cm, with thickened enhanced cyst walls, non-enhanced mural nodules, main pancreatic duct of 5-9 mm, an abrupt change in the main pancreatic duct caliber with distal pancreatic atrophy, and lymphadenopathy.

Peer-review

This valuable paper deals with the imaging features that are associated with meaningful progression of pancreatic cysts. Although there is not histological correlation, I believe that interesting data are derived from this research, and that it could be published after minor revision.

REFERENCES

- Laffan TA, Horton KM, Klein AP, Berlanstein B, Siegelman SS, Kawamoto S, Johnson PT, Fishman EK, Hruban RH. Prevalence of unsuspected pancreatic cysts on MDCT. *AJR Am J Roentgenol* 2008; **191**: 802-807 [PMID: 18716113 DOI: 10.2214/AJR.07.3340]
- de Jong K, Nio CY, Hermans JJ, Dijkgraaf MG, Gouma DJ, van Eijck CH, van Heel E, Klass G, Fockens P, Bruno MJ. High prevalence of pancreatic cysts detected by screening magnetic resonance imaging examinations. *Clin Gastroenterol Hepatol* 2010; **8**: 806-811 [PMID: 20621679 DOI: 10.1016/j.cgh.2010.05.017]
- Del Chiaro M, Verbeke C, Salvia R, Klöppel G, Werner J, McKay C, Friess H, Manfredi R, Van Cutsem E, Löhner M, Segersvärd R. European experts consensus statement on cystic tumours of the pancreas. *Dig Liver Dis* 2013; **45**: 703-711 [PMID: 23415799 DOI: 10.1016/j.dld.2013.01.010]
- de Jong K, Nio CY, Mearadji B, Phoa SS, Engelbrecht MR, Dijkgraaf MG, Bruno MJ, Fockens P. Disappointing interobserver agreement among radiologists for a classifying diagnosis of pancreatic cysts using magnetic resonance imaging. *Pancreas* 2012; **41**: 278-282 [PMID: 22015970 DOI: 10.1097/MPA.0b013e31822899b6]
- Sahani DV, Kambadakone A, Macari M, Takahashi N, Chari S, Fernandez-del Castillo C. Diagnosis and management of cystic pancreatic lesions. *AJR Am J Roentgenol* 2013; **200**: 343-354 [PMID: 23345356 DOI: 10.2214/AJR.12.8862]
- Farrell JJ, Fernández-del Castillo C. Pancreatic cystic neoplasms: management and unanswered questions. *Gastroenterology* 2013; **144**: 1303-1315 [PMID: 23622140 DOI: 10.1053/j.gastro.2013.01.073]
- de Castro SM, Houwert JT, van der Gaag NA, Busch OR, van Gulik TM, Gouma DJ. Evaluation of a selective management strategy of patients with primary cystic neoplasms of the pancreas. *Int J Surg* 2011; **9**: 655-658 [PMID: 21925294 DOI: 10.1016/j.ijsu.2011.08.007]
- Donahue TR, Hines OJ, Farrell JJ, Tomlinson JS, Eibl G, Reber HA. Cystic neoplasms of the pancreas: results of 114 cases. *Pancreas* 2010; **39**: 1271-1276 [PMID: 20717069 DOI: 10.1097/MPA.0b013e3181e1d6f4]
- Huang ES, Turner BG, Fernandez-Del-Castillo C, Brugge WR, Hur C. Pancreatic cystic lesions: clinical predictors of malignancy in patients undergoing surgery. *Aliment Pharmacol Ther* 2010; **31**: 285-294 [PMID: 19845568 DOI: 10.1111/j.1365-2036.2009.04173.x]
- Lee CJ, Scheiman J, Anderson MA, Hines OJ, Reber HA, Farrell J, Kochman ML, Foley PJ, Drebin J, Oh YS, Ginsberg G, Ahmad N, Merchant NB, Isbell J, Parikh AA, Stokes JB, Bauer T, Adams RB, Simeone DM. Risk of malignancy in resected cystic tumors of the pancreas ≤ 3 cm in size: is it safe to observe asymptomatic patients? A multi-institutional report. *J Gastrointest Surg* 2008; **12**: 234-242 [PMID: 18040749 DOI: 10.1007/s11605-007-0381-y]
- Walsh RM, Vogt DP, Henderson JM, Zuccaro G, Vargo J, Dumot J, Herts B, Biscotti CV, Brown N. Natural history of indeterminate pancreatic cysts. *Surgery* 2005; **138**: 665-670; discussion 670-671 [PMID: 16269295 DOI: 10.1016/j.surg.2005.07.019]
- Sahani DV, Saokar A, Hahn PF, Brugge WR, Fernandez-Del Castillo C. Pancreatic cysts 3 cm or smaller: how aggressive should treatment be? *Radiology* 2006; **238**: 912-919 [PMID: 16439566 DOI: 10.1148/radiol.2382041806]
- Soejima R. [Intractable airway infections and its management]. *Nihon Naika Gakkai Zasshi* 1991; **80**: 453-457 [PMID: 1856565 DOI: 10.1097/01.sla.0000237652.84466.54]
- Kim SY, Lee JM, Kim SH, Shin KS, Kim YJ, An SK, Han CJ, Han JK, Choi BI. Macrocytic neoplasms of the pancreas: CT differentiation of serous oligocystic adenoma from mucinous cystadenoma and intraductal papillary mucinous tumor. *AJR Am J Roentgenol* 2006; **187**: 1192-1198 [PMID: 17056905 DOI: 10.2214/AJR.05.0337]
- Tanaka M, Fernández-del Castillo C, Adsay V, Chari S, Falconi M, Jang JY, Kimura W, Levy P, Pitman MB, Schmidt CM, Shimizu M, Wolfgang CL, Yamaguchi K, Yamao K. International consensus guidelines 2012 for the management of IPMN and MCN of the pancreas. *Pancreatology* 2012; **12**: 183-197 [PMID: 22687371 DOI: 10.1016/j.pan.2012.04.004]
- Al-Haddad MA, Kowalski T, Siddiqui A, Mertz HR, Mallat D, Haddad N, Malhotra N, Sadowski B, Lybik MJ, Patel SN, Okoh E, Rosenkranz L, Karasik M, Golio M, Linder J, Catalano MF. Integrated molecular pathology accurately determines the malignant potential of pancreatic cysts. *Endoscopy* 2015; **47**: 136-142 [PMID: 25314329 DOI: 10.1055/s-0034-1390742]
- Hammond N, Miller FH, Sica GT, Gore RM. Imaging of cystic diseases of the pancreas. *Radiol Clin North Am* 2002; **40**: 1243-1262 [PMID: 12479709]
- Sarr MG, Murr M, Smyrk TC, Yeo CJ, Fernandez-del-Castillo C, Hawes RH, Freeny PC. Primary cystic neoplasms of the pancreas. Neoplastic disorders of emerging importance-current state-of-the-art and unanswered questions. *J Gastrointest Surg* 2003; **7**: 417-428 [PMID: 12654569]
- Talukdar R, Nageshwar Reddy D. Treatment of pancreatic cystic neoplasm: surgery or conservative? *Clin Gastroenterol Hepatol* 2014; **12**: 145-151 [PMID: 23978346 DOI: 10.1016/j.cgh.2013.08.031]
- Wu BU, Sampath K, Berberian CE, Kwok KK, Lim BS, Kao KT, Giap AQ, Kosco AE, Akmal YM, Diffranzo AL, Yu W, Ngor EW. Prediction of malignancy in cystic neoplasms of the pancreas: a population-based cohort study. *Am J Gastroenterol* 2014; **109**: 121-129; quiz 130 [PMID: 24080609 DOI: 10.1038/ajg.2013.334]

P- Reviewer: Luna A, Mathew S S- Editor: Yu J
L- Editor: Stewart G E- Editor: Ma S



Retrospective Study

Long-term outcomes after radical gastrectomy in gastric cancer patients with overt bleeding

Lei Wang, Xu-An Wang, Jia-Qi Hao, Li-Na Zhang, Mao-Lan Li, Xiang-Song Wu, Hao Weng, Wen-Jie Lv, Wen-Jie Zhang, Lei Chen, Hong-Gang Xiang, Jian-Hua Lu, Ying-Bin Liu, Ping Dong

Lei Wang, Xu-An Wang, Jia-Qi Hao, Mao-Lan Li, Xiang-Song Wu, Hao Weng, Wen-Jie Lv, Wen-Jie Zhang, Lei Chen, Hong-Gang Xiang, Jian-Hua Lu, Ying-Bin Liu, Ping Dong, Department of General Surgery, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

Li-Na Zhang, Department of Biostatistics, Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

Author contributions: Wang L, Wang XA and Hao JQ contributed equally to this work; Wang L, Wang XA, Hao JQ, Liu YB and Dong P conceived this research and participated in research design, writing of the manuscript, performance of the research, data analysis and manuscript revision; Zhang LN, Li ML, Wu XS, Lv WJ, Weng H, Zhang WJ, Chen L, Xiang HG and Lu JH participated in research design, data analysis and manuscript revision; all authors approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine.

Informed consent statement: Informed consent is given by patients preoperatively and registered in the electronic patient file. 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.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Ping Dong, MD, Associate Professor, Department of General Surgery, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, No. 1665 Kongjiang Road, Shanghai 200092, China. dongping1050@163.com
Telephone: +86-21-25076315
Fax: +86-21-65030840

Received: June 5, 2015

Peer-review started: June 8, 2015

First decision: August 26, 2015

Revised: August 31, 2015

Accepted: September 28, 2015

Article in press: September 30, 2015

Published online: December 21, 2015

Abstract

AIM: To investigate the difference in long-term outcomes between gastric cancer patients with and without a primary symptom of overt bleeding (OB).

METHODS: Consecutive patients between January 1, 2007 and March 1, 2012 were identified retrospectively by reviewing a gastric cancer database at Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. A follow-up examination was performed on patients who underwent a radical gastrectomy. OB due to gastric cancer included hematemesis, melena or hematochezia, and gastric cancer was confirmed as the source of bleeding by endoscopy. Patients without OB were defined as cases with occult bleeding and those with other initial presentations, including epigastric pain, weakness, weight loss and obstruction. The 3-year overall survival (OS) rate, age, gender, AJCC T stage, AJCC N stage, overall AJCC stage, tumor size, histological type, macroscopic (Borrmann) type, lymphovascular invasion and R status were compared between patients

with and without OB. Moreover, we carried out a subgroup analysis based on tumor location (upper, middle and lower).

RESULTS: We identified 939 patients. Of these, 695 (74.0%) were hospitalized for potential radical gastrectomy and another 244 received palliative resection, rerouting of the gastrointestinal tract, chemotherapy, radiotherapy or no treatment due to the presence of unresectable tumors. Notably, there was no significant difference in the percentage of OB patients between resectable cases and unresectable cases (20.3% *vs* 22.1%, $P = 0.541$). Follow-up examination was performed on 653 patients (94%) who underwent radical gastrectomy. We found no significant difference in 3-year OS rate (68.2% *vs* 61.2%, $P = 0.143$) or clinicopathological characteristics ($P > 0.05$) between these patients with and without OB. Subgroup analysis based on tumor location showed that the 3-year OS rate of upper gastric cancer was significantly higher in patients with OB (84.6%) than in those without OB (48.1%, $P < 0.01$) and that AJCC stages I - II (56.4% *vs* 35.1%, $P = 0.017$) and T1-T2 category tumors (30.8% *vs* 13%, $P = 0.010$) were more frequent in patients with OB than in those without OB. There was no significant difference in 3-year OS rate or clinicopathological characteristics between patients with and without OB ($P > 0.05$) for middle or lower gastric cancer.

CONCLUSION: Upper gastric cancer patients with OB exhibited tumors at less advanced pathological stages and had a better prognosis than upper gastric cancer patients without OB.

Key words: Gastric cancer; Overt bleeding; Tumor location; Prognosis; Pathological stage

© **The Author(s) 2015.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Data regarding the clinicopathological characteristics and long-term outcomes of gastric cancer patients presenting with overt bleeding (OB) are extremely limited. Our result showed that the prognosis of gastric cancer patients with OB was no worse than the prognosis of those without OB. In fact, upper gastric cancer patients with OB exhibited tumors at less advanced pathological stages and had a better prognosis than upper gastric cancer patients without OB. This provided a new insight into the intrinsic nature of gastric cancer with OB.

Wang L, Wang XA, Hao JQ, Zhang LN, Li ML, Wu XS, Weng H, Lv WJ, Zhang WJ, Chen L, Xiang HG, Lu JH, Liu YB, Dong P. Long-term outcomes after radical gastrectomy in gastric cancer patients with overt bleeding. *World J Gastroenterol* 2015; 21(47): 13316-13324 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13316.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13316>

INTRODUCTION

Gastrointestinal bleeding is classified as either overt bleeding (OB) or occult bleeding depending on the presence or absence of visible bleeding^[1]. OB is one of the most frequent complications in patients with gastric cancer^[2], as 1%-10% of hospitalized gastric cancer patients initially present with OB^[3-7].

Numerous studies on gastric cancer bleeding have focused on hemostasis and rebleeding^[2,4,5,7-9]. However, data regarding the clinicopathological characteristics and long-term outcomes of cases presenting with OB due to gastric cancer are extremely limited, and there are no reports comparing the clinical outcomes of patients with OB based on tumor location. The present study aimed to answer these questions, with a particular focus on OB in upper gastric cancer.

MATERIALS AND METHODS

Patients

Consecutive gastric cancer patients that presented between January 1, 2007 and March 1, 2012 were identified retrospectively by reviewing a gastric cancer database at Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. The eligibility criteria for the study included: (1) histologically proven primary adenocarcinoma of the stomach; (2) no history of gastrectomy or other previous malignancies; (3) no prior chemotherapy or radiation therapy; and (4) involvement of only one portion of the stomach [the stomach is anatomically divided into three portions, upper (U), middle (M), and lower (L), according to the lines connecting the trisected points on the lesser and greater curvatures^[10]]. This study was approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine.

Information collected from the database included the following: patient age and sex; the preoperative diagnosis; the surgical procedure performed; and the location, size, histological grade, macroscopic (Borrmann) type and stage of the primary tumor [according to the 7th edition of the AJCC (American Joint Committee on Cancer) cancer staging manual: stomach^[11]]. The depth of invasion, the presence or absence of lymph node metastasis and lymphovascular invasion, and the type of resection-complete (R0) or incomplete resection (R1 or R2)-were also recorded. All of these criteria were compared between patients with and without OB.

Definitions

OB due to gastric cancer included hematemesis, melena or hematochezia, and gastric cancer was confirmed as the source of bleeding by endoscopy. Patients without OB were defined as cases with occult bleeding and those with other initial presentations, including epigastric pain, weakness, weight loss and obstruction. A diagnosis of occult bleeding included

Table 1 Patient proportion according to tumor location *n* (%)

	OB	Non-OB	<i>P</i> value
All			0.541
Resectable	141 (20.3)	554 (79.7)	
Unresectable	54 (22.1)	190 (77.9)	
Upper			0.136
Resectable	41 (22.9)	138 (77.1)	
Unresectable	14 (15.2)	78 (84.8)	
Middle			0.887
Resectable	17 (24.3)	53 (75.7)	
Unresectable	9 (23.1)	30 (76.9)	
Lower			0.038 ^a
Resectable	83 (18.6)	363 (81.4)	
Unresectable	31 (27.4)	82 (72.6)	

^a*P* < 0.05, resectable *vs* unresectable. OB: Overt bleeding.

a positive fecal occult blood test result and/or iron-deficiency anemia without evidence of visible fecal blood according to the patient or the physician^[12-14].

Surgical management

Therapies for active bleeding included hemostatic drugs, endoscopic therapy and emergent embolization by interventional radiology. If hemostasis was not achieved, patients received emergency surgery both for bleeding and as a component of oncologic treatment. A careful pre- and intra-operative evaluation was performed on other patients. Those with unresectable tumors (poor performance status, distant metastasis and extensive abdominal metastasis) were treated *via* palliative resection, rerouting of the gastrointestinal tract, chemotherapy or radiotherapy. All patients with resectable tumors underwent radical gastrectomy and lymphadenectomy (D2+14v for advanced gastric cancer and D1 or D1+ for early gastric cancer) according to the guidelines of the Japanese Gastric Cancer Association^[15]. Histological evaluations were performed on all resected specimens. A final diagnosis of malignancy was established based on endoscopic biopsy or the histological assessment of the surgical specimens. Patients with an AJCC stages II-IV tumor received postoperative chemotherapy.

Follow-up

Patients who underwent radical gastrectomy were followed every 3 or 6 mo for 2 years and annually thereafter until death. The median follow-up duration for the entire cohort was 44 mo (range, 0-97 mo). A follow-up evaluation of all patients included in this study was completed by March 1, 2015. Ultrasonography, computed tomography, chest X-ray, and endoscopy were performed at each visit.

Statistical analysis

Consecutive variables were assessed by Student's *t*-test, and results are given as mean ± SD. Mann-Whitney *U* tests were adopted when consecutive variables were not normal. Categorical variables were

assessed by the Pearson's χ^2 test or Fisher's exact test. Survival curves were estimated by the Kaplan-Meier method and compared with the use of the log-rank test, with stratification according to tumor location. Multivariate analysis by a Cox's proportional hazards model was performed to identify independent prognostic factors of significance. All tests were two tailed. *P* values less than 0.05 were considered to indicate statistical significance. The analyses were performed with the use of IBM SPSS Statistics 19.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

During the study period, 939 consecutive gastric cancer patients were identified, including 195 (20.8%) with OB and 744 without OB. Of these, 695 (74.0%) were hospitalized for potential radical gastrectomy and another 244 received palliative resection, rerouting of the gastrointestinal tract, chemotherapy, radiotherapy or no treatment due to the presence of unresectable tumors accompanied by distant metastasis, extensive abdominal metastasis or poor performance status. Notably, there was no significant difference in the percentage of OB patients between resectable cases and unresectable cases (20.3% *vs* 22.1%, *P* = 0.541) (Table 1).

A follow-up examination was performed on 653 (94.0%) of the 695 patients who underwent a radical gastrectomy. The patients who underwent follow-up were aged 63.0 ± 12.0 years (range, 16-93 years). The male-to-female ratio among the 653 enrolled patients was 2.05 (439 males and 214 females). Of these patients, 132 (20.2%) were hospitalized with a primary symptom of OB. Additionally, 128 patients who achieved hemostasis underwent elective surgery, whereas 4 patients who failed to achieve hemostasis underwent emergency surgery. The characteristics of the 132 patients with OB and the 521 patients without OB were comparable (Table 2). There was no significant difference in the 3-year overall survival rate between patients with OB (68.2%, 319/521) and those without OB (61.2%, 90/132, *P* = 0.143) (Figure 1A).

Upper gastric cancer

Two hundred and seventy-one consecutive upper gastric cancer patients were identified. Of these, 179 (66.1%) underwent radical gastrectomy. There was no significant difference in the percentage of patients with OB between resectable cases and unresectable cases (22.9% *vs* 15.2%, *P* = 0.136) (Table 1). A follow-up examination was performed on 170 (95.0%) out of the 179 patients who underwent radical gastrectomy, including 39 (22.9%) with OB and 131 (77.1%) without OB. Of the 39 tumors in OB cases, 33 (82.1%) were found in the cardia and the lesser curvature and 6 (15.4%) were located in the fundus and the greater

Table 2 Patient characteristics for the whole patient group and upper gastric cancer patients *n* (%)

	All			Upper gastric cancer		
	Non-OB	OB	<i>P</i> value	Non-OB	OB	<i>P</i> value
Gender ¹			0.718			0.996
Male	352 (67.6)	87 (65.9)		94 (71.8)	28 (71.8)	
Female	169 (32.4)	45 (34.1)		37 (28.2)	11 (28.2)	
Age ²	63.2 ± 11.6	62.2 ± 13.4	0.497	64.8 ± 10.6	62 ± 12.8	0.276
AJCC stage ¹			0.081			0.017 ^a
I - II	240 (46.1)	72 (54.5)		46 (35.1)	22 (56.4)	
III-IV	281 (53.9)	60 (45.5)		85 (64.9)	17 (43.6)	
T category ¹			0.201			0.010 ^a
T1-T2	163 (31.3)	49 (37.1)		17 (13)	12 (30.8)	
T3-T4	358 (68.7)	83 (62.9)		114 (87)	27 (69.2)	
Involved lymph nodes ²	5.4 ± 7.8	4.7 ± 6.5	0.523	4.9 ± 5.9	4.2 ± 6.0	0.248
N category ¹			0.793			0.311
N0	187 (35.9)	49 (37.1)		36 (27.5)	14 (35.9)	
N1-N3	334 (64.1)	83 (62.9)		95 (72.5)	25 (64.1)	
Macroscopic (Borrmann) type ¹			0.357			0.036 ^a
EGC	90 (17.5)	19 (14.4)		8 (6.1)	4 (10.3)	
I	30 (5.8)	14 (10.6)		8 (6.1)	8 (20.5)	
II	325 (62.4)	81 (61.4)		89 (67.9)	23 (59)	
III	23 (4.4)	5 (3.8)		9 (6.9)	0 (0)	
IV	52 (10)	13 (9.8)		17 (13)	4 (10.3)	
Histological grade ¹			0.448			0.770
G1-G2	176 (33.8)	40 (30.3)		47 (35.9)	13 (33.3)	
G3-G4	345 (66.2)	92 (69.7)		84 (64.1)	26 (66.7)	
Size (cm) ²	4.7 ± 2.9	4.1 ± 2.1	0.083	5.4 ± 3.0	4.1 ± 2.0	0.018 ^a
R status ¹			0.729			0.114
R0	508 (97.5)	130 (98.5)		123 (93.9)	39 (100)	
R1	13 (2.5)	2 (1.5)		8 (6.1)	0 (0)	
Lymphovascular invasion ¹			0.785			0.484
Positive	437 (83.9)	112 (84.8)		108 (82.4)	34 (87.2)	
Negative	84 (16.1)	20 (15.2)		23 (17.6)	5 (12.8)	

¹Pearson's χ^2 test; ²Mann-Whitney *U*-test. ^a*P* < 0.05, non-OB vs OB. EGC: Early gastric cancer; OB: Overt bleeding.

curvature of the stomach. The clinicopathological characteristics are presented in Table 2. Characteristics including gender, age, AJCC N stage, the number of involved lymph nodes, histological grade, tumor size, R status and lymphovascular invasion status (*P* > 0.05) were similar between patients with and without OB. Early gastric cancer and Borrmann type I tumors were observed more frequently in patients with OB. Comparing the clinicopathological characteristics according to invasion depth and AJCC stage, the percentage of patients with AJCC T1-T2 category tumors was significantly higher among patients with OB than among those without OB (30.8% vs 13%, *P* = 0.010), and the proportion of patients with AJCC stages I - II tumors was significantly higher among patients with OB than among those without OB (56.4% vs 35.1%, *P* = 0.017). Moreover, patients presenting with OB had smaller primary tumors than those without OB (4.1 ± 2.0 cm vs 5.4 ± 3.0 cm, *P* = 0.018). The 3-year overall survival rate was significantly higher in patients with OB (84.6%) than in those without OB (48.1%, *P* < 0.01; Figure 1B).

Table 3 summarizes the results of univariate and multivariate analyses. The factors considered in the univariate and multivariate models of overall survival

included age, sex, AJCC T stage, AJCC N stage, overall AJCC stage, tumor size, histological type, macroscopic (Borrmann) type, lymphovascular invasion, R status and OB. Based on multivariate survival analysis, only overall AJCC stage (HR = 1.638, 95%CI: 1.366-1.966, *P* < 0.001), tumor size (HR = 1.090, 95%CI: 1.012-1.174, *P* = 0.023) and OB (vs non-OB) (HR = 0.346, 95%CI: 0.148-0.808, *P* = 0.014) were determined to be independent prognostic factors.

Middle gastric cancer

A total of 109 consecutive middle gastric cancer patients were included. Of these, 70 (64.2%) underwent radical gastrectomy. There was no difference in the percentage of patients with OB between resectable cases and unresectable cases (24.3% vs 23.1%, *P* = 0.887) (Table 1). A follow-up examination was performed on 66 (94.3%) of the radical gastrectomy patients. Of these, 16 (24.2%) were hospitalized with an initial manifestation of OB. The characteristics of the patients with or without OB were comparable (Table 4). No significant difference in age, sex, overall AJCC stage, AJCC T stage, AJCC N stage, the number of involved lymph nodes, macroscopic (Borrmann) type, histological grade, tumor size, R status or

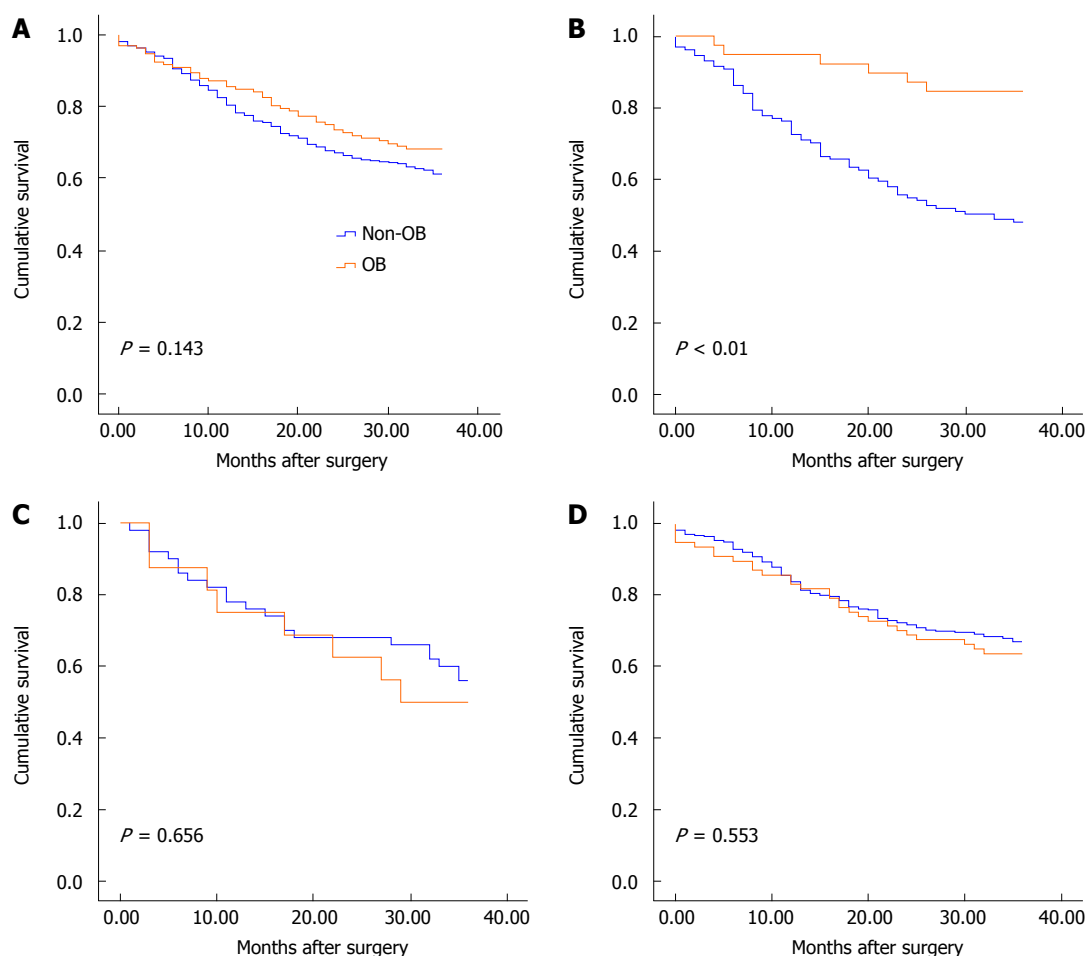


Figure 1 Three-year overall survival. A: The whole patient group; B: The upper gastric cancer; C: The middle gastric cancer; D: The lower gastric cancer. OB: Patients with a primary symptom of overt bleeding; Non-OB: Patients without a primary symptom of OB.

lymphovascular invasion status ($P > 0.05$) was observed between patients with and without OB. The 3-year overall survival rates were also similar between these groups (50% vs 56%, $P = 0.656$; Figure 1C). Multivariate survival analysis was not performed on the middle gastric cancer patients because of the small sample size.

Lower gastric cancer

A total of 559 consecutive lower gastric cancer patients were observed. Of these, 446 (79.9%) underwent radical gastrectomy. The percentage of patients with OB was significantly different between resectable and unresectable cases (18.6% vs 27.6%, $P = 0.038$) (Table 1). A follow-up examination was performed on 417 (93.5%) out of the 446 patients who underwent radical gastrectomy, including 77 (18.5%) patients with OB and 340 (81.5%) patients without OB. The characteristics of the patients with and without OB were comparable (Table 4). Similar 3-year overall survival rates were observed between these groups (63.6% vs 67.1%, $P = 0.553$; Figure 1D). The factors used in the univariate and multivariate models for lower gastric cancer patients were the same that were used for upper gastric cancer patients. The results of

univariate and multivariate analyses of the clinical and pathological characteristics are presented in Table 5. Based on multivariate survival analysis, only the overall AJCC stage (HR = 1.690, 95%CI: 1.508-1.894, $P < 0.001$), lymphovascular invasion status (positive vs negative, HR = 1.687, 95%CI: 1.160-2.455, $P = 0.006$) and age (HR = 1.023, 95%CI: 1.008-1.037, $P = 0.002$) were determined to be independent prognostic factors.

DISCUSSION

OB in patients with gastric cancer is a rare but serious condition with potentially dangerous effects. Although numerous studies have been conducted on the risk factors associated with gastric cancer bleeding and rebleeding, data regarding long-term outcomes following surgery and the pathological characteristics of gastric cancer patients with OB are extremely limited. The results of the current study provide information for clinical physicians to use when evaluating gastric cancer patients with OB. These findings also provide the following new perspectives relevant to our understanding of this group of patients: gastric cancer with OB is not synonymous with advanced gastric cancer, and its prognosis is no worse

Table 3 Univariate and multivariate survival analyses for upper gastric cancer

Variable	Univariate analysis P value	Multivariate analysis		
		HR	95%CI	P value
Age ¹				
Gender (<i>vs</i> male) ²	0.056			
OB <i>vs</i> non-OB ²	< 0.001 ^b	0.346	0.148-0.808	0.014 ^a
AJCC stage ³	< 0.001 ^b	1.638	1.366-1.966	< 0.001 ^b
AJCC T category ³	< 0.001 ^b			
AJCC N category ³	< 0.001 ^b			
Macroscopic (Borrmann) type ² (<i>vs</i> type I)	0.003 ^b			
II				
III				
IV				
Histological type ³	0.069			
Size ¹		1.090	1.012-1.174	0.023 ^a
R status ² (<i>vs</i> R0)	0.008 ^b			
R1				
Lymphovascular invasion ² (<i>vs</i> negative)	0.001 ^b			
Positive				

¹Consecutive variables; ²Unordered categorical variables; ³Ordinal categorical variables. ^a*P* < 0.05; ^b*P* < 0.01.

than the prognosis for gastric cancer without OB. In fact, patients with proximal gastric cancer with OB exhibit less advanced pathological stages and a better prognosis than patients with proximal gastric cancer without OB.

To investigate why the 3-year overall survival rate following surgery was significantly higher for upper gastric cancer patients presenting with OB than without OB, we compared clinicopathological characteristics between these two subgroups of upper gastric cancer patients. Our results revealed that compared to proximal gastric cancer patients without OB, those with OB exhibited not only less advanced tumors with respect to both overall AJCC stage and AJCC T stage but also smaller tumors. Further multivariate survival analysis demonstrated that the overall AJCC stage, tumor size and OB were independent factors affecting prognosis. An increment of one stage in the overall AJCC classification was associated with a 1.638-fold increased risk of death within 3 years of surgery. This result may explain why the former type of cancer exhibits a more positive prognosis. Fox *et al.*^[16] demonstrated that 62% of gastric cancer patients with bleeding were classified as overall AJCC stages I - II. Similarly, Kodama^[17] and colleagues and Moreno-Otero *et al.*^[6] found that 72.2% of these patients exhibited early stage tumors and an intraluminal growth pattern with irregularly distributed erosions or shallow, but not deep, ulcerations. These previous results support the findings of the current study. Gertsch *et al.*^[18] compared the prognosis of gastric cancer patients with bleeding and serosal invasion with the prognosis of gastric cancer patients without complications, but found no statistically significant difference in clinicopathological

characteristics or prognosis between the two groups. The following two considerations could explain why the results of Gertsch *et al.*^[18] were not consistent with the findings of the current study. On one hand, Gertsch *et al.*^[18] did not perform a stratified analysis that accounted for the differences in the locations of gastric cancer. On the other hand, the two studies assessed different subject populations: in particular, Gertsch *et al.*^[18] examined gastric carcinoma patients with serosal invasion, whereas we examined all gastric cancer patients at tumor stages T1-T4b.

Bleeding occurs as a result of gastric cancer for many reasons, including an increase in the size of the tumor body, insufficient blood supply to the central portion of the tumor, the softening, necrosis, and ulceration of tumor tissues, bleeding from the surface of the tumor ulcer, and the rupture of small and medium blood vessels^[6]. An interesting phenomenon discovered in this study was that in cases of upper gastric cancer, tumors accompanied by OB tended to be classified as an earlier pathological stage than tumors without OB; however, lower gastric adenocarcinomas did not follow this trend. Why did gastric adenocarcinomas with OB at different locations exhibit such different pathological presentations? Koh *et al.*^[2] examined the effects of different hemostasis treatment methods on bleeding resulting from gastric cancer and found that when transarterial embolization was performed after endoscopic hemostasis had failed, the left gastric artery was the predominant problematic artery. These results suggested that it would be challenging to stop gastric cancer bleeding from the left gastric artery. The current study also found that in 82.1% of upper gastric cancer patients with OB, the tumor was located in the cardia and the lesser curvature, for which the left gastric artery is the main feeding artery. Based on the combination of the results of the present study and the findings of Koh *et al.*^[2], we propose the following hypothesis. The left gastric artery and its branches are adjacent to the celiac artery and have high pressure and rich blood flow. Therefore, tumors in the cardia and the lesser curvature grow rapidly and readily display invasion and necrosis. When tumor tissue infiltration destroys branches of the left gastric artery, any resulting bleeding may be resistant to self-coagulation because these branches have high vascular pressure; thus, OB can easily occur in these cases. Therefore, OB provides an opportunity for the early diagnosis and treatment of upper gastric cancer patients. Lower gastric adenocarcinomas typically arise at the gastric antrum. The blood supply to this location is primarily provided by the right gastric artery and the right gastroepiploic artery, which originate from the proper hepatic artery and the gastroduodenal artery, respectively. Both of these arteries are far from the celiac artery and have relatively low pressure. When tumor tissues invade the branches of these arteries, the resulting bleeding can relatively rapidly

Table 4 Patient characteristics for middle and lower gastric cancer patients *n* (%)

	Middle gastric cancer			Lower gastric cancer		
	Non-OB	OB	<i>P</i> value	Non-OB	OB	<i>P</i> value
Gender ¹			0.902			0.684
Male	29 (58)	9 (56.3)		229 (67.4)	50 (64.9)	
Female	21 (42)	7 (43.8)		111 (32.6)	27 (35.1)	
Age ²	62.3 ± 11.2	61.3 ± 14.8	0.616	62.8 ± 11.9	62.6 ± 13.6	1.000
AJCC stage ¹			0.319			0.659
I - II	18 (36.0)	8 (50.0)		176 (51.8)	42 (54.5)	
III-IV	32 (64.0)	8 (50.0)		164 (48.2)	35 (45.5)	
T category ¹			0.291			0.891
T1-T2	12 (24)	6 (37.5)		134 (39.4)	31 (40.3)	
T3-T4	38 (76)	10 (62.5)		206 (60.6)	46 (59.7)	
Involved lymph nodes ²	8.5 ± 10.6	7.1 ± 9.0	0.432	5.1 ± 7.9	4.5 ± 6.2	0.844
N category ¹			0.104			0.396
N0	14 (28.0)	8 (50)		137 (40.3)	27 (35.1)	
N1-N3	36 (72.0)	8 (50)		203 (59.7)	50 (64.9)	
Macroscopic (Borrmann) type ¹			0.577			0.742
EGC	7 (14.0)	3 (18.8)		76 (22.4)	12 (15.6)	
I	4 (8.0)	2 (12.5)		18 (5.3)	4 (5.2)	
II	29 (58.0)	7 (43.8)		207 (60.9)	51 (66.2)	
III	2 (4.0)	2 (12.5)		12 (3.5)	3 (3.9)	
IV	8 (16.0)	2 (12.5)		27 (7.9)	7 (9.1)	
Histological grade ¹			0.554			0.282
G1-G2	10 (20)	5 (31.3)		119 (35.0)	22 (28.6)	
G3-G4	40 (80)	11 (68.8)		221 (65)	55 (71.4)	
Size (cm) ²	5.0 ± 3.0	4.0 ± 2.5	0.246	4.4 ± 2.8	4.1 ± 2.1	0.745
R status ¹			1.000			0.559
R0	48 (96.0)	15 (93.8)		337 (99.1)	76 (98.7)	
R1 + R2	2 (4.0)	1 (6.3)		3 (0.9)	1 (1.3)	
Lymphovascular invasion ¹			0.266			0.445
Positive	39 (78.0)	15 (93.8)		290 (85.3)	63 (81.8)	
Negative	11 (22.0)	1 (6.3)		50 (14.3)	14 (18.2)	

¹Pearson's χ^2 test; ²Mann-Whitney *U*-test. EGC: Early gastric cancer; OB: Overt bleeding.

coagulate, and the subsequent recurrence and non-OB cause fibrosis and thrombosis near the blood vessels. Thus, OB does not readily occur in these cases. This hypothesis requires additional validation by future studies. Another process could cause OB resulting from a proximal gastric adenocarcinoma. The cardia is narrower than other parts of the stomach, and tumor tissue infiltration increases vascular fragility at this location. When food passes the cardia, it is in a rough, undigested state. Therefore, mechanical stimuli can easily cause the rupture of blood vessels at this site.

Bleeding resulting from gastric cancer is closely associated with the vascular composition of the tumor. In 1971, Folkman^[19] proposed that tumor growth is dependent on angiogenesis. Furthermore, he suggested that tumor cells and blood vessels constitute a highly integrated environment. Angiogenesis plays an important role in the process of cancer development^[20,21]. High intratumor microvessel density correlates with high AJCC stage and lymph node metastasis. Intratumor microvessel density was found to have independent prognostic significance to cancer^[22], including gastric cancer^[23], compared to traditional prognostic markers based on multivariate analysis. Studies in Japan and China^[24] showed that the vascular composition of gastric cancer can be

categorized into hypovascular and hypervascular tumors, corresponding to Borrmann types II-III and Borrmann types I and IV gastric adenocarcinomas, respectively. In the current study, among upper gastric cancer cases, OB was observed more frequently in cases of early gastric cancer and Borrmann type I tumors. There was no difference in the percentage of Borrmann types II-IV tumors between upper gastric cancer patients with and without OB. Is the microvessel density or the Borrmann type of gastric adenocarcinomas associated with hemorrhaging from these tumors? Additional large-scale studies are warranted to precisely determine the impact of intratumor microvessel density on hemorrhaging from gastric cancer.

To determine whether selection bias may have influenced the conclusions of the current study, we investigated all patients receiving all types of therapies from our database and found that the rate of OB was similar between resectable and unresectable tumors. Therefore, selection bias was not possible for the whole patient group and upper gastric cancer cohort. But in the lower gastric cancer cases, the percentage of patients with OB in patients who underwent radical gastrectomy was lower than the percentage of patients with OB in patients who did not undergo radical

Table 5 Univariate and multivariate survival analyses for lower gastric cancer

Variable	Univariate analysis	Multivariate analysis		
	P value	HR	95%CI	P value
Age ¹		1.023	1.008-1.037	0.002
Gender (<i>vs</i> male) ²	0.930			
OB <i>vs</i> non-OB ²	0.553			
AJCC stage ³	< 0.001 ^b	1.690	1.508-1.894	< 0.001
AJCC T category ³	< 0.001 ^b			
AJCC N category ³	< 0.001 ^b			
Macroscopic (Borrmann) type ² (<i>vs</i> type I)	< 0.001 ^b			
II				
III				
IV				
Histological type ³	0.001 ^b			
Size ¹				
R status ² (<i>vs</i> R0)	0.003 ^b			
R1				
Lymphovascular invasion ² (<i>vs</i> negative)	< 0.001 ^b	1.687	1.160-2.455	0.006
Positive				

¹Consecutive variables; ²Unordered categorical variables; ³Ordinal categorical variables; ^bP < 0.01.

gastrectomy. Including patients who did not undergo radical gastrectomy, the overall 3-year survival rate may be lower in patients with OB than in patients without OB.

Cases of proximal gastric cancer, particularly cancer of the gastric cardia, only manifest as significant symptoms such as epigastric pain, weakness, weight loss and obstruction after the tumor has deeply infiltrated the gastric tissue. Therefore, most patients already have mid- to late-stage cancer by the time that they seek treatment to relieve the symptoms of eating difficulties and abdominal pain. As a result, these patients exhibit a poor prognosis. A subset of patients without OB have occult bleeding; however, this type of bleeding is generally not discovered in a timely fashion, resulting in the delay of disease treatment. Because there is currently no reliable tumor screening system in China, early diagnoses are difficult to obtain. Thus, OB provides an opportunity to diagnose proximal gastric cancer at an earlier pathological stage, thereby significantly improving patient prognosis.

COMMENTS

Background

Gastrointestinal bleeding is classified as either overt bleeding (OB) or occult bleeding depending on the presence or absence of visible bleeding. OB is one of the most frequent complications in patients with gastric cancer, as 1%-10% of hospitalized gastric cancer patients initially present with OB.

Research frontiers

Numerous studies on gastric cancer bleeding have focused on hemostasis and rebleeding. However, data regarding the clinicopathological characteristics and long-term outcomes of cases presenting with OB due to gastric cancer are extremely limited, and there are no reports comparing the clinical outcomes of

patients with OB based on tumor location. The present study aimed to answer these questions and provide new perspectives relevant to our understanding of this group of patients.

Innovations and breakthroughs

The results of the current study provide information for clinical physicians to use when evaluating gastric cancer patients with OB. These findings also provide the following new perspectives relevant to our understanding of this group of patients: gastric cancer with OB is not synonymous with advanced gastric cancer, and its prognosis is no worse than the prognosis for gastric cancer without OB. In fact, patients with proximal gastric cancer with OB exhibit less advanced pathological stages and a better prognosis than patients with proximal gastric cancer without OB.

Applications

OB provides an opportunity to diagnose proximal gastric cancer at an earlier pathological stage, thereby significantly improving patient prognosis.

Peer-review

The paper is an interesting study which provided a new perspective relevant to our understanding of gastric cancer patients with overt bleeding: overt bleeding provides an opportunity to diagnose proximal gastric cancer at an earlier pathological stage, thereby significantly improving patient prognosis. It is necessary to conduct a prospective clinical study to confirm the result and to find out the intrinsic difference between gastric cancer with overt bleeding and gastric cancer without overt bleeding.

REFERENCES

- 1 **Leung WK**, Ho SS, Suen BY, Lai LH, Yu S, Ng EK, Ng SS, Chiu PW, Sung JJ, Chan FK, Lau JY. Capsule endoscopy or angiography in patients with acute overt obscure gastrointestinal bleeding: a prospective randomized study with long-term follow-up. *Am J Gastroenterol* 2012; **107**: 1370-1376 [PMID: 22825363 DOI: 10.1038/ajg.2012.212]
- 2 **Koh KH**, Kim K, Kwon DH, Chung BS, Sohn JY, Ahn DS, Jeon BJ, Kim SH, Kim IH, Kim SW, Lee SO, Lee ST, Kim DG. The successful endoscopic hemostasis factors in bleeding from advanced gastric cancer. *Gastric Cancer* 2013; **16**: 397-403 [PMID: 23053826 DOI: 10.1007/s10120-012-0200-3]
- 3 **Webb WA**, McDaniel L, Johnson RC, Haynes CD. Endoscopic evaluation of 125 cases of upper gastrointestinal bleeding. *Ann Surg* 1981; **193**: 624-627 [PMID: 6972198 DOI: 10.1097/00000658-198105000-00013]
- 4 **Savides TJ**, Jensen DM, Cohen J, Randall GM, Kovacs TO, Pelayo E, Cheng S, Jensen ME, Hsieh HY. Severe upper gastrointestinal tumor bleeding: endoscopic findings, treatment, and outcome. *Endoscopy* 1996; **28**: 244-248 [PMID: 8739741 DOI: 10.1055/s-2007-1005436]
- 5 **Loftus EV**, Alexander GL, Ahlquist DA, Balm RK. Endoscopic treatment of major bleeding from advanced gastroduodenal malignant lesions. *Mayo Clin Proc* 1994; **69**: 736-740 [PMID: 8035627 DOI: 10.1016/s0025-6196(12)61090-8]
- 6 **Moreno-Otero R**, Rodriguez S, Carbó J, Mearin F, Pajares JM. Acute upper gastrointestinal bleeding as primary symptom of gastric carcinoma. *J Surg Oncol* 1987; **36**: 130-133 [PMID: 3498860 DOI: 10.1002/jso.2930360212]
- 7 **Sheibani S**, Kim JJ, Chen B, Park S, Saberi B, Keyashian K, Buxbaum J, Laine L. Natural history of acute upper GI bleeding due to tumours: short-term success and long-term recurrence with or without endoscopic therapy. *Aliment Pharmacol Ther* 2013; **38**: 144-150 [PMID: 23710797 DOI: 10.1111/apt.12347]
- 8 **Pereira J**, Phan T. Management of bleeding in patients with advanced cancer. *Oncologist* 2004; **9**: 561-570 [PMID: 15477642 DOI: 10.1634/theoncologist.9-5-561]
- 9 **Kasakura Y**, Ajani JA, Mochizuki F, Morishita Y, Fujii M, Takayama T. Outcomes after emergency surgery for gastric perforation or severe bleeding in patients with gastric cancer. *J Surg Oncol* 2002; **80**:

- 181-185 [PMID: 12210031 DOI: 10.1002/jso.10127]
- 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 **Washington K.** 7th edition of the AJCC cancer staging manual: stomach. *Ann Surg Oncol* 2010; **17**: 3077-3079 [PMID: 20882416 DOI: 10.1245/s10434-010-1362-z]
- 12 **Zuckerman GR,** Prakash C, Askin MP, Lewis BS. AGA technical review on the evaluation and management of occult and obscure gastrointestinal bleeding. *Gastroenterology* 2000; **118**: 201-221 [PMID: 10611170 DOI: 10.1016/s0016-5085(00)70430-6]
- 13 **Rockey DC.** Occult and obscure gastrointestinal bleeding: causes and clinical management. *Nat Rev Gastroenterol Hepatol* 2010; **7**: 265-279 [PMID: 20351759 DOI: 10.1038/nrgastro.2010.42]
- 14 **Spraycar M.** Stedman's medical dictionary. 26th ed. Baltimore, MD: Williams & Wilkins, 1995
- 15 **Japanese Gastric Cancer Association.** Japanese gastric cancer treatment guidelines 2010 (ver. 3). *Gastric Cancer* 2011; **14**: 113-123 [PMID: 21573742 DOI: 10.1007/s10120-011-0042-4]
- 16 **Fox JG,** Hunt PS. Management of acute bleeding gastric malignancy. *Aust N Z J Surg* 1993; **63**: 462-465 [PMID: 8498915 DOI: 10.1111/j.1445-2197.1993.tb00428.x]
- 17 **Kodama Y,** Inokuchi K, Soejima K, Matsusaka T, Okamura T. Growth patterns and prognosis in early gastric carcinoma. Superficially spreading and penetrating growth types. *Cancer* 1983; **51**: 320-326 [PMID: 6295599]
- 18 **Gertsch P,** Chow LW, Yuen ST, Chau KY, Lauder IJ. Long-term survival after gastrectomy for advanced bleeding or perforated gastric carcinoma. *Eur J Surg* 1996; **162**: 723-727 [PMID: 8908454]
- 19 **Folkman J.** Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971; **285**: 1182-1186 [PMID: 4938153 DOI: 10.1056/NEJM197111182852108]
- 20 **Kolev Y,** Uetake H, Iida S, Ishikawa T, Kawano T, Sugihara K. Prognostic significance of VEGF expression in correlation with COX-2, microvessel density, and clinicopathological characteristics in human gastric carcinoma. *Ann Surg Oncol* 2007; **14**: 2738-2747 [PMID: 17687613 DOI: 10.1245/s10434-007-9484-7]
- 21 **Zhang X,** Zheng Z, Shin YK, Kim KY, Rha SY, Noh SH, Chung HC, Jeung HC. Angiogenic factor thymidine phosphorylase associates with angiogenesis and lymphangiogenesis in the intestinal-type gastric cancer. *Pathology* 2014; **46**: 316-324 [PMID: 24798152 DOI: 10.1097/pat.0000000000000094]
- 22 **Weidner N.** Intratumor microvessel density as a prognostic factor in cancer. *Am J Pathol* 1995; **147**: 9-19 [PMID: 7541613]
- 23 **Isozaki H,** Fujii K, Nomura E, Mabuchi H, Hara H, Sako S, Nishiguchi K, Ohtani M, Tenjo T, Nohara T, Tanigawa N. Prognostic value of tumor cell proliferation and intratumor microvessel density in advanced gastric cancer treated with curative surgery. *Int J Oncol* 1998; **13**: 255-259 [PMID: 9664119 DOI: 10.3892/ijo.13.2.255]
- 24 **Chen JQ,** Zhang WF, Shan JX. Some characteristics of the stomach carcinoma haemorrhage and their clinical significance. *Zhongguo Yike Daxue Xuebao* 1985; **19**: 19-22

P- Reviewer: Berkane S, Cottam DR

S- Editor: Gong ZM **L- Editor:** Wang TQ **E- Editor:** Liu XM





Retrospective Study

Feasible endoscopic therapy for early gastric cancer

Tian-Jiao Guo, Jin-Yu Qin, Lin-Lin Zhu, Jin Wang, Jin-Lin Yang, Yi-Ping Wang

Tian-Jiao Guo, Jin-Yu Qin, Lin-Lin Zhu, Jin Wang, Jin-Lin Yang, Yi-Ping Wang, Gastroenterology Department of West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Author contributions: Qin JY, Zhu LL and Wang J collected the data; Guo TJ analyzed and drafted the manuscript; Wang YP provided analytical oversight; Yang JL designed the study and edited the manuscript; all authors have read and approved the final version to be published.

Supported by Science and Technology Department of Sichuan Province for Scientific Research, No. 2015SZ0123.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of West China Hospital of Sichuan University.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Jin-Lin Yang, MD, Gastroenterology Department of West China Hospital, Sichuan University, No. 37 GuoXue Xiang, Chengdu 610041, Sichuan Province, China. mouse-577@163.com
Telephone: +86-28-85423387
Fax: +86-28-85423387

Received: May 14, 2015

Peer-review started: May 15, 2015

First decision: July 10, 2015

Revised: July 28, 2015

Accepted: October 12, 2015

Article in press: October 13, 2015

Published online: December 21, 2015

Abstract

AIM: To analyze the relationship between lymph node metastasis and clinical pathology of early gastric cancer (EGC) in order to provide criteria for a feasible endoscopic therapy.

METHODS: Clinical data of the 525 EGC patients who underwent surgical operations between January 2009 and March 2014 in the West China Hospital of Sichuan University were analyzed retrospectively. Clinical pathological features were compared between different EGC patients with or without lymph node metastasis, and investigated by univariate and multivariate analyses for possible relationships with lymph node metastasis.

RESULTS: Of the 2913 patients who underwent gastrectomy with lymph node dissection, 529 cases were pathologically proven to be EGC and 525 cases were enrolled in this study, excluding 4 cases of gastric stump carcinoma. Among 233 patients with mucosal carcinoma, 43 (18.5%) had lymph node metastasis. Among 292 patients with submucosal carcinoma, 118 (40.4%) had lymph node metastasis. Univariate analysis showed that gender, tumor size, invasion depth, differentiation type and lymphatic involvement correlated with a high risk of lymph node metastasis. Multivariate analysis revealed that gender (OR = 1.649, 95%CI: 1.091-2.492, $P = 0.018$), tumor size (OR = 1.803, 95%CI: 1.201-2.706, $P = 0.004$), invasion depth (OR = 2.566, 95%CI: 1.671-3.941, $P = 0.000$), histological differentiation (OR = 2.621, 95%CI: 1.624-4.230, $P = 0.000$) and lymphatic involvement (OR = 3.505, 95%CI: 1.590-7.725, $P = 0.002$) were

independent risk factors for lymph node metastasis. Comprehensive analysis showed that lymph node metastasis was absent in patients with tumor that was limited to the mucosa, size ≤ 2 cm, differentiated and without lymphatic involvement.

CONCLUSION: We propose an endoscopic therapy for EGC that is limited to the mucosa, size ≤ 2 cm, differentiated and without lymphatic involvement.

Key words: Early gastric cancer; Clinical pathological features; Risk factor; Endoscopic therapy; Lymph node metastasis

© **The Author(s) 2015.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Early gastric cancer (EGC) is defined as invasive gastric cancer that invades no more deeply than the submucosa, irrespective of lymph node metastasis. Gastrectomy/endoscopic resection can be used for the treatment of patients meeting appropriate criteria. In this study, we retrospectively evaluated the relationship between lymph node metastasis and clinical pathological features of 525 EGC cases. Univariate and multivariate analyses were applied to confirm the risk factors for lymph node metastasis, and to establish indications for a feasible individualized endoscopic therapy for EGC.

Guo TJ, Qin JY, Zhu LL, Wang J, Yang JL, Wang YP. Feasible endoscopic therapy for early gastric cancer. *World J Gastroenterol* 2015; 21(47): 13325-13331 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13325.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13325>

INTRODUCTION

Early gastric cancer (EGC) is defined by the Japanese Society of Gastroenterological Endoscopy as invasive gastric adenocarcinoma confined to the mucosa or submucosa, irrespective of lymph node metastasis (T1, any N)^[1]. Worldwide, gastrectomy remains the most widely used approach for the treatment of EGC, and the 5-year survival rate of patients who undergo curative surgery exceeds 90%^[2]. Hence, it is important to detect and treat the cancer at early stage. In recent years, with the constant development of new endoscopy technology, such as chromoendoscopy, magnifying endoscopy, endoscopic ultrasonography (EUS) and the improvement of endoscopic diagnosis, more and more EGC are detected and accurately diagnosed^[3]. Approximately 50% of gastric cancer cases currently found in Japan are at early stage, while in China, less than 10% of EGC is diagnosed^[4]. The percentage has been gradually improving recently^[5]. Meanwhile, minimally invasive techniques,

especially endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) for EGC, have dramatically grown. Compared with traditional radical surgical procedure, endoscopic treatment has many unique features, including being equally effective, less invasive, and having fewer complications and faster postoperative recovery, which make it possibly a better option for EGC patients^[6,7]. For EGC with lymph node metastasis, however, EMR and ESD are not ideal since they are unable to remove lymph nodes and thus achieve a radical cure effect. So accurate judgment of lymph node metastasis in patients with EGC is very important for the selection of appropriate therapy and prognosis of patients^[8]. This study retrospectively analyzed the clinical data of 525 patients with EGC who underwent gastrectomy with lymph node dissection in our hospital over the last five years. The purpose is to evaluate the relationship between lymph node metastasis and clinical pathological features of EGC, and to propose criteria for feasible individualized endoscopic therapy.

MATERIALS AND METHODS

Patient characteristics

We collected 2913 cases of patients who underwent gastrectomy with lymph node dissection at West China Hospital of Sichuan University between January 2009 and March 2014. Of these, 529 cases were pathologically proven to be EGCs. A total of 525 cases were enrolled in this study, excluding 4 cases of gastric stump carcinoma. Patient characteristics, including age and gender, were collected. In addition, information on tumor size, histological type, invasion depth, ulceration and lymphatic invasion was also retrieved from medical records.

Methods

According to the 7th edition of Tumor-Node-Metastasis stage criteria by the American Joint Committee on Cancer, we subdivided EGC into: T1a-invasion of lamina propria or muscularis mucosae; T1b-invasion of submucosa^[9]. The depth of tumor invasion was classified as mucosa (T1a) and submucosa carcinoma (T1b). The maximum diameter of tumor was recorded as tumor size according to the operation records and pathologic description. Tumor histology was classified into two groups according to Japanese classification of gastric carcinoma: the differentiated group, which included papillary adenocarcinoma and well or moderately differentiated adenocarcinoma; and the undifferentiated group, which included poorly differentiated adenocarcinoma, mucinous, and signet ring cell carcinoma^[10]. Associations between lymph node metastasis and clinical pathological features were assessed by univariate (Table 1) and multivariate (Table 2) analyses, and the lymph node metastasis status of T1a tumors according to gender, tumor size,

Table 1 Clinical pathological features of 525 early gastric cancer cases and univariate analysis of risk factors for lymph node metastasis *n* (%)

Variables	Lymph node metastasis		<i>P</i> value
	Negative (<i>n</i> = 364)	Positive (<i>n</i> = 161)	
Gender			
Male	241 (73.5)	87 (26.5)	0.008
Female	123 (62.4)	74 (37.6)	
Age (yr)			
< 60	207 (67.2)	101 (32.8)	0.208
≥ 60	157 (72.4)	60 (27.6)	
Tumor size (cm)			
≤ 2	202 (76.5)	62 (23.5)	0.000
> 2	162 (62.1)	99 (37.9)	
Invasion depth			
Mucosa (T1a)	190 (81.5)	43 (18.5)	0.000
Submucosa (T1b)	174 (59.6)	118 (40.4)	
Histology			
Differentiated	144 (80.9)	34 (19.1)	0.000
Undifferentiated	220 (63.4)	127 (36.6)	
Ulceration			
Present	79 (65.8)	41 (34.2)	0.344
Absent	285 (70.4)	120 (29.6)	
Lymphatic involvement			
Present	11 (33.3)	22 (66.7)	0.000
Absent	353 (71.7)	139 (28.3)	

histological differentiation and lymphatic involvement was analyzed (Table 3).

Statistical analysis

All the data were analyzed with SPSS 22.0 statistics software (Chicago, IL, United States). Univariate analysis was performed by χ^2 test (or Fisher's exact test when appropriate) to compare the clinicopathological factors between patients with and without lymph node metastasis. Significant factors noted by univariate analysis were subsequently entered into a multivariate logistic regression model to assess the independent risk factors for lymph node metastasis. OR and 95%CI were calculated, and statistical significance was established at $P < 0.05$.

RESULTS

Univariate analysis of risk factors for lymph node metastasis

Among the 525 patients with EGC, 161 (30.7%) were shown to have lymph node metastasis and 364 (69.3%) had no lymph node metastasis. This study included 233 mucosal carcinomas and 292 submucosal carcinomas. Among 233 patients with mucosal carcinoma, 43 (18.5%) had lymph node metastasis. Among 292 patients with submucosal carcinoma, 118 (40.4%) had lymph node metastasis. The relationship between lymph node metastasis and various clinicopathological factors was analyzed first by χ^2 test (Table 1). Female gender ($P = 0.008$), tumor size > 2 cm ($P = 0.000$), invasion depth (submucosal

Table 2 Multivariate analysis of risk factors of 525 early gastric cancer cases for lymph node metastasis

Variables	OR	95%CI	<i>P</i> value
Gender (female/male)	1.649	1.091-2.492	0.018
Tumor size (> 2 cm/ ≤ 2 cm)	1.803	1.201-2.706	0.004
Invasion depth (T1b/T1a)	2.566	1.671-3.941	0.000
Histology (undifferentiated/differentiated)	2.621	1.624-4.230	0.000
Lymphaticinvolvement (present/absent)	3.505	1.590-7.725	0.002

invasion) ($P = 0.000$), undifferentiated histology ($P = 0.000$) and presence of lymphatic involvement ($P = 0.000$) were significantly associated with a higher rate of lymph node metastasis. In contrast, no significant relationship between lymph node metastasis and age or ulceration was found.

Multivariate analysis of risk factors for lymph node metastasis

Using multivariate analysis, we found that all five risk factors identified above demonstrated significant correlation with lymph node metastasis. Specifically, female gender (OR = 1.649, 95%CI: 1.091-2.492, $P = 0.018$), tumor size > 2 cm (OR = 1.803, 95%CI: 1.201-2.706, $P = 0.004$), submucosal invasion (OR = 2.566, 95%CI: 1.671-3.941, $P = 0.000$), histological differentiation (OR = 2.621, 95%CI: 1.624-4.230, $P = 0.000$) and presence of lymphatic involvement (OR = 3.505, 95%CI: 1.590-7.725, $P = 0.002$) were found to be significantly and independently related to lymph node metastasis by multivariate logistic regression analysis (Table 2).

Comprehensive analysis of T1a tumors for lymph node metastasis

According to four independent factors including gender, tumor size, lymphatic involvement and histological differentiation, we analyzed 233 cases of T1a tumor. Table 3 shows that lymph node metastasis could not be found in patients with tumor that was limited to the mucosa, size ≤ 2 cm, differentiated and without lymphatic involvement, irrespective of the gender.

DISCUSSION

For low risk of lymph node metastasis in EGC which has *en bloc* resection, EMR or ESD has become the first choice of treatment^[5]. How to accurately predict lymph node metastasis of EGC in the early stage is the main problem, which determines whether ESD/EMR treatment can be chosen. Clinically, we usually use EUS, a combination of endoscopy and ultrasound to obtain images of the gastrointestinal (GI) tract and adjacent structures^[11], and/or spiral computed tomography (CT) as major screening methods for lymph node metastasis of EGC in preoperative assessment^[12]. For the use

Table 3 Comprehensive analysis of 233 mucosal carcinomas (T1a) for lymph node metastasis *n* (%)

Gender	Tumor size (cm)	Lymphatic involvement	Histology	Lymph node metastasis	
				Negative	Positive
Female (93)	≤ 2	Absent	Differentiated	14	0 (0)
			Undifferentiated	32	8 (20)
	> 2	Present	Differentiated	0	1 (100)
			Undifferentiated	0	0 (0)
		Absent	Differentiated	5	1 (16.7)
			Undifferentiated	17	15 (46.9)
Male (140)	≤ 2	Present	Differentiated	0	0 (0)
			Undifferentiated	0	0 (0)
		Absent	Differentiated	36	0 (0)
			Undifferentiated	31	9 (22.5)
	> 2	Present	Differentiated	0	0 (0)
			Undifferentiated	1	1 (50)
		Absent	Differentiated	28	2 (6.7)
			Undifferentiated	26	6 (18.75)
		Present	Differentiated	0	0 (0)
			Undifferentiated	0	0 (0)

of EUS in the preoperative determination of lymph node status in patients with gastric cancer can have a significant impact on patient management^[13]. Although the specificity to predict lymph node metastasis is as high as 96.3%, the sensitivity of EUS is only 66.7%^[14]. Moreover, the accuracy of EUS depends on the diameter size of metastasis lymph nodes and the technique of operators^[14]. The specificity and sensitivity of spiral CT in the diagnosis of lymph node metastasis are 65% and 75%, respectively^[15]. Additionally, it is difficult to distinguish the metastasis lymph nodes from small normal lymph nodes by spiral CT, although it has certain clinical value for EGC^[13]. As the supplement of EUS and spiral CT, clinical pathology is one of the effective methods to determine lymph node metastasis in EGC^[16].

The reported rates of lymph node metastasis were 2.6%-4.8% for mucosal carcinoma and 16.5%-23.6% for submucosal carcinoma^[17]. However, our data showed that the positive rates of lymph node metastasis in mucosal and submucosal lesions were 18.5% and 40.4%, respectively. This difference may be due to the limitations of our sample collection. We simply retrospectively analyzed radical surgery patients as the research subjects over the most recent five-year period. It should be noted that some EGC patients, who were judged without lymph node metastasis by EUS/spiral CT, received ESD/EMR treatment instead of surgery. These patients were excluded from our study, which might lead to the high positive rates of lymph node metastasis.

Many studies have evaluated the risk of lymph node metastasis in gastric cancer. For example, Lim *et al*^[8] found that tumor size, invasion depth and lymphatic involvement were closely related to lymph node metastasis in EGC. In addition, Ye *et al*^[18] and Abe *et al*^[19] reported that histological differentiation and gender were independent risk factors for lymph node metastasis in EGC. Consistent with these reports,

in our study involving 525 patients and univariate/multivariate analyses, we found that tumor size, invasion depth, lymphatic involvement, histological differentiation and gender were independent risk factors for lymph node metastasis.

Multivariate analysis found that the risk of lymph node metastasis in EGC patients differed according to tumor size and invasion depth: tumor size > 2 cm had 1.803 times (95%CI: 1.201-2.706) greater risk than tumor size ≤ 2 cm; submucosal carcinoma had 2.566 times (95%CI: 1.671-3.941) greater risk than mucosa carcinoma. These data are consistent with previous reports that tumor size and invasion depth are considered to be independent risk factors for lymph node metastasis^[20,21]. Specifically, when the tumor is larger than 2 cm in diameter and infiltrates into the submucosa, the risk for lymph node metastasis increases significantly. It would be better to choose surgery instead of endoscopic therapy^[22]. Multivariate analysis also found that the risk for lymph node metastasis was 3.505 times greater with lymphatic invasion. Liu *et al*^[23] and Nakamura *et al*^[24] analyzed 188 cases and 73 cases of EGC respectively, and reported that lymphatic involvement was an independent risk factor for lymph node metastasis in EGC, which was consistent with our conclusion.

We found that histological differentiation was significantly correlated with lymph node metastasis. Undifferentiated EGC had a much higher risk than differentiated EGC in lymph node metastasis. Ye *et al*^[18] previously reported the same conclusion. It should be noted, however, that there has been evidence showing no correlation between histological differentiation and lymph node metastasis for EGC^[19,25]. Gotoda *et al*^[26] divided 5265 cases of EGC into mucosal carcinoma and submucosal carcinoma, and analyzed them retrospectively. They reported that lymph node metastasis in mucosal carcinoma was associated with histological differentiation, while there was no

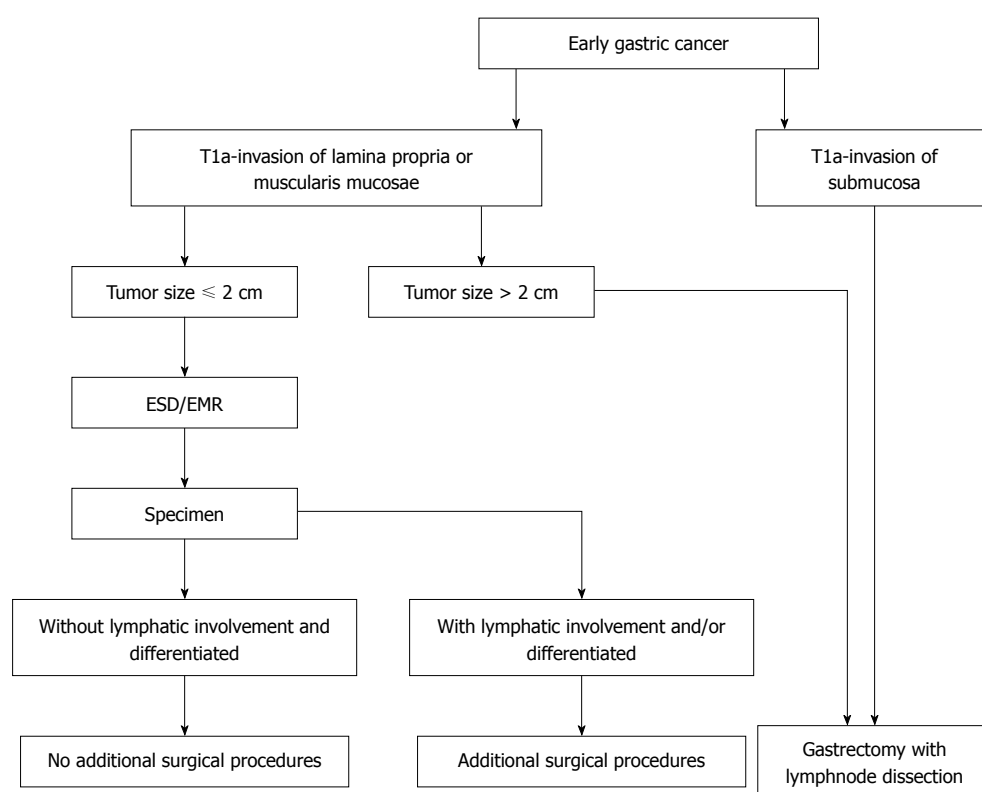


Figure 1 A feasible endoscopic therapy for early gastric cancer. EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

clear relationship between lymph node metastasis in submucosal carcinoma and histological differentiation.

We also showed that gender was associated with lymph node metastasis. Females were more likely to have lymph node metastasis than males. This finding was consistent with previous reports that the growth of tumor results from the estrogen produced by women^[27,28]. However, the specific biological mechanism remains unclear. Therefore, the specific link between gender and lymph node metastasis for EGC needs to be further examined.

After further analysis of 233 T1a carcinomas based on four independent factors including gender, tumor size, lymphatic involvement and histological differentiation, we propose an endoscopic therapy for T1a carcinoma that is limited to tumor size ≤ 2 cm, differentiated and without lymphatic involvement. In the latest edition of Japanese gastric cancer treatment guidelines, the indications for endoscopic treatment of T1a carcinoma are: tumor size ≤ 2 cm, differentiated and no ulcer^[29]. Different from the guidelines, we consider “no lymphatic involvement” rather than “no ulcer” as an indication for endoscopic treatment. Consistent with the reports from Park *et al.*^[25] and Sung *et al.*^[30], our study revealed that ulceration was not an independent risk factor for lymph node metastasis in EGC. Additionally, ulceration is mainly judged by endoscopy and pathology examinations; both have certain subjective bias. If patients had previously undergone mucosa biopsy, it would be quite difficult to identify primary ulcers from biopsy injuries under

the endoscope^[31]. So it is difficult to judge precisely whether there are ulcers or not in clinical practice. In addition, gastritis profunda cystica has been alongside EGC and mimicked cancer^[32].

In recent years, lymphatic involvement is considered to be the most powerful factor for the prediction of lymph node metastasis in EGC^[33,34]. However, there are no effective methods to estimate lymphatic involvement preoperatively. Only through pathologic examination of resection specimens after surgery or endoscopic resection can we identify lymphatic involvement. Therefore, once postoperative specimens of EMR/ESD indicate lymphatic invasion, we should undertake additional surgical procedures as soon as possible, even for T1a patients^[34].

In summary, gender, tumor size, invasion depth, histology and lymphatic involvement are significantly correlated with lymph node metastasis. Further studies on the specific links between gender and lymph node metastasis are still needed. Patients with EGC that is limited to the mucosa, tumor size ≤ 2 cm, differentiated and without lymphatic involvement, have low risk for lymph node metastasis. For this kind of EGC, endoscopic therapy, which is safe and as effective as surgery, is proposed and recommended (Figure 1).

COMMENTS

Background

Early gastric cancer (EGC) is defined as invasive gastric cancer that invades no more deeply than the submucosa, irrespective of lymph node

metastasis. Treatment modalities for EGC include endoscopic resection, surgery (gastrectomy) and adjuvant therapies. Endoscopic resection, by either endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD), is an option for selected patients with EGC without known lymph node metastasis who meet specific criteria. This study analyzed the predictive factors for lymph node metastasis in EGC, and established an indication for endoscopic treatment for EGC.

Research frontiers

Several studies have attempted to identify risk factors predictive of lymph node metastasis in EGC. Few reports, however, have proposed criteria for endoscopic resection for EGC.

Innovations and breakthroughs

Guidelines for endoscopic therapy remain uncertain in areas outside of Japan and Korea. The authors analyzed clinical pathology of lymph node metastasis in EGC and established a criterion of endoscopic therapy for EGC.

Applications

Endoscopic resection could be an alternative treatment in EGC patients without risk factors for lymph node metastasis.

Terminology

Endoscopic resection (ER) is an endoscopic alternative to surgical resection of mucosal and submucosal neoplastic lesions and intramucosal cancers; EMR: An ER technique providing a minimally invasive treatment for removal of superficial malignancies; ESD: An ER technique using a specialized needle-knife to dissect lesions from the submucosa, which offers the potential to remove mucosal and submucosal tumors *en bloc*.

Peer-review

This study analyzed the relationship between lymph node metastasis and clinical pathology of 525 EGC patients who underwent surgical operations. This is a good study about endoscopic therapy for EGC.

REFERENCES

- Kunisaki C, Akiyama H, Nomura M, Matsuda G, Otsuka Y, Ono H, Nagahori Y, Hosoi H, Takahashi M, Kito F, Shimada H. Significance of long-term follow-up of early gastric cancer. *Ann Surg Oncol* 2006; **13**: 363-369 [PMID: 16485155 DOI: 10.1245/ASO.2006.03.061]
- Choi JH, Kim ES, Lee YJ, Cho KB, Park KS, Jang BK, Chung WJ, Hwang JS, Ryu SW. Comparison of quality of life and worry of cancer recurrence between endoscopic and surgical treatment for early gastric cancer. *Gastrointest Endosc* 2015; **82**: 299-307 [PMID: 25892060 DOI: 10.1016/j.gie.2015.01.019]
- Hamashima C, Shibuya D, Yamazaki H, Inoue K, Fukao A, Saito H, Sobue T. The Japanese guidelines for gastric cancer screening. *Jpn J Clin Oncol* 2008; **38**: 259-267 [PMID: 18344316 DOI: 10.1093/jjco/hyn017]
- Nomura S, Kaminishi M. Surgical treatment of early gastric cancer. *Dig Surg* 2007; **24**: 96-100 [PMID: 17460412 DOI: 10.1159/000101895]
- Yada T, Yokoi C, Uemura N. The current state of diagnosis and treatment for early gastric cancer. *Diagn Ther Endosc* 2013; **2013**: 241320 [PMID: 23533320 DOI: 10.1155/2013/241320]
- Soetikno R, Kaltenbach T, Yeh R, Gotoda T. Endoscopic mucosal resection for early cancers of the upper gastrointestinal tract. *J Clin Oncol* 2005; **23**: 4490-4498 [PMID: 16002839 DOI: 10.1200/jco.2005.19.935]
- Choi KS, Jung HY, Choi KD, Lee GH, Song HJ, Kim do H, Lee JH, Kim MY, Kim BS, Oh ST, Yook JH, Jang SJ, Yun SC, Kim SO, Kim JH. EMR versus gastrectomy for intramucosal gastric cancer: comparison of long-term outcomes. *Gastrointest Endosc* 2011; **73**: 942-948 [PMID: 21392757 DOI: 10.1016/j.gie.2010.12.032]
- Lim MS, Lee HW, Im H, Kim BS, Lee MY, Jeon JY, Yang DH, Lee BH. Predictable factors for lymph node metastasis in early gastric cancer-analysis of single institutional experience. *J Gastrointest Surg* 2011; **15**: 1783-1788 [PMID: 21796460 DOI: 10.1007/s11605-011-1624-5]
- Washington K. 7th edition of the AJCC cancer staging manual: stomach. *Ann Surg Oncol* 2010; **17**: 3077-3079 [PMID: 20882416 DOI: 10.1245/s10434-010-1362-z]
- 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]
- Ge N, Sun S. Endoscopic ultrasound: An all in one technique vibrates virtually around the whole internal medical field. *J Transl Intern Med* 2014; **2**: 104-106 [DOI: 10.4103/2224-4018.141829]
- Ren G, Zhao JX, Cai R, Qi TY. Value of contrast-enhanced multiphasic spiral CT in detection of early gastric cancer and clinicopathologic features of early gastric cancer. *Shijie Huaren Xiaohua Zazhi* 2015; **23**: 110 [DOI: 10.11569/wjcd.v23.i1.110]
- Sharma M, Rai P, Rameshbabu CS. Techniques of imaging of nodal stations of gastric cancer by endoscopic ultrasound. *Endosc Ultrasound* 2014; **3**: 179-190 [PMID: 25184125 DOI: 10.4103/2303-9027.138793]
- Yan C, Zhu ZG, Zhu Q, Yan M, Chen J, Liu BY, Yin HR, Lin YZ. [A preliminary study of endoscopic ultrasonography in the preoperative staging of early gastric carcinoma]. *Zhonghua Zhongliu Xue* 2003; **25**: 390-393 [PMID: 12921574]
- Habermann CR, Weiss F, Riecken R, Honarpisheh H, Bohnacker S, Staedtler C, Dieckmann C, Schoder V, Adam G. Preoperative staging of gastric adenocarcinoma: comparison of helical CT and endoscopic US. *Radiology* 2004; **230**: 465-471 [PMID: 14752188 DOI: 10.1148/radiol.2302020828]
- Barreto SG, Windsor JA. Redefining early gastric cancer. *Surg Endosc* 2015; Epub ahead of print [PMID: 25829068 DOI: 10.1007/s00464-015-4184-z]
- Chen R, He Q, Cui J, Bian S, Chen L. Lymph node metastasis in early gastric cancer. *Chin Med J (Engl)* 2014; **127**: 560-567 [PMID: 24451967]
- Ye BD, Kim SG, Lee JY, Kim JS, Yang HK, Kim WH, Jung HC, Lee KU, Song IS. Predictive factors for lymph node metastasis and endoscopic treatment strategies for undifferentiated early gastric cancer. *J Gastroenterol Hepatol* 2008; **23**: 46-50 [PMID: 18171341 DOI: 10.1111/j.1440-1746.2006.04791.x]
- Abe N, Watanabe T, Suzuki K, Machida H, Toda H, Nakaya Y, Masaki T, Mori T, Sugiyama M, Atomi Y. Risk factors predictive of lymph node metastasis in depressed early gastric cancer. *Am J Surg* 2002; **183**: 168-172 [PMID: 11918883]
- Kunisaki C, Takahashi M, Nagahori Y, Fukushima T, Makino H, Takagawa R, Kosaka T, Ono HA, Akiyama H, Moriaki Y, Nakano A. Risk factors for lymph node metastasis in histologically poorly differentiated type early gastric cancer. *Endoscopy* 2009; **41**: 498-503 [PMID: 19533552 DOI: 10.1055/s-0029-1214758]
- Ren G, Cai R, Zhang WJ, Ou JM, Jin YN, Li WH. Prediction of risk factors for lymph node metastasis in early gastric cancer. *World J Gastroenterol* 2013; **19**: 3096-3107 [PMID: 23716990 DOI: 10.3748/wjg.v19.i20.3096]
- Kim JH, Lee YC, Kim H, Yoon SO, Kim H, Youn YH, Park H, Lee SI, Choi SH, Noh SH. Additive lymph node dissection may be necessary in minute submucosal cancer of the stomach after endoscopic resection. *Ann Surg Oncol* 2012; **19**: 779-785 [PMID: 21964889 DOI: 10.1245/s10434-011-2081-9]
- Liu C, Zhang R, Lu Y, Li H, Lu P, Yao F, Jin F, Xu H, Wang S, Chen J. Prognostic role of lymphatic vessel invasion in early gastric cancer: a retrospective study of 188 cases. *Surg Oncol* 2010; **19**: 4-10 [PMID: 19042124 DOI: 10.1016/j.suronc.2008.10.003]
- Nakamura Y, Yasuoka H, Tsujimoto M, Kurozumi K, Nakahara M, Nakao K, Kakudo K. Importance of lymph vessels in gastric cancer: a prognostic indicator in general and a predictor for lymph node metastasis in early stage cancer. *J Clin Pathol* 2006; **59**: 77-82 [PMID: 16394285 DOI: 10.1136/jcp.2005.028779]
- Park YD, Chung YJ, Chung HY, Yu W, Bae HI, Jeon SW, Cho CM, Tak WY, Kweon YO. Factors related to lymph node metastasis

- and the feasibility of endoscopic mucosal resection for treating poorly differentiated adenocarcinoma of the stomach. *Endoscopy* 2008; **40**: 7-10 [PMID: 18210339 DOI: 10.1055/s-2007-966750]
- 26 **Gotoda T**, Yanagisawa A, Sasako M, Ono H, Nakanishi Y, Shimoda T, Kato Y. Incidence of lymph node metastasis from early gastric cancer: estimation with a large number of cases at two large centers. *Gastric Cancer* 2000; **3**: 219-225 [PMID: 11984739]
 - 27 **Lee JH**, Choi MG, Min BH, Noh JH, Sohn TS, Bae JM, Kim S. Predictive factors for lymph node metastasis in patients with poorly differentiated early gastric cancer. *Br J Surg* 2012; **99**: 1688-1692 [PMID: 23023388 DOI: 10.1002/bjs.8934]
 - 28 **Abe N**, Sugiyama M, Masaki T, Ueki H, Yanagida O, Mori T, Watanabe T, Atomi Y. Predictive factors for lymph node metastasis of differentiated submucosally invasive gastric cancer. *Gastrointest Endosc* 2004; **60**: 242-245 [PMID: 15278052]
 - 29 **Sano T**, Aiko T. New Japanese classifications and treatment guidelines for gastric cancer: revision concepts and major revised points. *Gastric Cancer* 2011; **14**: 97-100 [PMID: 21573921 DOI: 10.1007/s10120-011-0040-6]
 - 30 **Sung CM**, Hsu CM, Hsu JT, Yeh TS, Lin CJ, Chen TC, Su MY, Chiu CT. Predictive factors for lymph node metastasis in early gastric cancer. *World J Gastroenterol* 2010; **16**: 5252-5256 [PMID: 21049560 DOI: 10.3748/wjg.v16.i41.5252]
 - 31 **Haruta H**, Hosoya Y, Sakuma K, Shibusawa H, Satoh K, Yamamoto H, Tanaka A, Niki T, Sugano K, Yasuda Y. Clinicopathological study of lymph-node metastasis in 1,389 patients with early gastric cancer: assessment of indications for endoscopic resection. *J Dig Dis* 2008; **9**: 213-218 [PMID: 18959593 DOI: 10.1111/j.1751-2980.2008.00349.x]
 - 32 **Butt MO**, Luck NH, Hassan SM, Zaigham Abbas Z, Mubarak M. Gastritis profunda cystica presenting as gastric outlet obstruction and mimicking cancer: A case report. *J Transl Intern Med* 2015; **3**: 35-38 [DOI: 10.4103/2224-4018.154296]
 - 33 **Kim H**, Kim JH, Park JC, Lee YC, Noh SH, Kim H. Lymphovascular invasion is an important predictor of lymph node metastasis in endoscopically resected early gastric cancers. *Oncol Rep* 2011; **25**: 1589-1595 [PMID: 21455589 DOI: 10.3892/or.2011.1242]
 - 34 **Park WY**, Shin N, Kim JY, Jeon TY, Kim GH, Kim H, Park do Y. Pathologic definition and number of lymphovascular emboli: impact on lymph node metastasis in endoscopically resected early gastric cancer. *Hum Pathol* 2013; **44**: 2132-2138 [PMID: 23806525 DOI: 10.1016/j.humpath.2013.04.006]

P- Reviewer: Chorny M, Pasricha PJ **S- Editor:** Yu J

L- Editor: Logan S **E- Editor:** Ma S





Clinical Trials Study

Robotic gastrectomy with transvaginal specimen extraction for female gastric cancer patients

Shu Zhang, Zhi-Wei Jiang, Gang Wang, Xiao-Bo Feng, Jiang Liu, Jian Zhao, Jie-Shou Li

Shu Zhang, Zhi-Wei Jiang, Gang Wang, Xiao-Bo Feng, Jiang Liu, Jian Zhao, Jie-Shou Li, Department of General Surgery, Jinling Hospital, Medical School, Nanjing University, Nanjing 210002, Jiangsu Province, China

Author contributions: Zhang S and Jiang ZW contributed equally to this work; Jiang ZW and Li JS designed the research; Wang G, Feng XB and Liu J collected the data; Wang G, Feng XB and Zhao J analyzed the data; and Zhang S wrote the paper.

Institutional review board statement: This clinical study was approved by the Ethic Committee of Jinling Hospital, Nanjing University.

Informed consent statement: All patients involved in this study gave their written informed consent authorizing use and disclosure of their protected health information.

Conflict-of-interest statement: We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled "Robotic Gastrectomy with Transvaginal Specimen Extraction for Female Gastric Cancer Patients".

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Zhi-Wei Jiang, MD, Department of General Surgery, Jinling Hospital, Medical School, Nanjing University, 305 East Zhongshan Rd., Nanjing 210002, Jiangsu Province, China. surgery34@163.com
Telephone: +86-25-80860034
Fax: +86-25-80860034

Received: May 23, 2015

Peer-review started: May 23, 2015

First decision: June 19, 2015

Revised: July 7, 2015

Accepted: September 13, 2015

Article in press: September 14, 2015

Published online: December 21, 2015

Abstract

AIM: To describe the application of complete robotic gastrectomy with transvaginal specimen extraction (TVSE) for gastric cancer patients.

METHODS: Between July and November 2014, eight female patients who were diagnosed with gastric adenocarcinoma underwent a TVSE following a full robot-sewn gastrectomy. According to the tumor location, the patients were allocated to two different groups; two patients received robotic total gastrectomy with TVSE and the other six received robotic distal gastrectomy with TVSE.

RESULTS: Surgical procedures were successfully performed in all eight cases without conversion. The mean age was 55.3 (range, 42-69) years, and the mean body mass index was 23.2 (range, 21.6-26.0) kg/m². The mean total operative time and blood loss were 224 (range, 200-298) min and 62.5 (range, 50-150) mL, respectively. The mean postoperative hospital stay was 3.6 (range, 3-5) d. The mean number of lymph nodes resected was 23.6 (range, 17-27). None was readmitted within 30 d of postoperation. During the follow-up, no stricture developed nor was any anastomotic leakage detected.

CONCLUSION: It is possible to perform a TVSE following a full robot-sewn gastrectomy with standard D2 lymph node resection for female gastric cancer patients.

Key words: Gastric cancer; Robotic surgery; Transvaginal; Natural orifice specimen extraction

© **The Author(s) 2015.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: It is widely recognized that the natural orifice specimen extraction (NOSE) could reduce postoperative pain, length of stay, and morbidity. Although NOSE has been performed in different institutions, there has not been any report of transvaginal specimen extraction following full robot-sewn gastrectomy for female gastric cancer. This study describes the new application of complete robotic gastrectomy with transvaginal specimen extraction in eight patients with gastric cancer in Jinling Hospital. There were two different surgeries performed, robotic total gastrectomy and robotic distal gastrectomy.

Zhang S, Jiang ZW, Wang G, Feng XB, Liu J, Zhao J, Li JS. Robotic gastrectomy with transvaginal specimen extraction for female gastric cancer patients. *World J Gastroenterol* 2015; 21(47): 13332-13338 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13332.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13332>

INTRODUCTION

Gastric cancer is one of the most prevalent cancer in China^[1]. Nearly a million new cases are diagnosed each year in the world, and it is the fourth most common type of cancer and the second leading cause of death worldwide^[2]. For generations, surgeons have been contriving new surgical methods for inner organs, especially the stomach. These methods include open gastrectomy, laparoscopic gastrectomy, robotic gastrectomy, and natural orifice specimen extraction (NOSE)^[3,4].

It is widely recognized that NOSE could reduce postoperative pain, length of stay, and morbidity. Although NOSE has been performed in a lot of institutions, there has not been any report of transvaginal specimen extraction (TVSE) following full robot-sewn gastrectomy for gastric cancer. This article aims to report robotic gastrectomy following a TVSE in eight patients with gastric cancer in Jinling Hospital. There were two different surgeries performed, robotic total gastrectomy (RTG) and robotic distal gastrectomy (RDG).

MATERIALS AND METHODS

Patients

Eight female patients with gastric cancer were enrolled in this study between July and November 2014. All patients were newly diagnosed with gastric cancer pathologically. The study was approved by the Research Ethics Committee of Nanjing University. All

eight specimen extraction case series following full robot-sewn gastrectomy were performed in Jinling Hospital. Patients with a tumor that is too large or too difficult to resect were excluded in this study. Only multipara with no other underlying diseases was enrolled. Other organ (lung or kidney) dysfunction and abnormal clinical test results were not considered in this study. Patients who had taken chemotherapy or radiotherapy preoperatively were also excluded. According to the tumor location, the patients were primarily allocated to two different groups; two patients received RTG with TVSE and the other six received RDG with TVSE.

Surgical techniques

All eight robotic gastrectomies were carried out by the same surgeon (Jiang ZW) with full intracorporeal robot-sewn anastomosis^[5]. The surgical procedure was carried out in four stages: (1) six of these patients underwent robotic distal gastrectomy followed by Billroth II reconstruction, and two underwent robotic total gastrectomy followed by Roux-en-Y reconstruction; (2) all the eight patients went through a standard D2 lymph node dissection (distal gastrectomy: 1, 3, 4Sb, 4d, 5, 6, 7, 8a, 9, 11p, 12a; total gastrectomy: 1, 2, 3, 4Sa, 4Sb, 4d, 5, 6, 7, 8a, 9, 10, 11, 12a)^[6]; (3) partial omentectomy might be conducted if necessary; and (4) the TVSE and suture of wound were conducted.

Positioning and settlement of robotic arms

After general anesthesia, a 12-mm trocar was inserted into the abdomen through the sub-umbilical area, and a pneumoperitoneum was formed by insufflations of carbon dioxide. The intraperitoneal pressure was maintained at 11-13 mmHg. With patients placed in the reverse Trendelenburg position, distribution of trocars was as follows: a 12-mm camera port, three 8-mm robotic working ports, and one additional (12 mm) on-table assistant port (1-5; Figure 1). During the procedure, one ultrasonic scalpel, two Cadere forceps, and one mega needle driver were used.

Surgical procedure

The Da Vinci Robotic System was installed with its arms settled in position. Afterwards, the operator first inspected the abdominal cavity for any sign of tumor seeding or metastasis. The greater curvature of the stomach was mobilized by the ultrasonic scalpel. The gastrocolic ligament was cut with the ascending colon lifted by robotic arms. The left gastroepiploic artery was divided at its root after clipping. The dissection then proceeded towards the right gastroepiploic vessels, and then the left gastroepiploic vessels were ligated. The 4th set of lymph nodes along the greater curve of the stomach were dissected with the 6th set of the intrapyloric lymph nodes. Then the right gastric artery was dissected, clipped, cut and ligated, and the 12a lymph nodes were resected with the 5th set of suprapyloric lymph node. The duodenal stump was

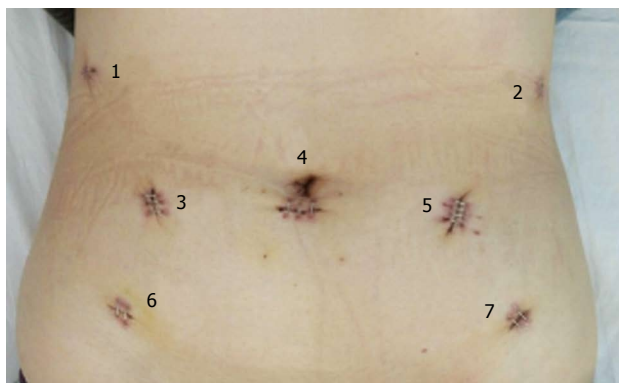


Figure 1 Placement of trocars.

mobilized using an endo-GIA 60 (Covidien, Mansfield, MA, United States) to allow clipping at the root of the left gastric vessel. After the 7th lymph nodes were harvested, the 11p set of lymph nodes near the splenic artery were also resected with the 9th set of celiac trunk lymph nodes, and then, the 8th set of lymph nodes along the common hepatic artery. The 1st and 3rd sets of perigastric lymph nodes along the lesser curvature were dissected up to the site of the right paracardial region and the lesser omentum. The proximal stomach was divided using an endo-GIA 60 (Covidien, Mansfield, MA, United States). In RTG, additional lymph nodes should be dissected. The 2nd sets of lymph nodes along the left paracardial region, the 11d sets of lymph nodes over the spleen artery and the 10th sets of splenic hilar lymph nodes were dissected. Then, the division on the lower end of esophagus was made by an endo-GIA 60. A retrieval bag was introduced through the transabdominal trocar, and the specimen was transferred into it. The retrieval bag was then transferred into the pelvic cavity for transvaginal extraction (Figure 2).

Reconstruction

According to the location of tumor, reconstructions were performed differently. Six patients who underwent RDG received a Billroth II reconstruction while the other two patients who underwent RTG received a Roux-en-Y reconstruction. All lymph nodes dissection as well as the digestive restoration were performed fully by robotic arms intracorporeally as described in previous literature^[5].

TVSE

The robotic system was moved from the headside to the footside with trocars reallocated; the trocar holes (1 and 2; Figure 1) left on the costal arch were sutured in case of air leakage. Additional trocars (6 and 7; Figure 1) were inserted. After the intraperitoneal pressure had been maintained back to 12 mmHg, the robotic arms were introduced into the abdominal cavity with the patients in lithotomy position. By purse-string suture, the uterus was lifted by a robotic arm. A 4-cm posterior colpotomy was performed with

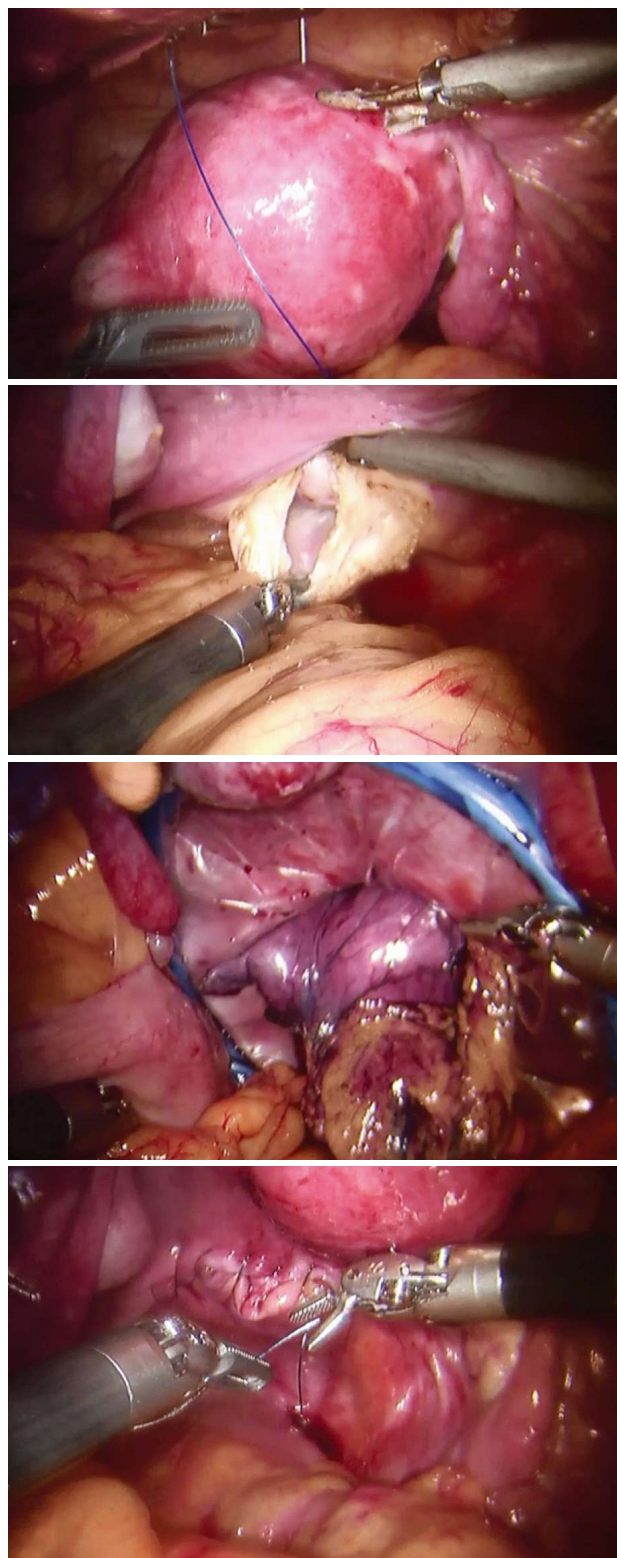


Figure 2 Procedure of robotic transvaginal specimen extraction.

a robotic harmonic knife. Like others, we used a two-layer retrieval bag to deposit the specimen^[7-9]. Then the retrieval bag containing the specimen was gently withdrawn until it reached the intra-abdominal tip of the transvaginal incision. The withdrawal of retrieval bag specimen was assisted by a tenaculum placed on the anterior lip of the cervix (3; Figure 2). After

Table 1 Basic information of robotic transvaginal specimen extraction patients

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Mean
Age/yr	42	54	49	59	65	69	54	50	55.3
BMI	23.25	23.1	20.8	25.7	26	21.6	22.2	23.25	23.2
Approach	RTG	RTG	RDG	RDG	RDG	RDG	RDG	RDG	
Reconstruction	Roux-en-Y	Roux-en-Y	Billroth II	Billroth II	Billroth II	Billroth II	Billroth II	Billroth II	
Number of retrieved LNs	16/17	0/22	0/27	0/24	0/27	0/25	0/22	0/25	2/23.6
WHO classification	PD	PD	PD	PD	PD	PD	PD	PD	
Lauren classification	Less curvature	Less curvature	Less curvature	Less curvature	Less curvature	Less curvature	Less curvature	Less curvature	
	Antrum	Antrum	Body	Body	Body	Body	Body	Body	
Histopathology	adenoma	adenoma	adenoma	adenoma	adenoma	adenoma	adenoma	adenoma	
History	Null	Null	Hemorrhoids 4 yr ago	Appendectomy 10 yr ago	Null	Null	Null	Caesarean section 22 yr ago	
Postoperative stay/d	5	4	3	3	3	4	3	4	3.6
Stage									
TNM	T4bN3M0	T1N0M0	T1N0M0	T4bN0M0	T1N0M0	T1N0M0	T1N0M0	T1N0M0	
Gross type	III C	IA	IA	III B	IA	IA	IA	IA	
During Operation									
Blood loss/mL	50	50	100	100	50	50	50	50	62.5
Blood transfusion/mL	0	0	0	0	0	0	0	0	0
Urine/mL	600	400	500	300	300	400	300	450	406.3
Operation time/min	225	215	298	200	230	200	185	235	224
Time to first flatus/h	30	32	27	33	36	35	28	33	31.8

PD: Poorly differentiated; RTG: Robotic total gastrectomy; RDG: Robotic distal gastrectomy.

the removal of the transvaginal incision protector, the posterior incisions were closed with an self-anchoring barbed suture (Covidien, United States) suture (4; Figure 2). Then over 1000 mL of warm water was poured into the abdominal cavity through one of the trocar port to clean the cavity. It was carefully examined to ensure that no spot was left untreated. One drain was placed in the abdominal cavity through the ancillary port. Closure of the port incisions with subcuticular suture completed the operation.

RESULTS

Eight patients participated in the study. The mean age of the women was 55.3 (range, 42-69) years (See Table 1), and the mean body mass index (BMI) was 23.2 (range, 21.6-26.0) kg/m². Histopathology confirmed poorly differentiated adenocarcinoma in all cases. The mean number of retrieved lymph nodes was 23.6 (range, 17-27). In case 1, 16 of 17 lymph nodes were found positive. Nevertheless, no metastatic lymph nodes were found in the other seven patients. Only the node-positive patients (case 1) underwent six cycles of postoperative chemotherapy.

Because the length of the incision was adapted to the size of the specimen and the tumor, with all tumors under 4 cm in diameter, the incision on the vagina was no larger than 4 cm. The mean operating time was 224 (range, 200-298) min, and the average blood loss was 62.5 (range, 50-150) mL and none of them needed blood transfusion. No intraoperative complications were noticed in any of the eight patients during the follow-up. The mean postoperative stay was

3.6 (range, 3-5) d. The first flatus of patients occurred at mean 28.5 (range, 24-33) h after surgery (Table 1).

There was no anastomotic leakage reported both within the hospital stay and 30 d after discharge from the hospital. During the follow-up, neither was there a sign of surgical site infection nor lung infection. No other complications were reported during the hospital stay. Thus, there was no one who needed re-admission in hospital. That is to say that both the mortality and morbidity were zero in 30 d (Table 2). The average VAS of six patients in the TVSE-RDG group decreased from 2.8 on postoperative day (POD) 1 to 1.7 on POD 2, and then 1.0 on POD 3 while the average VAS of the two patients in the TVSE-RTG group was 2, 1 and 1 on the POD 1, POD 2 and POD 3, respectively. The trend of postoperative mobility was also related to the VAS score.

All eight patients showed great progress in postoperative activity; their steps increased greatly for the TVSE-RDG group patients from 62 (range, 40-75) steps on POD 1, to 336 (range, 116-465) on POD 2, and then maybe with pain relief, the activity steps took a great leap to 1078 (range, 985-1200) steps on POD 3, nearing a normal daily level. The statistics from the TVSE-RTG group also showed the same trend (Table 2). There were 55 steps on POD 1, 342 on POD 2 and 1100 on POD 3 in the TVSE-RTG group. The sleep time measured by Fitbit Flex indicated an improvement on POD 2 to POD 3 by 0.5 h in the TVSE-RTG group and 1.1 h in the TVSE-RDG group.

During the perioperative course, the SpO₂ showed a slight fluctuation (Table 3). The SpO₂ decreased from preoperative 100% to 98.3% (range, 96%-99%)

Table 2 Clinical features of robotic transvaginal specimen extraction patients

	Adverse event			Pain/ VAS			activity/ step			Sleep/h		
	Anastomotic leakage	30 d re-admission	SSI or LI	POD 1	POD 2	POD 3	POD 1	POD 2	POD 3	POD 1	POD 2	POD 3
TVSE-RTG	0/2	0/2	0/2	2	1	1	55	342	1100	7	6	6.5
TVSE-RDG	0/6	0/6	0/6	2.8	1.7	1	62	336	1078	7.1	6.8	7.9

SSI: Surgical site infection; LI: Lung infection; TVSE: Transvaginal specimen extraction.

Table 3 SpO₂ fluctuation during the perioperative course

	SpO ₂ /%			HR/min			Hand grip strength/(left/right) kg	
	Pre-	POD 1	POD 3	Pre-	POD 1	POD 3	Admission	Discharge
TVSE-RTG	100	98.5	99	60	62	60	25.6/25	27.8/26.3
TVSE-RDG	100	98.3	99.3	66.2	77.5	72.5	23.3/21.7	25.6/24.5

Pre-: Pre-operation; POD: Postoperative day; TVSE: Transvaginal specimen extraction; RTG: Robotic total gastrectomy; RDG: Robotic distal gastrectomy.

on POD 1, and then 99.3% (range, 98%-100%) on POD 3 in the TVSE-RDG group, while the SpO₂ was 100, 98.5% (range, 98%-99%) and 99% (range, 98%-100%) on preoperative day, POD 1 and POD 3, respectively. Similarly, the heart rate (HR) also fluctuated from preoperative 66.2 (range, 58-69) to 77.5 (range, 62-102) on POD 1, and then down back to 72.5 (range, 60-85) on POD 3 in the TVSE-RDG group. However, we noticed that the grip strength strangely increased in 7 of the 8 patients, causing the average strength to increase from 25.6/25 kg to 27.8/26.3 kg in the TVSE-RTG group and 23.3/21.7 kg to 25.6/24.5 kg in the TVSE-RDG group. We also measured the body mass with a body machine (Inbody 230, South Korea). The body machine detected nearly no change in the bone (10.3%-10.2%), body fat (30.5%-30.2%), water (47.9%-48.1%) or muscle (32.0%-31.4%), which was also an indication for stability in keeping weight.

DISCUSSION

Thanks to the development of new technology and the enterprise of generations of surgeons in minimally invasive surgery, innovations and progresses have evolved to another level in the field of NOSE surgery.

There are four potential routes, namely, transesophageal, transrectal, transvaginal, and transurethral. The robotic surgery makes it possible for NOSE to become more and more feasible and thereby the optimal way of approaching gastric cancer.

Although NOSE is now relatively widely performed, it does not give any importance to gastric resection with specimen extraction through the transvaginal route for gastric cancer, especially robotic gastrectomy.

TVSE using a posterior colpotomy has extensively been reported during gynecologic procedures^[10,11]. In 1993, Delvaux *et al.*^[12] performed the first transvaginal extraction. The gallbladder containing a stone was extracted through the transvaginal route following laparoscopic cholecystectomy in female

patients. In 1996, Redwine *et al.*^[13] first described a segmental colectomy with transvaginal extraction for bowel endometriosis. Kim *et al.*^[14] in 1996 reported transvaginal extraction of the rectum in four patients following low anterior resection. In 2002, Gill *et al.*^[15] described vaginal extraction of the intact specimen following laparoscopic radical nephrectomy. Ghezzi *et al.*^[10] reported 60 cases of vaginal extraction of pelvic masses following operative laparoscopy in the same year. In 2007, a Chinese surgeon, Yuan *et al.*^[16] presented a 65-year-old female with invasive urothelial carcinoma of the urinary bladder and end-stage renal disease who underwent laparoscopic radical cystectomy combined with bilateral nephroureterectomy, where the specimen was extracted transvaginally. In 2008, Palanivelu *et al.*^[17] reported extraction of total colon and rectum following totally laparoscopic proctocolectomy. Also in 2008, a swine model for TVSE following total gastrectomy was successfully constructed by Nakajima, who later also succeeded in human the following year^[18,19]. Despite the difficulty of transvaginal laparoscopic/endoscopic gastrectomy described by Lacy *et al.*^[20] in 2009, Jeong *et al.*^[21] succeeded in repeating the laparoscopic transvaginal extraction of gastric cancer in 2011.

Nevertheless, there have not been previous reports of transvaginal extraction following robotic gastrectomy, nor any transvaginal NOSE following gastrectomy with standard D2 lymph node resection for gastric cancer (Table 4). It was not until June 2014 that the first NOSE following gastrectomy was performed by Dostalík *et al.*^[22]. However, the natural orifice specimen was extracted through the oral-esophageal route. Even when carefully washed with water, the bad smell of blood mixed with stomach contents could not be disserved within a couple of weeks. Damage to the esophageal wall during extraction occurred in our previous experience. Moreover, in single port laparoscopic gastrectomy, lymph node resection was not as easy as the robotic surgery or the open abdomen surgery, nor was

Table 4 Laboratory features of robotic transvaginal specimen extraction patients

	CRP (mg/L)			HB (g/L)			ALB (g/L)			PAB (g/L)			TFN (g/L)		
	Pre-	POD 1	POD 3	Pre-	POD 1	POD 3	Pre-	POD 1	POD 3	Pre-	POD 1	POD 3	Pre-	POD 1	POD 3
TVSE-RTG	0.4	1.8	32.2	132	131	123	41.6	34.8	28.4	321	227	200	2.7	2.6	2.2
TVSE-RDG	0.5	19.4	35	133	124	116.3	41.4	33.5	29.6	273.7	236.8	205.8	2.8	2.5	2.3

POD: Postoperative day; CRP: C-reactive protein; HB: Haemoglobin; ALB: Albumin; PAB: Prealbumin; TFN: Transferrin; HR: Heart rate; TVSE: Transvaginal specimen extraction; RTG: Robotic total gastrectomy; RDG: Robotic distal gastrectomy.

the specimen extraction. Colpotomy is considered safe and does not lead to surgical site infections or dyspareunia^[23,24]. Transvaginal NOSE using a posterior colpotomy is considered a mature procedure used in gynecology. Not only is the robotic gastrectomy valued in the surgical safety but also in oncological performance as it is a safe way to perform the standard D2 lymph node harvest in gastric cancer^[25,26].

In 2013, we have succeeded in publishing an investigation on robotic procedure which provides us a safe and feasible approach to robotic gastrectomy^[5]. This new procedure for gastrectomy is performed daily in our hospital with the anastomosis sewn fully by robot intracorporeally. This daily training provides us a possibility to perform the TVSE following full robot-sewn gastrectomy.

We are glad to see a decrease in the morbidity and mortality in patients with TVSE following full intracorporeal robot-sewn gastrectomy. This study showed the safety of eight cases of TVSE following gastrectomy, indicating that it could be a more feasible way of approaching gastric cancer.

In comparison with transrectal extraction, the transvaginal extraction is feasible and carries a low risk of infection and postoperative leakage^[27]. Despite the potential advantages, there are still potential risks in transvaginal NOSE, for example, infertility or dyspareunia to some extent. The complicated surgical procedure requires more technique and consumes more time to perform the transvaginal NOSE. The mean time of TVSE following robotic gastrectomy was 224 min in the eight patients. The mean operative times were 229 min and 212 min for Awad and McKenzie, even though they are laparoscopic colectomy which seems easier than those in gastrectomy^[28,29]. However, there were no significant side effects clinically detected in the post-vaginal-resection patients^[30,31].

Robotic TVSE in gastric cancer might be a feasible and alternative operative procedure for patients with gastric cancer. It showed minimal postoperative pain with small incisions; minimal invasiveness as well as less severe postoperative complications such as anastomotic fistulae, stenosis, and bleeding.

This innovation with robotic surgery provides a new approach to gastric cancer with pure NOSE approach. However, we must realize that this TVSE following standard D2 gastric surgery study with eight patients is small. The follow-up period of six months is a little

too short for the judgement of oncological safety. Extensive studies with larger sample size of patients focusing on the oncologic safety with long-term surveillance are needed for adequate confirmation.

COMMENTS

Background

Despite the advantages of robotic gastrectomy, the need for an incision in the abdominal wall to remove the surgical specimen is a morbid factor.

Research frontiers

The development of natural orifice transluminal robotic surgery and natural orifice specimen extraction (NOSE) appears to be the next major frontier in robotic surgery.

Innovations and breakthroughs

Present research showed that robotic gastrectomy with transvaginal extraction is a safe and effective procedure. This technique is feasible and simple to perform, and avoids the abdominal wall incision and its potential complications.

Applications

NOSE may provide both an attractive way to reduce abdominal wall morbidity and a bridge to pure natural orifice transluminal endoscopic surgery for robotic gastrectomy.

Terminology

NOSE in robotic gastrectomy prevents the need for an enlarged port site or minilaparotomy to extract the surgical specimen. The current trend to develop less invasive techniques by reducing the number and size of abdominal incisions has spurred new interest in practice.

Peer-review

Authors conducted the case series analysis about the transvaginal specimen extraction following robotic total or partial gastrectomy for gastric cancer. Authors concluded that this surgical procedure is safe and feasible for patients with gastric cancer. This paper is very interesting and well written.

REFERENCES

- 1 Guo P, Huang ZL, Yu P, Li K. Trends in cancer mortality in China: an update. *Ann Oncol* 2012; **23**: 2755-2762 [PMID: 22492700 DOI: 10.1093/annonc/mds069]
- 2 Kamangar F, Dores GM, Anderson WF. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. *J Clin Oncol* 2006; **24**: 2137-2150 [PMID: 16682732 DOI: 10.1200/JCO.2005.05.2308]
- 3 Franklin ME, Ramos R, Rosenthal D, Schuessler W. Laparoscopic colonic procedures. *World J Surg* 1993; **17**: 51-56 [PMID: 8447141]
- 4 Darzi A, Super P, Guillou PJ, Monson JR. Laparoscopic sigmoid colectomy: total laparoscopic approach. *Dis Colon Rectum* 1994; **37**: 268-271 [PMID: 8137674]
- 5 Liu XX, Jiang ZW, Chen P, Zhao Y, Pan HF, Li JS. Full robot-

- assisted gastrectomy with intracorporeal robot-sewn anastomosis produces satisfying outcomes. *World J Gastroenterol* 2013; **19**: 6427-6437 [PMID: 24151361 DOI: 10.3748/wjg.v19.i38.6427]
- 6 **Japanese Gastric Cancer Association.** Japanese gastric cancer treatment guidelines 2010 (ver. 3). *Gastric Cancer* 2011; **14**: 113-123 [PMID: 21573742 DOI: 10.1007/s10120-011-0042-4]
 - 7 **Boni L, Tenconi S, Beretta P, Cromi A, Dionigi G, Rovera F, Dionigi R, Ghezzi F.** Laparoscopic colorectal resections with transvaginal specimen extraction for severe endometriosis. *Surg Oncol* 2007; **16** Suppl 1: S157-S160 [PMID: 18024017 DOI: 10.1016/j.suronc.2007.10.003]
 - 8 **Tarantino I, Linke GR, Lange J, Siercks I, Warschkow R, Zerz A.** Transvaginal rigid-hybrid natural orifice transluminal endoscopic surgery technique for anterior resection treatment of diverticulitis: a feasibility study. *Surg Endosc* 2011; **25**: 3034-3042 [PMID: 21487875 DOI: 10.1007/s00464-011-1666-5]
 - 9 **Torres RA, Orban RD, Tocaimaza L, Vallejos Pereira G, Arévalo JR.** Transvaginal specimen extraction after laparoscopic colectomy. *World J Surg* 2012; **36**: 1699-1702 [PMID: 22374544 DOI: 10.1007/s00268-012-1528-x]
 - 10 **Ghezzi F, Raio L, Mueller MD, Gyr T, Buttarelli M, Franchi M.** Vaginal extraction of pelvic masses following operative laparoscopy. *Surg Endosc* 2002; **16**: 1691-1696 [PMID: 12140631 DOI: 10.1007/s00464-002-9043-z]
 - 11 **Diana M, Perretta S, Wall J, Costantino FA, Leroy J, Demartines N, Marescaux J.** Transvaginal specimen extraction in colorectal surgery: current state of the art. *Colorectal Dis* 2011; **13**: e104-e111 [PMID: 21564461 DOI: 10.1111/j.1463-1318.2011.02599.x]
 - 12 **Delvaux G, Devroey P, De Waele B, Willems G.** Transvaginal removal of gallbladders with large stones after laparoscopic cholecystectomy. *Surg Laparosc Endosc* 1993; **3**: 307-309 [PMID: 8269249]
 - 13 **Redwine DB, Koning M, Sharpe DR.** Laparoscopically assisted transvaginal segmental resection of the rectosigmoid colon for endometriosis. *Fertil Steril* 1996; **65**: 193-197 [PMID: 8557141]
 - 14 **Kim J, Shim M, Kwun K.** Laparoscopic-assisted transvaginal resection of the rectum. *Dis Colon Rectum* 1996; **39**: 582-583 [PMID: 8620813]
 - 15 **Gill IS, Cherullo EE, Meraney AM, Borsuk F, Murphy DP, Falcone T.** Vaginal extraction of the intact specimen following laparoscopic radical nephrectomy. *J Urol* 2002; **167**: 238-241 [PMID: 11743316]
 - 16 **Yuan LH, Chung HJ, Chen KK.** Laparoscopic radical cystectomy combined with bilateral nephroureterectomy and specimen extraction through the vagina. *J Chin Med Assoc* 2007; **70**: 260-261 [PMID: 17591588 DOI: 10.1016/S1726-4901(09)70371-5]
 - 17 **Palanivelu C, Rangarajan M, Jategaonkar PA, Anand NV.** An innovative technique for colorectal specimen retrieval: a new era of „natural orifice specimen extraction“ (N.O.S.E). *Dis Colon Rectum* 2008; **51**: 1120-1124 [PMID: 18481149 DOI: 10.1007/s10350-008-9316-2]
 - 18 **Nakajima K, Takahashi T, Souma Y, Shinzaki S, Yamada T, Yoshio T, Nishida T.** Transvaginal endoscopic partial gastrectomy in porcine models: the role of an extra endoscope for gastric control. *Surg Endosc* 2008; **22**: 2733-2736 [PMID: 18813999 DOI: 10.1007/s00464-008-0122-7]
 - 19 **Nakajima K, Nishida T, Takahashi T, Souma Y, Hara J, Yamada T, Yoshio T, Tsutsui T, Yokoi T, Mori M, Doki Y.** Partial gastrectomy using natural orifice transluminal endoscopic surgery (NOTES) for gastric submucosal tumors: early experience in humans. *Surg Endosc* 2009; **23**: 2650-2655 [PMID: 19357916 DOI: 10.1007/s00464-009-0474-7]
 - 20 **Lacy AM, Delgado S, Rojas OA, Ibarzabal A, Fernandez-Esparrach G, Taura P.** Hybrid vaginal MA-NOS sleeve gastrectomy: technical note on the procedure in a patient. *Surg Endosc* 2009; **23**: 1130-1137 [PMID: 19242758 DOI: 10.1007/s00464-008-0292-3]
 - 21 **Jeong SH, Lee YJ, Choi WJ, Paik WY, Jeong CY, Park ST, Choi SK, Hong SC, Jung EJ, Joo YT, Ha WS.** Trans-vaginal specimen extraction following totally laparoscopic subtotal gastrectomy in early gastric cancer. *Gastric Cancer* 2011; **14**: 91-96 [PMID: 21264485 DOI: 10.1007/s10120-011-0006-8]
 - 22 **Dostalík J, Gunkova P, Gunka I, Mazur M, Mrazek T.** Laparoscopic gastric resection with natural orifice specimen extraction for postulcer pyloric stenosis. *Wideochir Inne Tech Maloinwazyjne* 2014; **9**: 282-285 [PMID: 25097701 DOI: 10.5114/wiitm.2014.41622]
 - 23 **Lomanto D, Chua HC, Myat MM, So J, Shabbir A, Ho L.** Microbiological contamination during transgastric and transvaginal endoscopic techniques. *J Laparoendosc Adv Surg Tech A* 2009; **19**: 465-469 [PMID: 19575634 DOI: 10.1089/lap.2009.0007]
 - 24 **Wood SG, Panait L, Duffy AJ, Bell RL, Roberts KE.** Complications of transvaginal natural orifice transluminal endoscopic surgery: a series of 102 patients. *Ann Surg* 2014; **259**: 744-749 [PMID: 23598384 DOI: 10.1097/SLA.0b013e3182916138]
 - 25 **Patriti A, Ceccarelli G, Bellocchi R, Bartoli A, Spaziani A, Di Zitti L, Casciola L.** Robot-assisted laparoscopic total and partial gastric resection with D2 lymph node dissection for adenocarcinoma. *Surg Endosc* 2008; **22**: 2753-2760 [PMID: 18813994 DOI: 10.1007/s00464-008-0129-0]
 - 26 **Liao G, Chen J, Ren C, Li R, Du S, Xie G, Deng H, Yang K, Yuan Y.** Robotic versus open gastrectomy for gastric cancer: a meta-analysis. *PLoS One* 2013; **8**: e81946 [PMID: 24312610 DOI: 10.1371/journal.pone.0081946]
 - 27 **Wolthuis AM, de Buck van Overstraeten A, D'Hoore A.** Laparoscopic natural orifice specimen extraction-colectomy: a systematic review. *World J Gastroenterol* 2014; **20**: 12981-12992 [PMID: 25278692 DOI: 10.3748/wjg.v20.i36.12981]
 - 28 **Awad ZT, Qureshi I, Seibel B, Sharma S, Dobbertien MA.** Laparoscopic right hemicolectomy with transvaginal colon extraction using a laparoscopic posterior colpotomy: a 2-year series from a single institution. *Surg Laparosc Endosc Percutan Tech* 2011; **21**: 403-408 [PMID: 22146161 DOI: 10.1097/SLE.0b013e31823945ac]
 - 29 **McKenzie S, Baek JH, Wakabayashi M, Garcia-Aguilar J, Pigazzi A.** Totally laparoscopic right colectomy with transvaginal specimen extraction: the authors' initial institutional experience. *Surg Endosc* 2010; **24**: 2048-2052 [PMID: 20108143 DOI: 10.1007/s00464-009-0870-z]
 - 30 **Paraíso MF, Barber MD, Muir TW, Walters MD.** Rectocece repair: a randomized trial of three surgical techniques including graft augmentation. *Am J Obstet Gynecol* 2006; **195**: 1762-1771 [PMID: 17132479 DOI: 10.1016/j.ajog.2006.07.026]
 - 31 **Roovers JP, van der Bom JG, van der Vaart CH, van Leeuwen JH, Scholten PC, Heintz AP.** A randomized comparison of post-operative pain, quality of life, and physical performance during the first 6 weeks after abdominal or vaginal surgical correction of descensus uteri. *Neurourol Urodyn* 2005; **24**: 334-340 [PMID: 15924355 DOI: 10.1002/nau.20104]

P- Reviewer: Murata A S- Editor: Ma YJ

L- Editor: Wang TQ E- Editor: Liu XM



Clinical Trials Study

Enhanced recovery after surgery with laparoscopic radical gastrectomy for stomach carcinomas

Ikram Abdikarim, Xue-Yuan Cao, Shou-Zhen Li, Yin-Quan Zhao, Yerlan Taupyk, Quan Wang

Ikram Abdikarim, Xue-Yuan Cao, Shou-Zhen Li, Yin-Quan Zhao, Yerlan Taupyk, Quan Wang, Department of Gastric and Colorectal Surgery, First Hospital of Jilin University, Changchun 130021, Jilin Province, China

Author contributions: Abdikarim I and Cao XY contributed equally to this work; Wang Q, Abdikarim I and Cao XY designed the study and carried out most of the research; Abdikarim I, Cao XY and Wang Q wrote the first draft, critically revised and approved the final version of the manuscript; Li SZ, Zhao YQ, and Taupyk Y provided valuable suggestions for the study.

Institutional review board statement: The study protocol was approved by the Ethical Committee of First Hospital of Jilin University.

Informed consent statement: Written informed consent was provided by all study participants prior to study enrollment.

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

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Quan Wang, MD, PhD, Department of Gastric and Colorectal Surgery, First Hospital of Jilin University, No. 71 Xinmin Street, Changchun 130021, Jilin Province, China. wangquan-jlcc@hotmail.com
Telephone: +86-431-81875617

Received: April 30, 2015

Peer-review started: May 7, 2015

First decision: July 10, 2015

Revised: July 27, 2015

Accepted: October 12, 2015

Article in press: October 13, 2015

Published online: December 21, 2015

Abstract

AIM: To study the efficacy of the enhanced recovery after surgery (ERAS) program in laparoscopic radical gastrectomy for stomach carcinomas.

METHODS: From June 2010 to December 2012, 61 gastric cancer patients who underwent laparoscopic-assisted radical gastrectomy with D2 lymphadenectomy at First Hospital of Jilin University were enrolled in this randomized controlled trial. (Clinical Trials.gov, registration ID: NCT01955096). The subjects were divided into the ERAS program group and the conventional control group. The clinical characteristics, recovery variables, and complications of patients were analyzed.

RESULTS: The time to first ambulation, oral food intake, and time to defecation were significantly shorter in the ERAS group ($n = 30$), compared to the conventional group ($n = 31$; $P = 0.04$, 0.003 , and 0.01 , respectively). The postoperative hospital stay was less in the ERAS group (6.8 ± 1.1 d) compared to the conventional group (7.7 ± 1.1 d) ($P = 0.002$). There was no significant difference in postoperative complications between the ERAS (1/30) and conventional care groups (2/31) ($P = 1.00$). There were no readmissions or mortality during the 30-d follow-up period.

CONCLUSION: The ERAS program is associated with a shorter hospital stay in gastric cancer patients undergoing laparoscopic radical gastrectomy. The ERAS protocol is useful in the treatment of gastric cancer.

Key words: Enhanced recovery after surgery; Laparoscopic; Gastrectomy; Gastric cancer

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: This randomized controlled trial enrolled 61 consecutive laparoscopic-assisted gastrectomy patients, who were divided into the enhanced recovery after surgery (ERAS) group and the conventional group. Compared to the conventional group, the ERAS group showed earlier postoperative food intake, earlier defecation time, and shorter postoperative hospital stay. ERAS was safe and feasible in patients with advanced gastric cancers.

Abdikarim I, Cao XY, Li SZ, Zhao YQ, Taupyk Y, Wang Q. Enhanced recovery after surgery with laparoscopic radical gastrectomy for stomach carcinomas. *World J Gastroenterol* 2015; 21(47): 13339-13344 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13339.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13339>

INTRODUCTION

Annually, almost one million cases of gastric cancer are diagnosed worldwide, of which, 400000 cases alone are diagnosed in China^[1,2]. Surgical resection of gastric cancer and regional lymphadenectomy are the only options for curability in these patients. However, it is sometimes associated with significant morbidity, mortality, and has an impact on the quality of life of patients after surgery^[3-5].

Since its introduction in 1994 by Kitano *et al.*^[6] laparoscopic-assisted gastrectomy (LAG) has become the standard treatment for early gastric cancer. LAG has significant advantages over conventional open gastrectomy such as faster recovery, shorter hospital stay and an overall improvement in quality of life^[7-9]. In recent years, as a result of advancements in the design of surgical instruments and increased surgeon experience, LAG is increasingly being used in the treatment of advanced gastric cancer, although there is still controversy surrounding the extent of lymphadenectomy that should be performed.

The combination of LAG with Enhanced Recovery After Surgery (ERAS), an evidence based multi-disciplinary perioperative and postoperative care program, leads to additional improvements in outcome such as decreased complications, length of hospital stay and hospital cost^[10]. ERAS constitutes components such as optimized pain control, restricted intravenous fluids, early oral nutrition and enforced mobilization with the aim of reducing surgical stress response hence optimizing patient recovery.

Despite the successful adoption of ERAS by several centers in the United Kingdom, Scandinavia and Germany, for colorectal cancer^[11], this is not the case with gastric cancer. Studies reporting on fast track

rehabilitation for gastric cancer are scarce^[12,13]. Thus, we undertook this study to evaluate the efficacy and safety of the ERAS program in patients undergoing elective laparoscopic gastric resection for advanced cancer.

MATERIALS AND METHODS

Patient population

This prospective study was conducted in the First Hospital of Jilin University, Department of Gastric and Colorectal Surgery between 2010 and 2012 (Trial registration number: NCT01955096). Only those diagnosed with advanced gastric cancer were enrolled into the study after undergoing a diagnostic workup consisting of endoscopy with biopsy, total-body CT scan, and endoscopic ultrasound in selected patients. Inclusion criteria were as follows: diagnosis of advanced gastric cancer, elective laparoscopic surgery and age under 75 years. Patients with early gastric cancer received neoadjuvant chemotherapy, and those with pyloric obstruction or with distant metastasis were excluded from the study.

After obtaining written consent from all patients, we randomly assigned them into two groups: 30 patients underwent LAG with the ERAS program and 31 underwent LAG, but received conventional postoperative care (Table 1). All operations were performed by an independent surgical group and the study protocol was approved by the hospital's ethical committee.

Laparoscopic procedure

There was no difference in the surgical procedures between the two groups. Laparoscopy-assisted gastrectomy was carried out and depending on the location of the primary tumor, total, proximal subtotal, or distal subtotal gastrectomy was performed. After resection, a supraumbilical midline incision was made for restoration of intestinal continuity and tumor retrieval. Lymph node dissection was performed according to the Guidelines of Japanese Gastric Cancer Association with D2^[14,15].

Postoperative care

Briefly, during the postoperative period, fluid intake was from POD 1 or POD 2 in the ERAS group, patients were advised to begin cautiously and increase intake according to tolerance. Furthermore, they were encouraged to take full semi-liquid diet on POD 2, and normal food as soon as possible after surgery. In the conventional group, postoperative oral intake was restricted. In the ERAS group, patients were encouraged to mobilize early from POD 1, and meet daily targets for mobilization. The conventional group received traditional postoperative care including bed rest. Urinary bladder drainage was routinely used in the conventional group, but limited to POD 1 in the ERAS group (Table 1).

Table 1 Perioperative protocols in the enhanced recovery after surgery and conventional groups

	ERAS group	Conventional group
PreOp	Patient education regarding FTS Solid food allowed until 6 h before surgery and carbohydrate drinks until 2 h before surgery No bowel preparation	Patient education No solid food 24 h before surgery and no liquids 12 h before surgery Mechanical bowel preparation and nasogastric placement performed
Intraoperative	5-trocar laparoscopy-assisted procedure. Non-opioid analgesia after induction of anesthesia. No nasogastric tube or drainage tube used. I.V. fluids were restricted (Ringer's lactate 20 mL/kg in the first h, after that 6 mL/kg per hour). Vasoactive drugs used if needed	5-trocar laparoscopy-assisted procedure. Routine use of abdominal drainage tubes and placement of catheters I.V. fluids not restricted (Ringer's lactate 20 mL/kg in the first h, then at the rate of 10-12 mL/kg per hour)
1 st PostOp Day	Soluble contrast swallow study is done to check the anastomosis. If intact, fluids are started. Adequate pain control maintained Urinary catheter removed Patient mobilized out of bed	Keep NPO Removal of urinary catheter Mobilization in bed
2 nd PostOp Day	Patient started on soft food Continue and increase ambulation. Pain control maintained	Patient is advised to get out of bed
3 rd PostOp Day	Patient progresses to solid food Epidural stopped and acetaminophen started Mobilization continued	Remove nasogastric tube and Liquids started Encouraged to walk in the ward
4 th PostOp Day	Check discharge criteria	Drains removed solid food intake

FTS: Fast-track surgery; PostOp: Postoperative; ERAS: Enhanced recovery after surgery.

Outcome parameters (data collection)

Collected data included: age, sex, tumor location, type of gastrectomy and reconstruction, T stage, number of lymph nodes retrieved, duration of operation, complications and postoperative outcomes (time to return to diet, first defecation and ambulation). Patients were monitored for the next 30 d for readmission.

Discharge criteria

The criteria for discharge were as follows: tolerance of solid diet, return of bowel habits and ability to walk on their own. To avoid influences from the clinicians, the discharge administration was managed by independent clinicians who were not involved in the study.

Statistical analysis

Measurement data are expressed as mean \pm SD. The data were analyzed using the Independent Student's *t* test, whereas categorical data were calculated using the χ^2 test or Fisher's exact test. Data analysis was performed using SPSS® software package version 16.0 (SPSS Inc., Chicago, IL, United States). *P* < 0.05 was considered statistically significant.

RESULTS

Sixty-one consecutive patients were included in the current study and were followed for 30 d. Mean age was 62 years in both patient groups, with a 2:1 male to female ratio. Table 2 summarizes the clinical data of the patients. According to the TNM classification system of the Union for International Cancer Control, 26 patients were stage II, and 35 cases were stage III. D2 lymphadenectomy was routinely performed in

all patients. The number of lymph nodes retrieved, duration of surgery, amount of blood loss and type of surgery are shown in Table 3.

Compared to the conventional group, the ERAS group showed faster recovery with a shorter postoperative hospital stay (7.7 ± 1.1 d and 6.8 ± 1.1 d, respectively). Earlier postoperative food intake was observed in the ERAS group (2.9 ± 0.7 d) compared with the conventional group (3.5 ± 0.8 d). There was also a significant difference in defecation time between the ERAS (3.1 ± 0.7 d) and conventional (3.6 ± 0.8 d) groups. Time to ambulation (2.6 ± 0.9 d for the ERAS group and 3.1 ± 1.0 d for the conventional group) was also significantly different (Table 4).

There were two cases of ileus complications, one in each group. They were managed by fasting, nasogastric decompression and other measures. In the ERAS group, one patient developed a wound infection. The incidence of complications between the ERAS group and the conventional group was not significantly different (*P* = 1.0). Table 5 details these complications. There were no readmissions during the 30-d follow-up period. In addition, no mortality was reported.

DISCUSSION

This study set out to explore the safety and outcomes of ERAS in patients diagnosed with advanced gastric cancer. Our findings showed that the ERAS program was feasible and safe compared to conventional postoperative care. Earlier studies by Kehlet and colleagues confirmed that ERAS in colon cancer was beneficial and included reduced hospital stay, avoided or minimized short-term complications and patients had a speedier recovery and returned to their normal

Table 2 Comparison of clinical characteristics of the patients

Variables	ERAS group (n = 30)	Conventional group (n = 31)	P value
Gender (male/female)	21/9	20/11	0.79
Median age (range in years)	63 ± 12	62 ± 11	0.95
Tumor location			0.94
Upper	10	9	
Middle	10	11	
Lower	10	11	
Length of hospital stay (d)	8.3 ± 1.3	9.9 ± 1.1	< 0.001

ERAS: Enhanced recovery after surgery.

Table 3 Comparison of surgical and oncological factors between the enhanced recovery after surgery group and the conventional group

Variables	ERAS group (n = 30)	Conventional group (n = 31)	P value
Type of surgery			0.72
Distal subtotal gastrectomy D2	21	23	
Total gastrectomy D2	9	8	
TNM stage			0.92
I	0	0	
II	13	13	
III	17	18	
IV	0	0	
Blood loss (mL)	54.5 ± 71.8	64.5 ± 89.7	0.67
Operation time (min)	137.4 ± 28.7	141.5 ± 30.5	0.74
Reconstruction			0.50
Billroth I	7	8	
Billroth II	14	10	
Roux-en-Y	9	13	
Lymph nodes retrieved	39.6 ± 2.3	41.2 ± 3.3	0.42

ERAS: Enhanced recovery after surgery.

Table 4 Postoperative outcomes in days (d)

Variables	ERAS group (n = 30)	Conventional group (n = 31)	P value
Ambulation time	2.6 ± 0.9	3.1 ± 1.0	0.04
Defecation time	3.1 ± 0.7	3.6 ± 0.8	0.01
Food intake	2.9 ± 0.7	3.5 ± 0.8	0.003
POS	6.8 ± 1.1	7.7 ± 1.1	0.002

ERAS: Enhanced recovery after surgery.

way of life including eating a solid diet and earlier defecation time^[16]. Moreover, another recent study demonstrated that ERAS enhanced postoperative recovery, and resulted in a shorter hospital stay and lower morbidity rate^[17]. Consistent with these studies, Wang *et al.*^[12] verified that ERAS can improve the stress reaction, decrease the patient's resting energy expenditure during the postoperative period and hasten the rehabilitation of gastric cancer patients. To date, studies investigating the efficacy of this multi-modal rehabilitation program in gastric cancer

Table 5 Complications and readmission in the two groups

Variables	ERAS group (n = 30)	Conventional group (n = 31)	P value
Morbidity			
Wound infection	0	1	1.00
Bleeding	0	0	-
Ileus	1	1	1.00
Stenosis	0	0	-
Leakage	0	0	-
Others			
Readmission	0	0	-
Mortality	0	0	-

ERAS: Enhanced recovery after surgery.

patients are scarce. Our study demonstrated that ERAS outweighed conventional care in terms of safety, hospital stay, defecation time, ambulation time and postoperative recovery. These results are consistent with those reported by Feng *et al.*^[13].

ERAS, unlike conventional care, does not require long postoperative fasting periods. Based on the concept that eating too early after surgery may cause intestinal obstruction and anastomotic disruption, surgeons routinely restrict oral intake in patients receiving conventional care. A recent prospective study confirmed that early oral food intake after laparoscopic gastric surgery is safe and might be associated with enhanced recovery with shorter hospital stay^[11]. In the same context, patients with gastric cancer planning to receive conventional care have been advised to fast for 12 to 24 h before gastrectomy. Conversely, gastric cancer patients receiving fast track rehabilitation are required to fast for only 2 h before the operation. Therefore, in this study, patients were allowed carbohydrate-rich drinks up to 2 h before surgery. Some recent reports have indicated that a nasogastric tube is not necessary as it may instigate pulmonary complications^[18-20]. Furthermore, ERAS advocates restriction of fluid therapy during the perioperative period. Recently published articles have shown that the infusion of extra fluid needed to sustain intra-operative infusion in conventional perioperative care augments the threat of pulmonary interstitial edema and postoperative hypoxia; but it can also raise the threat of cardiopulmonary complications. In addition, the infusion of extra fluid exacerbates gastrointestinal edema and as a consequence can lead to delayed recovery of gastrointestinal function. In the present study, this was the reason that the quantity of fluid infused in the ERAS group was restricted^[21,22].

The results of this study confirm that ERAS accelerates postoperative recovery compared with conventional care. In the ERAS group, average hospital stay was 6.8 ± 1.1 d after laparoscopic gastric resection and was 7.7 ± 1.1 d in the conventional group. Because drainage tubes increase the risk of complications including accumulation of intra-

abdominal fluid, infection and fistula, surgical drainage was not used in the ERAS group, and this facilitated earlier ambulation in this group compared with the conventional group (2.6 ± 0.9 d and 3.1 ± 1.0 d, respectively). In addition, our results also showed that defecation time was less in the ERAS group (3.1 ± 0.7 d, $P < 0.002$) than in the conventional group (3.6 ± 0.8 d).

Three patients developed postoperative complications, one in the ERAS group and two in the conventional group, the difference was not significant. Moreover, there were no readmissions or mortality reported during the follow-up period.

Importantly, if a patient is randomized to the ERAS program, clinicians may be influenced to accelerate the discharge rather than to judge the patient's condition. Thus, in order to ensure the randomness of the study, the discharge criteria together with the clinical decisions regarding oral intake and drainage extraction should be managed by clinicians who are not involved in the study.

In conclusion, although the occurrence of postoperative complications between the two groups was not statistically significant, overall, ERAS was safe and feasible compared to conventional care in patients with advanced gastric cancer^[23-27].

ACKNOWLEDGMENTS

The authors would like to thank Shaahidah Nur Sulaiman for providing valuable suggestions in the preparation of the manuscript.

COMMENTS

Background

Recently, the concept of enhanced recovery after surgery (ERAS) has been used in a variety of operations. There are many international studies on ERAS in colorectal surgery. However, there are few studies on ERAS in gastric surgery.

Research frontiers

Many evidence-based clinical trials have confirmed the efficacy of ERAS; however, the wide application of ERAS requires further introduction in the laparoscopic gastric surgery field.

Innovations and breakthroughs

This randomized controlled trial demonstrated that ERAS was safe and effective in advanced gastric cancer patients. This study represents a new evaluation of ERAS in laparoscopic gastric surgery.

Applications

This study demonstrates the new use of ERAS in laparoscopic gastric surgery and will hopefully promote the clinical application of ERAS in gastric cancer.

Terminology

ERAS or fast-track surgery is a series of perioperative multidisciplinary approaches including preoperative, intraoperative and postoperative techniques, which could reduce complications, postoperative pain, costs and the length of hospital stay.

Peer-review

The authors show the analysis of ERAS in laparoscopic-assisted gastrectomy for advanced gastric cancer using randomized controlled trial study. The topic of the manuscript is very interesting, with many important issues that could be considered relevant for the clinical practice.

REFERENCES

- 1 Yearbook of health in the people's republic of China 2010. China Yearbook Press, 2011
- 2 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]
- 3 Etoh T, Inomata M, Shiraishi N, Kitano S. Minimally invasive approaches for gastric cancer-Japanese experiences. *J Surg Oncol* 2013; **107**: 282-288 [PMID: 22504947 DOI: 10.1002/jso.23128]
- 4 Sasako M, Saka M, Fukagawa T, Katai H, Sano T. Surgical treatment of advanced gastric cancer: Japanese perspective. *Dig Surg* 2007; **24**: 101-107 [PMID: 17446702 DOI: 10.1159/000101896]
- 5 Park DJ, Lee HJ, Kim HH, Yang HK, Lee KU, Choe KJ. Predictors of operative morbidity and mortality in gastric cancer surgery. *Br J Surg* 2005; **92**: 1099-1102 [PMID: 15931657 DOI: 10.1002/bjs.4952]
- 6 Kitano S, Iso Y, Moriyama M, Sugimachi K. Laparoscopy-assisted Billroth I gastrectomy. *Surg Laparosc Endosc* 1994; **4**: 146-148 [PMID: 8180768]
- 7 Jeong SH, Lee YJ, Park ST, Choi SK, Hong SC, Jung EJ, Joo YT, Jeong CY, Ha WS. Risk of recurrence after laparoscopy-assisted radical gastrectomy for gastric cancer performed by a single surgeon. *Surg Endosc* 2011; **25**: 872-878 [PMID: 21072670 DOI: 10.1007/s00464-010-1286-5]
- 8 Kim YW, Baik YH, Yun YH, Nam BH, Kim DH, Choi IJ, Bae JM. Improved quality of life outcomes after laparoscopy-assisted distal gastrectomy for early gastric cancer: results of a prospective randomized clinical trial. *Ann Surg* 2008; **248**: 721-727 [PMID: 18948798 DOI: 10.1097/SLA.0b013e318185e62e]
- 9 Adachi Y, Suematsu T, Shiraishi N, Katsuta T, Morimoto A, Kitano S, Akazawa K. Quality of life after laparoscopy-assisted Billroth I gastrectomy. *Ann Surg* 1999; **229**: 49-54 [PMID: 9923799]
- 10 Wilmore DW, Kehlet H. Management of patients in fast track surgery. *BMJ* 2001; **322**: 473-476 [PMID: 11222424]
- 11 Scatizzi M, Kröning KC, Boddì V, De Prizio M, Feroci F. Fast-track surgery after laparoscopic colorectal surgery: is it feasible in a general surgery unit? *Surgery* 2010; **147**: 219-226 [PMID: 19892383 DOI: 10.1016/j.surg.2009.09.035]
- 12 Wang D, Kong Y, Zhong B, Zhou X, Zhou Y. Fast-track surgery improves postoperative recovery in patients with gastric cancer: a randomized comparison with conventional postoperative care. *J Gastrointest Surg* 2010; **14**: 620-627 [PMID: 20108171 DOI: 10.1007/s11605-009-1139-5]
- 13 Feng F, Ji G, Li JP, Li XH, Shi H, Zhao ZW, Wu GS, Liu XN, Zhao QC. Fast-track surgery could improve postoperative recovery in radical total gastrectomy patients. *World J Gastroenterol* 2013; **19**: 3642-3648 [PMID: 23801867 DOI: 10.3748/wjg.v19.i23.3642]
- 14 Japanese Gastric Cancer Association. Japanese gastric cancer treatment guidelines 2010 (ver. 3). *Gastric Cancer* 2011; **14**: 113-123 [PMID: 21573742 DOI: 10.1007/s10120-011-0042-4]
- 15 Nakajima T. Gastric cancer treatment guidelines in Japan. *Gastric Cancer* 2002; **5**: 1-5 [PMID: 12021853 DOI: 10.1007/s101200200000]
- 16 Kehlet H, Kennedy RH. Laparoscopic colonic surgery--mission accomplished or work in progress? *Colorectal Dis* 2006; **8**: 514-517 [PMID: 16784473 DOI: 10.1111/j.1463-1318.2006.00955.x]
- 17 Scharfenberg M, Raue W, Junghans T, Schwenk W. "Fast-track" rehabilitation after colonic surgery in elderly patients--is it feasible? *Int J Colorectal Dis* 2007; **22**: 1469-1474 [PMID: 17446702]

- 17483956 DOI: 10.1007/s00384-007-0317-8]
- 18 **Suchihiro T**, Matsumata T, Shikada Y, Sugimachi K. Accelerated rehabilitation with early postoperative oral feeding following gastrectomy. *Hepatogastroenterology* 2004; **51**: 1852-1855 [PMID: 15532842]
 - 19 **Carrère N**, Seulin P, Julio CH, Bloom E, Gouzi JL, Pradère B. Is nasogastric or nasojejunal decompression necessary after gastrectomy? A prospective randomized trial. *World J Surg* 2007; **31**: 122-127 [PMID: 17186430 DOI: 10.1007/s00268-006-0430-9]
 - 20 **Yoo CH**, Son BH, Han WK, Pae WK. Nasogastric decompression is not necessary in operations for gastric cancer: prospective randomised trial. *Eur J Surg* 2002; **168**: 379-383 [PMID: 12463426]
 - 21 **Ibrahim MS**, Khan MA, Nizam I, Haddad FS. Peri-operative interventions producing better functional outcomes and enhanced recovery following total hip and knee arthroplasty: an evidence-based review. *BMC Med* 2013; **11**: 37 [PMID: 23406499 DOI: 10.1186/1741-7015-11-37]
 - 22 **Wang Q**, Suo J, Jiang J, Wang C, Zhao YQ, Cao X. Effectiveness of fast-track rehabilitation vs conventional care in laparoscopic colorectal resection for elderly patients: a randomized trial. *Colorectal Dis* 2012; **14**: 1009-1013 [PMID: 21985126 DOI: 10.1111/j.1463-1318.2011.02855.x]
 - 23 **Aoyama T**, Yoshikawa T, Hayashi T, Hasegawa S, Tsuchida K, Yamada T, Cho H, Ogata T, Fujikawa H, Yukawa N, Oshima T, Rino Y, Masuda M. Randomized comparison of surgical stress and the nutritional status between laparoscopy-assisted and open distal gastrectomy for gastric cancer. *Ann Surg Oncol* 2014; **21**: 1983-1990 [PMID: 24499830 DOI: 10.1245/s10434-014-3509-9]
 - 24 **Pędziwiatr M**, Matłok M, Kisielewski M, Migaczewski M, Major P, Winiarski M, Budzyński P, Zub-Pokrowiecka A, Budzyński A. Short hospital stays after laparoscopic gastric surgery under an Enhanced Recovery After Surgery (ERAS) pathway: experience at a single center. *Eur Surg* 2014; **46**: 128-132 [PMID: 24971087 DOI: 10.1007/s10353-014-0264-x]
 - 25 **Gustafsson UO**, Scott MJ, Schwenk W, Demartines N, Roulin D, Francis N, McNaught CE, Macfie J, Liberman AS, Soop M, Hill A, Kennedy RH, Lobo DN, Fearon K, Ljungqvist O. Guidelines for perioperative care in elective colonic surgery: Enhanced Recovery After Surgery (ERAS®) Society recommendations. *World J Surg* 2013; **37**: 259-284 [PMID: 23052794 DOI: 10.1007/s00268-012-1772-0]
 - 26 **Mortensen K**, Nilsson M, Slim K, Schäfer M, Mariette C, Braga M, Carli F, Demartines N, Griffin SM, Lassen K. Consensus guidelines for enhanced recovery after gastrectomy: Enhanced Recovery After Surgery (ERAS®) Society recommendations. *Br J Surg* 2014; **101**: 1209-1229 [PMID: 25047143 DOI: 10.1002/bjs.9582]
 - 27 **Kehlet H**. Enhanced Recovery After Surgery (ERAS): good for now, but what about the future? *Can J Anaesth* 2015; **62**: 99-104 [PMID: 25391731 DOI: 10.1007/s12630-014-0261-3]

P- Reviewer: Bencini L **S- Editor:** Yu J
L- Editor: Webster JR **E- Editor:** Liu XM



Observational Study

Colorectal resection in deep pelvic endometriosis: Surgical technique and post-operative complications

Marco Milone, Andrea Vignali, Francesco Milone, Giusto Pignata, Ugo Elmore, Mario Musella, Giuseppe De Placido, Antonio Mollo, Loredana Maria Sosa Fernandez, Guido Coretti, Umberto Bracale, Riccardo Rosati

Marco Milone, Francesco Milone, Mario Musella, Giuseppe De Placido, Antonio Mollo, Loredana Maria Sosa Fernandez, Guido Coretti, Department of Advanced Biomedical Science, University of Naples "Federico II", 80131 Naples, Italy

Andrea Vignali, Ugo Elmore, Riccardo Rosati, Department of Gastrointestinal Surgery, San Raffaele Scientific Institute, University Vita Salute, 20132 Milan, Italy

Giusto Pignata, Umberto Bracale, General and Mini-invasive Surgery, "San Camillo" Hospital, 38122 Trento, Italy

Author contributions: All authors contributed equally to this paper.

Institutional review board statement: Approval of Local Ethics Committee obtained.

Informed consent statement: Obtained from patients.

Conflict-of-interest statement: All authors have nothing to declare.

Data sharing statement: The depositary database is located in the Department of Advanced Biomedical Science, and all data are available for any further analysis. Data is stored anonymously to respect patient privacy.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Marco Milone, MD, Surgery Unit, Department of Advanced Biomedical Science, University of Naples "Federico II", Via Pansini 5, 80131 Naples, Italy. milone.marco.md@gmail.com
Telephone: +39-81-7463064

Fax: +39-81-7462896

Received: June 26, 2015

Peer-review started: June 27, 2015

First decision: July 10, 2015

Revised: July 30, 2015

Accepted: September 28, 2015

Article in press: September 30, 2015

Published online: December 21, 2015

Abstract

AIM: To investigate the impact of different surgical techniques on post-operative complications after colorectal resection for endometriosis.

METHODS: A multicenter case-controlled study using the prospectively collected data of 90 women (22 with and 68 without post-operative complications) who underwent laparoscopic colorectal resection for endometriosis was designed to evaluate any risk factors of post-operative complications. The prospectively collected data included: gender, age, body mass index, American Society of Anesthesiologists risk class, endometriosis localization (from anal verge), operative time, conversion, intraoperative complications, and post-operative surgical complications such as anastomotic dehiscence, bleeding, infection, and bowel dysfunction.

RESULTS: A similar number of complicated cases have been registered for the different surgical techniques evaluated (laparoscopy, single access, flexure mobilization, mesenteric artery ligation, and transvaginal specimen extraction). A multivariate regression analysis showed that, after adjusting for major clinical, demographic, and surgical characteristics, complicated cases were only associated with

endometriosis localization from the anal verge (OR = 0.8, 95%CI: 0.74-0.98, $P = 0.03$). After analyzing the association of post-operative complications and each different surgical technique, we found that only bowel dysfunction after surgery was associated with mesenteric artery ligation (11 out of 44 dysfunctions in the mesenteric artery ligation group vs 2 out of 36 cases in the no mesenteric artery ligation group; $P = 0.03$).

CONCLUSION: Although further randomized clinical trials are needed to give a definitive conclusion, laparoscopic colorectal resection for deep infiltrating endometriosis appears to be both feasible and safe. Surgical technique cannot be considered a risk factor of post-operative complications.

Key words: Endometriosis; Bowel; Complication; Technique; Laparoscopy; Mesenteric artery

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: To the best of our knowledge, this is the first study evaluating the impact of different surgical techniques on the occurrence of post-operative complications. We have evaluated the potential influence of the most relevant surgical differences, including: laparoscopic approach, single access laparoscopy, flexure mobilization, mesenteric artery ligation, specimen extraction site, and diverting ileostomy creation.

Milone M, Vignali A, Milone F, Pignata G, Elmore U, Musella M, De Placido G, Mollo A, Fernandez LMS, Coretti G, Bracale U, Rosati R. Colorectal resection in deep pelvic endometriosis: Surgical technique and post-operative complications. *World J Gastroenterol* 2015; 21(47): 13345-13351 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13345.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13345>

INTRODUCTION

Endometriosis is a common condition that affects up to 10% of women in their reproductive years^[1]. Deep infiltrating endometriosis (DIE) is characterized by endometriosis implants that penetrate more than 5 mm into the affected tissue. Although the disease is limited in most patients to the genital organs, endometriosis may diffusely involve pelvic structures such as the bowels and urinary tract^[2-5]. The estimated incidence of bowel endometriosis is between 3% and 36%^[6], while rectal and rectosigmoid junction involvement together account for 70%-93% of all intestinal endometriotic lesions^[7].

Due to the limited efficacy of medical therapy and symptom recurrence rates as high as 76%^[8], surgical excision is frequently advocated as the treatment of

choice^[9,10].

Although it is well known that laparoscopic segmental colorectal resection is preferred for the treatment of colorectal endometriosis, little is known about the impact of different surgical techniques on post-operative complications.

MATERIALS AND METHODS

Utilizing prospectively maintained endometriosis databases, all consecutive women who underwent colorectal resection for endometriosis from January 2005 to December 2013 were identified for inclusion in a multicenter study after obtaining local ethics committee approval and signed informed consent. A case-controlled study was designed, including 22 women with and 68 women without post-operative complications, to identify potential risk factors for complications after surgery, with a focus on surgical technique.

Only institutions with a high volume of colorectal surgeries were included, and only prospectively recorded data were analyzed. All consecutive procedures were included in our analyses, according to strict inclusion criteria; all patients operated on by expert surgeons with standardized indications for surgery were included in our analyses. The laparoscopic colectomy learning curve can be considered completed after between 30 and 70 procedures. Thus, only procedures performed by an expert surgeon (*i.e.*, with experience of more than 70 laparoscopic colectomies) were included in the study^[11]; furthermore, all procedures were performed by a multidisciplinary surgical team, including an expert colorectal surgeon and a gynecologist. Indications for colorectal resection included the presence of colorectal involvement in deep bowel endometriosis diagnosed by laparoscopy^[12] and of endometriosis-related symptoms (*i.e.*, pelvic pain, dyschezia, rectal bleeding, obstruction, and dyspareunia).

Different surgical approaches were performed according to the clinical advice of each individual surgeon. A propensity score analysis was performed to exclude any bias related to the allocation of each patient into the different surgical technique groups.

Dissection is performed through the rectovaginal septum, where endometriosis implants are frequently found and must be removed. The rectum is mobilized at least 2 cm below the nodule of the endometriosis. A stapler is introduced into the peritoneal cavity, and then the rectum is sectioned. After extracting the rectal stump, the rectum or rectosigmoid (depending on the disease extension) is resected. Then, the head of the EEA (end-to-end) stapler (usually 29 mm) is positioned, the pneumoperitoneum is reconstituted, and a transanal end-to-end colorectal anastomosis is performed according to the Knight-Griffen technique. According to the clinical advice of each surgeon, flexure mobilization, mesenteric artery ligation,

Table 1 Patient and disease characteristics *n* (%)

	Complicated cases (22)	Uncomplicated cases (68)	<i>P</i> value
Age (yr)	33 ± 5.5	35.2 ± 5.3	0.11
BMI	24 ± 4	24 ± 2.9	0.96
Symptoms (pts)	17 (77.7)	54 (79.4)	1.00
Pain	7 (31.8)	15 (22)	0.46
Dyspareunia	5 (22.7)	17 (25)	0.78
Rectal bleeding	9 (40.9)	30 (44.1)	1.00
Constipation			
localization (cm from anal verge)	12.4 ± 4.8	13.9 ± 4.4	0.17

Table 2 Complication occurrence and different surgical technique *n* (%)

	Complicated cases (22)	Uncomplicated cases (68)	<i>P</i> value
Laparoscopy	19 (86.3)	58 (85.2)	0.73
Single access laparoscopy	3 (13.6)	7 (10.2)	0.71
Ileostomy	4 (18.1)	18 (26.4)	0.41
Flexure mobilization	15 (68.1)	46 (67.6)	0.79
Mesenteric artery ligation	16 (72.7)	36 (52.9)	0.22
Transvaginal extraction	7 (31.8)	15 (22)	0.57

diverting ileostomy, or transvaginal colon extraction may be performed.

To minimize the bias related to the use of differing post-surgical managements, the post-operative period was homogenized to exclude patients who received different medical and nursing care. Specifically, on post-operative day 1, endovenous hydration was suspended, and the patients were allowed to drink liquids and consume oral medicines. Criteria for discharge included symptom absence, tolerance of a minimum of three meals without restrictions, and stool passage.

Prospectively collected data included gender, age, body mass index (BMI), American Society of Anesthesiologists risk class, endometriosis localization (from anal verge), operative time, conversion, intra-operative complications, and post-operative surgical complications such as anastomotic dehiscence, bleeding, infection, and bowel dysfunction.

Short-term follow-up was conducted at 5 and 30 d after discharge. All adverse events that occurred within 90 d after surgery were considered to be complications.

The term anastomotic leakage defines all conditions with clinical or radiologic anastomotic dehiscence, with or without the need for surgical revision. Any bleeding led to an evaluation to determine if a blood transfusion was required. Bowel dysfunction was considered if any problems with the frequency, consistency, and/or ability to control bowel movements occurred after surgery.

Statistical analysis was performed with SPSS 16 (SPSS Inc., Chicago, IL, United States). Continuous data are expressed as the mean ± SD, with categorical

variables expressed as a percentage. To compare continuous variables, an independent sample *t*-test was performed. The Wilcoxon test for paired samples was employed as a non-parametric equivalent of the paired sample *t*-test used for continuous variables. The χ^2 test was employed to analyze categorical data. When the minimum expected value was < 5, Fisher's exact test was used. All results are presented as 2-tailed values with statistical significance if *P* values were < 0.05. To adjust for all the other variables and to make predictions, multivariate analyses were performed with post-operative complication occurrence (logistic regression) as dependent variables, and with major clinical and demographic characteristics, as well as surgical approach, as independent variables.

RESULTS

Demographics and disease-related data for each cohort are shown in Table 1. There were no significant differences in terms of age, BMI, or symptoms between the two groups.

Operative time (207.1 ± 53.3 min in complicated cases vs 206.7 ± 8 min in uncomplicated cases) was similar in both groups (*P* = 0.98). Interestingly, multivariate analysis (linear regression) showed that, after adjusting for different surgical techniques (laparoscopy, single access laparoscopy, flexure mobilization, ileostomy creation, mesenteric artery ligation, and transvaginal extraction), operative time was significantly longer only in cases of flexure mobilization (β = 0.3, *P* = 0.01). Although time to flatus was similar in both complicated and uncomplicated cases (36.1 ± 18.2 h vs 29.6 ± 17 h, *P* = 0.13), the length of hospital stay was statistically shorter in uncomplicated cases (7.9 ± 3.1 in complicated vs 6.4 ± 1.5 in uncomplicated cases, *P* < 0.001).

All of the different surgical techniques are summarized in Table 2. We registered a similar number of complicated cases. A multivariate regression analysis (stepwise method) showed that, after adjusting for major clinical, demographic, and surgical characteristics, complicated cases were associated only with endometriosis localization from the anal verge (OR = 0.8, 95%CI: 0.74-0.98, *P* = 0.03). Interestingly, analysis of the association between post-operative complications and each different surgical technique (Figures 1, 2, 3 and 4) revealed that only bowel dysfunction after surgery was associated with mesenteric artery ligation (11 out of 44 dysfunctions in the mesenteric artery ligation group vs 2 out of 36 cases in the no mesenteric artery ligation group, *P* = 0.03). However, a trend toward less infection and dysfunction was obtained after laparoscopic surgery (infection: 5% in the laparoscopic group vs 15% in the open group; dysfunction: 14% in the laparoscopic group vs 23% in the open group). Similarly, a trend toward more bleeding was obtained after flexure mobilization (5% in the flexure mobilization group

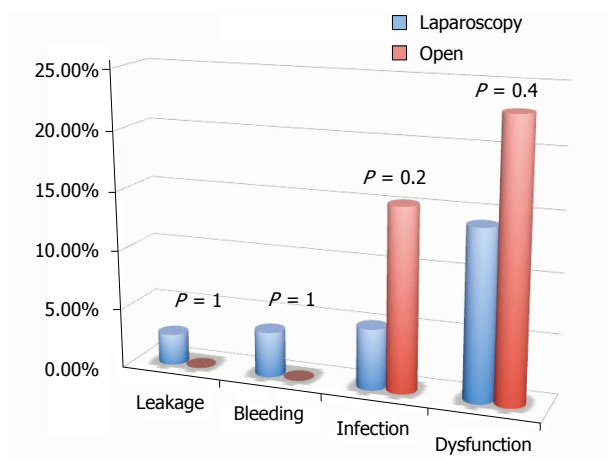


Figure 1 Complications in laparoscopic vs open procedures.

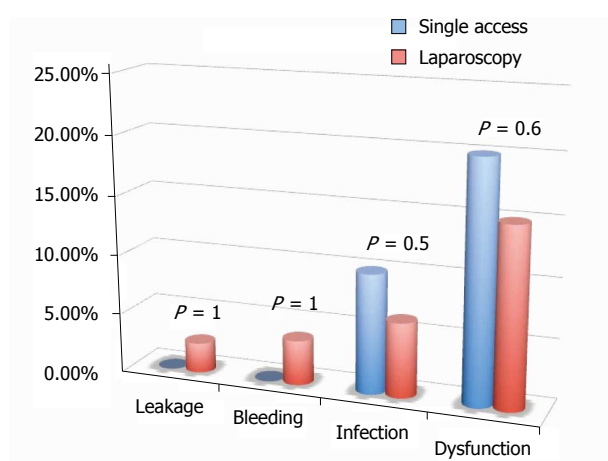


Figure 2 Complications in laparoscopic vs single-access laparoscopic procedures.

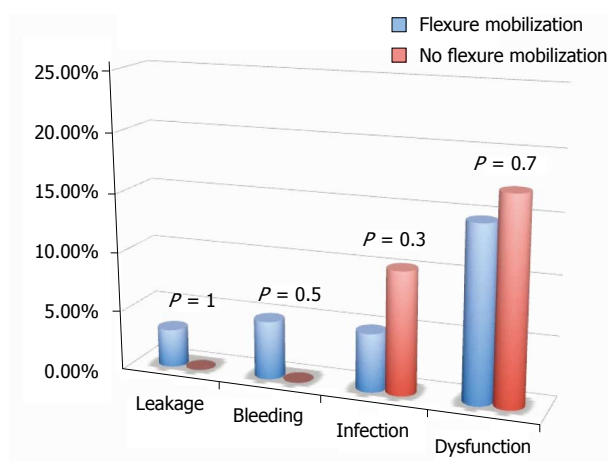


Figure 3 Complications in flexure mobilization vs no flexure mobilization procedures.

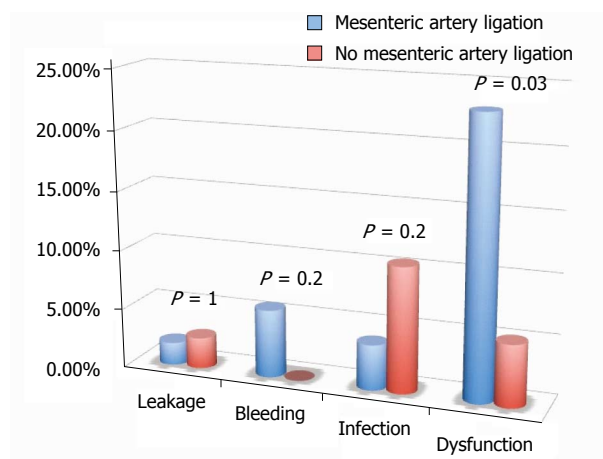


Figure 4 Complications in mesenteric ligation vs no mesenteric ligation procedures.

vs 0% in the no flexure mobilization group). Finally, a trend toward more bleeding was observed after mesenteric artery ligation (6% in the mesenteric ligation group vs 0% in the no mesenteric ligation group).

Interestingly, the incidence of leakage was very low (2.2%), and the negative predictive value (those without leakage and without diverting ileostomy/ those without leakage either with or without diverting ileostomy) of having a leakage in the absence of diverting ileostomy was quite high (97%). Similarly, no leakage occurred after single access laparoscopy.

DISCUSSION

Deep endometriosis invading the bowel constitutes a major challenge for gynecologists. In addition to a greater impact on pain^[13,14], the high incidence of surgical morbidity involved with the bowel^[2,15,16] poses a therapeutic dilemma for the surgeon^[17,18]. Intestinal involvement of deep endometriotic nodules has been estimated to occur in 8%-12% of women with endometriosis^[19,20], with colorectal disease involvement representing almost 90% of these cases^[4,7,21-23]. The complete excision of all endometriotic lesions is the main objective of both laparoscopic and laparotomic surgeries that require a multidisciplinary approach^[24,25] and highly skilled surgeons. Laparoscopic excision of deep infiltrating bowel endometriosis has become a frequently used treatment modality, and segmental bowel resection has been performed in many cases, despite the relatively high morbidity rate.

Both major and minor surgical complications have

been reported after the excision of deep endometriosis involving the bowel, including fistula (0%-14%)^[25-27], hemorrhage (1%-11%)^[19,28], infection (1%-3%)^[27,29], laparoconversion (up to 12%), and bladder (1%-71%) and bowel (1%-15%)^[27,30,31] dysfunction such as post-operative severe constipation^[32].

Brouwer and Woods^[33] reported that the type of surgical approach, including full-thickness excision of the rectal wall and segmental resection, does not change the rate of complications. However, many factors are affected by the surgeon's learning curve, such as the conversion rate, operating time, complication rate, and surgical effectiveness^[34]. Nevertheless, complications can occur, even among experienced surgeons^[35]. Accordingly, only procedures performed by expert surgeons were included in our analysis.

There are three frequently observed risk factors for major complications: opening of the vagina at the time of the bowel surgical procedure^[36], excessive use of electrocoagulation that may increase the risk of rectovaginal fistulae and abscesses (potentially leading to necrosis of the posterior vaginal cuff)^[30], and surgical treatment of low rectal lesions (< 5-8 cm from the anal verge), which increases the risk of anastomotic leaks^[28,37].

To the best of our knowledge, this is the first study to evaluate the impact of different surgical techniques on the occurrence of post-operative complications. We have evaluated the potential influence of the most relevant surgical differences, including laparoscopic approach, single access laparoscopy, flexure mobilization, mesenteric artery ligation, specimen extraction site, and diverting ileostomy creation.

Interestingly, we found that the occurrence of post-operative complications was not influenced by surgical technique. Only mesenteric artery ligation was associated with a higher incidence of bowel dysfunction after surgery ($P = 0.03$). Thus, based on current knowledge, this study confirms that preservation of the inferior mesenteric artery should be recommended to reduce the incidence of defecatory disorders after left hemicolectomy for benign disease^[38]. The sectioning of sigmoid arteries close to the colonic wall without sectioning the inferior mesenteric artery may preserve innervations of the neosigmoid and could reduce defecatory disorders.

As the incidence of leakage was very low (2.2%), we cannot justify the routine use of diverting ileostomy. Similarly, the negative predictive value (probability of leakage in the absence of diverting ileostomy) was quite high (97%), and thus we can exclude the need for diverting ileostomy.

Furthermore, the absence of a higher incidence of post-operative complications after single access laparoscopy encourages this approach's introduction in daily practice.

Finally, in contrast with previous publications, we did not find any association between the opening

of the vagina and the occurrence of post-operative complications. This could be related to cooperation between the surgeon and the gynecologist.

Some limitations of this study must be addressed. The most major limitation lies in the study design; because this study was an evaluation of a prospective maintained database, there was a lack of patient randomization. However, multivariate analyses including all patients' characteristics were performed to adjust the results for all other variables. Furthermore, although the surgical approach was dependent on the clinical advice of each individual surgeon, a propensity score was calculated to exclude any bias. Although a relatively small sample size was obtained in this multicenter study, certain biases were excluded, as only procedures performed by expert surgeons in standardized surgical indications with standard post-operative management were included.

Thus, our results encourage the consideration of laparoscopic colorectal resection for deep infiltrating endometriosis owing to its feasibility, safety, and low occurrence of post-operative complications, if performed by experienced surgeons. Furthermore, we cannot identify any surgical technique or approach that should be avoided to reduce major complications after surgery.

This study clearly provides the rationale for a future randomized clinical trial comparing different surgical approaches in order to provide a definitive conclusion.

COMMENTS

Background

Although laparoscopic segmental colorectal resection is an established technique for the treatment of colorectal endometriosis, little is known about the impact of different surgical techniques on post-operative complications.

Innovations and breakthroughs

The results encourage the consideration of laparoscopic colorectal resection for deep infiltrating endometriosis owing to its feasibility, safety, and low incidence of post-operative complications, if performed by experienced surgeons. Furthermore, we cannot identify any surgical technique or approach that should be avoided to reduce major complications after surgery.

Applications

This study clearly provides the rationale for a future randomized clinical trial comparing different surgical approaches in order to provide a definitive conclusion.

Peer-review

This paper is well constructed, with good data and statistical analysis. Further randomized clinical trials are needed to give more a definitive conclusion.

REFERENCES

- 1 **Emmanuel KR**, Davis C. Outcomes and treatment options in rectovaginal endometriosis. *Curr Opin Obstet Gynecol* 2005; **17**: 399-402 [PMID: 15976546]
- 2 **Ruffo G**, Sartori A, Crippa S, Partelli S, Barugola G, Manzoni A, Steinasserer M, Minelli L, Falconi M. Laparoscopic rectal resection for severe endometriosis of the mid and low rectum: technique and operative results. *Surg Endosc* 2012; **26**: 1035-1040

- [PMID: 22038165 DOI: 10.1007/s00464-011-1991-8]
- 3 **Darai E**, Bazot M, Rouzier R, Houry S, Dubernard G. Outcome of laparoscopic colorectal resection for endometriosis. *Curr Opin Obstet Gynecol* 2007; **19**: 308-313 [PMID: 17625410]
 - 4 **Bailey HR**, Ott MT, Hartendorp P. Aggressive surgical management for advanced colorectal endometriosis. *Dis Colon Rectum* 1994; **37**: 747-753 [PMID: 8055717]
 - 5 **Azioni G**, Bracale U, Scala A, Capobianco F, Barone M, Rosati M, Pignata G. Laparoscopic ureteroneocystostomy and vesicopsoas hitch for infiltrative ureteral endometriosis. *Minim Invasive Ther Allied Technol* 2010; **19**: 292-297 [PMID: 20868303 DOI: 10.3109/13645706.2010.507345]
 - 6 **Jerby BL**, Kessler H, Falcone T, Milsom JW. Laparoscopic management of colorectal endometriosis. *Surg Endosc* 1999; **13**: 1125-1128 [PMID: 10556452]
 - 7 **Remorgida V**, Ferrero S, Fulcheri E, Ragni N, Martin DC. Bowel endometriosis: presentation, diagnosis, and treatment. *Obstet Gynecol Surv* 2007; **62**: 461-470 [PMID: 17572918]
 - 8 **Shaw RW**. Treatment of endometriosis. *Lancet* 1992; **340**: 1267-1271 [PMID: 1359330]
 - 9 **Fanfani F**, Fagotti A, Gagliardi ML, Ruffo G, Ceccaroni M, Scambia G, Minelli L. Discoid or segmental rectosigmoid resection for deep infiltrating endometriosis: a case-control study. *Fertil Steril* 2010; **94**: 444-449 [PMID: 19394600 DOI: 10.1016/j.fertnstert.2009.03.066]
 - 10 **Kamergorodsky G**, Lemos N, Rodrigues FC, Asanuma FY, D'Amora P, Schor E, Girão MJ. Evaluation of pre- and post-operative symptoms in patients submitted to linear stapler nodulectomy due to anterior rectal wall endometriosis. *Surg Endosc* 2015; **29**: 2389-2393 [PMID: 25380710 DOI: 10.1007/s00464-014-3945-4]
 - 11 **Milone M**, Elmore U, Di Salvo E, Delrio P, Bucci L, Ferulano GP, Napolitano C, Angiolini MR, Bracale U, Clemente M, D'Ambra M, Luglio G, Musella M, Pace U, Rosati R, Milone F. Intracorporeal versus extracorporeal anastomosis. Results from a multicentre comparative study on 512 right-sided colorectal cancers. *Surg Endosc* 2015; **29**: 2314-2320 [PMID: 25414066 DOI: 10.1007/s00464-014-3950-7]
 - 12 **Milone M**, Mollo A, Musella M, Maietta P, Sosa Fernandez LM, Shatalova O, Conforti A, Barone G, De Placido G, Milone F. Role of colonoscopy in the diagnostic work-up of bowel endometriosis. *World J Gastroenterol* 2015; **21**: 4997-5001 [PMID: 25945014 DOI: 10.3748/wjg.v21.i16.4997]
 - 13 **Fauconnier A**, Fritel X, Chapron C. [Endometriosis and pelvic pain: epidemiological evidence of the relationship and implications]. *Gynecol Obstet Fertil* 2009; **37**: 57-69 [PMID: 19128998 DOI: 10.1016/j.gyobfe.2008.08.016]
 - 14 **Jacobson TZ**, Duffy JM, Barlow D, Koninckx PR, Garry R. Laparoscopic surgery for pelvic pain associated with endometriosis. *Cochrane Database Syst Rev* 2009; (4): CD001300 [PMID: 19821276 DOI: 10.1002/14651858]
 - 15 **Vercellini P**, Crosignani PG, Abbiati A, Somigliana E, Viganò P, Fedele L. The effect of surgery for symptomatic endometriosis: the other side of the story. *Hum Reprod Update* 2009; **15**: 177-188 [PMID: 19136455 DOI: 10.1093/humupd/dmn062]
 - 16 **Roman H**, Vassilief M, Gourcerol G, Savoye G, Leroi AM, Marpeau L, Michot F, Tuech JJ. Surgical management of deep infiltrating endometriosis of the rectum: pleading for a symptom-guided approach. *Hum Reprod* 2011; **26**: 274-281 [PMID: 21131296 DOI: 10.1093/humrep/deq332]
 - 17 **Chapron C**, Chopin N, Borghese B, Malartic C, Decuypere F, Foulot H. Surgical management of deeply infiltrating endometriosis: an update. *Ann N Y Acad Sci* 2004; **1034**: 326-337 [PMID: 15731323]
 - 18 **Abrao MS**, Gonçalves MO, Dias JA, Podgaec S, Chamie LP, Blasbalg R. Comparison between clinical examination, transvaginal sonography and magnetic resonance imaging for the diagnosis of deep endometriosis. *Hum Reprod* 2007; **22**: 3092-3097 [PMID: 17947378]
 - 19 **Seracchioli R**, Poggioli G, Pierangeli F, Manuzzi L, Gualerzi B, Savelli L, Remorgida V, Mabrouk M, Venturoli S. Surgical outcome and long-term follow up after laparoscopic rectosigmoid resection in women with deep infiltrating endometriosis. *BJOG* 2007; **114**: 889-895 [PMID: 17501958]
 - 20 **Wills HJ**, Reid GD, Cooper MJ, Morgan M. Fertility and pain outcomes following laparoscopic segmental bowel resection for colorectal endometriosis: a review. *Aust N Z J Obstet Gynaecol* 2008; **48**: 292-295 [PMID: 18532961 DOI: 10.1111/j.1479-828X.2008.00871.x]
 - 21 **Coronado C**, Franklin RR, Lotze EC, Bailey HR, Valdés CT. Surgical treatment of symptomatic colorectal endometriosis. *Fertil Steril* 1990; **53**: 411-416 [PMID: 2307243]
 - 22 **Tran KT**, Kuijpers HC, Willemsen WN, Bulten H. Surgical treatment of symptomatic rectosigmoid endometriosis. *Eur J Surg* 1996; **162**: 139-141 [PMID: 8639727]
 - 23 **De Cicco C**, Corona R, Schonman R, Mailova K, Ussia A, Koninckx P. Bowel resection for deep endometriosis: a systematic review. *BJOG* 2011; **118**: 285-291 [PMID: 21040395 DOI: 10.1111/j.1471-0528.2010.02744.x]
 - 24 **Possover M**, Diebolder H, Plaul K, Schneider A. Laparoscopically assisted vaginal resection of rectovaginal endometriosis. *Obstet Gynecol* 2000; **96**: 304-307 [PMID: 10960302]
 - 25 **Keckstein J**, Wiesinger H. Deep endometriosis, including intestinal involvement--the interdisciplinary approach. *Minim Invasive Ther Allied Technol* 2005; **14**: 160-166 [PMID: 16754158]
 - 26 **Duepre HJ**, Senagore AJ, Delaney CP, Marcello PW, Brady KM, Falcone T. Laparoscopic resection of deep pelvic endometriosis with rectosigmoid involvement. *J Am Coll Surg* 2002; **195**: 754-758 [PMID: 12495306]
 - 27 **Ruffo G**, Scopelliti F, Scioscia M, Ceccaroni M, Mainardi P, Minelli L. Laparoscopic colorectal resection for deep infiltrating endometriosis: analysis of 436 cases. *Surg Endosc* 2010; **24**: 63-67 [PMID: 19466489 DOI: 10.1007/s00464-009-0517-0]
 - 28 **Darai E**, Ackerman G, Bazot M, Rouzier R, Dubernard G. Laparoscopic segmental colorectal resection for endometriosis: limits and complications. *Surg Endosc* 2007; **21**: 1572-1577 [PMID: 17342560]
 - 29 **Meuleman C**, D'Hoore A, Van Cleynenbreugel B, Beks N, D'Hooghe T. Outcome after multidisciplinary CO2 laser laparoscopic excision of deep infiltrating colorectal endometriosis. *Reprod Biomed Online* 2009; **18**: 282-289 [PMID: 19192351]
 - 30 **Dubernard G**, Piketty M, Rouzier R, Houry S, Bazot M, Darai E. Quality of life after laparoscopic colorectal resection for endometriosis. *Hum Reprod* 2006; **21**: 1243-1247 [PMID: 16439504]
 - 31 **Mangler M**, Loddenkemper C, Lanowska M, Bartley J, Schneider A, Köhler C. Histopathology-based combined surgical approach to rectovaginal endometriosis. *Int J Gynaecol Obstet* 2008; **103**: 59-64 [PMID: 18721921 DOI: 10.1016/j.ijgo.2008.06.009]
 - 32 **Armengol-Debeir L**, Savoye G, Leroi AM, Gourcerol G, Savoye-Collet C, Tuech JJ, Vassilief M, Roman H. Pathophysiological approach to bowel dysfunction after segmental colorectal resection for deep endometriosis infiltrating the rectum: a preliminary study. *Hum Reprod* 2011; **26**: 2330-2335 [PMID: 21705371 DOI: 10.1093/humrep/der190]
 - 33 **Brouwer R**, Woods RJ. Rectal endometriosis: results of radical excision and review of published work. *ANZ J Surg* 2007; **77**: 562-571 [PMID: 17610695]
 - 34 **Carmona F**, Martínez-Zamora A, González X, Ginés A, Buñesch L, Balasch J. Does the learning curve of conservative laparoscopic surgery in women with rectovaginal endometriosis impair the recurrence rate? *Fertil Steril* 2009; **92**: 868-875 [PMID: 18829016 DOI: 10.1016/j.fertnstert.2008.07.1738]
 - 35 **Haggag H**, Solomayer E, Juhasz-Böss I. The treatment of rectal endometriosis and the role of laparoscopic surgery. *Curr Opin Obstet Gynecol* 2011; **23**: 278-282 [PMID: 21666468 DOI: 10.1097/GCO.0b013e328348a25b]
 - 36 **Meuleman C**, Tomassetti C, D'Hoore A, Van Cleynenbreugel B, Koninckx F, Vergote I, D'Hooghe T. Surgical treatment of deeply infiltrating endometriosis with colorectal involvement. *Hum Reprod Update* 2011; **17**: 311-326 [PMID: 21233128 DOI: 10.1093/humupd/dmq057]

- 37 **Trencheva K**, Morrissey KP, Wells M, Mancuso CA, Lee SW, Sonoda T, Michelassi F, Charlson ME, Milsom JW. Identifying important predictors for anastomotic leak after colon and rectal resection: prospective study on 616 patients. *Ann Surg* 2013; **257**: 108-113 [PMID: 22968068 DOI: 10.1097/SLA.0b013e318262a6cd]
- 38 **Masoni L**, Mari FS, Nigri G, Favi F, Gasparrini M, Dall'Oglio A, Pindozzi F, Pancaldi A, Brescia A. Preservation of the inferior mesenteric artery via laparoscopic sigmoid colectomy performed for diverticular disease: real benefit or technical challenge: a randomized controlled clinical trial. *Surg Endosc* 2013; **27**: 199-206 [PMID: 22733197 DOI: 10.1007/s00464-012-2420-3]

P-Reviewer: La Torre F **S-Editor:** Ma YJ **L-Editor:** Rutherford A
E-Editor: Liu XM



Observational Study

Characteristics of symptomatic reflux episodes in Japanese proton pump inhibitor-refractory non-erosive reflux disease patients

Kenichiro Nakagawa, Tomoyuki Koike, Katsunori Iijima, Masahiro Saito, Hiroki Kikuchi, Waku Hatta, Nobuyuki Ara, Kaname Uno, Naoki Asano, Tooru Shimosegawa

Kenichiro Nakagawa, Tomoyuki Koike, Katsunori Iijima, Masahiro Saito, Hiroki Kikuchi, Waku Hatta, Nobuyuki Ara, Kaname Uno, Naoki Asano, Tooru Shimosegawa, Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai Miyagi 980-8574, Japan

Author contributions: Nakagawa K and Koike T performed the literature search and wrote the manuscript; Koike T and Iijima K participated in drafting the paper and performing critical revision for important intellectual content; Nakagawa K, Koike T and Iijima K performed the statistical analysis; Iijima K, Saito M, Kikuchi H, Hatta W, Ara N, Uno K, Asano N and Shimosegawa T interpreted the data; Shimosegawa T approved the final draft submitted.

Institutional review board statement: This study was approved by the Tohoku University Hospital Ethics Committee.

Informed consent statement: Informed consent was obtained from all subjects.

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

Data sharing statement: The technical appendix, statistical code, and dataset are available from the corresponding author at tkoike@rd5.so-net.ne.jp.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Tomoyuki Koike, MD, PhD, Division of Gastroenterology, Tohoku University Graduate School of

Medicine, 1-1 Seiryō-machi Aoba-ku, Sendai Miyagi 980-8574, Japan. tkoike@rd5.so-net.ne.jp
Telephone: +81-22-7177171
Fax: +81-22-7177177

Received: February 23, 2015
Peer-review started: February 24, 2015
First decision: March 26, 2015
Revised: April 13, 2015
Accepted: September 30, 2015
Article in press: September 30, 2015
Published online: December 21, 2015

Abstract

AIM: To clarify the pathogenesis of gastroesophageal reflux disease symptoms in non-erosive reflux disease (NERD) patients.

METHODS: Thirty-five NERD patients with persistent symptoms, despite taking rabeprazole 10 mg twice daily for at least 8 wk, were included in this study. All patients underwent 24 h combined impedance - pH on rabeprazole. The symptom index (SI) was considered to be positive if $\geq 50\%$, and proximal reflux episodes were determined when reflux reached 15 cm above the proximal margin of the lower esophageal sphincter.

RESULTS: In 14 (40%) SI-positive patients, with liquid weakly acid reflux, the occurrence rate of reflux symptoms was significantly more frequent in proximal reflux episodes (46.7%) than in distal ones (5.7%) ($P < 0.001$). With liquid acid reflux, there were no significant differences in the occurrence rate of reflux symptoms between proximal reflux episodes (38.5%) and distal ones (20.5%) (NS). With mixed liquid-

gas weakly acid reflux, the occurrence rate of reflux symptoms in proximal reflux episodes was significantly more frequent (31.0%) than in distal reflux ones (3.3%) ($P < 0.001$). With mixed liquid-gas acid reflux, there were no significant differences in the occurrence rate of reflux symptoms between proximal reflux episodes (29.4%) and distal ones (14.3%) (NS).

CONCLUSION: The proximal extent of weakly acidic liquid and mixed liquid-gas reflux is a major factor associated with reflux perception in SI-positive patients on proton pump inhibitor therapy.

Key words: Proton pump inhibitor; Symptomatic reflux episodes; Proximal reflux; Non-erosive reflux disease; Impedance-pH monitoring

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Thirty-five non-erosive reflux disease patients with persistent symptoms, despite taking rabeprazole 10 mg twice daily, were included. In 14 (40%) symptom index (SI)-positive patients, with liquid weakly acid reflux, the occurrence rate of reflux symptoms in proximal reflux episodes was significantly more frequent (46.7%) than in distal ones (5.7%) ($P < 0.001$). With mixed liquid-gas weakly acid reflux, the occurrence rate of reflux symptoms in proximal reflux episodes was significantly more frequent (31.0%) than in distal reflux ones (3.3%) ($P < 0.001$). The proximal extent of weakly acidic liquid and mixed liquid-gas reflux is a major factor of reflux perception in SI-positive patients on proton pump inhibitor (PPI).

Nakagawa K, Koike T, Iijima K, Saito M, Kikuchi H, Hatta W, Ara N, Uno K, Asano N, Shimosegawa T. Characteristics of symptomatic reflux episodes in Japanese proton pump inhibitor-refractory non-erosive reflux disease patients. *World J Gastroenterol* 2015; 21(47): 13352-13359. Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13352.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13352>

INTRODUCTION

The reflux of gastric juice containing acid, bile, and pepsin into the esophagus is a causal factor of gastroesophageal reflux disease (GERD). It was reported that the incidence of GERD has increased significantly in each of the last two decades in Western countries^[1]. The incidence of GERD is also increasing in Japan as well^[2] because of the decreasing prevalence of *Helicobacter pylori* (*H. pylori*) infection and the Westernization of the diet in recent decades.

Proton pump inhibitors (PPIs) are commonly used as the first-line treatment because of their high effectiveness and prolonged duration in suppressing of gastric acid secretion. Several studies have demonstrated that non-erosive reflux disease (NERD)

patients are less sensitive to PPI treatment than patients with erosive reflux disease (ERD)^[3-5]. Patients with NERD are hypersensitive to visceral stimulation, which is characterized by a reduced pain threshold to experimental stimulation^[6,7].

With a combined pH and multiple intraluminal impedance (MII-pH) technique that can detect GERD irrespective of acidity, reflux with a nadir pH between 4 and 7 (weakly acidic reflux) and with a nadir pH above 7 (alkaline reflux) can be detected^[8]. In Western countries, several studies have reported using MII-pH monitoring in GERD patients^[9-11], but the pathogenesis of heartburn and acid regurgitation has not yet been fully elucidated in PPI-refractory NERD patients on PPI.

Few studies have evaluated the association between reflux symptoms and reflux episodes, and reflux episodes that are liquid reflux and mixed liquid-gas reflux in PPI-refractory NERD patients on PPI. Therefore, the aim of this study was to clarify reflux episodes, the occurrence of reflux symptoms, and the pH values at each impedance site by classifying them into acid reflux and weakly acid reflux.

MATERIALS AND METHODS

From August 2011 to April 2014, 35 consecutive NERD patients [14 men; median age, 65 (21-76) years] with persistent symptoms suggestive of gastro-esophageal reflux despite taking rabeprazole 10 mg twice daily for at least 8 wk were included in this study. Patients with troublesome GERD symptoms in the absence of visible esophageal mucosal injury at endoscopy without taking off PPI were defined as NERD. In fact, patients' symptoms were assessed using a questionnaire for the diagnosis of reflux disease, the frequency scale for symptoms of gastro-esophageal reflux disease (FSSG)^[12]. PPI-refractory NERD patients were so defined if their FSSG scores were higher than 8, and they had persistently troublesome GERD symptoms, such as heartburn or regurgitation, even while taking a double dose of PPI (rabeprazole, 10 mg bid) for at least 8 wk. All PPI-refractory NERD patients were administered 24 h combined MII-pH on PPI. "Heavy alcohol use" was defined as consuming more than 75 mL ethanol per day.

Subjects with peptic ulcer disease, malignant disease, a history of previous esophagogastric surgery, or who had taken antibiotics and prokinetic drugs prior to the endoscopic examination were excluded from the analysis. This study was approved by the Tohoku University Hospital Ethics Committee. Informed consent was obtained from all subjects, and the study was conducted according to the provisions of the Declaration of the Helsinki. No complications occurred in any patients during this study.

Endoscopic findings

All patients underwent esophagogastroduodenoscopy. Hiatus hernia was diagnosed when the distance between

the esophagogastric junction and the diaphragmatic hiatus was ≥ 2 cm. Gastric atrophy was endoscopically assessed by the pattern system described by Kimura and Takemoto^[13]. The extent of atrophy was classified as either closed or open according to their classification. The closed type is defined as having an atrophic border restricted to the lesser curvature of the stomach, whereas the open type is when the atrophic border is absent on the lesser curvature but extends along the anterior and posterior walls of the stomach. In this study, "Gastric atrophy" was defined as the open type.

Evaluation of *H. pylori* infection

Serum IgG antibodies against *H. pylori* were measured by enzyme linked immunosorbent assay using an EIA kit (E Plate "Eiken" *H. pylori*-antibody, Eiken Chemical Co. Ltd., Tokyo, Japan). Patients were considered to be infected with *H. pylori* if their serum test values were greater than 9.90 U/mL.

MII-pH catheter characteristics and placement

The data of intraluminal electrical impedance were recorded with a 2.1 mm in diameter polyvinyl assembly containing a series of cylindrical electrodes spaced at 2 cm intervals (ConforTec MII/pH, Sandhill Scientific, Highland Ranch, CO, United States). Each pair of electrodes formed a measuring segment corresponding to one recording channel (Sandhill Scientific, Highland Ranch, CO, United States). The signals from six impedance channels and two pH channels were recorded at 50 samples per second.

The data were stored in a portable recorder and saved on a 256 Mb Compact Flash card (San Disk, Milpitas, CA, United States). The esophageal pH level and gastric pH level were measured using an antimony pH electrode. pH electrodes were calibrated using pH 4.0 and pH 7.0 buffer solutions before beginning the recording.

Patients were studied after an overnight fast of at least 10 h. After stationary esophageal manometry to locate the lower esophageal sphincter (LES), the pH-MII was passed through the nose under topical anesthesia and positioned with the pH electrodes at 5 cm above the proximal border of the LES and 10 cm below the LES. In this position, impedance was measured at 3, 5, 7, 9, 15, and 17 cm proximal to the LES. Once the desired location was achieved, the catheter was taped to the patient's nose and connected to a recording device on the patient's belt, and the impedance and pH data were recorded and stored. Patients were encouraged to maintain normal activity, sleep schedules, and usual meals at regular times. Subjects were in the upright position as much as possible during the daytime and were allowed free movement and one recumbent period. All subjects had three usual meals at regular times without between-meal eating during the measurement period. All

events during testing, including meals, symptoms, and body position (upright/recumbent), were entered by the patient directly into the monitor. Portable MII-pH monitoring was performed over 24 h, after which patients returned to the testing laboratory for catheter removal.

MII-pH interpretation

The data stored on the Compact Flash card were downloaded onto a personal computer and visually analyzed with the assistance of dedicated software, Bioview analysis version 5.4.3 (Sandhill Scientific). Each recording was manually reviewed, edited, and analyzed independently by two authors (K.N. and T.K.). Discordant readings were reviewed until consensus was reached. Reflux episodes were classified according to previously described criteria^[14]. Liquid reflux was defined as a retrograde 50% drop in impedance starting distally (above the LES) and propagating at least to the next two more proximal impedance measuring segments. Gas reflux was defined as a rapid (3000 Ω /s) increase in impedance, occurring simultaneously in at least two esophageal measuring segments, in the absence of swallowing. Mixed liquid-gas reflux was defined as gas reflux occurring immediately before or during a liquid reflux. Reflux episodes consisting of only gaseous reflux, which appears as a simultaneous increase in impedance $> 3000 \Omega$, in any two consecutive sites were excluded from the study. Reflux events were divided into three subcategories based on the pH level at the point of reflux: acid reflux (reflux episodes that reduced the pH of the esophagus to below 4 or that occurred when the esophageal pH was already below 4), weakly acid reflux (an impedance-detected reflux event occurring when the esophageal pH was between $4 < \text{pH} < 7$), and weakly alkaline reflux (a reflux episode during which the pH did not drop below 7)^[8]. The numbers and duration of total reflux events, acid reflux, and weakly acid reflux were analyzed, whereas meal periods (three periods of about 20 min each) were excluded from the analysis. A reflux episode was considered to be proximal reflux if the impedance indicated liquid and mixed liquid-gas reflux at 15 cm above the LES, and distal reflux if reflux was below that level.

Symptoms were considered to be associated with reflux if reflux episodes were detected 5 min prior to the symptom occurring. The symptom index (SI) was calculated for each patient in relation to liquid reflux and mixed liquid-gas reflux. SI was defined as the ratio of the number of symptoms related to the reflux to the total number of symptoms^[15]. The relationship between reflux symptoms and reflux episodes was analyzed only in SI positive patients.

Pathological acid reflux is present when the intra-esophageal pH is below 4 for greater than 4.2% of the time^[16].

Table 1 Clinicopathological features *n* (%)

	SI positive	SI negative	<i>P</i> value
No. patients	14 (40.0)	21 (60.0)	NS ¹
Age (SD)	61.9 (12.3)	59.3 (15.6)	NS ¹
Gender (male/female)	6/8	7/14	NS ²
Heavy drinker	3 (21.4)	4 (19.0)	NS ²
Current smoker	2 (14.3)	1 (4.8)	NS ²
BMI	21.1 (2.1)	21.0 (2.3)	NS ²
Hiatus hernia	3 (21.4)	8 (38.1)	NS ²
<i>Helicobacter pylori</i>	4 (28.5)	6 (28.6)	NS ²
Gastric atrophy	6 (42.9)	7 (33.3)	NS ²
FSSG score (SD)	16.7 (8.7)	23.1 (9.0)	0.0437 ³

¹Unpaired *t* test; ²Fisher's exact test; ³Mann-Whitney *U*-test; SI: Symptom index; SD: Standard deviation; BMI: Body mass index.

Statistical analysis

Parametric data were expressed as mean \pm standard deviation (SD) and non-parametric data as median (interquartile range). The frequencies of different impedance/pH patterns of reflux in each group were analyzed and compared using Mann - Whitney *U*-test. Correlations among intragastric pH levels and the numbers of total reflux events, acid reflux, and non-acid reflux, were analyzed by Spearman's rank correlation. Fisher's exact test was used to compare the categorical variables in the clinicopathological features between SI positive and negative. A *P* value of < 0.05 was considered to be significant. Analyses were carried out using SPSS software (version 11.0; Chicago, IL, United States).

RESULTS

Patient population

Comparisons of related parameters between the SI-positive group and the SI-negative group are shown in Table 1. Of the 35 NERD patients, 14 (40.0%) were SI-positive, and 21 (60.0%) were SI-negative. There were no significant differences in gender, heavy alcohol use, current smokers, body mass index, presence of esophageal hiatus hernia, gastric atrophy, or prevalence of *H. pylori* infection. The mean FSSG score of the SI-negative group (23.1 ± 9.0) was significantly higher than that of the SI-positive group (16.7 ± 8.7) ($P = 0.0437$). Five patients (three patients with SI positive and two patients with SI negative) had abnormal esophageal acid exposure times.

In the 14 patients with a positive SI, 589 liquid and mixed liquid-gas reflux episodes were recorded. Amongst these, 94 (16.0%) were acid reflux, 477 (80.9%) were weakly acidic reflux, and 18 (3.1%) were weakly alkaline reflux.

In the SI-positive patients, a total of 383 liquid reflux episodes with acid reflux and weakly acid reflux were detected. Overall, 51 (13.3%) reflux episodes were symptomatic, including 14 (27.5%) acid and 37 (72.5%) weakly acidic. The total reflux episodes and symptoms above the proximal margin of the LES at

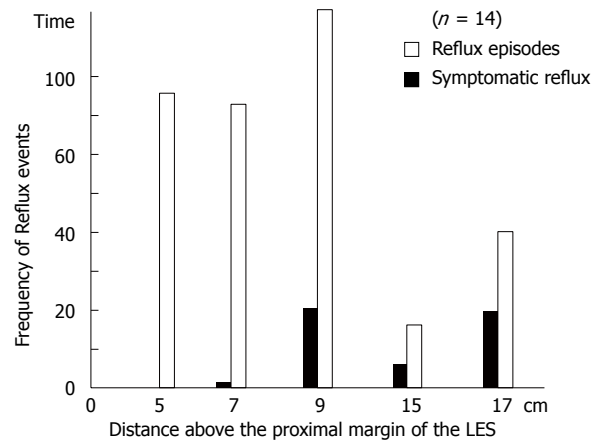


Figure 1 Total liquid reflux episodes and symptoms above the proximal margin of the lower esophagogastric junction at each impedance site. The 325 distal reflux episodes were significantly more frequent (84.9%) than the 58 proximal ones (15.1%) ($P < 0.001$, distal reflux episodes vs proximal ones). LES: Lower esophagogastric junction.

each impedance site are shown in Figure 1. The 325 distal reflux episodes were significantly more frequent (84.9%) than the 58 proximal ones (15.1%) ($P < 0.001$). The occurrence rate of reflux symptoms above the proximal margin of the LES at each impedance site is shown in Figure 2A. The occurrence rate of reflux symptoms in proximal reflux episodes was significantly more frequent (44.8%) than in distal ones (7.7%) ($P < 0.001$). With liquid acid reflux, there were no significant differences in the occurrence rate of reflux symptoms between proximal reflux episodes (38.5%) and distal ones (20.5%) (NS) (Figure 2B). On the other hand, with liquid weakly acid reflux, the occurrence rate of reflux symptoms in proximal reflux episodes was significantly more frequent (46.7%) than in distal ones (5.7%) ($P < 0.001$) (Figure 2C).

A total of 188 mixed liquid-gas reflux episodes with acid reflux and weakly acidic were detected, and 29 (15.4%) were symptomatic. The total reflux episodes and symptoms above the proximal margin of the LES at each impedance site are shown in Figure 3. Distal reflux episodes were significantly more frequent (60.1%) than proximal reflux ones (39.9%) ($P = 0.0047$). The occurrence rate of reflux symptoms above the proximal margin of the LES at each impedance site is shown in Figure 2D. The occurrence rate of reflux symptoms in proximal reflux episodes was significantly more frequent (30.7%) than in distal ones (5.3%) ($P < 0.001$). In addition, when classifying acid reflux and weakly acid reflux, as simply acid reflux, there were no significant differences in the occurrence rate of reflux symptoms between proximal reflux episodes (29.4%) and distal ones (14.3%) (NS) (Figure 2E). On the other hand, with weakly acid reflux, the occurrence rate of reflux symptoms in proximal reflux episodes was significantly more frequent (31.0%) than in distal ones (3.3%) ($P < 0.001$) (Figure 2F).

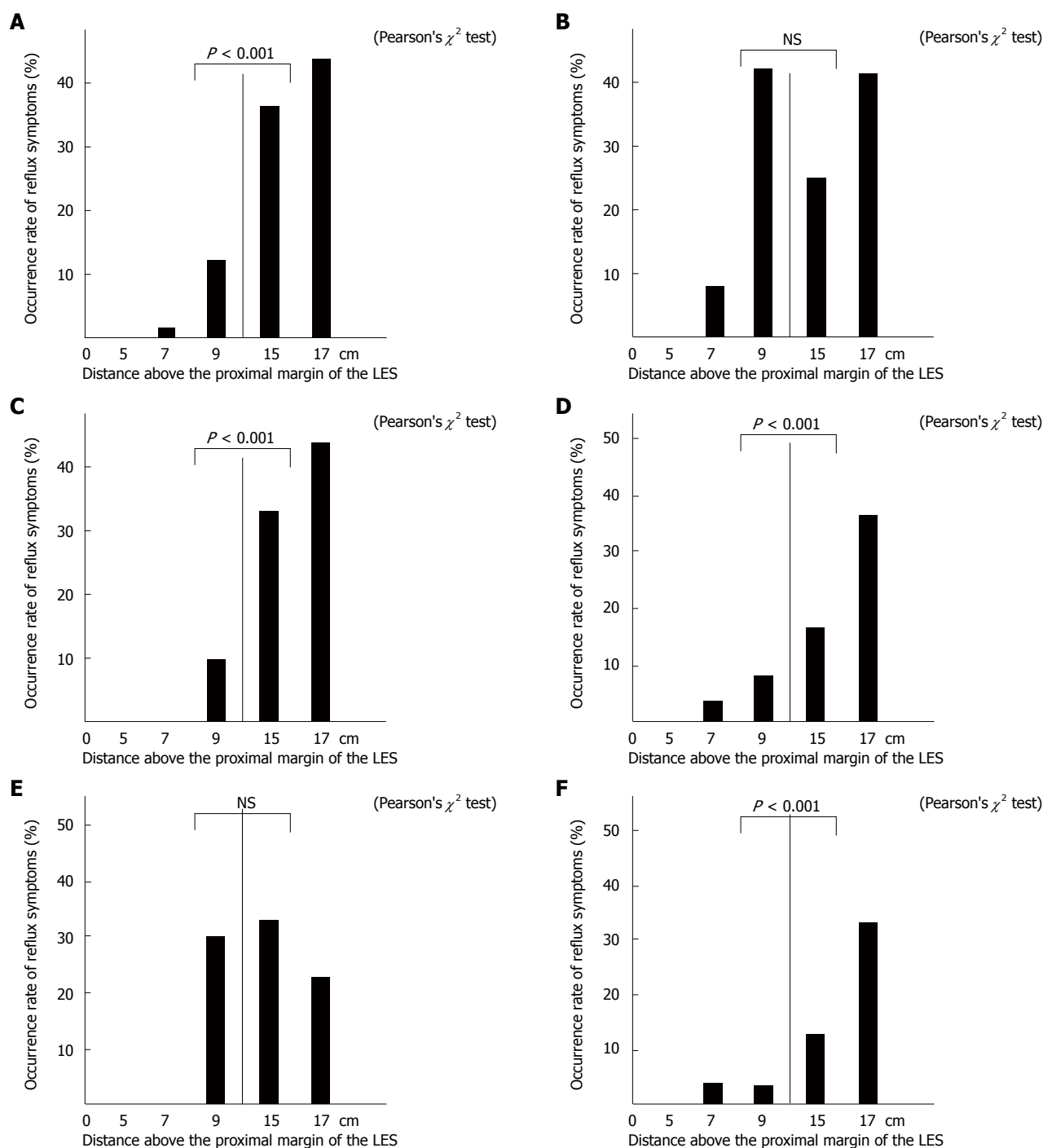


Figure 2 Occurrence rates of reflux symptoms. A: Occurrence rates of reflux symptoms above the proximal margin of the lower esophagogastric junction at each impedance site with liquid reflux. The occurrence rate of reflux symptoms in proximal reflux episodes was significantly more frequent (44.8%) than in distal ones (7.7%) ($P < 0.001$, proximal reflux episodes vs distal ones); B: Occurrence rates of reflux symptoms above the proximal margin of the lower esophagogastric junction at each impedance site with acid reflux. There were no significant differences in the occurrence rate of reflux symptoms between proximal reflux episodes (38.5%) and distal ones (20.5%) (NS); C: Occurrence rates of reflux symptoms above the proximal margin of the lower esophagogastric junction at each impedance site with liquid weakly acid reflux. The occurrence rate of reflux symptoms in proximal reflux episodes was significantly more frequent (46.7%) than in distal ones (5.7%) ($P < 0.001$, proximal reflux episodes vs distal ones); D: Occurrence rates of reflux symptoms above the proximal margin of the lower esophagogastric junction at each impedance site with mixed gas-liquid reflux. The occurrence rate of reflux symptoms in proximal reflux episodes was significantly more frequent (30.7%) than in distal ones (5.3%) ($P < 0.001$, proximal reflux episodes vs distal ones); E: Occurrence rates of reflux symptoms above the proximal margin of the lower esophagogastric junction at each impedance site with mixed liquid-gas acid reflux. There were no significant differences in the occurrence rate of reflux symptoms between the proximal reflux episodes (29.4%) and distal ones (14.3%) (NS); F: Occurrence rates of reflux symptoms above the proximal margin of the lower esophagogastric junction at each impedance site with mixed liquid-gas weakly acid reflux. The occurrence rate of reflux symptoms in proximal reflux episodes was significantly more frequent (31.0%) than in distal ones (3.3%) ($P < 0.001$, proximal reflux episodes vs distal ones). LES: Lower esophagogastric junction.

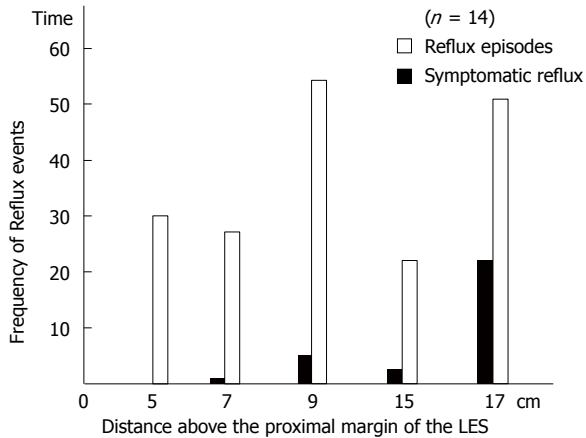


Figure 3 Total mixed liquid-gas reflux episodes and symptoms above the proximal margin of the lower esophagogastric junction at each impedance site. The 113 distal reflux episodes were significantly more frequent (60.1%) than 35 proximal reflux (39.9%) ($P = 0.0047$, distal reflux episodes vs proximal reflux). LES: Lower esophagogastric junction.

DISCUSSION

In Western countries, 20 mg of rabeprazole is the standard dose for GERD patients, whereas in Japan, 20 mg rabeprazole is used as a double dose PPI therapy for such patients. Therefore, patients with persistent symptoms despite using 20 mg rabeprazole were considered to be PPI-refractory NERD patients in Japan.

In this study, the relationship between reflux symptoms and reflux episodes were analyzed only in SI positive patients. SI negative patients did not show a relationship between reflux symptoms and reflux episodes because they were not analyzed.

Of the 35 NERD patients, 14 (40.0%) were SI-positive and 21 (60.0%) were SI-negative. SI-negative is characterized by functional heartburn (FH), which is generally defined according to the Rome III criteria as the presence of reflux symptoms with no evidence of symptomatic reflux by MII-pH monitoring^[17].

The FSSG score, which is widely used for the diagnosis of GERD in Japan, assesses the 12 symptoms that Japanese patients with GERD complain of most often, not only heartburn but also dyspeptic symptoms, such as "heavy stomach" and "feeling full quickly"^[12,18,19]. In the present study, the FSSG score in the SI-negative group was significantly higher than that in the SI-positive group because patients with FH had a tendency to be more sensitive both to heartburn and dyspeptic symptoms than those who were SI positive.

In our study, 94 of total reflux episodes (16.0%) were acid reflux, 477 (80.9%) were weakly acidic reflux, and 18 (3.1%) were weakly alkaline reflux. Therefore, weakly acid reflux is a major factor in the reflux symptoms of SI positive patients with NERD, despite taking a double-dose of PPI. These results are similar to previous results reported from Western countries^[20,21] and Japan^[22,23].

A previous study suggested that the level of reflux might play an important role in the symptoms of GERD, including NERD patients^[10,20-23]. In Western countries, it has been reported that a high proximal extent of the refluxate is an important factor associated with reflux perception in patients with GERD off-PPI therapy^[10,13] and on-PPI therapy^[24,25]. In Japan, it was reported that proximal liquid migration was associated with symptomatic reflux in PPI-refractory NERD patients^[22,23]. However, few studies have evaluated in Japan the relationship between reflux symptoms and reflux episodes by comparing liquid reflux and mixed liquid-gas reflux in PPI-refractory NERD. In addition, few studies have classified reflux episodes and the occurrence of reflux symptoms at each impedance site into acid reflux and weakly acid reflux.

In our study, with liquid reflux, the occurrence rate of reflux symptoms in the proximal reflux episodes was significantly more frequent (44.8%) than in distal ones (7.7%). With liquid weakly acid reflux, the occurrence rate of reflux symptoms in proximal reflux episodes was significantly more frequent (46.7%) than in distal ones (5.7%). With mixed liquid-gas weakly acid reflux, the occurrence rate of reflux symptoms in proximal reflux episodes was significantly more frequent (31.0%) than in distal ones (3.3%). As supported by previous studies, the proximal extent of reflux with liquid weakly acid reflux is a major factor in the perception of reflux^[22,23]. The etiology of the association between the symptoms and the proximal extent of gastroesophageal reflux still remains to be fully elucidated. In a previous study, a secondary mechanical hyperalgesia of the proximal esophagus (not exposed to acid) was demonstrated following acid perfusion of the distal esophagus^[26]. Additionally, dilation of the intercellular spaces of the esophageal epithelium, first demonstrated in the distal esophagus and proposed as a mechanism of reflux perception in NERD, has since been demonstrated in the proximal esophagus by Caviglia *et al*^[27]. Additionally, the proximal esophagus might have a larger number of mechanoreceptors than the distal esophagus as a result of dilatation of the intercellular spaces of the epithelium^[28]. In NERD patients, the presence of gas in the refluxate significantly enhances the probability of reflux perception^[13]. Concerning mixed liquid-gas reflux, clarification of these mechanisms could be useful. However, with liquid acid reflux, there were no significant differences in the occurrence rate of reflux symptoms between proximal reflux episodes (38.5%) and distal ones (20.5%). With mixed liquid-gas acid reflux, there were no significant differences in the occurrence rate of reflux symptoms between proximal reflux episodes (29.4%) and distal ones (14.3%). The occurrence rate of reflux symptoms was more frequent in the proximal esophagus with weakly acid reflux, although it was more frequent in distal esophagus as well as proximal esophagus with acid reflux. Therefore, acid reflux is an important factor even in distal

esophageal reflux in PPI-refractory NERD patients. Acid reflux causes macroscopic damage to the esophageal wall, which is a stimulating factor that contributes to the reflux symptoms^[29]. Even if the proportion of acid reflux is less than that of weakly acid reflux, acid reflux is one of the important factors in the perception of reflux with insufficient acid suppressive therapy in NERD patients.

In this study, the subjects did not have standardized meals during the measurement period because we wanted to evaluate reflux conditions in their usual daily activity.

Reflux episodes consisting of gas reflux events without liquid (belches) and weakly alkaline were not considered for the purpose of this study. We did not include the relationship between gas-only reflux episodes and symptoms because of differences in the mechanisms and therapeutic approaches for liquid-containing and gas-only reflux episodes. Furthermore, the frequency of symptomatic pure gas reflux and weakly alkaline was very low and, therefore, not further analyzed.

In conclusion, the proximal extent of weakly acidic liquid and mixed liquid-gas reflux are major factors associated with reflux perception in SI-positive patients on PPI therapy. In acid reflux that includes gas, reflux to the distal esophagus is associated with reflux perception in SI-positive patients with PPI-refractory NERD.

COMMENTS

Background

The pathogenesis of heartburn and acid regurgitation in non-erosive reflux disease (NERD) patients still remains to be fully elucidated.

Research frontiers

Several studies have demonstrated that NERD patients are less sensitive to proton pump inhibitor (PPI) treatment than patients with erosive reflux disease. With a combined pH and multiple intraluminal impedance (MII-pH) technique that can detect gastroesophageal reflux disease (GERD) irrespective of acidity, reflux with a nadir pH between 4 and 7 (weakly acidic reflux) and with a nadir pH above 7 (alkaline reflux) can also be detected.

Innovations and breakthroughs

In Western countries, several studies have reported using MII-pH monitoring in GERD patients, but the pathogenesis of heartburn and acid regurgitation has not yet been fully elucidated in PPI-refractory NERD patients on PPI. Few studies have evaluated the association between reflux symptoms and reflux episodes, and reflux episodes that are liquid reflux and mixed liquid-gas reflux in these patients. The proximal extent of weakly acidic liquid and mixed liquid-gas reflux is a major factor associated with reflux perception in symptom index (SI)-positive patients on PPI therapy.

Applications

The proximal extent of weakly acidic liquid and mixed liquid-gas reflux is a major factor associated with reflux perception in SI-positive patients on PPI therapy. Additionally, with acid reflux that includes gas, reflux to the distal esophagus is associated with reflux perception in SI-positive patients with PPI-refractory NERD.

Terminology

PPIs are commonly used as the first-line treatment because of their high

effectiveness and prolonged duration in suppressing gastric acid secretion. Several studies have demonstrated that NERD patients are less sensitive to PPI treatment than patients with erosive reflux disease. Patients with NERD are hypersensitive to visceral stimulation, which is characterized by a reduced pain threshold to experimental stimulation. With a combined MII-pH technique that can detect GERD irrespective of acidity, reflux with a nadir pH between 4 and 7 (weakly acidic reflux) and with a nadir pH above 7 (alkaline reflux) can also be detected. The SI was calculated for each patient in relation to liquid reflux and mixed liquid-gas reflux. SI was defined as the ratio of the number of symptoms related to the reflux to the total number of symptoms.

Peer-review

This is an interesting and carefully conducted study, which sheds some light into the nature of NERD. The aim of this study was to clarify reflux episodes, the occurrence of reflux symptoms, and the pH values at each impedance site by classifying them into acid reflux and weakly acid reflux. The authors concluded that the proximal extent of weakly acidic liquid and mixed liquid-gas reflux is a major factor associated with reflux perception in SI-positive patients on PPI therapy.

REFERENCES

- 1 **El-Serag HB.** Time trends of gastroesophageal reflux disease: a systematic review. *Clin Gastroenterol Hepatol* 2007; **5**: 17-26 [PMID: 17142109 DOI: 10.1016/j.cgh.2006.09.016]
- 2 **Fujiwara Y, Arakawa T.** Epidemiology and clinical characteristics of GERD in the Japanese population. *J Gastroenterol* 2009; **44**: 518-534 [PMID: 19365600 DOI: 10.1007/s00535-009-0047-5]
- 3 **Tew S, Jamieson GG, Pilowsky I, Myers J.** The illness behavior of patients with gastroesophageal reflux disease with and without endoscopic esophagitis. *Dis Esophagus* 1997; **10**: 9-15 [PMID: 9079267]
- 4 **Lind T, Havelund T, Carlsson R, Anker-Hansen O, Glise H, Hernqvist H, Junghard O, Lauritsen K, Lundell L, Pedersen SA, Stubberød A.** Heartburn without oesophagitis: efficacy of omeprazole therapy and features determining therapeutic response. *Scand J Gastroenterol* 1997; **32**: 974-979 [PMID: 9361168 DOI: 10.3109/00365529709011212]
- 5 **Richter JE, Kovacs TO, Greski-Rose PA, Huang section sign B, Fisher R.** Lansoprazole in the treatment of heartburn in patients without erosive oesophagitis. *Aliment Pharmacol Ther* 1999; **13**: 795-804 [PMID: 10383510 DOI: 10.1046/j.1365-2036.1999.00558.x]
- 6 **Trimble KC, Pryde A, Heading RC.** Lowered oesophageal sensory thresholds in patients with symptomatic but not excess gastro-oesophageal reflux: evidence for a spectrum of visceral sensitivity in GORD. *Gut* 1995; **37**: 7-12 [PMID: 7672684 DOI: 10.1136/gut.37.1.7]
- 7 **Rodriguez-Stanley S, Robinson M, Earnest DL, Greenwood-Van Meerveld B, Miner PB.** Esophageal hypersensitivity may be a major cause of heartburn. *Am J Gastroenterol* 1999; **94**: 628-631 [PMID: 10086642 DOI: 10.1111/j.1572-0241.1999.00925.x]
- 8 **Sifrim D, Castell D, Dent J, Kahrilas PJ.** Gastro-oesophageal reflux monitoring: review and consensus report on detection and definitions of acid, non-acid, and gas reflux. *Gut* 2004; **53**: 1024-1031 [PMID: 15194656 DOI: 10.1136/gut.2003.033290]
- 9 **Vela MF, Camacho-Lobato L, Srinivasan R, Tutuian R, Katz PO, Castell DO.** Simultaneous intraesophageal impedance and pH measurement of acid and nonacid gastroesophageal reflux: effect of omeprazole. *Gastroenterology* 2001; **120**: 1599-1606 [PMID: 11375942 DOI: 10.1053/gast.2001.24840]
- 10 **Bredenoord AJ, Weusten BL, Curvers WL, Timmer R, Smout AJ.** Determinants of perception of heartburn and regurgitation. *Gut* 2006; **55**: 313-318 [PMID: 16120760 DOI: 10.1136/gut.2005.074690]
- 11 **Sifrim D, Holloway R, Silny J, Xin Z, Tack J, Lerut A, Janssens J.** Acid, nonacid, and gas reflux in patients with gastroesophageal reflux disease during ambulatory 24-hour pH-impedance recordings. *Gastroenterology* 2001; **120**: 1588-1598 [PMID: 11375942 DOI: 10.1053/gast.2001.24840]

- 11375941 DOI: 10.1053/gast.2001.24841]
- 12 **Kusano M**, Shimoyama Y, Sugimoto S, Kawamura O, Maeda M, Minashi K, Kuribayashi S, Higuchi T, Zai H, Ino K, Horikoshi T, Sugiyama T, Toki M, Ohwada T, Mori M. Development and evaluation of FSSG: frequency scale for the symptoms of GERD. *J Gastroenterol* 2004; **39**: 888-891 [PMID: 15565409 DOI: 10.1007/s00535-004-1417-7]
 - 13 **Kimura K**, Takemoto T. An endoscopic recognition of the atrophic border and its significance in chronic gastritis. *Endoscopy* 1969; **3**: 87-97 [DOI: 10.1055/s-0028-1098086]
 - 14 **Emerenziani S**, Sifrim D, Habib FI, Ribolsi M, Guarino MP, Rizzi M, Caviglia R, Petitti T, Cicala M. Presence of gas in the refluxate enhances reflux perception in non-erosive patients with physiological acid exposure of the oesophagus. *Gut* 2008; **57**: 443-447 [PMID: 17766596 DOI: 10.1136/gut.2007.130104]
 - 15 **Wiener GJ**, Richter JE, Copper JB, Wu WC, Castell DO. The symptom index: a clinically important parameter of ambulatory 24-hour esophageal pH monitoring. *Am J Gastroenterol* 1988; **83**: 358-361 [PMID: 3348191]
 - 16 **Johnson LF**, Demeester TR. Twenty-four-hour pH monitoring of the distal esophagus. A quantitative measure of gastroesophageal reflux. *Am J Gastroenterol* 1974; **62**: 325-332 [PMID: 4432845]
 - 17 **Galmiche JP**, Clouse RE, Bálint A, Cook IJ, Kahrilas PJ, Paterson WG, Smout AJ. Functional esophageal disorders. *Gastroenterology* 2006; **130**: 1459-1465 [PMID: 16678559 DOI: 10.1053/j.gastro.2005.08.060]
 - 18 **Shimoyama Y**, Kusano M, Sugimoto S, Kawamura O, Maeda M, Minashi K, Kuribayashi S, Higuchi T, Zai H, Ino K, Horikoshi T, Moki F, Sugiyama T, Toki M, Ohwada T, Mori M. Diagnosis of gastroesophageal reflux disease using a new questionnaire. *J Gastroenterol Hepatol* 2005; **20**: 643-647 [PMID: 15836717 DOI: 10.1111/j.1440-1746.2005.03776.x]
 - 19 **Kusano M**, Shimoyama Y, Kawamura O, Maeda M, Kuribayashi S, Nagoshi A, Zai H, Moki F, Horikoshi T, Toki M, Sugimoto S, Mori M. Proton pump inhibitors improve acid-related dyspepsia in gastroesophageal reflux disease patients. *Dig Dis Sci* 2007; **52**: 1673-1677 [PMID: 17385034 DOI: 10.1007/s10620-006-9674-3]
 - 20 **Savarino E**, Zentilin P, Tutuian R, Pohl D, Gemignani L, Malesci A, Savarino V. Impedance-pH reflux patterns can differentiate non-erosive reflux disease from functional heartburn patients. *J Gastroenterol* 2012; **47**: 159-168 [PMID: 22038553 DOI: 10.1007/s00535-011-0480-0]
 - 21 **Mainie I**, Tutuian R, Shay S, Vela M, Zhang X, Sifrim D, Castell DO. Acid and non-acid reflux in patients with persistent symptoms despite acid suppressive therapy: a multicentre study using combined ambulatory impedance-pH monitoring. *Gut* 2006; **55**: 1398-1402 [PMID: 16556669 DOI: 10.1136/gut.2005.087668]
 - 22 **Kohata Y**, Fujiwara Y, Machida H, Okazaki H, Yamagami H, Tanigawa T, Watanabe K, Watanabe T, Tominaga K, Arakawa T. Pathogenesis of proton-pump inhibitor-refractory non-erosive reflux disease according to multichannel intraluminal impedance-pH monitoring. *J Gastroenterol Hepatol* 2012; **27** Suppl 3: 58-62 [PMID: 22486873 DOI: 10.1111/j.1440-1746.2012.07074.x]
 - 23 **Iwakiri K**, Sano H, Tanaka Y, Kawami N, Umezawa M, Futagami S, Hoshihara Y, Nomura T, Miyashita M, Sakamoto C. Characteristics of symptomatic reflux episodes in patients with non-erosive reflux disease who have a positive symptom index on proton pump inhibitor therapy. *Digestion* 2010; **82**: 156-161 [PMID: 20588027 DOI: 10.1159/000309483]
 - 24 **Zerbib F**, Duriez A, Roman S, Capdepon M, Mion F. Determinants of gastro-oesophageal reflux perception in patients with persistent symptoms despite proton pump inhibitors. *Gut* 2008; **57**: 156-160 [PMID: 17951358 DOI: 10.1136/gut.2007.133470]
 - 25 **Tutuian R**, Vela MF, Hill EG, Mainie I, Agrawal A, Castell DO. Characteristics of symptomatic reflux episodes on Acid suppressive therapy. *Am J Gastroenterol* 2008; **103**: 1090-1096 [PMID: 18445095 DOI: 10.1111/j.1572-0241.2008.01791.x]
 - 26 **Sarkar S**, Hobson AR, Furlong PL, Woolf CJ, Thompson DG, Aziz Q. Central neural mechanisms mediating human visceral hypersensitivity. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G1196-G1202 [PMID: 11668028]
 - 27 **Caviglia R**, Ribolsi M, Gentile M, Rabitti C, Emerenziani S, Guarino MP, Petitti T, Cicala M. Dilated intercellular spaces and acid reflux at the distal and proximal oesophagus in patients with non-erosive gastro-oesophageal reflux disease. *Aliment Pharmacol Ther* 2007; **25**: 629-636 [PMID: 17305764 DOI: 10.1111/j.1365-2036.2006.03237.x]
 - 28 **Cicala M**, Habib FI, Emerenziani S. Proximal oesophagus: the added value in understanding GORD symptoms. *Neurogastroenterol Motil* 2009; **21**: 790-795 [PMID: 19624384 DOI: 10.1111/j.1365-2982.2009.01355.x]
 - 29 **Tobey NA**, Carson JL, Alkiek RA, Orlando RC. Dilated intercellular spaces: a morphological feature of acid reflux--damaged human esophageal epithelium. *Gastroenterology* 1996; **111**: 1200-1205 [PMID: 8898633 DOI: 10.1053/gast.1996.v111.pm8898633]

P- Reviewer: Slomiany BL **S- Editor:** Qi Y **L- Editor:** Filipodia
E- Editor: Zhang DN



Observational Study

Development of *Fok*-I based nested polymerase chain reaction-restriction fragment length polymorphism analysis for detection of hepatitis B virus X region V5M mutation

Hong Kim, Seok-Hyun Hong, Seoung-Ae Lee, Jeong-Ryeol Gong, Bum-Joon Kim

Hong Kim, Seok-Hyun Hong, Seoung-Ae Lee, Jeong-Ryeol Gong, Bum-Joon Kim, Department of Biomedical Sciences, Microbiology and Immunology, and Liver Research Institute, Seoul National University College of Medicine, Seoul 110-799, South Korea

Author contributions: Kim H and Hong SH contributed equally to this work; Kim BJ was the guarantor and designed the study; Kim H and Hong SH participated in the acquisition, analysis, and interpretation of the data, and drafted the initial manuscript; Lee SA, Gong JR revised the article critically for important intellectual content.

Supported by a National Research Foundation (NRF) of Korea grant funded by the Korean government (Ministry of Education, Science, and Technology, MEST), Grant No. 2013-005810.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Seoul National University Hospital (Seoul) (IRB No. 1404-070-572).

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

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Bum-Joon Kim, Professor, Department

of Biomedical Sciences, Microbiology and Immunology, and Liver Research Institute, Seoul National University College of Medicine, Seoul 110-799, South Korea. kbumjoon@snu.ac.kr
Telephone: +82-2-7408316
Fax: +82-2-7430881

Received: June 8, 2015
Peer-review started: June 11, 2015
First decision: July 10, 2015
Revised: July 17, 2015
Accepted: September 30, 2015
Article in press: September 30, 2015
Published online: December 21, 2015

Abstract

AIM: To develop a *Fok*-I nested polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis (PRA) method for the detection of hepatitis B virus X region (HBx) V5M mutation.

METHODS: Nested PCR was applied into DNAs from 198 chronic patients at 2 different stages [121 patients with hepatocellular carcinoma (HCC) and 77 carrier patients]. To identify V5M mutants, digestion of nested PCR amplicons by the restriction enzyme *Fok*-I (GGA TGN9↓) was done. For size comparison, the enzyme-treated products were analyzed by electrophoresis on 2.5% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator.

RESULTS: The assay enabled the identification of 69 patients (sensitivity of 34.8%; 46 HCC patients and 23 carrier patients). Our data also showed that V5M prevalence in HCC patients was significantly higher than in carrier patients (47.8%, 22/46 patients vs 0%, 0/23 patients, $P < 0.001$), suggesting that HBxAg V5M mutation may play a pivotal role in HCC generation in

chronic patients with genotype C infections.

CONCLUSION: The *Fok*-I nested PRA developed in this study is a reliable and cost-effective method to detect HBxAg V5M mutation in chronic patients with genotype C2 infection.

Key words: Hepatitis B virus; X antigen; Polymerase chain reaction-restriction fragment length polymorphism analysis; V5M mutation; Hepatocellular carcinoma

© **The Author(s) 2015.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: In the present study, we developed a reliable and cost-effective *Fok*-I nested polymerase chain reaction-restriction fragment length polymorphism analysis (PRA) method for the detection of V5M from chronic patients with genotype C2 infection. In addition, our epidemiological data based on the *Fok*-I nested PRA method strongly support the previous reports that V5M may play a very pivotal role in hepatocarcinogenesis, at least in chronic patients infected with genotype C2.

Kim H, Hong SH, Lee SA, Gong JR, Kim BJ. Development of *Fok*-I based nested polymerase chain reaction-restriction fragment length polymorphism analysis for detection of hepatitis B virus X region V5M mutation. *World J Gastroenterol* 2015; 21(47): 13360-13367 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13360.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13360>

INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem, and more than 350 million people are chronic carriers of the virus^[1]. Korea is a recognized endemic area of HBV infection, and an extraordinary prevalence of genotype C2 was also reported in this area, which is known to be more prone to mutations and related to more severe liver diseases and a lower antiviral response compared with genotype B^[2,3]. Furthermore, the high prevalence of basal core promoter (BCP) double mutations and the presence of a distinct immune response against HBV proteins in the Korean population could lead to the generation of distinct HBV variants that are rarely encountered in other areas, resulting in distinct clinical manifestations in Korean chronic patients^[4-19].

Despite its small size, the HBV genome contains four partially overlapping open reading frames producing at least seven viral gene products^[20]. Among these, the HBV X antigen (HBxAg) has been the focus of much attention in recent years because it is implicated strongly in hepatocarcinogenesis. HBxAg is a 154-amino-acid protein with an N-terminal negative regulatory domain and a C-terminal transactivation

domain. The HBxAg is multifunctional and affects gene transcription, signaling pathways, genotoxic stress responses, cell-cycle control, and apoptosis, and it also plays an essential role in viral replication^[21-24].

During the natural course of HBV infection, naturally occurring mutations may occur, and these could be HBV variants that affect prognosis^[25-27]. Several reports have demonstrated that specific point mutations, deletions or insertions in the HBxAg gene were related to severe forms of liver disease, such as cirrhosis of the liver and/or HCC^[17,28,29]. In particular, our previous study based on a direct sequencing protocol had introduced a novel N-terminal mutation type, V5M/L, with a mutation in codon 5 of HBxAg from Korean chronic patients with genotype C infections^[12]. This mutation type was found significantly more frequently in HCC patients than in patients in other disease groups. This finding suggests that it may play a pivotal role in HCC generation during the natural course of HBV chronic infection. For its monitoring among chronic patients, particularly with genotype C2 infections, the development of a new molecular diagnostic method for its detection is needed. For this purpose, in the present study, we developed a new nested PCR-restriction fragment length polymorphism analysis (PRA) method that can detect V5M and applied it to DNAs from Korean chronic patients.

MATERIALS AND METHODS

Study subjects

Among the patients visiting the Jeju National University Hospital from March to November and the Seoul National University Hospital from January to December in 2005, hepatitis B patients were selected in this study. The patients with a history of anti-viral therapy, an alcoholic liver disease, or hepatitis C involving liver disorders were excluded from this research. For an analysis of the correlation between V5M mutants and hepatocellular carcinoma (HCC), 121 serum samples from HCC patients and 77 samples from carriers were collected and stored at -80 °C. The basic biochemical tests for the serum samples were performed, and hepatitis B e antigen (HBeAg), anti-HBe, HBV-DNA and alpha-fetoprotein (AFP) assays were performed in the case of hepatitis B surface antigen (HBsAg)-positive samples. The detection of HBsAg, HBeAg, anti-HBs, and anti-HBe were performed by a chemiluminescence immunoassay (Abbott ARCHITECT, Abbott, IL, United States), and the determination of the serum HBV DNA was quantified by using a Versant HBV DNA Assay version 3.0 (bDNA, Siemens, NY, United States). This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB No. 1404-070-572).

Diagnosis of liver disease

A definitive diagnosis of liver disease was made according to the overall findings, including clinical,

biochemical, and radiological data. Among patients having positive HBsAg, the subjects with positive HBeAg, anti-HBe negative, HBV DNA positive, and normal serum transaminase were clinically defined as HBeAg-positive healthy carriers if there was no evidence of chronic liver disease in the radiologic findings^[30]. Hepatocellular carcinoma was diagnosed by clinical findings such as more than 400 pg/mL in a typical value of the serum alpha-fetoprotein (AFP), typical hyper blood vessels upon computed tomography (CT) or biopsy from the liver^[31].

HBV DNA extraction

Viral HBV DNA was extracted from 200 μ L of the serum obtained from 121 HCC and 77 carrier patients using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Briefly, 20 μ L of QIAGEN Protease (or proteinase K) was added into the 1.5 mL microcentrifuge tube containing 200 μ L of serum sample. Then, 200 μ L of buffer AL was added into the sample and was mixed by pulse-vortexing for 15 s. To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution. After the sample was incubated at 56 °C for 10 min, 200 μ L of ethanol (96%-100%, DukSan, Seoul, Korea) was loaded into the sample and was mixed again by pulse-vortexing for 15 s. After mixing, the mixture was carefully applied to the QIAamp Minispin column and was centrifuged at 8000 rpm for 1 min. Then, 500 μ L of buffer AW1 was carefully added into the column, and the column was centrifuged at 8000 rpm for 1 min. Next, 500 μ L of buffer AW2 was added into the column and was centrifuged at full speed (14000 rpm) for 3 min. After the collection tube containing the filtrate was discarded, 50 μ L of buffer AE was added into the column and was incubated at room temperature for 1 min; it was then centrifuged at 8000 rpm for 1 min. The eluted DNA was stored at -20 °C and used for polymerase chain reaction (PCR) mixtures as the template.

PCR amplification and direct sequencing analysis of HBx region

To analyze the mutation patterns in the HBx gene from 69 patients amplified by nested PCR, the PCR product was directly sequenced for sequence analysis. The first round of PCR was carried out using the sense primer X-pro-F1 (5'-CTC TGC CAA GTG TTT GCT GA-3'; GenBank accession number AY641558, positions 1171-1190 nt) and the antisense primer X-pro-R1 (5'-CAA GGC ACA GCT TGG AGG CT-3'; positions 1886-1905 nt), which amplify a HBV X region, while the second round of amplification was performed using the sense primer X-pro-F2 (5'-TTG CTC GCA GCC GGT CTG GA-3'; positions 1295-1314 nt) and the antisense primer X-pro-R2 (5'-TGA ACA GTA GGA CAT GAA CA-3'; antisense positions 1866-1885 nt) (Figure 1A). PCR was initiated using the i-MAX

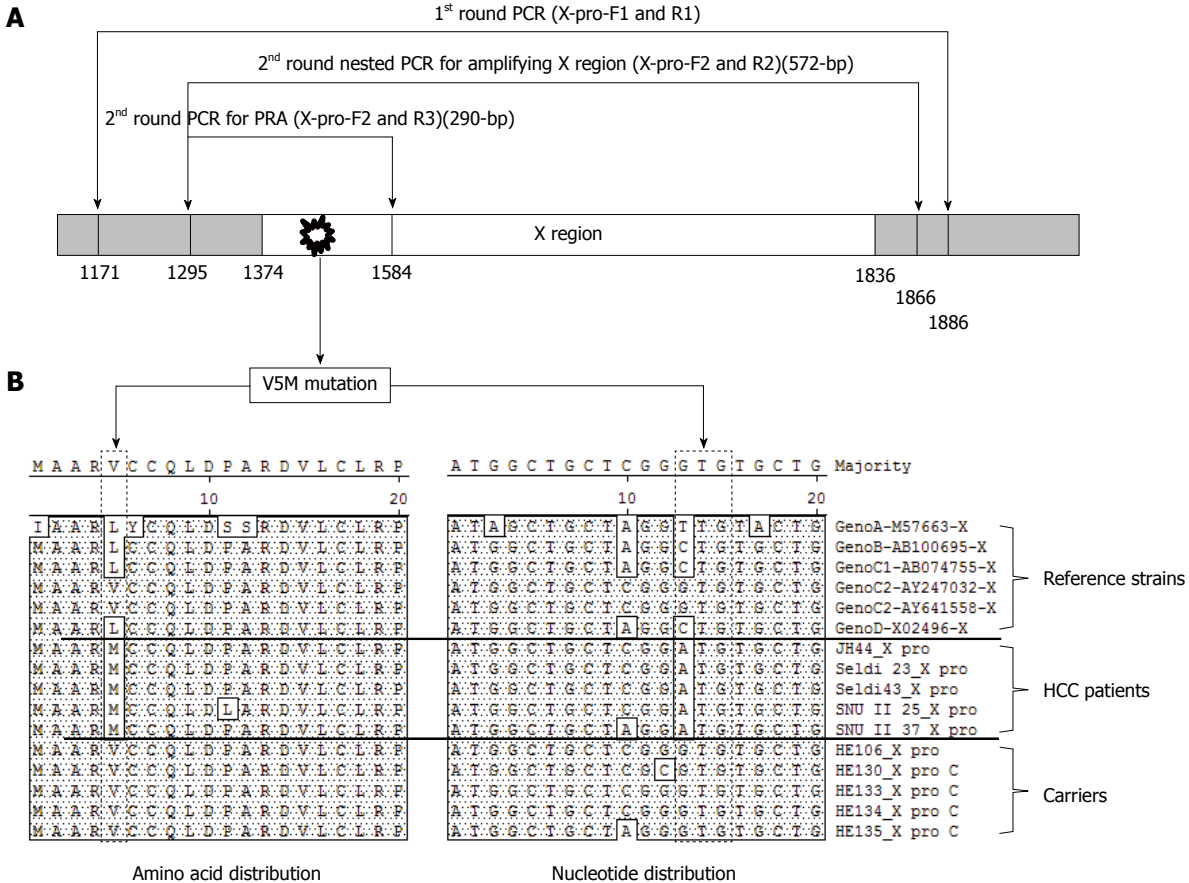
II DNA polymerase (iNtRON Bio, Seoul, Korea) in a 20 μ L mixture containing 1X PCR buffer containing 2 mmol/L MgCl₂, 2.5 mmol/L dNTP each, 5 pmol primer each, and 5 U of i-MAX II DNA polymerase. The 2 μ L of Template DNA extracted from patients' serum was added into the mixture. The final reaction mixture was subjected to 30 cycles of amplification (60 s at 95 °C, 45 s at 60 °C, and 90 s at 72 °C) followed by a 5 min extension at 72 °C. A 96-well thermocycler (Model 9600 thermocycler, Perkin-Elmer Cetus, Norwalk, United States) was also used. The obtained PCR products were analyzed by electrophoresis on 1% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator. For direct sequence analysis, an Applied Biosystems model 373A automatic sequencer and a BigDye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, Norwalk, United States) were used for the sequencing. For sequencing reactions, 60 ng of PCR-amplified DNA, 5 pmol of reverse primer (X-pro-R2), and 4 μ L of BigDye terminator v2.0 100 RR mix (Perkin-Elmer Applied Biosystems, Norwalk, United States) were mixed. Contents were adjusted to a final volume of 10 μ L by adding distilled water, and the reaction was run for 30 cycles of 10 s at 96 °C, 5 s at 60 °C, and 4 min at 60 °C. Determined sequences were aligned with the sequences of five HBV references (GenBank accession no. M57663, AB100695, AB074755, AY247032, AY641558, and X02496) using the multiple-alignment algorithm in the MegAlign package (DNASTAR, WS, United States).

Detection of V5M mutation by Fok-I PCR-restriction analysis

To identify V5M mutants conveniently from PCR product, the restriction enzyme *Fok-I* (GGA TGN9↓) was used in this study. Amplification of the targeted X region was performed under the same conditions in the first round of the nested PCR using the same primer set (X-pro-F1 and X-pro-R1). The second round of PCR was conducted using the sense primer X-pro-F2 (5'-TTG CTC GCA GCC GGT CTG GA-3'; positions 1295-1314 nt) and the antisense primer X-pro-R3 (5'-CGT GCA GAG GTG AAG CGA AG-3'; antisense 1584-1603 nt), which could amplify the size of the 290-bp product (Figure 1A). After amplification, 5 units of *Fok-I* (New England Biolabs, MA, United States) restriction enzyme were treated with 10x buffer and PCR product at 37 °C for 1 h. For size comparison, the enzyme-treated products were analyzed by electrophoresis on 2.5% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator (Figure 2).

Statistical analysis

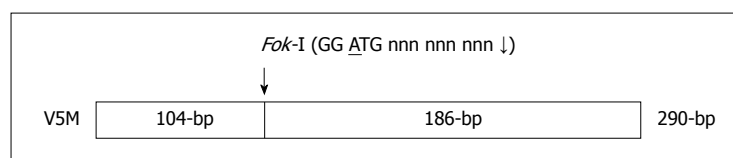
Statistical analysis of the data in this study was conducted using the SPSS version 21.0 software program (Professional Statistic, Chicago, IL), and the results are expressed as percentages. Frequency



1st Nested PCR amplicons (298-bp) using two primer sets (X-pro-F1 and R1 → X-pro-F2 and R3)



2nd Detection of V5M mutation by PRA of nested PCR amplicons



3rd Analysis of different types of PRA band patterns from gel electrophoresis

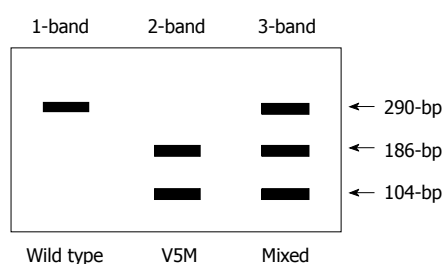


Figure 2 Algorithm of *Fok-I* nested polymerase chain reaction-restriction fragment length polymorphism analysis method for the detection of V5M mutation in the HBV X antigen region used in the present study.

Table 1 Sensitivity of nested polymerase chain reaction assay for the amplification of the 572-bp HBV X antigen fragment *n* (%)

	No. of samples (<i>n</i> = 198)		Sensitivity
	HCC (<i>n</i> = 121)	Carrier (<i>n</i> = 77)	
PCR amplification	46 (38.0)	23 (29.9)	34.8%

PCR: Polymerase chain reaction.

X5M type in an agarose gel (Figures 2 and 3).

Application of *Fok-I* nested PRA into DNAs from 198 chronic patients

Our *Fok-I* nested PRA protocol designed in this study was applied to the DNA from 198 chronic patients of 2 different stages (121 patients with hepatocellular carcinoma and 77 carrier patients). We produced the nested PCR amplicons from 69 patients (sensitivity of 34.8%; 46 HCC patients and 23 carrier patients) (Table 1). In 46 HCC patients with PCR-positive amplicons, 24 (52.2%), 17 (37.0%) and 5 (10.8%) were identified as wild-type infection alone, V5M mutant type infection alone, and coinfection with both types, wild and mutant, respectively (Figure 3). A total of 22 (47.8 %) from 46 HCC patients with positive PCR amplicons were identified as infection with the V5M mutant. All 23 carriers with PCR positive amplicons were identified as wild-type infection alone. No patients with V5M mutant infection were found in the carriers. This result was completely concordant with that obtained from a

direct sequencing protocol (specificity of 100%). The prevalence of infection of the V5M mutant type was significantly higher in HCC compared with carriers [47.8% (22/46 patients) vs 0 % (0/23 patients), *P* < 0.001] (Table 2).

DISCUSSION

Previously, we introduced a total of 5 types of mutations in the X gene (V5M/L, P38S, H94Y, I127T/N, and K130M and V131I) that were significantly related to the clinical severity of chronic patients infected with genotype C2 *via* a molecular epidemiologic study of Korean chronic patients^[12]. Of these, the V5M/L type was first introduced by us and has some properties that are distinct from other mutations. First, this is a genuine HCC-specific mutation as its prevalence in HCC patients was significantly higher than even that of cirrhosis patients or in chronic hepatitis or carriers. Recently, the combination of both BCP double mutations and both types of the V5M mutation, V5M and V5L, has also been reported to increase the risk of HCC by 5.34 times compared with wild type, suggesting that V5M with HBV genotype C2 is a risk factor for the development of HCC and can be used to predict the clinical impact of chronic HBV infection^[32]. Second, it is more prevalent in HBeAg-negative patients than in HBeAg-positive patients, suggesting that it may be generated from host immune pressure^[12]. Third, of note, it has a positive correlation with the BCP

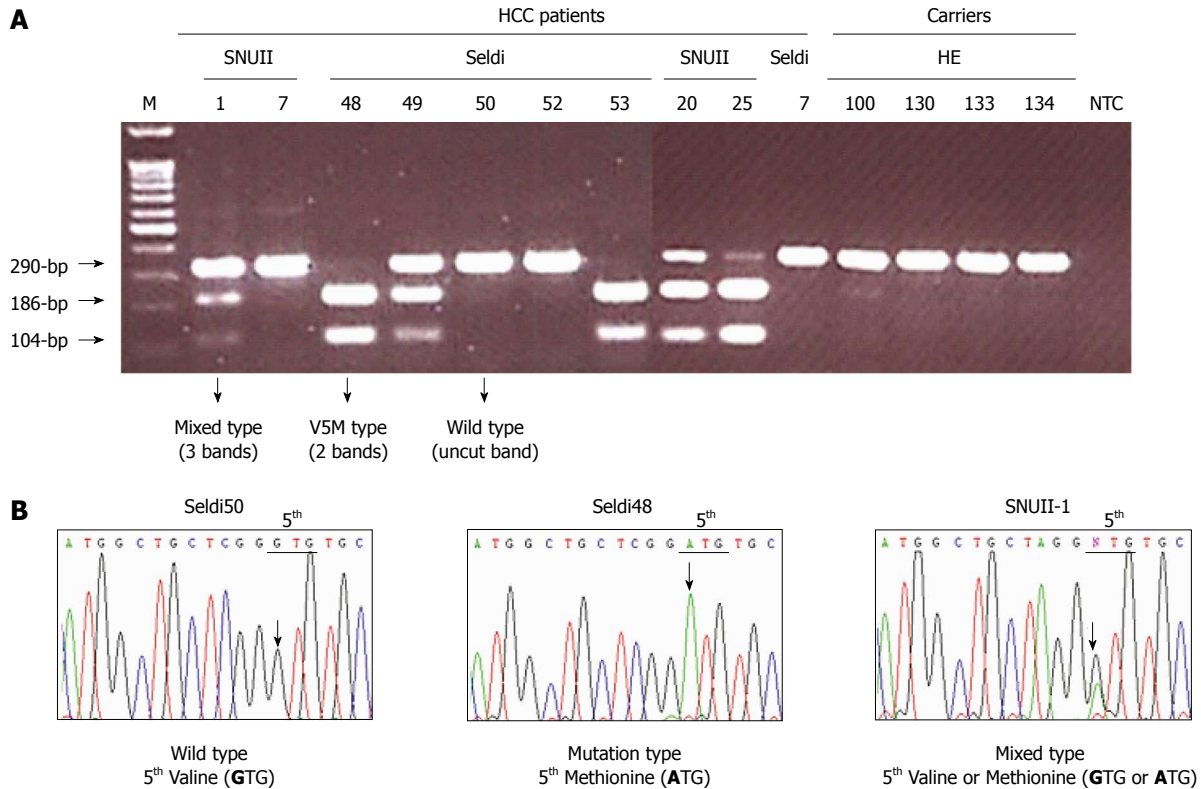


Figure 3 Application of *Fok-I* nested polymorphism analysis method (A) and direct sequencing analysis (B) into serum DNAs. A: The *Fok-I* PRA application of nested PCR products could produce three types of PRA pattern on an agarose gel (2.5%): an undigested 290-bp from the wild type, V5(GTG); two bands of complete digestion, 186-bp and 104-bp, from the V5M mutant and three bands, an undigested one of 290-bp from the wild type and 2 digested bands, 186-bp and 104-bp, from V5M mutant. B: Direct sequencing could also produce three types: wild type only [V5(GTG)], mutation only [M5(ATG)], and mixed types with the wild type V5(GTG) and M5(ATG). The mixed types showed the mixed peaks (G and A) at the first nucleotide of the 5th codon of HBxAg (NTT). The results of the *Fok-I* nested PRA method were completely concordant with those obtained by direct sequencing analysis. Lane M, 100-bp DNA ladder marker.

Table 2 Evaluation of *Fok-I* nested polymorphism analysis assay for the detection of V5M mutations from 69 samples amplified by nested PCR by comparing with the results of the direct sequencing method *n* (%)

Type of HBxAg 5 th codon	Result for			
	<i>Fok-I</i> nested PRA		Direct sequencing	
	HCC (<i>n</i> = 46)	Carrier (<i>n</i> = 23)	HCC (<i>n</i> = 46)	Carrier (<i>n</i> = 23)
Wild type	24 (52.2)	23 (100)	24 (52.2)	23 (100)
V5M	17 (37.0)	0 (0)	17 (37.0)	0 (0)
Wild type + V5M	5 (10.8)	0 (0)	5 (10.8)	0 (0)

PRA: Polymorphism analysis; HCC: Hepatocellular carcinoma; HBxAg: HBV X antigen.

mutations K130M and V131I^[12]. Taken together, it may play a very pivotal role in hepatocarcinogenesis during the HBeAg-negative immune active stage in the natural course of genotype C2 HBV infection. The development of molecular diagnosis for the detection of V5M must therefore be accomplished in genotype C2 endemic areas such as South Korea and China.

For the molecular detection of V5M mutants of the HBV X region, we developed a molecular-based approach, *Fok-I* nested PRA, which allowed for the rapid detection of the V5M mutant type without

sequence analysis. There are some noteworthy advantages in our *Fok-I* nested PRA method. First, the 5-bp consecutive HBV sequences (GGATG) corresponding to the *Fok-I* (GGATGN9↓) recognition site of the V5M mutant type are highly conserved among the HBV genotype C2 strains having the V5M mutation, as shown in Figure 1B. When we analyzed the HBxAg sequences from 45 independent chronic patients infected with genotype C2 who had been identified as having the V5M mutation by direct sequencing analysis^[12], no mutations were found in the 5-bp consecutive HBV sequences (GGATG) (data not shown). This result could guarantee the enhanced specificity of our *Fok-I* nested PRA method. Actually, our *Fok-I* PRA method can detect all of the V5M mutations with 100% specificity compared with the direct sequencing protocol (Table 2). Second, this method can also detect mixed type infections consisting of both the V5M mutant and the wild type in addition to being able to detect V5M mutation infections alone and wild type infections alone. However, some concerns regarding this assay should also be noted. First, this method may underestimate the frequency of the V5M/L mutation because it is blind to the L(CTG) genotype starting from the CTG in the 5th codon of the X region, which is found in wild-

type genotype A, B, C1 or D, but very rare in genotype C2 as shown in Figure 1B. However, given that the V5L genotype is the wild type in genotype A, B or D, it seems more likely and reasonable that unlike the V5M type, it may be one of the wild types in genotype C2 rather than the mutation generated by host immune pressure. Second, despite the nested PCR protocol, our assay had a low level of sensitivity (34.8 %, 69/198 patients) (Table 1). This low sensitivity may be due to the absence of the target X region rather than a defect of our assay as previously reported^[33] because in 129 samples not amplified by our assay, our repeated attempts to amplify a partial X gene also failed, but the nested PCR protocol targeting the partial S gene could amplify most of them (data not shown). Our epidemiological data based on our *Fok-I* nested PRA method showed that V5M mutations are significantly more prevalent in HCC patients than in carrier stage patients [47.8% (22/46 patients) vs 0% (0/23 patients), $P < 0.001$], strongly supporting previous reports that this mutation may play a very pivotal role in hepatocarcinogenesis, at least in chronic patients infected with genotype C2^[12,32].

In the present study, we developed a reliable and cost-effective *Fok-I* nested PRA method for the detection of V5M from chronic patients with genotype C2 infection. In addition, our epidemiological data based on the *Fok-I* nested PRA method strongly support the previous reports that V5M may play a very pivotal role in hepatocarcinogenesis, at least in chronic patients infected with genotype C2.

COMMENTS

Background

The HBxAg V5M mutation proved to be associated with liver disease progression in chronic subjects with genotype C2 using our previous molecular epidemiologic study based on direct sequencing protocol. So, for its monitoring among genotype C2 infected chronic patients, the development of a new molecular diagnostic method for its detection is needed.

Research frontiers

For molecular epidemiologic study for detection of V5M mutation, in this study, we developed the novel nested polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis (PRA) method that can detect V5M and applied it to DNAs from Korean chronic patients.

Innovations and breakthroughs

The present epidemiological data based on the *Fok-I* nested PRA method strongly support the previous reports that V5M may play a very pivotal role in hepatocarcinogenesis, at least in chronic patients infected with genotype C2.

Applications

The FRET-based real-time PCR for detection of the preS1 deletion developed in this study might have potential for early prediction for risk of liver disease progression in chronic subjects.

Terminology

The nested PRA method that can detect V5M could be used for molecular epidemiologic purposes. It can permit not only the simultaneous identification of coexisting quasispecies of both wild type and variant, but also the direct

identification of target mutations from primary specimens such as serum.

Peer-review

The authors developed a reliable and cost-effective *Fok-I* nested PRA method for the detection of V5M from chronic patients with genotype C2 infection, which could be effectively used for its screening instead of direct sequencing protocols. Furthermore, they also confirmed that V5M may play a very pivotal role in hepatocarcinogenesis, at least in chronic patients infected with genotype C2.

REFERENCES

- 1 **Orito E**, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, Okanoue T, Yotsuyanagi H, Iino S. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. *Hepatology* 2001; **33**: 218-223 [PMID: 11124839 DOI: 10.1053/jhep.2001.20532]
- 2 **Kao JH**, Chen PJ, Lai MY, Chen DS. Genotypes and clinical phenotypes of hepatitis B virus in patients with chronic hepatitis B virus infection. *J Clin Microbiol* 2002; **40**: 1207-1209 [PMID: 11923332]
- 3 **Lavanchy D**. Worldwide epidemiology of HBV infection, disease burden, and vaccine prevention. *J Clin Virol* 2005; **34** Suppl 1: S1-S3 [PMID: 16461208]
- 4 **Kim BJ**. Hepatitis B virus mutations related to liver disease progression of Korean patients. *World J Gastroenterol* 2014; **20**: 460-467 [PMID: 24574714 DOI: 10.3748/wjg.v20.i2.460]
- 5 **Kim DW**, Lee SA, Hwang ES, Kook YH, Kim BJ. Naturally occurring precore/core region mutations of hepatitis B virus genotype C related to hepatocellular carcinoma. *PLoS One* 2012; **7**: e47372 [PMID: 23071796 DOI: 10.1371/journal.pone.0047372]
- 6 **Kim DW**, Lee SA, Kim H, Won YS, Kim BJ. Naturally occurring mutations in the nonstructural region 5B of hepatitis C virus (HCV) from treatment-naïve Korean patients chronically infected with HCV genotype 1b. *PLoS One* 2014; **9**: e87773 [PMID: 24489961 DOI: 10.1371/journal.pone.0087773]
- 7 **Kim H**, Jee Y, Mun HS, Park JH, Yoon JH, Kim YJ, Lee HS, Hyun JW, Hwang ES, Cha CY, Kook YH, Kim BJ. Characterization of two hepatitis B virus populations in a single Korean hepatocellular carcinoma patient with an HBeAg-negative serostatus: a novel X-Gene-deleted strain with inverted duplication sequences of upstream enhancer site II. *Intervirology* 2007; **50**: 273-280 [PMID: 17570929 DOI: 10.1159/000103915]
- 8 **Kim H**, Jee Y, Mun HS, Song BC, Park JH, Hyun JW, Hwang ES, Cha CY, Kook YH, Kim BJ. Comparison of full genome sequences between two hepatitis B virus strains with or without preC mutation (A1896) from a single Korean hepatocellular carcinoma patient. *J Microbiol Biotechnol* 2007; **17**: 701-704 [PMID: 18051288]
- 9 **Kim H**, Jee YM, Song BC, Hyun JW, Mun HS, Kim HJ, Oh EJ, Yoon JH, Kim YJ, Lee HS, Hwang ES, Cha CY, Kook YH, Kim BJ. Analysis of hepatitis B virus quasispecies distribution in a Korean chronic patient based on the full genome sequences. *J Med Virol* 2007; **79**: 212-219 [PMID: 17245716 DOI: 10.1002/jmv.20789]
- 10 **Kim H**, Jee YM, Song BC, Shin JW, Yang SH, Mun HS, Kim HJ, Oh EJ, Yoon JH, Kim YJ, Lee HS, Hwang ES, Cha CY, Kook YH, Kim BJ. Molecular epidemiology of hepatitis B virus (HBV) genotypes and serotypes in patients with chronic HBV infection in Korea. *Intervirology* 2007; **50**: 52-57 [PMID: 17164558 DOI: 10.1159/000096313]
- 11 **Kim H**, Lee SA, Kim DW, Lee SH, Kim BJ. Naturally occurring mutations in large surface genes related to occult infection of hepatitis B virus genotype C. *PLoS One* 2013; **8**: e54486 [PMID: 23349904 DOI: 10.1371/journal.pone.0054486]
- 12 **Kim HJ**, Park JH, Jee Y, Lee SA, Kim H, Song BC, Yang S, Lee M, Yoon JH, Kim YJ, Lee HS, Hwang ES, Kook YH, Kim BJ. Hepatitis B virus X mutations occurring naturally associated with clinical severity of liver disease among Korean patients with chronic genotype C infection. *J Med Virol* 2008; **80**: 1337-1343

- [PMID: 18551606 DOI: 10.1002/jmv.21219]
- 13 **Lee SA**, Cho YK, Lee KH, Hwang ES, Kook YH, Kim BJ. Gender disparity in distribution of the major hydrophilic region variants of hepatitis B virus genotype C according to hepatitis B e antigen serostatus. *J Med Virol* 2011; **83**: 405-411 [PMID: 21264860 DOI: 10.1002/jmv.21988]
 - 14 **Lee SA**, Kim H, Won YS, Seok SH, Na Y, Shin HB, Inn KS, Kim BJ. Male-specific hepatitis B virus large surface protein variant W4P potentiates tumorigenicity and induces gender disparity. *Mol Cancer* 2015; **14**: 23 [PMID: 25645622 DOI: 10.1186/s12943-015-0303-7]
 - 15 **Lee SA**, Kim K, Kim H, Kim BJ. Nucleotide change of codon 182 in the surface gene of hepatitis B virus genotype C leading to truncated surface protein is associated with progression of liver diseases. *J Hepatol* 2012; **56**: 63-69 [PMID: 21827734 DOI: 10.1016/j.jhep.2011.06.028]
 - 16 **Lee SA**, Kim KJ, Kim DW, Kim BJ. Male-specific W4P/R mutation in the pre-S1 region of hepatitis B virus, increasing the risk of progression of liver diseases in chronic patients. *J Clin Microbiol* 2013; **51**: 3928-3936 [PMID: 24025913 DOI: 10.1128/jcm.01505-13]
 - 17 **Lee SA**, Mun HS, Kim H, Lee HK, Kim BJ, Hwang ES, Kook YH, Kim BJ. Naturally occurring hepatitis B virus X deletions and insertions among Korean chronic patients. *J Med Virol* 2011; **83**: 65-70 [PMID: 21108340 DOI: 10.1002/jmv.21938]
 - 18 **Mun HS**, Lee SA, Jee Y, Kim H, Park JH, Song BC, Yoon JH, Kim YJ, Lee HS, Hyun JW, Hwang ES, Kook YH, Kim BJ. The prevalence of hepatitis B virus preS deletions occurring naturally in Korean patients infected chronically with genotype C. *J Med Virol* 2008; **80**: 1189-1194 [PMID: 18461612 DOI: 10.1002/jmv.21208]
 - 19 **Mun HS**, Lee SA, Kim H, Hwang ES, Kook YH, Kim BJ. Novel F141L pre-S2 mutation in hepatitis B virus increases the risk of hepatocellular carcinoma in patients with chronic genotype C infections. *J Virol* 2011; **85**: 123-132 [PMID: 20962085 DOI: 10.1128/jvi.01524-10]
 - 20 **Summers J**, Mason WS. Replication of the genome of a hepatitis B--like virus by reverse transcription of an RNA intermediate. *Cell* 1982; **29**: 403-415 [PMID: 6180831]
 - 21 **Murakami S**. [Expression and function of hepatitis B virus (HBV) X protein]. *Seikagaku* 1999; **71**: 1309-1326 [PMID: 10614179]
 - 22 **Murakami S**. Hepatitis B virus X protein: a multifunctional viral regulator. *J Gastroenterol* 2001; **36**: 651-660 [PMID: 11686474]
 - 23 **Tang SG**, Yang BC. [Clone of hepatitis B virus X gene and its protein expression]. *Zhongnandaxue Xuebao Yixueban* 2005; **30**: 525-528 [PMID: 16320580]
 - 24 **Yang Y**, Ma Y, Zhen L, Chen Y, Ma W, Murakami S. HBV X protein (HBX) interacts with general transcription factor TFIIB both in vitro and in vivo. *Chin Med Sci J* 1999; **14**: 152-157 [PMID: 12903814]
 - 25 **Fattovich G**, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. *J Hepatol* 2008; **48**: 335-352 [PMID: 18096267 DOI: 10.1016/j.jhep.2007.11.011]
 - 26 **Lapiński TW**, Pogorzelska J, Flisiak R. HBV mutations and their clinical significance. *Adv Med Sci* 2012; **57**: 18-22 [PMID: 22430043 DOI: 10.2478/v10039-012-0006-x]
 - 27 **Xia L**, Huang W, Tian D, Zhu H, Zhang Y, Hu H, Fan D, Nie Y, Wu K. Upregulated FoxM1 expression induced by hepatitis B virus X protein promotes tumor metastasis and indicates poor prognosis in hepatitis B virus-related hepatocellular carcinoma. *J Hepatol* 2012; **57**: 600-612 [PMID: 22613004 DOI: 10.1016/j.jhep.2012.04.020]
 - 28 **Kim S**, Park SY, Yong H, Famulski JK, Chae S, Lee JH, Kang CM, Saya H, Chan GK, Cho H. HBV X protein targets hBubR1, which induces dysregulation of the mitotic checkpoint. *Oncogene* 2008; **27**: 3457-3464 [PMID: 18193091 DOI: 10.1038/sj.onc.1210998]
 - 29 **Park EH**, Koh SS, Srisuttee R, Cho IR, Min HJ, Jhun BH, Lee YS, Jang KL, Kim CH, Johnston RN, Chung YH. Expression of HBX, an oncoprotein of hepatitis B virus, blocks reoviral oncolysis of hepatocellular carcinoma cells. *Cancer Gene Ther* 2009; **16**: 453-461 [PMID: 19096445 DOI: 10.1038/cgt.2008.95]
 - 30 **Lok AS**, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000--summary of a workshop. *Gastroenterology* 2001; **120**: 1828-1853 [PMID: 11375963]
 - 31 **Bruix J**, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodés J. Clinical Management of Hepatocellular Carcinoma. Conclusions of the Barcelona-2000 EASL Conference. *J Hepatol* 2001; **35**: 421-430 [DOI: 10.1016/S0168-8278(01)00130-1]
 - 32 **Lee JH**, Han KH, Lee JM, Park JH, Kim HS. Impact of hepatitis B virus (HBV) x gene mutations on hepatocellular carcinoma development in chronic HBV infection. *Clin Vaccine Immunol* 2011; **18**: 914-921 [PMID: 21490166 DOI: 10.1128/cvi.00474-10]
 - 33 **Datta S**, Banerjee A, Chandra PK, Biswas A, Panigrahi R, Mahapatra PK, Panda CK, Chakrabarti S, Bhattacharya SK, Chakravarty R. Analysis of hepatitis B virus X gene phylogeny, genetic variability and its impact on pathogenesis: implications in Eastern Indian HBV carriers. *Virology* 2008; **382**: 190-198 [PMID: 18952249 DOI: 10.1016/j.virol.2008.09.007]

P- Reviewer: Haruki K S- Editor: Yu J

L- Editor: A E- Editor: Liu XM



Observational Study

Epidemiological study of elderly constipation in Beijing

Mei Zhang, Xiao-Jiao Yang, Hong-Ming Zhu, Zhe Tang, Bang-Yi Li, Dan-Dan Zhao

Mei Zhang, Hong-Ming Zhu, Zhe Tang, Bang-Yi Li, Dan-Dan Zhao, Department of Gastroenterology, Xuanwu Hospital, Capital Medical University, Beijing 100053, China

Xiao-Jiao Yang, McGill University, 845 Sherbrooke Street West, Montreal, Quebec H3A 0G4, Canada

Author contributions: Zhang M designed the research; Tang Z and Zhu HM performed the research; Yang XJ performed data and statistical analyses; Zhao DD and Li BY analyzed data; Zhang M wrote the paper.

Institutional review board statement: The study was reviewed and approved by the Department of Gastroenterology of Xuanwu Hospital, Capital Medical University.

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

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Dr. Mei Zhang, Department of Gastroenterology, Xuanwu Hospital, Capital Medical University, No. 45 Changchun Street, Xuanwu District, Beijing 100053, China. zhang2955@sina.com
Telephone: +86-10-83198899
Fax: +86-10-83198899

Received: June 12, 2015

Peer-review started: June 12, 2015

First decision: August 26, 2015

Revised: September 9, 2015

Accepted: November 13, 2015

Article in press: November 13, 2015

Published online: December 21, 2015

Abstract

AIM: To investigate the present situation of elderly constipation in urban and rural areas of the Beijing region.

METHODS: A total of 1942 cases (≥ 60 years) were selected in the Beijing region for investigation. Constipation-related data collection was carried out *via* hierarchical status, segmentation, and random cluster sampling. Patient data concerning constipation-related demographic indicators, education level, occupation, economic status, and history of gastrointestinal disease was obtained *via* questionnaires and surveys. Constipation was defined according to the Rome III criteria, with the following constipation judgment indicators: defecation less than 3 times per week, stool weight less than 35 g/d, dry and hard stool, and difficulty in defecating during more than 25% of evacuation attempts.

RESULTS: Of the 1942 cases, 634 were diagnosed with constipation, and the total prevalence rate was 32.6%, which increased with age. There was a statistically insignificantly higher prevalence of constipation in females (compared to males) and urban areas (compared to rural areas). There was a statistically insignificantly higher prevalence in the illiterate group compared to the literacy group. Those engaged in mental work suffered from statistically significantly higher constipation prevalence than those engaged in physical labor. A total of 1847 cases did not suffer from gastritis, of which 595 cases were constipated; although the prevalence rate was 32.2%, showing a higher incidence of constipation in patients with gastritis, no significant statistical difference between the two groups was found. A total of 59 cases

with a past history of biliary tract disease were found, of which 26 had constipation; constipation prevalence was 44.1% (far higher than other groups), which was a statistically significant difference.

CONCLUSION: The prevalence of elderly constipation in the Beijing region closely resembles Western countries, and is significantly affected by region, age, and past history of other related illnesses.

Key words: Constipation; Elderly; Epidemiology; Prevalence; Factors

© **The Author(s) 2015.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Constipation is a common clinical symptom of gastrointestinal dysfunction. Only a few people suffering from constipation problems will seek medical advice. This article concerns the latest epidemiological studies on elderly constipation in Beijing, China.

Zhang M, Yang XJ, Zhu HM, Tang Z, Li BY, Zhao DD. Epidemiological study of elderly constipation in Beijing. *World J Gastroenterol* 2015; 21(47): 13368-13373 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13368.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13368>

INTRODUCTION

Constipation is a common clinical symptom of gastrointestinal dysfunction. Since only a few people suffering from constipation will seek medical advice^[1], the exact epidemiological prevalence is difficult to determine. A recent study of evidence-based medicine reported a total global prevalence of 0.7%-79%, with an average of 16%^[2]. The prevalence rate was 3%-11% in our country of China^[3-9]. Constipation can be acute, with mild symptoms and acute onset, but defecation difficulty and other symptoms can soon be completely relieved. Constipation can also be chronic and develop from acute constipation, with chronic constipation mainly referring to symptoms persisting for at least six months. As an individual's age increases, so too does their constipation prevalence. Older individuals are therefore significantly more frequently affected than younger individuals. Elderly constipation is caused by a variety of factors, all of which interrelate to cause a complex problem for geriatric medicine^[10]. Constipation prevalence has recently been on rise throughout the world. Therefore, in order to investigate the prevalence of elderly constipation and its related factors in Beijing, China, we carried out an epidemiological investigation into elderly constipation in different regions of Beijing in 2010.

MATERIALS AND METHODS

Research subjects

In 2010, Beijing patients aged ≥ 60 years from rural (Huairou, Daxing) and urban (Xuanwu) centers were stratified, segmented, and randomized, using the cluster sampling method. The target survey objective was 1959 cases, with an actual survey of 1942 cases and a response rate of 99.1%. Patient characteristics were as follows: 822 urban (42.3%), 1120 rural (57.7%), 921 male (47.4%), and 1,021 female (52.6%). Although there were no significant differences ($P > 0.05$) between urban/rural, elderly age, and gender, there was a significant difference with regards to cultural background ($P < 0.01$).

Research method

Questionnaires and surveys were carefully designed *via* centralized and household questionnaires. Survey questions covered demographic-related indicators, constipation-related indicators, education level, occupation, economic status, and medical history (including any history of other digestive diseases). The Rome III criteria for constipation were taken as standard reference with the following constipation judgement indicators: defecation less than 3 times per week, stool weight of less than 35 g/d, dry and hard stool, and difficulty in defecating during more than 25% of evacuation attempts.

Statistical method

SPSS 11.5 software was used for calculations. The χ^2 test was performed to analyze the data. $P < 0.05$ was considered statistically significant.

RESULTS

Age

Of a total of 1942 patients from both urban and rural areas, 634 were diagnosed with constipation. The total prevalence rate was 32.6%, which increased with age: 21.1% for 60- to 65-year-olds; 21.3% for 65- to 70-year-olds; 30.4% for 70- to 75-year-olds; 37.2% for 75- to 80-year-olds; and 44.8% for ≥ 80 -year-olds. The difference was statistically significant ($\chi^2 = 81.779$, $P = 0.00$) (Table 1).

Area

Of the 634 cases of patients with constipation, 297 cases were from urban areas and 337 were from rural areas; prevalence rates were 36.1% and 30.1%, respectively. In the urban regions, 152 cases of acute constipation and 145 cases of chronic constipation were found, with a prevalence rate of 18.5% and 17.6%, respectively. In rural areas, 180 cases of acute constipation and 157 cases of chronic constipation

Table 1 Urban and rural prevalence of different age groups

Age (yr)	Cases	Constipation	Prevalence (%)	Constipation frequency, <i>n</i> (%)	
				Acute	Chronic
60-65	261	55	21.1 ^b	36 (13.8)	19 (7.3)
65-70	301	64	21.3	46 (15.3)	18 (6.0)
70-75	444	135	30.4	71 (16.0)	64 (14.4)
75-80	519	193	37.2	90 (17.3)	103 (19.8)
80-	417	187	44.8	89 (21.3)	98 (23.5)
Total	1942	634	32.6	332 (17.1)	302 (15.6)

^b*P* < 0.01.**Table 2** Different gender, age, region, and education level epidemiological characteristics of elderly patients with chronic constipation

Item	Cases	Constipation	Prevalence (%)	Constipation frequency, <i>n</i> (%)	
				Acute	Chronic
Sex					
Male	921	284	30.8	145 (15.7)	139 (15.1)
Female	1021	350	34.3	187 (18.3)	163 (16.0)
Age (yr)					
< 75	1006	254	25.2 ^a	153 (15.2)	101 (10.0)
≥ 75	936	380	40.6	179 (19.1)	201 (21.5)
Area					
Urban	822	297	36.1 ^a	152 (18.5)	145 (17.6)
Rural	1120	337	30.1	180 (16.1)	157 (14.0)
Education					
Literate	1217	385	31.6 ^a	212 (17.4)	173 (14.2)
Illiterate	725	249	34.3	120 (16.6)	129 (17.8)

^a*P* < 0.05.

were found, with a prevalence rate of 16.1% and 14.0%, respectively. Constipation prevalence was higher in urban than for rural areas, but the difference was not statistically significant ($\chi^2 = 8.193$, *P* = 0.017) (Table 2).

Gender

Of the 1942 total patients, 921 were male with 284 cases of constipation (prevalence: 30.8%) and 1021 were female with 350 cases of constipation (prevalence: 34.3%). Although there was a higher female prevalence compared to males, the difference was not statistically significant ($\chi^2 = 2.612$, *P* = 0.105) (Table 2).

Literacy

Of the 1942 total patients, 1217 were literate and 725 were illiterate. The literate group had 385 cases of constipation (prevalence: 31.6%), of which 212 were acute (prevalence: 17.4%) and 173 were chronic (prevalence: 14.2%). The illiterate group had 249 cases of constipation (prevalence: 34.3%), of which 129 were acute (prevalence: 16.6%) and 129 were chronic (prevalence: 17.8%). Although there was a higher prevalence in the illiterate group compared to the literate group, the difference was not statistically significant ($\chi^2 = 7.86$, *P* = 0.02) (Table 2).

Occupation

Of the 1942 total patients, 959 were engaged in occupations that mostly required mental effort, while 983 had occupations that mostly required physical effort. The mental group had 342 cases of constipation (prevalence: 35.7%), of which 177 were acute (prevalence: 18.5%) and 165 were chronic (prevalence: 17.2%). The physical group had 292 cases of constipation (prevalence: 29.7%), of which 155 were acute (prevalence: 15.8%) and 137 were chronic (prevalence: 13.9%). The mental group showed a statistically significantly higher constipation prevalence than the physical group ($\chi^2 = 7.86$, *P* = 0.02) (Table 3).

Relationship between elderly constipation and gastrointestinal tract diseases

Of the 1942 total patients, 1934 completed the gastrointestinal disease history questionnaire. There were 87 cases with a history of gastritis, of which 37 had constipation; a prevalence of 42.5%. Of the 1847 total cases that did not suffer from gastritis, 595 had constipation; a prevalence of 32.2%. There was a higher incidence of constipation in patients with gastritis, but no significant statistical difference between the two groups was found. There were 59 cases with a past history of biliary tract disease, out

Table 3 Prevalence of constipation affected by different occupations

	Cases	Constipation	Prevalence (%)	Constipation frequency, <i>n</i> (%)	
				Acute	Chronic
Mental work	959	342	35.7 ^a	177 (18.5)	165 (17.2)
Manual work	983	292	29.7	155 (15.8)	137 (13.9)
Total	1942	634	32.6	332 (17.1)	302 (15.6)

^a*P* < 0.05.**Table 4** Prevalence of constipation affected by gastritis

Chronic gastritis	Cases	constipation	Prevalence (%)	Constipation frequency, <i>n</i> (%)	
				Acute	Chronic
No	1847	595	32.2	312 (16.9)	283 (15.3)
Yes	87	37	42.5	18 (20.7)	19 (21.8)
Total	1934	632	32.7	330 (17.1)	302 (15.6)

Table 5 Prevalence of constipation affected by biliary tract disease

Biliary tract disease	Cases	Constipation	Prevalence (%)	Constipation frequency, <i>n</i> (%)	
				Acute	Chronic
No	1875	605	32.3 ^a	320 (17.1)	285 (15.2)
Yes	59	26	44.1	10 (16.9)	16 (27.1)
Total	1934	631	32.6	330 (17.1)	301 (15.6)

^a*P* < 0.05.

of which 26 had constipation; a prevalence of 44.1%, which was far higher than other groups and was a statistically significant difference. ($\chi^2 = 6.378$, *P* = 0.041) (Table 4 and Table 5).

DISCUSSION

Elderly constipation has recently become a worldwide problem. It is related to many factors, including such as pelvic floor aging, decreased social activity, psychological disorders, co-morbidity, and effects from multiple drug usage. A recent evidence-based study has shown an incidence rate of 33% in Western countries^[2]. Patients with chronic constipation constitute an elderly population with a high disease burden. The mean annual constipation-related health care cost, adjusted for potentially confounding factors, was €951 per patient in Sweden^[11]. Constipation not only causes mental distress to elderly patients, but also leads to a variety of sub-health symptoms and is a major factor in inducing gastrointestinal tumors^[12]. Previous epidemiological studies have shown a difference in constipation prevalence rates between the East and the West; Asian countries had a lower prevalence rate than Europe and the United States^[2], which may be due to differences in culture, diet, genetics, environment, socio-economic conditions, and health-care system. With the improvement of living standards and social aging process, the prevalence of constipation in our country also seems to be increasing

rapidly. An epidemiological survey implemented in 1997 by Yu *et al.*^[3] with 1434 elderly patients in Beijing showed the constipation prevalence rate of individuals over 60 years to be 20.3%. However, our research shows a prevalence rate of 32.6%. Over the past decade, the prevalence of elderly constipation in Beijing was found to be rising to levels almost equivalent to those found in the West, which is deeply concerning.

In the Beijing region's elderly population (> 60-years-old), constipation prevalence was 32.6% and the epidemiological characteristics are as follows: (1) there are regional differences for elderly constipation, with urban areas having a higher prevalence than rural areas. There is no specific conclusion in either domestic or international research to explain this difference. Studies by Johanson found that the highest prevalence was in rural areas, the lowest was in urban areas, and the prevalence in cities was in-between. A study by Li *et al.*^[13] has shown that the constipation prevalence in rural areas was higher than that in cities. However, a study by Kan *et al.*^[8] showed that there was no difference in the constipation prevalence between urban and rural areas, and that the regional differences in urban and rural areas had little impact on prevalence. The difference in regional prevalence for constipation requires further studies; (2) the prevalence of constipation increases with age^[3,14,15], which was consistent with domestic and international reports. While aging is obviously a risk factor for

constipation, the prevalence in females is higher than males, but not to a significant degree; (3) education level has a negative correlation with constipation. Research by Johanson in the United States found that a population with a lower education level had a higher prevalence of constipation. However, there is no clear conclusion concerning the nature of the relationship between constipation prevalence with literacy level. A large sample of clinical studies is required for further confirmation; and (4) in elderly patients with constipation, the prevalence of gastritis and biliary tract diseases is higher than normal. Constipation is often associated with a variety of other digestive diseases. Previous studies have shown that patients with constipation often have associated diseases, such as functional dyspepsia and gastro-esophageal reflux disease. Physicians should pay special attention to such patients during clinical work.

Constipation has a serious effect on the quality of life, social functions, and daily living activities of the elderly^[16]. Choung *et al*^[17] randomly selected 4850 cases in various regions of different countries and carried a longitudinal cohort study on constipation for 20 years, of which there were 2853 respondents. His study found that patients with persistent constipation for 20 years were only 3% of cases, while non-persistent constipation was 21%. Choung *et al*^[18] surveyed the incidence of chronic constipation in a definite population in Olmsted Village in the United States from 1988 to 2003 *via* questionnaire letters and telephone interviews. The criteria for chronic constipation was defined as symptoms such as difficulty in defecation, straining stool, hard stool, and poor bowel, with at least two symptoms out of four and symptoms occurring at least 25% of the time. At the beginning of the survey there were a total of 4235 respondents (79%), while a reinvestigation of this population in 2003-2004 had only 2298 (55%) respondents. The cumulative total incidence of chronic constipation was 17.4%.

Our research shows that there is a difference in the prevalence rate of elderly constipation for different age groups, regions, and education levels. The incidence of acute constipation was higher than chronic constipation. The incidence of constipation increased with age, as primary constipation can persist. With increasing age, the incidence of acute constipation, increased co-morbidities, and increased secondary diseases (such as Parkinson's and diabetes) may increase^[19-21]. Chronic constipation rarely occurs during a long period of time, with previous cross-sectional studies apparently appearing to have exaggerated its prevalence. In order to reduce elderly constipation and its adverse effects, more attention from clinicians, early intervention, treatment, and improvement of the quality of life of the elderly is of utmost importance.

In conclusion, the prevalence of constipation was higher in the elderly population in the Beijing area. The prevalence of constipation closely resemble that found

in Western countries, and is significantly affected by region, age, and past history of other related illnesses.

COMMENTS

Background

With the increasing life expectancy of society, more elderly people will suffer from constipation. Constipation not only brings mental distress to the elderly, but also leads to a variety of sub-health symptoms and is a major factor in inducing gastrointestinal tumors. Therefore, to investigate the prevalence of elderly constipation and its related factors in Beijing, the authors carried out an epidemiological investigation into elderly constipation in different regions of Beijing in 2010. The Rome III criteria for constipation were taken as standard reference.

Research frontiers

Over the past decade, the prevalence of elderly constipation in Beijing was found to be rising, almost to the levels found in the West, suggesting we should be more concerned about the increasing prevalence of elderly constipation in the region. With regards to the differing prevalence between urban and rural areas, there as of yet no specific explanation to be found in either domestic or international research. This is also the case with the relationship between constipation prevalence and literacy level. A large sample of clinical studies is required for further confirmation.

Innovations and breakthroughs

In the Beijing area, there has been no large sample epidemiological investigation of elderly constipation in the past 10 years. There is little research on the epidemiology of constipation according to the Rome III criteria. The authors carried out an epidemiological investigation into elderly constipation in the Beijing area using the Rome III criteria.

Applications

The constipation prevalence in the Beijing region elderly closely resembles that found in Western countries. Elderly constipation is significantly affected by region, age, and past history of other related illnesses.

Terminology

The Rome III criteria for constipation was taken as standard reference, with the following constipation judgment indicators: defecation less than 3 times per week, stool weight less than 35 g/d, dry and hard stool, and difficulty in defecating during more than 25% of evacuation attempts.

Peer-review

This is a very interesting study about the epidemiology of elderly constipation in Beijing. In this study, the authors investigated the present situation of elderly constipation in urban and rural areas in the Beijing region.

REFERENCES

- 1 Fang X, Lu S, Pan G. [An epidemiologic study of bowel habit in adult non-patient population in Beijing area]. *Zhonghua Yixue Zazhi* 2001; **81**: 1287-1290 [PMID: 16200717]
- 2 Mugie SM, Benninga MA, Di Lorenzo C. Epidemiology of constipation in children and adults: a systematic review. *Best Pract Res Clin Gastroenterol* 2011; **25**: 3-18 [PMID: 21382575 DOI: 10.1016/j.bpg.2010.12.010]
- 3 Yu P, Li Z, Zheng H, Zhu H, Li X, He Q, Wang J, Yuan K, Jiang Z, Duan C, Gao F. Elderly constipation preliminary analysis of epidemiological characteristics. *Zhongguo Laonianxue Zazhi* 2001; **20**: 132-134 [DOI: 10.3760/j.issn:0254-9026.2001.02.015]
- 4 Yu X, Chen M. Guangzhou residents epidemiological investigation of functional constipation. *Weichangbingxue He Ganbingxue Zazhi* 2001; **10**: 150-155 [DOI: 10.3969/j.issn.1006-5709.2001.02.015]
- 5 Guo X, Ke M, Pan G, Han S, Fang X, Lu S, Guo H. Beijing area adult chronic constipation cluster, stratified, randomized

- epidemiological survey and analysis of relevant factors. *Zhonghua Xiaohua Zazhi* 2002; **22**: 637-638 [DOI: 10.3760/j.issn.0254-1432.2002.10.025]
- 6 **Xiong S**, Chen M, Chen H, Xu A, Wang W, Hu P. Guangdong Province community population epidemiological study of chronic constipation. *Zhonghua Xiaohua Zazhi* 2004; **24**: 448-449 [DOI: 10.3760/j.issn.0254-1432.2004.08.011]
 - 7 **Lv N**, Xie Y, Huang D, Wang C, Yuan Z, Hu J, Li G, Xiong W. Nanchang part of the population in the epidemiological investigation of chronic constipation. *Zhongguo Shiyong Neike Zazhi* 2005; **25**: 236-237 [DOI: 10.3969/j.issn.1005-2194.2005.03.021]
 - 8 **Kan Z**, Yao H, Long Z, Liu Z, Han Y, Zhang Z, Wang D, Yang Q, Ding G. Tianjin adult chronic constipation investigation and related factors. *Zhonghua Xiaohua Zazhi* 2004; **24**: 612-613 [DOI: 10.3760/j.issn.0254-1432.2004.10.011]
 - 9 **Liu Z**, Yang G, Shen Z, He W, He F, Yuan Y. Hangzhou City epidemiological survey of constipation. *Zhonghua Xiaohua Zazhi* 2004; **24**: 435-436 [DOI: 10.3760/j.issn.0254-1432.2004.07.019]
 - 10 **Costilla VC**, Foxx-Orenstein AE. Constipation: understanding mechanisms and management. *Clin Geriatr Med* 2014; **30**: 107-115 [PMID: 24267606 DOI: 10.1016/j.cger.2013.10.001]
 - 11 **Bruce Wirta S**, Hodgkins P, Joseph A. Economic burden associated with chronic constipation in Sweden: a retrospective cohort study. *Clinicoecon Outcomes Res* 2014; **6**: 369-379 [PMID: 25143749 DOI: 10.2147/CEOR.S61985]
 - 12 **Wald A**, Sigurdsson L. Quality of life in children and adults with constipation. *Best Pract Res Clin Gastroenterol* 2011; **25**: 19-27 [PMID: 21382576 DOI: 10.1016/j.bpg.2010.12.004]
 - 13 **Li Z**, Yu P, Shi Q, Jiang Z, Chu D, Lv X. Beijing urban and rural parts of the current status of investigations elderly constipation. *Zhongguo Laonianxue Zazhi* 2000; **20**: 1-2 [DOI: 10.3969/j.issn.1005-9202.2000.01.001]
 - 14 **Campbell AJ**, Busby WJ, Horwath CC. Factors associated with constipation in a community based sample of people aged 70 years and over. *J Epidemiol Community Health* 1993; **47**: 23-26 [PMID: 8382251 DOI: 10.1136/jech.47.1.23]
 - 15 **Stewart RB**, Moore MT, Marks RG, Hale WE. Correlates of constipation in an ambulatory elderly population. *Am J Gastroenterol* 1992; **87**: 859-864 [PMID: 1615939]
 - 16 **Belsey J**, Greenfield S, Candy D, Geraint M. Systematic review: impact of constipation on quality of life in adults and children. *Aliment Pharmacol Ther* 2010; **31**: 938-949 [PMID: 20180788 DOI: 10.1111/j.1365-2036.2010.04273.x]
 - 17 **Choung RS**, Locke GR, Rey E, Schleck CD, Baum C, Zinsmeister AR, Talley NJ. Factors associated with persistent and nonpersistent chronic constipation, over 20 years. *Clin Gastroenterol Hepatol* 2012; **10**: 494-500 [PMID: 22289877 DOI: 10.1016/j.cgh.2011.12.041]
 - 18 **Choung RS**, Locke GR, Schleck CD, Zinsmeister AR, Talley NJ. Cumulative incidence of chronic constipation: a population-based study 1988-2003. *Aliment Pharmacol Ther* 2007; **26**: 1521-1528 [PMID: 17919271 DOI: 10.1111/j.1365-2036.2007.03540.x]
 - 19 **Gage H**, Kaye J, Kimber A, Storey L, Egan M, Qiao Y, Trend P. Correlates of constipation in people with Parkinson's. *Parkinsonism Relat Disord* 2011; **17**: 106-111 [PMID: 21130017 DOI: 10.1016/j.parkreldis.2010.11.003]
 - 20 **Chen H**, Zhao EJ, Zhang W, Lu Y, Liu R, Huang X, Ciesielski-Jones AJ, Justice MA, Cousins DS, Peddada S. Meta-analyses on prevalence of selected Parkinson's nonmotor symptoms before and after diagnosis. *Transl Neurodegener* 2015; **4**: 1 [PMID: 25671103 DOI: 10.1186/2047-9158-4-1]
 - 21 **Rodrigues ML**, Motta ME. Mechanisms and factors associated with gastrointestinal symptoms in patients with diabetes mellitus. *J Pediatr (Rio J)* 2012; **88**: 17-24 [PMID: 22344626 DOI: 10.2223/JPED.2153]

P- Reviewer: Mullan MJ **S- Editor:** Gong ZM

L- Editor: Rutherford A **E- Editor:** Liu XM



Endoscopic stenting for inoperable malignant biliary obstruction: A systematic review and meta-analysis

Leonardo Zorrón Pu, Eduardo Guimarães Hourneaux de Moura, Wanderley Marques Bernardo, Felipe Iankelevich Baracat, Ernesto Quaresma Mendonça, André Kondo, Gustavo Oliveira Luz, Carlos Kiyoshi Furuya Júnior, Everson Luiz de Almeida Artifon

Leonardo Zorrón Pu, Eduardo Guimarães Hourneaux de Moura, Felipe Iankelevich Baracat, Ernesto Quaresma Mendonça, André Kondo, Gustavo Oliveira Luz, Carlos Kiyoshi Furuya Júnior, Everson Luiz de Almeida Artifon, Gastrointestinal Endoscopy Division, Department of Gastroenterology, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo 05403-000, Brazil

Wanderley Marques Bernardo, Guidelines Program Coordinator, Brazilian Medical Association, São Paulo 01333-001, Brazil

Author contributions: Zorrón Pu L did the electronic database search, revised and read all selected studies, analysed all data and graphs, wrote the paper; de Moura EGH scientifically coordinated the study; Bernardo WM performed a paired database search, peer-reviewed the paper and scientifically coordinated the study (biostatistics and evidence-based guidance); Baracat FI helped with the development of tables and graphs and, through weekly reunions, provided cues for improvements; Mendonça EQ helped with the development of tables and graphs and, through weekly reunions, provided cues for improvements; Kondo A helped with the revision of the article; Luz GO provided technical observations and evaluated bias and applicability of the therapies; Furuya Júnior CK provided technical observations and evaluated bias and applicability of the therapies; Artifon ELA scientifically coordinated the study; all authors reviewed and approved the final manuscript as submitted.

Conflict-of-interest statement: All the authors declare that they have no competing interests.

Data sharing statement: The technical appendix, statistical code, and dataset are available from the corresponding author at leozorron@gmail.com. No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on

different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Leonardo Zorrón Pu, MD, Fellow of the Gastrointestinal Endoscopy Division, Department of Gastroenterology, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo 05403-000, Brazil. leozorron@gmail.com
 Telephone: +55-11-26616467
 Fax: +55-11-26616467

Received: May 21, 2015
 Peer-review started: May 23, 2015
 First decision: July 10, 2015
 Revised: July 22, 2015
 Accepted: September 28, 2015
 Article in press: September 30, 2015
 Published online: December 21, 2015

Abstract

AIM: To analyze through meta-analyses the benefits of two types of stents in the inoperable malignant biliary obstruction.

METHODS: A systematic review of randomized clinical trials (RCT) was conducted, with the last update on March 2015, using EMBASE, CINAHL (EBSCO), MEDLINE, LILACS/CENTRAL (BVS), SCOPUS, CAPES (Brazil), and gray literature. Information of the selected studies was extracted in sight of six outcomes: primarily regarding dysfunction, complication and re-intervention rates; and secondarily costs, survival, and patency time. The data about characteristics of trial participants, inclusion and exclusion criteria and types of stents were also extracted. The bias was mainly assessed through the JADAD scale. This meta-

analysis was registered in the PROSPERO database by the number CRD42014015078. The analysis of the absolute risk of the outcomes was performed using the software RevMan, by computing risk differences (RD) of dichotomous variables and mean differences (MD) of continuous variables. Data on RD and MD for each primary outcome were calculated using the Mantel-Haenszel test and inconsistency was qualified and reported in χ^2 and the Higgins method (I^2). Sensitivity analysis was performed when heterogeneity was higher than 50%, a subsequent assay was done and other findings were compiled. Student's *t*-test was used for the comparison of weighted arithmetic means regarding secondary outcomes.

RESULTS: Initial searching identified 3660 studies; 3539 were excluded through title, repetition, and/or abstract, while 121 studies were fully assessed and were excluded mainly because they did not compare self-expanding metal stents (SEMS) and plastic stents (PS), leading to thirteen RCT selected, with 13 articles and 1133 subjects meta-analyzed. The mean age was 69.5 years old, that were affected mostly by bile duct (proximal) and pancreatic tumors (distal). The preferred SEMS diameter used was the 10 mm (30 Fr) and the preferred PS diameter used was 10 Fr. In the meta-analysis, SEMS had lower overall stent dysfunction compared to PS (21.6% vs 46.8%, $P < 0.00001$) and fewer re-interventions (21.6% vs 56.6%, $P < 0.00001$), with no difference in complications (13.7% vs 15.9%, $P = 0.16$). In the secondary analysis, the mean survival rate was higher in the SEMS group (182 d vs 150 d, $P < 0.0001$), with a higher patency period (250 d vs 124 d, $P < 0.0001$) and a lower cost per patient (4193.98 vs 4728.65 Euros, $P < 0.0985$).

CONCLUSION: SEMS are associated with lower stent dysfunction, lower re-intervention rates, better survival, and higher patency time. Complications and costs showed no difference.

Key words: Biliary tract neoplasms; Malignant biliary obstruction; Jaundice; Palliative care; Endoscopic retrograde cholangiopancreatography; Stent; Systematic review; Meta-analysis

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Endoscopic stenting is accepted worldwide as the first choice palliative treatment for malignant biliary obstruction. There are still two types of materials currently being used, which are plastic and metal. Therefore, many doubts are raised as to which one is the most beneficial to the patient. This review gathers the highest quality information available about these two types of stent, giving information in regards to dysfunction, complication, re-intervention rates, costs, survival, and patency time; and intend to help handle clinical practice nowadays, especially in countries where the availability of metallic stents is scarce and

cannot be offered to all patients.

Zorrón Pu L, de Moura EGH, Bernardo WM, Baracat FI, Mendonça EQ, Kondo A, Luz GO, Furuya Júnior CK, Artifon ELA. Endoscopic stenting for inoperable malignant biliary obstruction: A systematic review and meta-analysis. *World J Gastroenterol* 2015; 21(47): 13374-13385 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13374.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13374>

INTRODUCTION

Biliary tract neoplasms are uncommon yet important pathologies. Their main symptom (jaundice) can cause important disorders, such as immunosuppression^[1-4], and prognosis is usually poor^[5]. Despite their rarity, estimates from the SEER (Surveillance, Epidemiology and End Results) database from North America reveal increased incidence and a maintained poor prognosis^[6].

It is estimated that almost 20% of the subclinical jaundice is due to malignant bile duct obstruction^[7], divided about 2/3 to 1/3 between pancreatic and other biliary obstructive cancers, respectively^[8].

Because of its invasiveness and late symptom appearance and onset in elderly people, the majority of the diagnosed cases are not deemed curable by resection^[9-11]. According to INCA (Brazilian's National Institute of Cancer) pancreatic tumors accounted for 2% of the malignant tumors of Brazil, with an estimate of around 17000 new cases in 2015. Having in mind that only 15%-20% of these neoplasms are resectable, the number of inoperable malignant biliary obstruction (MBO) just in Brazil in 2015 has been estimated at over 13000^[12].

The use of palliative methods is essential in these cases and endoscopic biliary stenting is becoming more prevalent, especially in high operative risk cases or very ill patients, because it is minimally invasive^[13,14]. The subject of the quality and durability of the palliative methods is becoming more important due to skyrocketing survival in advanced stages biliary tract neoplasms. For instance, Kim *et al.*^[15] demonstrated a survival in metastatic biliary tract cancer of about 9 mo in a phase II study of gemcitabine and S-1 combination chemotherapy, in contrast of the 3-4 mo survival of earlier studies.

Two types of stents are routinely used in current practice: plastic stents (PS) and self-expanding metal stents (SEMS). Several randomized clinical trials (RCT) demonstrated that metal stents are associated with longer stent patency but survival is the same as when plastic stents are used. Some studies favored SEMS^[16-25] and some favored PS^[26,27], although the difference about survival has only been shown in one study, favoring SEMS^[28].

The latest meta-analysis regarding metal and plastic stenting in malignant biliary obstruction^[29],

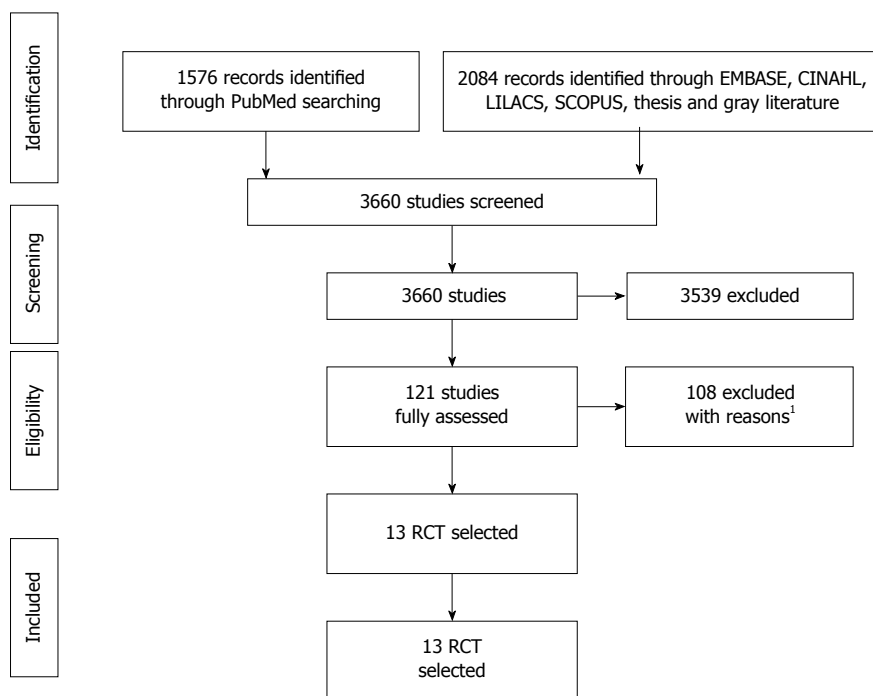


Figure 1 PRISMA's studies selection fluxogram. ¹The reasons for exclusions are displayed on Supplementary Table 1. RCT: Randomized clinical trials.

which involved stents inserted through percutaneous transhepatic drainage and endoscopic retrograde cholangiopancreatography (ERCP), published in January 2013 and involving ten RCT, demonstrated the same information about stent patency and re-intervention rates, although it also demonstrated a diminished survival in the PS group. This meta-analysis aims to use the 4 RCT not included in the last study.

Considering the acceptable use of either SEMS or PS in endoscopic stenting for inoperable MBO, this meta-analysis aims to gather applicable information, primarily regarding dysfunction, complication and re-intervention rates; and secondarily costs, survival, and patency time.

MATERIALS AND METHODS

Literature search

This meta-analysis was registered in the PROSPERO international prospective register of systematic review^[30], by the number CRD42014015078.

All assessed randomized clinical trials regarding comparisons between SEMS and PS that were endoscopically placed for MBO were collected from these databases: EMBASE, CINAHL (EBSCO), MEDLINE, LILACS and CENTRAL (BVS), SCOPUS, Master's and Doctorate's theses and dissertations on the CAPES database (Brazil)^[31], and gray literature. Any outcome was considered, from any date of publication until March 2015, with any number of subjects. Publications were accepted in any format, language, or publication status.

Besides searching through the tools mentioned

above, we conducted a direct approach by emailing authors, when necessary. The last date the databases were assessed for new releases was March 15, 2015.

The search strategies used for MEDLINE, SCOPUS and EMBASE databases are stated in supplementary material; the Brazilian Master's and Doctorate's thesis and dissertations strategy was the word "stent"; CINAHL, LILACS and CENTRAL strategies were "plastic stent OR metallic stent (filter - randomized controlled trials)".

Study selection

Initially, studies were excluded because clear information in the title or abstract stated that it did not compare stent techniques or did not study MBO. Furthermore, the abstracts and full articles were assessed and excluded if proven not to be RCT or the comparison was not between SEMS and PS.

Initial searching identified 3660 studies; 3539 were excluded through title, repetition, and/or abstract (Figure 1). One hundred and twenty-one studies were fully assessed and were excluded mainly because they did not compare PS and SEMS. One of the studies included in the last meta-analysis of Hong *et al.*^[29] was not used because it involved only percutaneous transhepatic drainage. Two authors were contacted through email regarding two studies that, later on, proved unfit for this systematic review; the reasons are shown in Supplementary Table 1, in the supplementary material. In addition, there were some studies that were previews of later ones, and thus were also excluded after confirmation. Detailed information about the findings by database and reasons for exclusion can

Table 1 Neoplasms distribution in individual studies

Ref.	No. of patients	Bile duct cancer (%)	Pancreatic tumor (%)	Papillae tumor (%)	Gallbladder cancer (%)	Metastatic tumor (%)	Other (%)
Walter <i>et al</i> 2014	240 ¹	0	83	0	0	0	17
Moses <i>et al</i> 2013	85	0	69	3	3	10	15
Mukai <i>et al</i> 2013	60	50	0	0	22	28	0
Sangchan <i>et al</i> 2012	108	100	0	0	0	0	0
Bernon <i>et al</i> 2011	22	NA	NA	NA	NA	NA	NA
Isayama <i>et al</i> 2011	120	0	100	0	0	0	0
Soderlund <i>et al</i> 2006	100	9	78	2	0	7	4
Katsinelos <i>et al</i> 2006	47	17	53	11	0	19	0
Kaassis <i>et al</i> 2003	118	15	75	0	0	0	10
Prat <i>et al</i> 1998	105 ¹	21	64	3	0	12	0
Wagner <i>et al</i> 1993	20	100	0	0	0	0	0
Knyrim <i>et al</i> 1993	62	3	69	5	0	0	23
Davids <i>et al</i> 1992	105	0	100	0	0	0	0

¹Not all patients were included in the meta-analysis.

be found in the supplementary material.

Data extraction

The data were extracted from all the databases mentioned by two independent authors, confirming the same eligible final studies. The eligible studies were confronted after both authors stopped article exclusion.

The dysfunction outcome was accessed through the number of patients who had occlusion, migration, or kinking of the first stent used; therefore any disorder, confirmed or presumed because of cholestasis, that needed a re-intervention.

Re-intervention was any procedure needed for a new drainage of the biliary tree, endoscopic or percutaneous, to replace a dysfunctional stent.

The complication outcome was any disorder attributed to the stent insertion that required a medical intervention at the time of diagnosis, but not obligatorily related to stent dysfunction (pancreatitis, cholangitis, bleeding, perforation, cholecystitis, liver abscess).

The outcome of costs was calculated using the data presented in the studies and converted to Euro (€), regardless of whether they concerned the full treatment, or using stent value and the number of stents exchanged only. Stent patency time was the mean time to stent dysfunction, calculated in days. Overall survival was calculated through the reported mean time of survival in days. If the study used months, each month was considered as having 30 d.

Risk of bias

The biases were individually assessed through the JADAD scale^[32] (Figure 2), a tool to assess a RCT quality through evaluation of blinding, randomization, and losses reported. In addition, biases were evaluated regarding intention to treat, prognosis characteristics, regional differences, amount of losses, and follow up; these were not used as exclusion criteria.

Although no restrictions were made for language

in the present study, it has a publication bias since the search databases used required that studies have abstracts in English or Portuguese. Therefore, studies in other languages that did not have at least an abstract in English were not assessed. The differences between the study population, type of cancer, and type and diameter of stent were considered as possible biases.

Statistical analysis

Regarding meta-analysis, the difference was calculated as the risk difference for dichotomic variables with a Cochran-Mantel-Haenszel test with a 95%CI, and as the mean difference with fixed effect using inverse variance, with a 95%CI, for continuous variables. The semi-quantitative measures were described as the weighted arithmetic mean using the patient number of each study, with standard deviation, and Student's *t*-test analysis. All data were addressed as intention to treat analysis (ITT).

RevMan 5 software (Review Manager version 5.3.5 - Cochrane Collaboration, Copyright © 2014) was used for meta-analysis of stent dysfunction, complication, and re-intervention rates. Student's *t*-test was used for the comparison of weighted arithmetic means regarding costs, survival, and stent patency, as we could not extract the standard deviation of this data from most of the studies selected. The heterogeneity was evaluated through a χ^2 test (I^2 , or χ^2), and modified up to 50% with sensitive analysis, when possible and necessary.

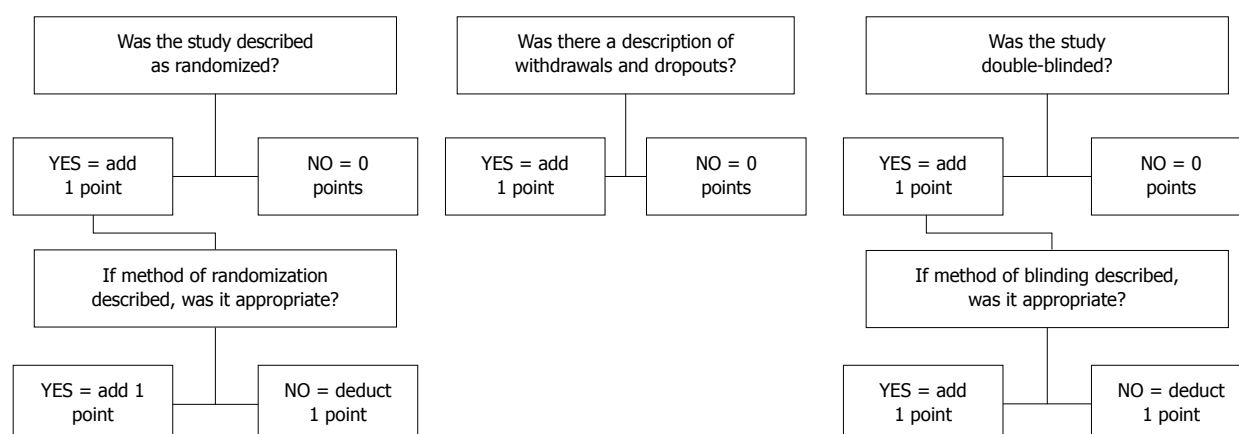
Additional analysis

The studies were further divided between proximal (MPBO) and distal (MDBO) obstruction, and JADAD ≥ 3 and JADAD < 3 for meta-analysis.

RESULTS

Studies characteristics

A total of 13 RCT were selected, with 1133 patients



Adapted from Jadad *et al*^[32]. 1996

Figure 2 JADAD scale.

(mean age of 69.5 years old) affected mostly by bile duct (MPBO) and pancreatic tumors (MDBO). Apart from the most common causes of biliary obstruction, 5 papers had description of metastatic tumors causing this obstruction, probably caused by extrinsic compression. Moses *et al*^[27] had around 10% of metastatic cancer from a primary location of colon and lung; Mukai *et al*^[17] had about 30% of metastatic cancer as cause for obstruction while Soderlund *et al*^[20] found 7%, although no information about the primary site was described for neither; Katsinelos *et al*^[21] describes compressive lymph nodes as cause for 19% of the obstructions, while Prat *et al*^[26] had 12%. The preferred SEMS diameter used was the 10 mm (30 Fr) and the preferred PS diameter used was 10 Fr. Detailed data regarding the etiology of the MBO can be found in Table 1. Supplementary Tables 2 and 3 have information about outcomes and specific stent used and can be found in the supplementary material. All the extractable data used in the calculations are exposed in Supplementary Table 4.

Risk of bias within studies

The maximum score in the JADAD scale^[32] was 3 because it is not possible to have a double-blind study in this field. In total, there were 6 studies with JADAD 1, 2 studies with JADAD 2, and 5 studies with JADAD 3. Overall, survival did not include information about neoplasm-specific mortality. The studies did not always give strict specifications about the stent's type, nor about inoperable criteria. The use of one abstract^[19] also negatively impacted the information that could be assessed; however, the information given was sufficient for some outcomes. Prat *et al*^[26] only had the number of ERCP performed because of dysfunction (accordingly, this was the number used for the dysfunction outcome) and excluded people who lived farther than 150 km from the hospital, leading to a possible bias; also, there was no information about

the currency used for cost calculation, but because the study was conducted in France, it was considered as the Euro. In addition, the study had three groups, one of which was not used in our meta-analysis because the outcomes analyzed would be biased (the group had scheduled stent exchange regardless of the patients' symptoms). Walter *et al*^[16] performed the randomization before the ERCP but excluded some individuals after it. Despite the intent of using ITT analysis for this study, the information on which randomized group the excluded patients were from was not available. Moreover, the information about the mean survival time specified per stent was not obtainable. Five studies^[16,17,20,23,25] used the combined approach (rendezvous) or PTC when the endoscopic approach alone was insufficient. All studies selected were RCT with no selection bias.

Results of individual studies

All studies had extractable information about stent dysfunction, ten about re-intervention (although Walter *et al*^[16] was not used because the lack of standard deviation data), and eleven about complication; these were used in the meta-analysis. Eleven studies had comparative information on overall survival, eight on costs and ten on stent patency time (Kaassis *et al*^[22] had information only about the PS group), although the information did not include the standard deviation (SD), which compromised our intent for quantitative analysis. Therefore, these three latest outcomes were evaluated semi-quantitatively. Overall, the results regarding stent dysfunction, time of stent patency, and re-intervention were homogeneously beneficial towards SEMS in all studies. Regarding costs, complications, and survival, the results were discrepant.

Synthesis of results

The results were divided between quantitative and semi-quantitative results because of the absence

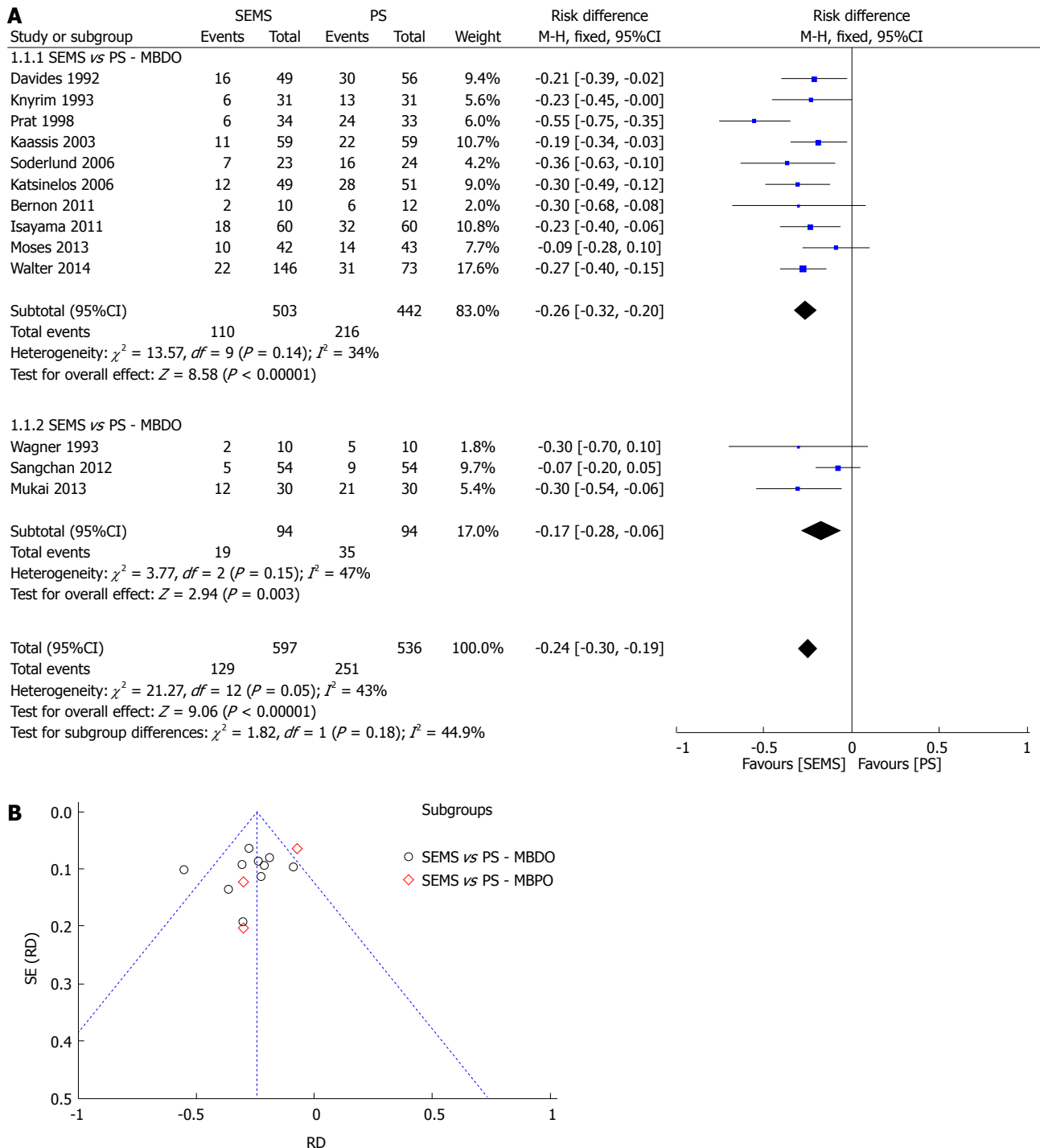


Figure 3 Stent dysfunction. A: Forest plot; B: Funnel plot.

of vital information for quantitative analysis of all outcomes.

Primary outcomes: The dysfunction rate was 24% lower in the SEMS group, with a NNT of 5. The sensitive analysis lowered the heterogeneity to 0% (withdrawing data from Prat *et al.*^[26]) with no change to the result. The meta-analysis graph was divided between MBDO (1.1.1) and MBPO (1.1.2) (Figure 3).

Re-intervention was separated into dichotomous and continuous data, corresponding to the data

reported by the author, except for Katsinelos *et al.*^[21], whose data were transformed into continuous for statistical analysis purposes. In both meta-analyses, SEMS had at least 30% fewer re-interventions and a NNT of 3. The meta-analysis graph was divided between MBDO (1.3.1/1.5.1) and MBPO (1.3.2/1.5.2) (Figure 4 and Figure 5).

MBO complication rates were not different between PS or SEMS. Even after sensitivity analysis (with the lowest heterogeneity of 0%, withdrawing 5 studies), the result remained the same. The meta-analysis

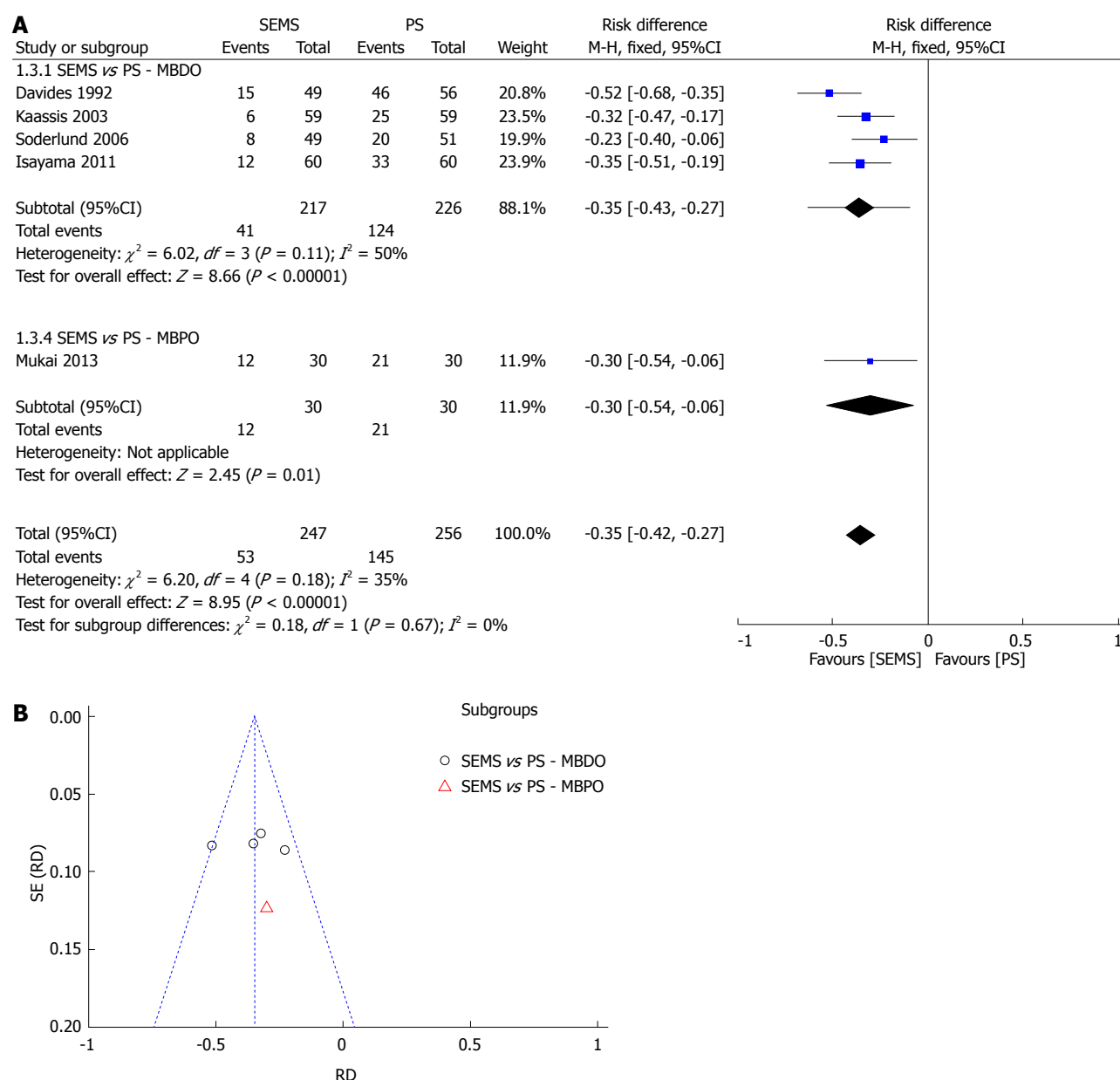


Figure 4 RE-INTERVENTION (dichotomic). A: Forest plot; B: Funnel plot.

graph was divided between MBDO (1.7.1) and MBPO (1.7.2) (Figure 6).

Secondary outcomes: In the costs analysis, the SEMS group had a less expensive result, although without statistical significance, with a mean of €4193.98 for SEMS *vs* €4728.65 for PS ($P = 0.0985$) (Table 2).

The time for stent dysfunction was measured in days and was statistically higher in the SEMS group (250 *vs* 124 d, $P < 0.0001$) (Table 3).

Regarding survival, also measured in days, SEMS had a better result. The outcome was divided in MBDO and MBPO because of the different causes of malignant obstruction between the two, therefore we tried to minimize the bias of the worse survival in hilar tumors. Nevertheless, apart from a global minor survival in

the MPBO for the both stents comparing to MBDO, SEMS had a longer survival, with statistical significance in MBPO and MBDO alike (Figure 7). Detailed data regarding survival of each study can be found in Supplementary Figures 1 and 2.

Risk of bias across studies

The definition of dysfunction varied from study to study, but always indicated an improper drainage requiring re-intervention. The costs were measured by the studies in Deutsche Marks, Japanese Yen, and Euros. The values were all converted to Euro to lessen the conversion error, as the majority of the results were in this currency. The fare of the month and year of the last included patient was used for Mukai *et al.*^[17], and the fare of February 1999 for earlier studies, using the database of Brazil's Central Bank^[33]. The

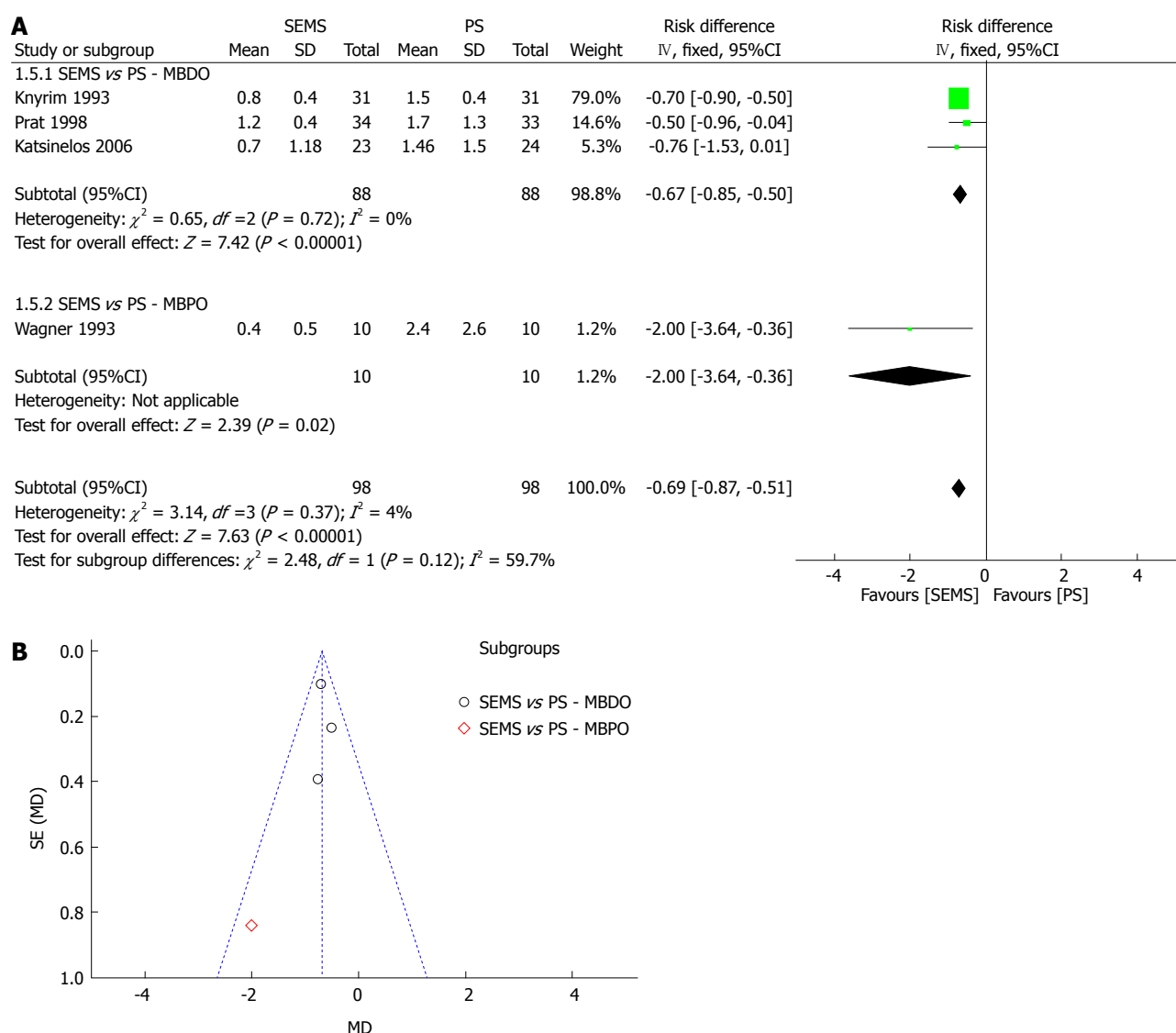


Figure 5 RE-INTERVENTION (continuous). A: Forest plot; B: Funnel plot.

costs have another bias, as most studies considered the whole treatment (*i.e.*, ICU, ERCP initial and re-intervention, stents, hostelry, drugs, and laboratory), while Katsinelos *et al.*^[21] accounted only for the prosthesis itself Katsinelos *et al.*^[21].

The invasiveness, other than “inoperable tumor” and the patient performance status, was not globally reported and the exclusion criteria differed between the studies, with populations from 28% Prat *et al.*^[26] to 71% Moses *et al.*^[27] with liver metastasis. This probably was a bias between studies for the overall survival and time to stent dysfunction comparisons, because of prognostic differences. Also, the younger mean age of the patients in Davids *et al.*^[23] could have influenced the prognosis.

There was also bias regarding the different types of stents used in the studies (uSEMS, pcSEMS and cSEMS; Amsterdam and Tannenbaum PS), and the number of stents used in the same procedure.

Additional analysis

The studies were divided between MBPO and MBDO and therefore analyzed in the same way. We also analyzed the subgroups with JADAD ≥ 3 and JADAD < 3 . The subgroup analysis did not change the previously-stated results, hence the graphs are located in the supplementary material. The meta-analysis graph was divided between JADAD ≥ 3 (1.2.1/1.4.1/1.6.1/1.8.1) and JADAD < 3 (1.2.2/1.4.2/1.6.2/1.8.2), found in Supplementary Figures 3-10.

DISCUSSION

The use of SEMS has fewer dysfunction, longer patency, and longer survival. It requires fewer re-interventions, with no differences in complication rate or costs, when compared to PS.

Regarding the patients' quality of life and adequate palliative care with the lowest hospital stay possible

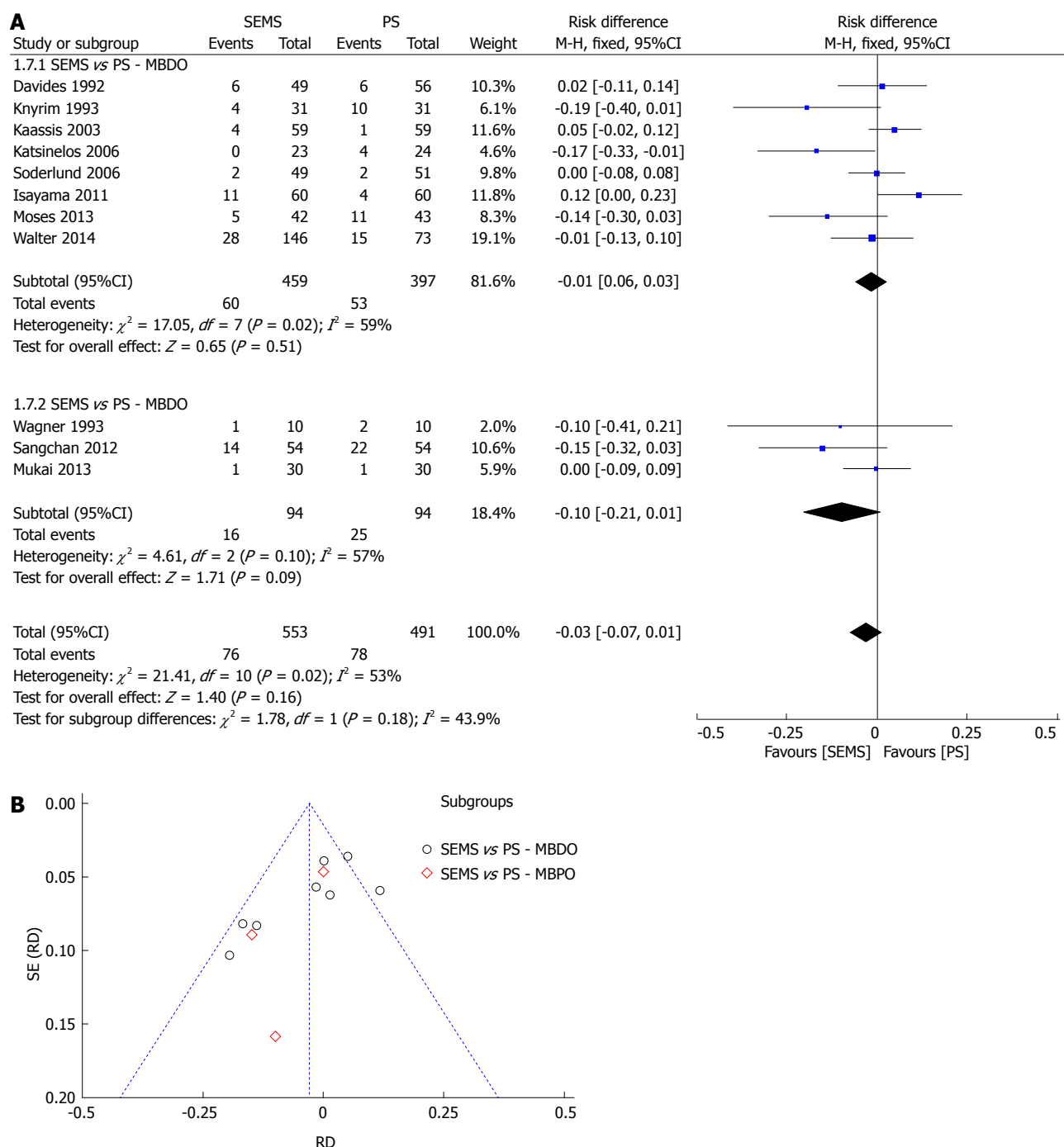


Figure 6 Complication. A: Forest plot; B: Funnel plot.

and minimal symptomatology, SEMS are always the first option, as there is no definitive way to determine one's life expectancy, despite the known mean survival rates according to the stage of the disease^[34-36]. From the perspective of the healthcare system and the rational use of materials, SEMS does not seem to have more to offer than PS for very ill patients (with a life expectancy of less than 4 mo), however with the innovations in chemo and radiotherapy, the life expectancy shall only increase^[15]. Therefore, the SEMS could be saved for cases that are more suitable, without compromising adequate palliative care, by

considering their mean survival (99-162 d vs 159-187 d, $P < 0.0001$) and mean patency (124 d vs 250 d, $P < 0.0001$), compared to PS. This is true especially in countries like Brazil, where SEMS are not widely available for every inoperable MBO, so we could make the most of our limited resources.

The results of sub-analysis did not differ from the main results, so the conclusions can be extrapolated to MPBO and MDBO alike.

New RCT that study the comparison between PS and SEMS, especially regarding quality of life and costs, with explicit tumor staging involved and

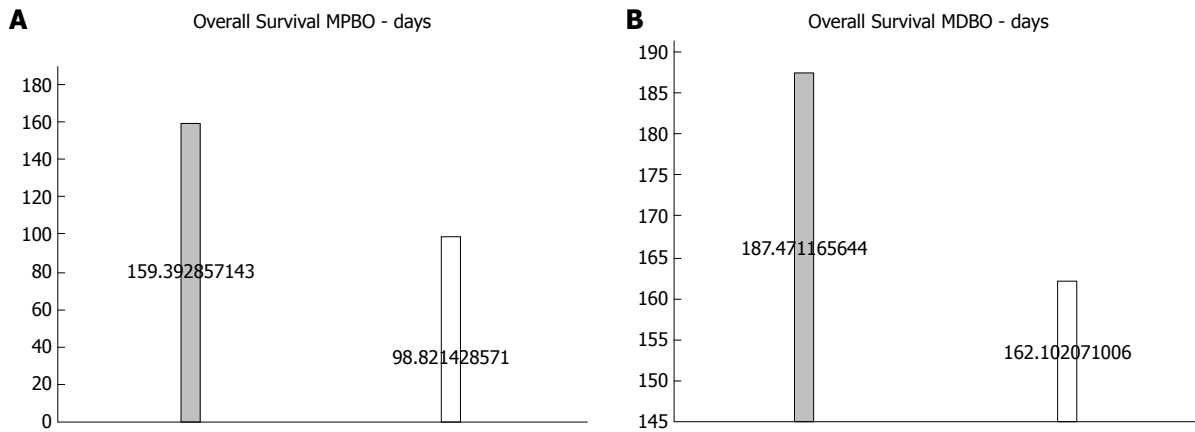


Figure 7 Mean survival by stent in patients ($P < 0.0001$). A: With proximal tumors; B: With distal tumors.

Table 2 Costs evaluation - per patient in Euros

Ref.	Costs (€)					
	PS			SEMS		
	No pts	PS cost (mean)	Total	No pts	SEMS cost (mean)	Total
Walter <i>et al</i> , 2014	73	6906	504138	146	6437	939802
Mukai <i>et al</i> , 2013	30	17170.19	515105.7	30	8935.68	268070.4
Soderlund <i>et al</i> , 2006	51	953.1372549	48610	49	940	46060
Katsinelos <i>et al</i> , 2006	24	737.5	17700	23	1308.695652	30100
Kaassis <i>et al</i> , 2003	59	1216.805085	71791.5	59	1208.116949	71278.9
Prat <i>et al</i> , 1998	33	5547	183051	34	4643	157862
Wagner <i>et al</i> , 1993	10	3511.31	35113.1	10	2552.57	25525.7
Knyrim <i>et al</i> , 1993	31	3067.75	95100.25	31	2045.17	63400.27
MEAN (per patient)		4728.648071			4193.977147	
SD		5434.740692			2905.677081	

$P = 0.0985$

SEMS: Self-expanding metal stents; PS: Plastic stents.

Table 3 Time for stent dysfunction - mean time per author in days

Time for dysfunction in days		
Ref.	PS	SEMS
Walter <i>et al</i> , 2014	172	293.3
Moses <i>et al</i> , 2013	153.3	385.3
Mukai <i>et al</i> , 2013	112	359
Sangchan <i>et al</i> , 2012	35	103
Isayama <i>et al</i> , 2011	202	285
Soderlund <i>et al</i> , 2006	54	108
Katsinelos <i>et al</i> , 2006	123.5	255
Prat <i>et al</i> , 1998	96	144
Davids <i>et al</i> , 1992	126	273
MEAN	123.7261792	250.2537988
SD	53.38757918	104.0647031

$P < 0.0001$

SEMS: Self-expanding metal stents; PS: Plastic stents.

presenting the means for a meta-analysis (data with standard deviation and tables), are required for advancing the knowledge of palliative care for MBO. These studies could be considered in very ill and poor prognosis patients, with no risk for their optimal palliative treatment.

The lack of information, such as the SD in the costs, overall survival, and patency time outcomes, made some data unsuitable for meta-analysis, despite being adequate for semi-quantitative analysis. The heterogeneity in the costs analysis of each study compromises its adequate comparison. Non-standard and subjective criteria for inoperable patients could have undermined the complication and costs significance, as these may have been different if only metastatic or non-metastatic patients could be clustered.

In conclusion, metal stents are associated with a lower rate of stent dysfunction and lower re-intervention rate, with no difference in complications. SEMS also have better survival rates and a higher patency period with no difference in costs from PS.

COMMENTS

Background

It is estimated that almost 20% of the subclinical jaundice is due to malignant bile duct obstruction, divided about 2/3 to 1/3 between pancreatic and other biliary obstructive cancers, respectively. According to INCA (Brazilian's National Institute of Cancer), the number of inoperable malignant biliary obstruction (MBO) just in Brazil in 2015 should be over 13000. The use of palliative

methods is essential in these cases and endoscopic biliary stenting is becoming more prevalent, especially in high operative risk cases or very ill patients, because it is minimally invasive. Endoscopic stenting is accepted worldwide as the first choice palliative treatment for malignant biliary obstruction. There are still in use two main types of material (plastic and metal), leaving doubts about what should be the best indication for one or the other. This review gathers the most recent quality information about these two types of stent, bringing information regarding dysfunction, complication, re-intervention rates, costs, survival, and patency time; and intend to help handling nowadays clinical practice especially in countries where the availability of metallic stents is scarce and cannot be offered to all patients.

Research frontiers

To choose the better stent for treatment of a patient while saving resources to the other patients with similar conditions is the main question of this meta-analysis.

Innovations and breakthroughs

There are still in use two main types of stent for MBO, and despite the loose belief that plastic stents (PS) are better for short survival patients and self-expanding metal stents (SEMS) are better for long survival patients, there is no consensus or guidelines to determine in what cases the endoscopist can use each.

Applications

This review aims to help with detailed and specific information about each stent to aid physicians and the Health's Departments around the globe, showing that sometimes the cheapest item can be not so advantageous when you look at the whole treatment. The discrepancy of measures in the selected studies is a limitation. Prospective randomized controlled studies with attention to a specific population (short expected survival) are needed to clarify if the SEMS is actually no better in this group.

Terminology

Malignant biliary obstruction is defined as a cancer being responsible, directly or indirectly, for the mechanical blockage of the bile output, produced by the hepatocytes. Clinically, it usually is suspected because of jaundice. When this cancer cannot be resected, due to the patient's clinical condition or due to the cancer invasion, it is nominated inoperable. Palliatively, the first choice to treat the symptoms is to place an endoscopic stent, that nowadays can be either made of plastic or metal.

Peer-review

In this meta-analysis, the authors have presented the costs and clinical outcomes of endoscopic stenting with SEMS and PS in patients with inoperable MBO, bringing the information to better understand the advantages and disadvantages of each.

REFERENCES

- 1 **Roughneen PT**, Didlake R, Kumar SC, Kahan BD, Rowlands BJ. Enhancement of heterotopic cardiac allograft survival by experimental biliary ligation. *Transplantation* 1987; **43**: 437-438 [PMID: 3547799 DOI: 10.1097/00007890-198703000-00023]
- 2 **Treglia-Dal Lago M**, Jukemura J, Machado MC, da Cunha JE, Barbuto JA. Phagocytosis and production of H2O2 by human peripheral blood mononuclear cells from patients with obstructive jaundice. *Pancreatol* 2006; **6**: 273-278 [PMID: 16636599 DOI: 10.1159/000092688]
- 3 **Kawarabayashi N**, Seki S, Hatsuse K, Kinoshita M, Takigawa T, Tsujimoto H, Kawabata T, Nakashima H, Shono S, Mochizuki H. Immunosuppression in the livers of mice with obstructive jaundice participates in their susceptibility to bacterial infection and tumor metastasis. *Shock* 2010; **33**: 500-506 [PMID: 19823116]
- 4 **Katz SC**, Ryan K, Ahmed N, Plitas G, Chaudhry UI, Kingham TP, Naheed S, Nguyen C, Somasundar P, Espat NJ, Junghans RP, Dematteo RP. Obstructive jaundice expands intrahepatic regulatory T cells, which impair liver T lymphocyte function but modulate liver cholestasis and fibrosis. *J Immunol* 2011; **187**: 1150-1156 [PMID: 21697460 DOI: 10.4049/jimmunol.1004077]
- 5 **Evans DB**, Farnell MB, Lillemoe KD, Vollmer C, Strasberg SM, Schulick RD. Surgical treatment of resectable and borderline resectable pancreas cancer: expert consensus statement. *Ann Surg Oncol* 2009; **16**: 1736-1744 [PMID: 19387741 DOI: 10.1245/s10434-009-0416-6]
- 6 **Siegel R**, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin* 2014; **64**: 9-29 [PMID: 24399786 DOI: 10.3322/caac.21208]
- 7 **Reisman Y**, Gips CH, Lavelle SM, Wilson JH. Clinical presentation of (subclinical) jaundice--the Euricterus project in The Netherlands. United Dutch Hospitals and Euricterus Project Management Group. *Hepatogastroenterology* 1996; **43**: 1190-1195 [PMID: 8908550]
- 8 **Carriaga MT**, Henson DE. Liver, gallbladder, extrahepatic bile ducts, and pancreas. *Cancer* 1995; **75**: 171-190 [PMID: 8000995]
- 9 **Ryan DP**, Hong TS, Bardeesy N. Pancreatic adenocarcinoma. *N Engl J Med* 2014; **371**: 1039-1049 [PMID: 25207767 DOI: 10.1056/NEJMr1404198]
- 10 **Burke EC**, Jarnagin WR, Hochwald SN, Pisters PW, Fong Y, Blumgart LH. Hilar Cholangiocarcinoma: patterns of spread, the importance of hepatic resection for curative operation, and a presurgical clinical staging system. *Ann Surg* 1998; **228**: 385-394 [PMID: 9742921 DOI: 10.1097/00000658-199809000-00011]
- 11 **Albores-Saavedra J**, Schwartz AM, Batich K, Henson DE. Cancers of the ampulla of vater: demographics, morphology, and survival based on 5,625 cases from the SEER program. *J Surg Oncol* 2009; **100**: 598-605 [PMID: 19697352 DOI: 10.1002/jso.21374]
- 12 Available from: URL: <http://www.inca.gov.br/>
- 13 **Scott EN**, Garcea G, Doucas H, Steward WP, Dennison AR, Berry DP. Surgical bypass vs. endoscopic stenting for pancreatic ductal adenocarcinoma. *HPB (Oxford)* 2009; **11**: 118-124 [PMID: 19590634]
- 14 **Glazer ES**, Hornbrook MC, Krouse RS. A meta-analysis of randomized trials: immediate stent placement vs. surgical bypass in the palliative management of malignant biliary obstruction. *J Pain Symptom Manage* 2014; **47**: 307-314 [PMID: 23830531 DOI: 10.1016/j.jpainsymman.2013.03.013]
- 15 **Kim HS**, Kim HY, Zang DY, Oh HS, Jeon JY, Cho JW, Park CK, Kim JH, Kim MJ, Ha HI, Kim JH, Han B, Song H, Kwon JH, Choi DR, Jung JY. Phase II study of gemcitabine and S-1 combination chemotherapy in patients with metastatic biliary tract cancer. *Cancer Chemother Pharmacol* 2015; **75**: 711-718 [PMID: 25630414 DOI: 10.1007/s00280-015-2687-x]
- 16 **Walter D**, Van Boeckel PG, Groenen M. Metal stent placement is cost-effective for palliation of malignant common bile duct obstruction: A randomized controlled trial. *Gastrointest Endosc* 2014; **79** (Suppl 1): 5 [DOI: 10.1016/j.gie.2014.02.144]
- 17 **Mukai T**, Yasuda I, Nakashima M, Doi S, Iwashita T, Iwata K, Kato T, Tomita E, Moriwaki H. Metallic stents are more efficacious than plastic stents in unresectable malignant hilar biliary strictures: a randomized controlled trial. *J Hepatobiliary Pancreat Sci* 2013; **20**: 214-222 [PMID: 22415652 DOI: 10.1007/s00534-012-0508-8]
- 18 **Isayama H**, Yasuda I, Ryoza S, Maguchi H, Igarashi Y, Matsuyama Y, Katanuma A, Hasebe O, Irisawa A, Itoi T, Mukai H, Arisaka Y, Okushima K, Uno K, Kida M, Tamada K. Results of a Japanese multicenter, randomized trial of endoscopic stenting for non-resectable pancreatic head cancer (JM-test): Covered Wallstent versus DoubleLayer stent. *Dig Endosc* 2011; **23**: 310-315 [PMID: 21951091 DOI: 10.1111/j.1443-1661.2011.01124.x]
- 19 **Bernon M**, Shaw J, Krige J, Bornman P. Malignant biliary obstruction: A prospective randomised trial comparing plastic and metal stents for palliation of symptomatic jaundice. *HPB* 2011; **13**: 139-145 [DOI: 10.1111/j.1477-2574.2011.00308.x]
- 20 **Soderlund C**, Linder S. Covered metal versus plastic stents for malignant common bile duct stenosis: a prospective, randomized, controlled trial. *Gastrointest Endosc* 2006; **63**: 986-995 [PMID: 16733114 DOI: 10.1016/j.gie.2005.11.052]
- 21 **Katsinelos P**, Paikos D, Kountouras J, Chatzimavroudis G,

- Paroutoglou G, Moschos I, Gatopoulou A, Beltsis A, Zavos C, Papaziogas B. Tannenbaum and metal stents in the palliative treatment of malignant distal bile duct obstruction: a comparative study of patency and cost effectiveness. *Surg Endosc* 2006; **20**: 1587-1593 [PMID: 16897286 DOI: 10.1007/s00464-005-0778-1]
- 22 **Kaassis M**, Boyer J, Dumas R, Ponchon T, Coumaros D, Delcenserie R, Canard JM, Fritsch J, Rey JF, Burtin P. Plastic or metal stents for malignant stricture of the common bile duct? Results of a randomized prospective study. *Gastrointest Endosc* 2003; **57**: 178-182 [PMID: 12556780 DOI: 10.1067/mge.2003.66]
- 23 **Daivids PH**, Groen AK, Rauws EA, Tytgat GN, Huibregtse K. Randomised trial of self-expanding metal stents versus polyethylene stents for distal malignant biliary obstruction. *Lancet* 1992; **340**: 1488-1492 [PMID: 1281903 DOI: 10.1016/0140-6736(92)92752-2]
- 24 **Knyrim K**, Wagner HJ, Pausch J, Vakil N. A prospective, randomized, controlled trial of metal stents for malignant obstruction of the common bile duct. *Endoscopy* 1993; **25**: 207-212 [PMID: 8519239 DOI: 10.1055/s-2007-1010294]
- 25 **Wagner HJ**, Knyrim K, Vakil N, Klose KJ. Plastic endoprotheses versus metal stents in the palliative treatment of malignant hilar biliary obstruction. A prospective and randomized trial. *Endoscopy* 1993; **25**: 213-218 [PMID: 7686100 DOI: 10.1055/s-2007-1010295]
- 26 **Prat F**, Chapat O, Ducot B, Ponchon T, Pelletier G, Fritsch J, Choury AD, Buffet C. A randomized trial of endoscopic drainage methods for inoperable malignant strictures of the common bile duct. *Gastrointest Endosc* 1998; **47**: 1-7 [PMID: 9468416 DOI: 10.1016/S0016-5107(98)70291-3]
- 27 **Moses PL**, Alnaamani KM, Barkun AN, Gordon SR, Mitty RD, Branch MS, Kowalski TE, Martel M, Adam V. Randomized trial in malignant biliary obstruction: plastic vs partially covered metal stents. *World J Gastroenterol* 2013; **19**: 8638-8646 [PMID: 24379581 DOI: 10.3748/wjg.v19.i46.8638]
- 28 **Sangchan A**, Kongkasame W, Pugkhem A, Jenwitheesuk K, Mairiang P. Efficacy of metal and plastic stents in unresectable complex hilar cholangiocarcinoma: a randomized controlled trial. *Gastrointest Endosc* 2012; **76**: 93-99 [PMID: 22595446 DOI: 10.1016/j.gie.2012.02.048]
- 29 **Hong WD**, Chen XW, Wu WZ, Zhu QH, Chen XR. Metal versus plastic stents for malignant biliary obstruction: an update meta-analysis. *Clin Res Hepatol Gastroenterol* 2013; **37**: 496-500 [PMID: 23333231 DOI: 10.1016/j.clinre.2012.12.002]
- 30 Available from: URL: <http://www.crd.york.ac.uk/PROSPERO/>
- 31 Available from: URL: <http://www.dominiopublico.gov.br/>
- 32 **Jadad AR**, Moore RA, Carroll D, Jenkinson C, Reynolds DJ, Gavaghan DJ, McQuay HJ. Assessing the quality of reports of randomized clinical trials: is blinding necessary? *Control Clin Trials* 1996; **17**: 1-12 [PMID: 8721797 DOI: 10.1016/0197-2456(95)00134-4]
- 33 Available from: URL: <http://www4.bcb.gov.br/pec/conversao/conversao.asp>
- 34 **Amikura K**, Kobari M, Matsuno S. The time of occurrence of liver metastasis in carcinoma of the pancreas. *Int J Pancreatol* 1995; **17**: 139-146 [PMID: 7622937]
- 35 **Kayahara M**, Nagakawa T, Ueno K, Ohta T, Takeda T, Miyazaki I. An evaluation of radical resection for pancreatic cancer based on the mode of recurrence as determined by autopsy and diagnostic imaging. *Cancer* 1993; **72**: 2118-2123 [PMID: 8104092]
- 36 **Mavros MN**, Economopoulos KP, Alexiou VG, Pawlik TM. Treatment and Prognosis for Patients With Intrahepatic Cholangiocarcinoma: Systematic Review and Meta-analysis. *JAMA Surg* 2014; **149**: 565-574 [PMID: 24718873 DOI: 10.1001/jamasurg.2013.5137]

P- Reviewer: Seicean A **S- Editor:** Ma YJ

L- Editor: Filipodia **E- Editor:** Liu XM



Effect of intraperitoneal local anesthetic on pain characteristics after laparoscopic cholecystectomy

Geun Joo Choi, Hyun Kang, Chong Wha Baek, Yong Hun Jung, Dong Rim Kim

Geun Joo Choi, Hyun Kang, Chong Wha Baek, Yong Hun Jung, Dong Rim Kim, Department of Anesthesiology and Pain Medicine, Chung-Ang University College of Medicine, Seoul 06911, South Korea

Published online: December 21, 2015

Author contributions: Choi GJ designed the study, conducted the study (selected study, extracted data), analysed and interpreted the data, wrote the manuscript; Kang H designed the study, conducted the study (extracted data), analysed and interpreted the data, wrote the manuscript; Baek CW conducted the study (helped to select study, assessed the risk of bias), provided critical revision of the manuscript; Jung YH conducted the study (helped to select study, assessed the risk of bias), provided critical revision of the manuscript; Kim DR conducted the study (selected study), analysed the data and wrote the manuscript; all authors approved the final manuscript.

Conflict-of-interest statement: The authors deny any conflict of interest.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Hyun Kang, MD, PhD, MPH, Associate Professor, Department of Anesthesiology and Pain Medicine, Chung-Ang University College of Medicine, 84 Heukseok-ro, Dongjak-gu, Seoul 06911, South Korea. roman00@naver.com
Telephone: +82-2-62992583
Fax: +82-2-62992585

Received: April 28, 2015
Peer-review started: May 6, 2015
First decision: August 31, 2015
Revised: September 14, 2015
Accepted: October 12, 2015
Article in press: October 13, 2015

Abstract

AIM: To systematically evaluate the effect of intraperitoneal local anesthetic on pain characteristics after laparoscopic cholecystectomy (LC).

METHODS: We searched MEDLINE, EMBASE, and the Cochrane Library. Randomized controlled trials in English that compared the effect of intraperitoneal administration of local anesthetics on pain with that of placebo or nothing after elective LC under general anesthesia were included. The primary outcome variables analyzed were the combined scores of abdominal, visceral, parietal, and shoulder pain after LC at multiple time points. We also extracted pain scores at resting and dynamic states.

RESULTS: We included 39 studies of 3045 patients in total. The administration of intraperitoneal local anesthetic reduced pain intensity in a resting state after laparoscopic cholecystectomy: abdominal [standardized mean difference (SMD) = -0.741; 95%CI: -1.001 to -0.48, $P < 0.001$]; visceral (SMD = -0.249; 95%CI: -0.493 to -0.006, $P = 0.774$); and shoulder (SMD = -0.273; 95%CI: -0.464 to -0.082, $P = 0.097$). Application of intraperitoneal local anesthetic significantly reduced the incidence of shoulder pain (RR = 0.437; 95%CI: 0.299 to 0.639, $P < 0.001$). There was no favorable effect on resting parietal or dynamic abdominal pain.

CONCLUSION: Intraperitoneal local anesthetic as an analgesic adjuvant in patients undergoing laparoscopic cholecystectomy exhibited beneficial effects on postoperative abdominal, visceral, and shoulder pain in a resting state.

Key words: Local anesthetic; Laparoscopic cholecystectomy;

Intraperitoneal; Meta-analysis; Pain

© **The Author(s) 2015.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Pain after laparoscopic cholecystectomy is located at abdomen or shoulder, and abdominal pain includes visceral and parietal pain. This characteristic pain is mainly because of pneumoperitoneum, which leads to visceral and shoulder pain. Intraperitoneal local anesthetics thus can be a beneficial strategy given the origin of various type of pain after laparoscopic cholecystectomy, which is evaluated systematically focused on the analgesic efficacy on pain characteristics. Intraperitoneal local anesthetics in patients undergoing laparoscopic cholecystectomy has the evidence to reduce postoperative abdominal, visceral, and shoulder pain. Further less heterogeneous evidence is necessary to draw definite conclusion.

Choi GJ, Kang H, Baek CW, Jung YH, Kim DR. Effect of intraperitoneal local anesthetic on pain characteristics after laparoscopic cholecystectomy. *World J Gastroenterol* 2015; 21(47): 13386-13395 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13386.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13386>

INTRODUCTION

Laparoscopic cholecystectomy (LC) is a widely performed surgical procedure that achieves superior outcomes in postoperative pain, recovery time, cosmetic issues, and morbidity^[1]. LC is associated with less postoperative pain than open cholecystectomy, but patients still experience significant pain. Pain after LC is characterized by body component, which is different from laparotomy^[2]. This difference is roughly divided into abdominal and shoulder pain, according to location^[3]. Abdominal pain consists of two components: visceral pain associated with tissue injury due to gallbladder dissection and the stretching of nerve endings in the peritoneal cavity; and parietal pain related to the incisional trauma at the port sites. Shoulder pain is referred by diaphragmatic stretching^[4].

A number of studies reported various treatment modalities to relieve pain after LC. A therapeutic approach using intraperitoneal local anesthetic (IPLA) is remarkable because the beneficial effect of this strategy is closely linked to pain characteristics after LC, which primarily arises from pneumoperitoneum. The results of the available data on the efficacy of IPLA in LC are inconsistent. Therefore, a systematic review would be informative to create evidence for IPLA use in LC. Several systematic reviews from a variety of perspectives based on postoperative pain or safety issues were published^[5-8]. However, there is no data on

the effect of IPLA on pain characteristics after LC.

This review investigated the effect of IPLA on pain after LC through a systematic evaluation of the available literature. Pain characteristics, including visceral, parietal, and shoulder pain, were the primary focus of this meta-analysis. Pain at resting and dynamic states was also assessed, and the limitations of the data were reviewed.

MATERIALS AND METHODS

Literature search

A meta-analysis of studies investigating the effect of IPLA in LC was conducted according to the protocol recommended by the Cochrane Collaboration^[9]. Two authors (Choi GJ and Kang H) independently performed database searches using EMBASE, MEDLINE, and the Cochrane Central Register of Controlled Trials (CENTRAL) in April 2014 and updated in March 2015. The reference lists of the identified literature were also searched manually. Search terms were used based on the following combination of keywords: local anesthetics, lidocaine, lignocaine, ropivacaine, bupivacaine, levobupivacaine, procaine, intraperitoneal, intra-abdominal, laparoscopic cholecystectomy, and randomized trials.

Study selection

Randomized controlled trials (RCTs) in English that compared the effects of the intraperitoneal administration of local anesthetic (IPLA group) with placebo or no treatment (control group) on pain after elective LC under general anesthesia were included. Studies that combined IPLA with other interventions were included if there were comparable intervention and control groups in which the only difference was the use of IPLA. Studies with more than one IPLA or control group were included if there were comparable groups that met the inclusion criteria. Two authors (Choi GJ and Kim DR) independently selected eligible studies and reached a consensus on study inclusion or exclusion. Disagreements over study inclusion or exclusion were settled by discussions with the two senior authors (Baek CW and Jung YH).

Data extraction

When studies did not provide detailed pain characteristics, *i.e.*, visceral or parietal pain and resting or dynamic pain were not clarified, we considered abdominal pain and pain at the resting state. Pain from coughing, moving, or inspiration was regarded as dynamic abdominal pain. We treated the intraperitoneal administration of normal saline and nothing as the control group. We combined all of the IPLA or control groups if a given study had more than one IPLA or control group to avoid multiple counting of the same individuals^[10]. We extracted data from partial groups that were eligible in a study with several groups if the groups

were comparable. Data from studies in which wound infiltration with local anesthetics was applied to both intervention and control groups, or not, in a single study with several groups greater than four were extracted to effectively yield two sub-studies of whether wound infiltration with local anesthetics was performed. Studies reporting pain severity on a visual analog scale (VAS) or numerical rating scale (NRS) were included. We selected the VAS if various scales including VAS were used. We considered the median pain evaluation value as the time point when pain evaluation times were presented as ranges. Means and standard deviations of pain scores for intervention and control groups were extracted from tables, graphs, or text. We attempted to contact the corresponding author to obtain data if the values were not reported. We calculated these values using the methods described in the Cochrane handbook when contact was unsuccessful^[9].

Two authors (Choi GJ and Kang H) independently extracted the following data: name of the first author; year of publication; number of participants and their respective allocation; type of local anesthesia; pain characteristics evaluated (abdominal, visceral, parietal, or shoulder pain; pain at resting or dynamic states); time points of pain score evaluations; pain scores at each time point; and incidence of shoulder pain.

Risk of bias assessment

Two authors (Baek CW and Jung YH) independently assessed the quality of eligible studies using the Cochrane Collaboration's Tool for assessment of risk of bias^[9]. Quality was evaluated based on the following seven potential sources of bias: random sequence generation; allocation concealment; blinding of the participants; blinding of the outcome assessors; incomplete outcome data; selective reporting; and other bias. The methodology of each trial was graded as 'high', 'low' or 'unclear' to reflect a high risk of bias, low risk of bias and uncertainty of bias, respectively.

Statistical analysis

The pooled risk ratio (RR) or standardized mean difference (SMD) and 95% CIs were calculated for each outcome. We used the χ^2 test for homogeneity and the I^2 test for heterogeneity. We regarded a level of 10% significance ($P < 0.100$) in the χ^2 statistic or an I^2 greater than 50% as considerable heterogeneity, and we used the Mantel-Haenszel random-effect model. Otherwise, we applied the Mantel-Haenszel fixed-effect model^[9,11]. We performed subgroup analyses based on the type of local anesthetics and wound infiltration. We also conducted sensitivity analyses to evaluate the influence of a single study on the overall effect estimate by the exclusion of one study. Data measured at multiple time-points were dependent on each other, and multiple comparisons at each time point would increase the possibility of type I error. Therefore,

we combined the outcomes of multiple time points and performed analyses using the pooled combined outcomes.

Publication bias was evaluated using a funnel plot and Egger's linear regression test. If the funnel plot was asymmetrical or the P value was < 0.100 by the Egger's test, we considered the presence of a publication bias and performed trim and fill analyses.

We performed all analyses using Comprehensive Meta-analysis software (version 3.0, Biostat, Englewood, NJ, United States).

RESULTS

Search results

A total of 998 records were identified using our electronic and manual search strategy. The titles and/or abstracts were screened, and 48 potentially eligible RCTs were retrieved. Full text assessments for eligibility excluded 9 studies. Finally, 39 studies were included in the present meta-analysis. Figure 1 presents the flow diagram of the literature selection process.

Study characteristics

A total of 3045 patients in 39 RCTs were included in this review: 1633 in the IPLA group and 1412 in the control group. Fourteen studies had more than one IPLA and/or control group based on following factors^[12-25]: specification of study solution, such as volume, concentration, or type of local anesthetics; timing of when IPLA was administered in relation to gallbladder dissection; and combination of study solution and adrenaline. Five studies with several groups had only two eligible groups for comparison^[26-30]. We produced two sub-studies from one study in which two independent investigations were performed^[24]. There were four studies that yielded two sub-studies based on whether wound infiltration with local anesthetics was applied^[18,19,31,32]. Dynamic abdominal pain was evaluated in the following states: moving^[19,25]; coughing^[15,19,24]; inspiration or deep breathing^[15,24]; sitting up and valsalva^[33]. Joris *et al*^[34] reported no information on the standard deviations of IPLA group except for a range of standard errors. Therefore, we estimated standard deviations from the most conservative value of standard errors to minimize the possibility of type I error. Limited data were reported on visceral and parietal pains in one study^[35] and the severity of abdominal and shoulder pain in another study^[36]. Only data of the incidences of shoulder pain in both studies were included in present meta-analysis. Table 1 summarizes the study characteristics.

Results of meta-analysis

Resting abdominal pain was evaluated in 30 studies (2263 patients)^[12-14,16,17,19-30,32,33,37-47]. There was a

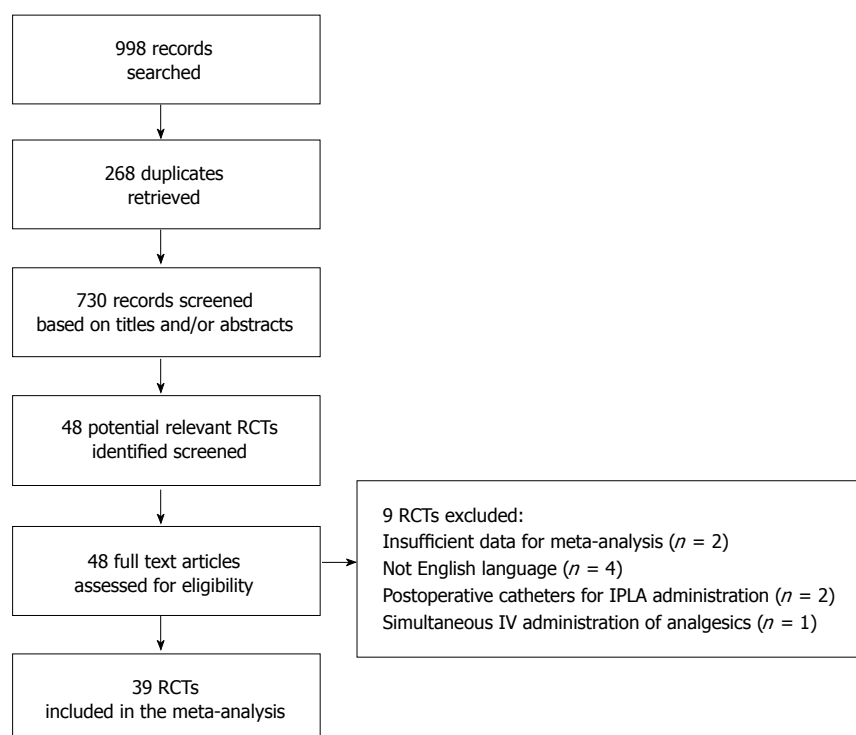


Figure 1 Flow diagram of studies identified and selected.

significant reduction in pain scores with the use of IPLA compared to the control group (SMD = -0.741; 95%CI: -1.001 to -0.48, $P < 0.001$; $I^2 = 87\%$; Figure 2A).

Dynamic abdominal pain was measured in five studies (335 patients)^[15,19,24,25,33]. IPLA administration did not exhibit a significant decrease in pain scores compared to the control group (SMD = -0.320; 95%CI: -0.649 to 0.010, $P = 0.002$; $I^2 = 66\%$; Figure 2B). The exclusion of Raetzell *et al.*^[24] changed the result (SMD = -0.402; 95%CI: -0.777 to -0.028, $P = 0.001$; $I^2 = 70\%$).

Visceral and parietal pain scores at a resting state were examined in three studies (148 patients)^[18,31,34]. There were no data for dynamic states. IPLA administration significantly reduced visceral pain scores compared to the control group (SMD = -0.249; 95%CI: -0.493 to -0.006, $P = 0.774$; $I^2 = 0\%$; Figure 2C), but there was no reduction in parietal pain score using IPLA (SMD = -0.305; 95%CI: -0.708 to 0.098, $P = 0.036$; $I^2 = 61\%$; Figure 2D).

Shoulder pain at a resting state was evaluated, including severity in eight studies (729 patients)^[12,20,27-29, 31,34,48] and incidences in 14 studies (1092 patients)^[15,17-19,35-38,41-43,46,49,50]. There were no data for dynamic states. IPLA administration significantly reduced shoulder pain severity (SMD = -0.273; 95%CI: -0.464 to -0.082, $P = 0.097$; $I^2 = 39\%$; Figure 2E) and incidence (RR = 0.437; 95%CI: 0.299 to 0.639, $P < 0.001$; $I^2 = 68\%$; Figure 2F).

Sensitivity analyses, except for one case in a dynamic abdominal pain and subgroup analyses,

based on type of local anesthesia used did not alleviate substantial heterogeneity or change the significance of the result.

Publication bias

A funnel plot was used for every comparison, and all data displayed a symmetrical appearance. The results of Egger's test indicated that publication bias was unlikely for all outcomes: resting abdominal pain ($P = 0.076$); dynamic abdominal pain ($P = 0.416$); visceral pain ($P = 0.143$); parietal pain ($P = 0.508$); shoulder pain severity ($P = 0.683$); and incidence of shoulder pain ($P = 0.239$). We performed trim and fill analyses on the assumption that publication bias was evident for resting abdominal pain to evaluate the influence of publication bias. The result of resting abdominal pain remained significant (SMD = -0.914; 95%CI: -1.182 to -0.646), which suggests that publication bias was unlikely (Figure 3).

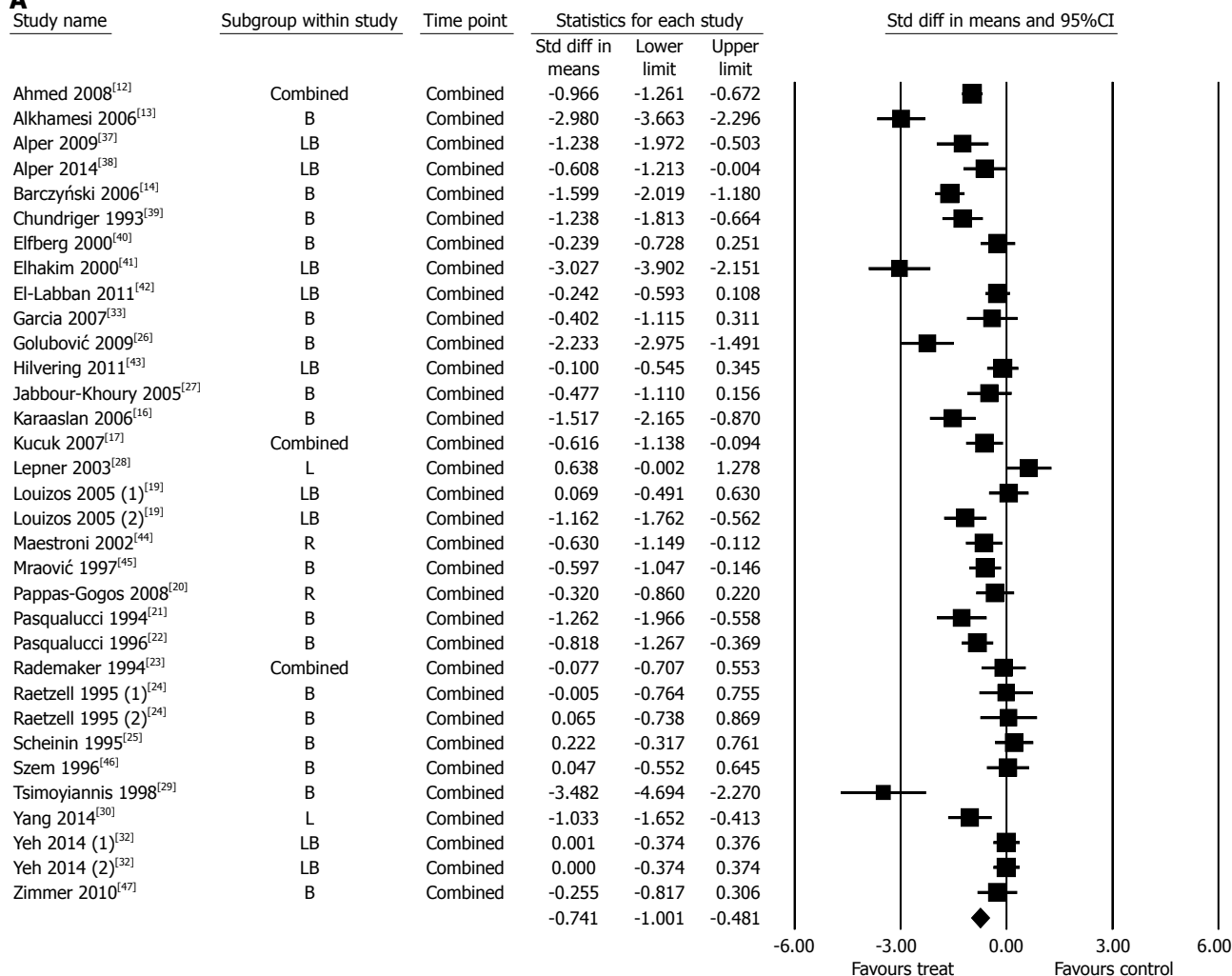
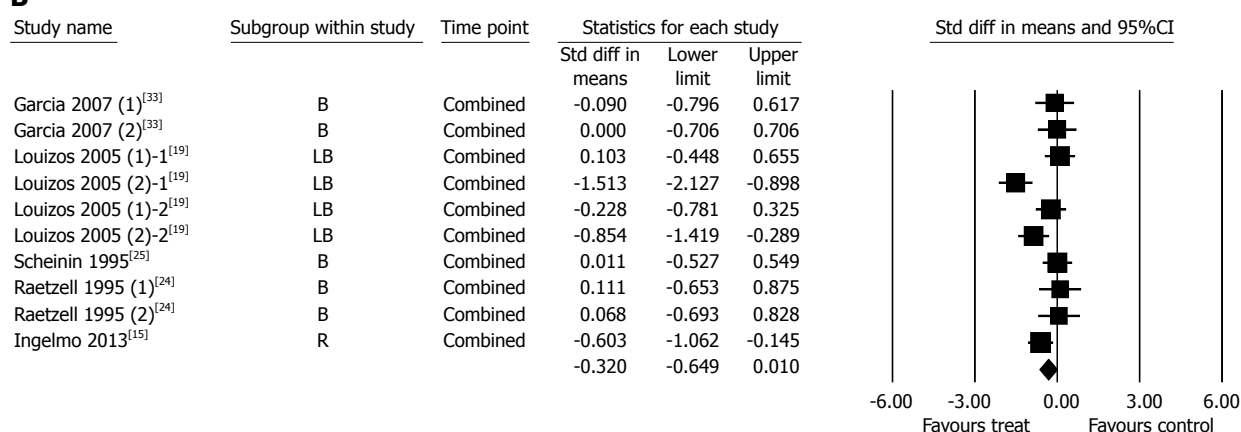
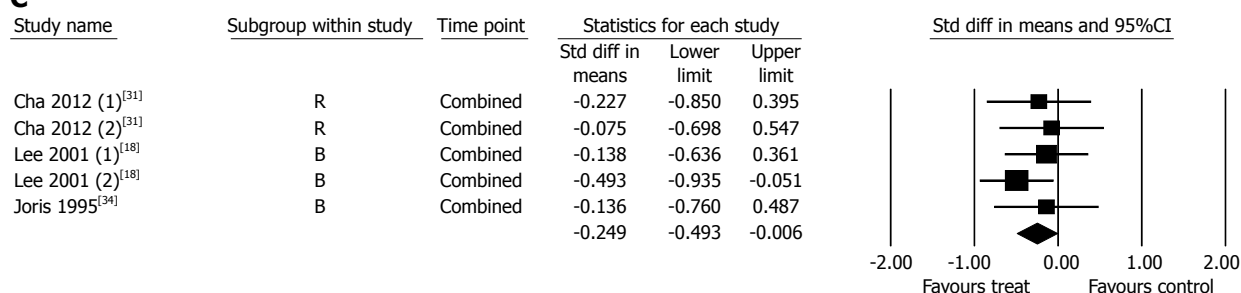
DISCUSSION

The results of our meta-analysis suggest that IPLA is effective for the control of resting abdominal, visceral, and shoulder pain. This effect may be explained by the mechanisms of pain development after LC and the action of IPLA, but these mechanisms are multifactorial and not clearly understood. Visceral pain may be initiated by tissue injury due to gallbladder removal from the liver bed and the stretching of nerve endings^[4]. Pneumoperitoneum causes a stretching of the peritoneum and the diaphragmatic muscle

Table 1 Study characteristics

Study	Year	Patients (n)	IPLA/control	Type of LA	Pain characteristics	Postoperative time point (h)
Chundrigar <i>et al</i> ^[39]	1993	28/30		B	Abdominal (R)	1, 2, 4, 8
Pasqualucci <i>et al</i> ^[21]	1994	28/14		B	Abdominal (R)	0, 4, 8, 12, 24
Rademaker <i>et al</i> ^[23]	1994	30/15		B, L	Abdominal(R)	0.5, 1, 2, 4
Joris <i>et al</i> ^[34]	1995	20/20		B	Visceral/Parietal (R) Shoulder (S)	1, 2, 4, 6, 8, 24, 48
Raetzell <i>et al</i> (1) ^[24]	1995	20/10		B	Abdominal (R, D)	4, 24, 48, 72
Raetzell <i>et al</i> (2) ^[24]	1995	12/12		B	Abdominal (R)	1, 2, 3, 4, 5, 6, 24
Scheinin <i>et al</i> ^[25]	1995	40/20		B	Abdominal (R, D)	24, 48, 72, 96, 120, 144, 168
Pasqualucci <i>et al</i> ^[22]	1996	82/27		B	Abdominal (R)	0, 4, 8, 12, 24
Szem <i>et al</i> ^[46]	1996	26/29		B	Abdominal (R) Shoulder (I)	3, 9, 15, 21
Mraović <i>et al</i> ^[45]	1997	40/40		R	Abdominal (R)	0.5, 4, 8, 12, 24
Cunniffe <i>et al</i> ^[49]	1998	55/50		B	Shoulder (I)	NA
Tsimoyiannis <i>et al</i> ^[29]	1998	50/50		B	Abdominal (R)	2, 6, 12, 24, 36, 48, 72
Elfberg <i>et al</i> ^[40]	2000	33/32		B	Abdominal (R)	2, 4, 8, 24, 48
Elhakim <i>et al</i> ^[41]	2000	25/25		L	Abdominal (R) Shoulder (I, S)	0, 1, 6, 12, 18, 24
Gharaibeh <i>et al</i> ^[50]	2000	37/38		B	Shoulder (I)	NA
Lee <i>et al</i> ^[18]	2001	80/68		B	Visceral/Parietal (R) Shoulder (I)	1, 3, 6, 9, 24, 48
Labaille <i>et al</i> ^[35]	2002	25/12		R	Visceral/Parietal (R, D) Shoulder (I)	0, 0.5, 1, 2, 4, 8, 12, 20
Maestroni <i>et al</i> ^[44]	2002	30/30		R	Abdominal (R)	0, 4, 8, 12, 24
Lepner <i>et al</i> ^[28]	2003	20/20		L	Abdominal (R) Shoulder (S)	1, 3, 6, 12, 18, 24
Ng <i>et al</i> ^[36]	2004	21/22		LB	Abdominal (R, D) Shoulder (I, S)	0, 1, 2, 3, 4
Jabbour-Khoury <i>et al</i> ^[27]	2005	20/20		B	Abdominal (R) Shoulder (S)	0, 1, 2, 6, 12, 24
Louizos <i>et al</i> ^[19]	2005	54/50		B	Abdominal (R, D) Shoulder (I, S)	0.5, 4, 8, 12, 24
Barczyński <i>et al</i> ^[14]	2006	60/60		B	Abdominal (R)	4, 8, 12, 24, 48
Karaaslan <i>et al</i> ^[16]	2006	50/15		B	Abdominal (R)	0, 4, 8, 12, 24
Alkhamesi <i>et al</i> ^[13]	2007	40/40		B	Abdominal (R)	0, 6, 12, 24
Garcia <i>et al</i> ^[33]	2007	19/13		B	Abdominal (R, D)	0, 2, 4, 8, 12, 24
Kucuk <i>et al</i> ^[17]	2007	60/20		B, R	Abdominal (R) Shoulder (I)	0, 1, 2, 4, 8, 12, 24
Ahmed <i>et al</i> ^[12]	2008	100/100		B, L	Abdominal (R)	0, 4, 8, 12, 24
Pappas-Gogos <i>et al</i> ^[20]	2008	40/20		R	Abdominal (R) Shoulder (S)	2, 4, 6, 12, 24, 48, 72
Alper <i>et al</i> ^[37]	2009	20/20		LB	Abdominal (R) Shoulder (I)	0, 0.5, 1, 2, 4, 6, 8, 12, 24
Golubović <i>et al</i> ^[26]	2009	30/30		B	Abdominal (R)	0.5, 1, 2, 4, 24
Zimmer <i>et al</i> ^[47]	2010	25/25		B	Abdominal (R) Shoulder (S)	1, 2, 24
El-Labban <i>et al</i> ^[42]	2011	63/63		LB	Abdominal (R) Shoulder (I)	0.5, 4, 8, 12, 24
Hilvering <i>et al</i> ^[43]	2011	39/39		LB	Abdominal (R) Shoulder (I)	0.5, 2, 4, 8, 24
Cha <i>et al</i> ^[31]	2012	40/40		R	Visceral/Parietal (R) Shoulder (S)	2, 4, 8, 12, 24, 48
Ingelmo <i>et al</i> ^[15]	2013	56/29		R	Abdominal (D) Shoulder (I)	4, 24, 48, 72
Alper <i>et al</i> ^[38]	2014	22/22		LB	Abdominal (R) Shoulder (I)	0, 0.5, 1, 2, 4, 6, 8, 12, 24
Niknam <i>et al</i> ^[48]	2014	84/85		R	Shoulder (S)	4, 72
Yang <i>et al</i> ^[30]	2014	22/24		L	Abdominal (R)	2, 4, 8, 12, 24, 48
Yeh <i>et al</i> ^[32]	2014	110/110		LB	Abdominal (R)	1, 6, 24

IPLA: Intraperitoneal local anesthetics; LA: Local anesthetics; R: Resting; D: Dynamic; I: Incidence; S: Severity; R: Ropivacaine; L: Lidocaine; B: Bupivacaine; LB: Levobupivacaine; NA: Not available.

A**B****C**

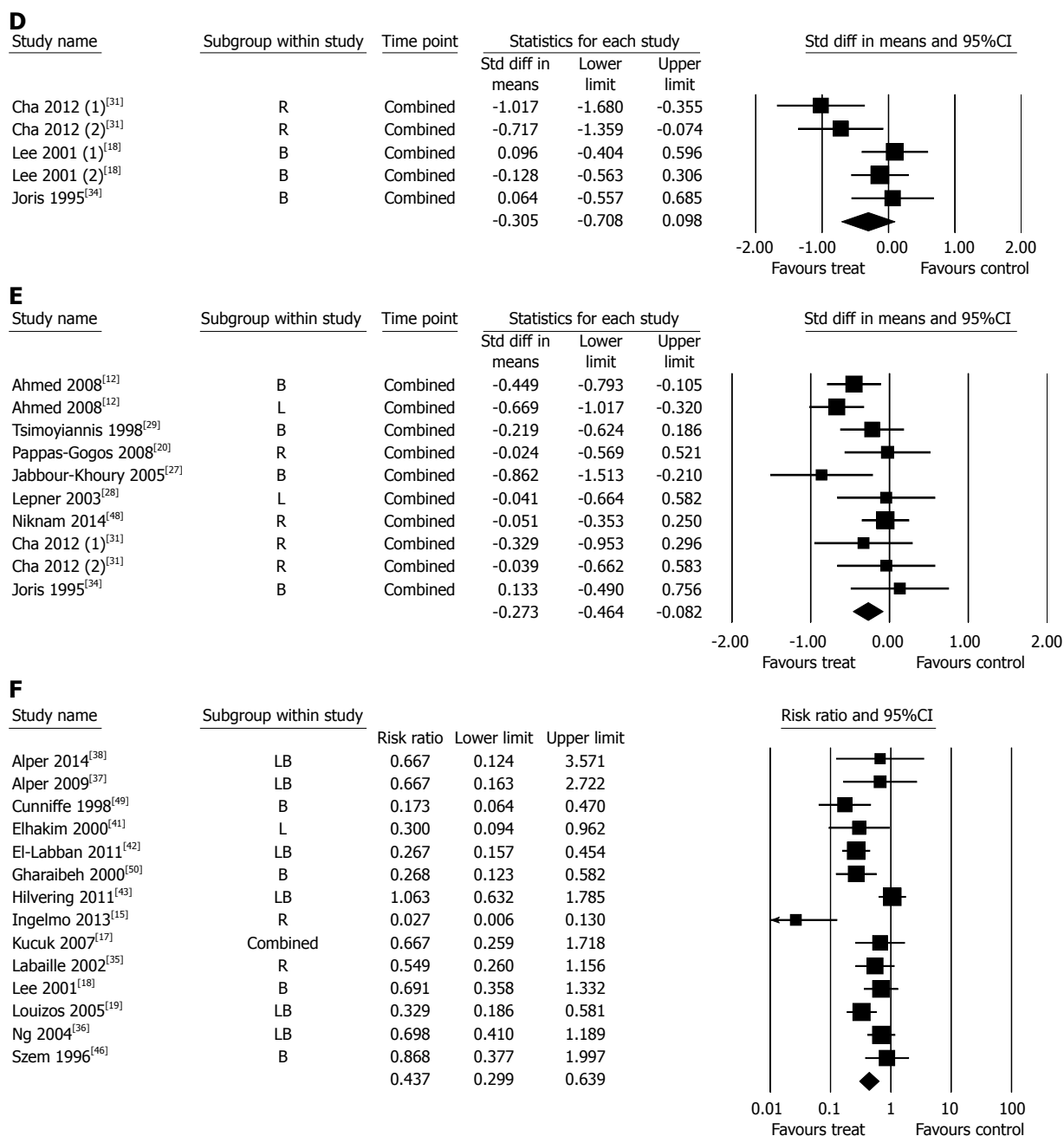


Figure 2 Forest plot of resting abdominal pain (A), dynamic abdominal pain (B), visceral pain (C), parietal pain (D), shoulder pain (severity) (E) and shoulder pain (incidence) (F).

fibers, which irritates phrenic nerve endings^[2,4]. The phrenic nerve innervates the gallbladder and liver, and this nerve shares a common route with nerves that innervate the shoulder^[51]. Dissolved carbon dioxide contributes to diaphragmatic irritation^[52]. Therefore, the pain induced by pneumoperitoneum leads to referred pain in the shoulder. The topical application of local anesthetic to the viscera, *i.e.*, IPLA, exhibits an analgesic effect by blocking visceral nociception from the area of tissue damage and the peritoneum. The systemic absorption of local anesthetics through the peritoneal surface may also play a role in the analgesic effect by attenuating nociception^[53].

Most studies included in present review evaluated

abdominal pain, not visceral pain. Visceral pain made up a large portion of abdominal pain after LC compared to parietal or shoulder pain in several studies^[18,34,35]. Therefore, we expected that the results of visceral and abdominal pain would exhibit a similar tendency. The administration of IPLA induced a significant reduction in visceral and abdominal pain at resting states after LC.

IPLA did not significantly reduce parietal pain in the present review. This result may be explained by the different origins of parietal and visceral pain. The analgesic effect of IPLA is favorable to visceral pain because IPLA is aimed at the injured viscera in the peritoneal cavity, not the abdominal wall. Parietal pain

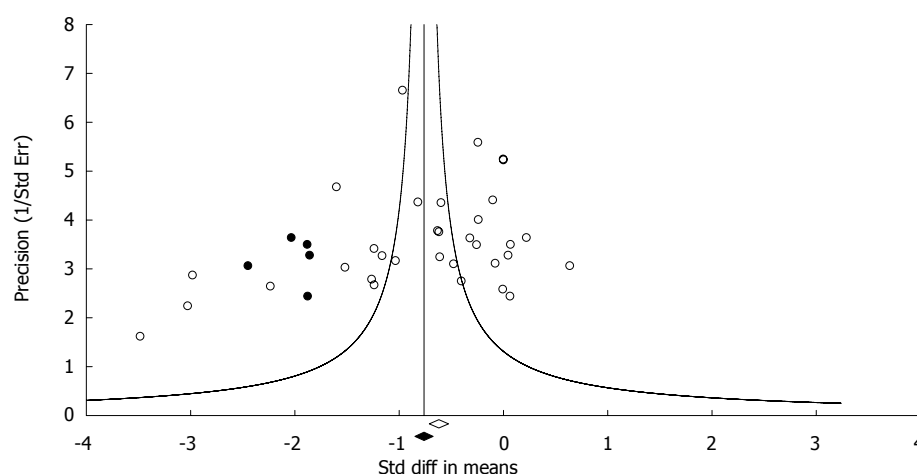


Figure 3 Funnel plot of publication bias of resting abdominal pain.

is a lesser component of the pain that is somatic origin and induced by the surgical incision in the abdominal wall for trocar insertion^[2]. The origin of parietal pain supports the application of local anesthetics to trocar insertion sites, *i.e.*, wound infiltration with local anesthetics would be beneficial. Several studies investigated the effect of wound infiltration on pain after LC^[54] and demonstrated favorable results in regard to pain control. Therefore, we performed subgroup analyses on wound infiltration. However, our meta-analysis demonstrated that parietal pain was not reduced in the wound infiltration subgroup, which is inconsistent with the results of previous studies.

Some of the included studies assessed abdominal pain in a dynamic state, such as moving, coughing, inspiration, or valsalva. Movement demands the contraction of primarily abdominal muscles, but coughing or deep inspiration is mediated by the movement of intraabdominal viscera^[34]. Each type of movement suggests an aggravation of parietal and visceral pain. IPLA did not significantly alleviate dynamic abdominal pain in the present meta-analysis. Our results revealed that IPLA was effective on visceral pain in a resting state. Abdominal pain may be represented by visceral pain, which is worsened by coughing or inspiration. Therefore, we performed a subgroup analysis of studies that investigated abdominal pain during coughing or inspiration on the assumption that our results of the effect of IPLA on dynamic pain would be altered. However, subgroup analyses did not alter this result.

The present review is limited by the substantial heterogeneity between studies and the quality of included studies. There are many potential sources of clinical and methodological heterogeneity, such as dose, concentration, or volume of IPLA, the timing or site of IPLA administration, the volume and pressure of pneumoperitoneum, and the analgesic method during the postoperative period. We tried to conduct sensitivity and subgroup analyses for some of the possible factors, but we could not consider all of these

factors in our analysis. Second, the quality of the included studies was limited. Notwithstanding this limitation, our study was the first meta-analysis to evaluate the effect of IPLA on pain characteristics after LC application using a rigorous methodology.

In conclusion, IPLA as an analgesic adjuvant in patients undergoing LC exhibited a favorable effect on postoperative abdominal, visceral, and shoulder pain during a resting state.

COMMENTS

Background

Laparoscopic cholecystectomy (LC) is widely performed because of the benefits associated with lower invasiveness, but patients still experience significant postoperative pain. Numerous studies demonstrated therapeutic strategies for pain after LC, including the administration of intraperitoneal local anesthetic (IPLA). There were three types of pain characteristics after LC: visceral, parietal, and shoulder pain. It would be beneficial for postoperative pain management to provide evidence of the effect of IPLA on pain after LC. Therefore, the authors systematically evaluated the effect of IPLA on pain characteristics after LC.

Research frontiers

Postoperative pain management is an important issue in LC. Recent recognition of different pain components suggests that strategies for pain therapy should focus on pain characteristics based on their origins. Currently, LC and pain characteristics are significantly promising subjects of research.

Innovations and breakthroughs

Several meta-analyses on LC were performed, but no studies systematically evaluated the effect of IPLA on pain components after LC. This study is the first report of a meta-analysis to investigate the effect of IPLA on pain characteristics after LC.

Applications

This meta-analysis provides evidence of the effect of IPLA on pain characteristics after LC and suggests a more effective therapeutic approach based on pain components after LC.

Terminology

Pain characteristics after LC are composed of visceral, parietal, and shoulder pain. Visceral and shoulder pain are associated with pneumoperitoneum during laparoscopic surgery. Parietal pain is linked to trocar incisions. The origins of these components are different, and distinct therapeutic approaches should

be distinguished. IPLA in the present study reduced visceral and shoulder pain after LC, which suggests that intraperitoneally administered local anesthetic exerts an analgesic effect on the viscera and peritoneum that are affected by surgery and pneumoperitoneum.

Peer-review

The authors of this meta-analysis present the application value of intraperitoneal local anesthetic on pain after LC. This article is valuable for clinical physicians.

REFERENCES

- 1 **Soper NJ**, Stockmann PT, Dunnegan DL, Ashley SW. Laparoscopic cholecystectomy. The new 'gold standard'? *Arch Surg* 1992; **127**: 917-921; discussion 921-923 [PMID: 1386505]
- 2 **Wills VL**, Hunt DR. Pain after laparoscopic cholecystectomy. *Br J Surg* 2000; **87**: 273-284 [PMID: 10718794 DOI: 10.1046/j.1365-2168.2000.01374.x]
- 3 **Ure BM**, Troidl H, Spangenberg W, Dietrich A, Lefering R, Neugebauer E. Pain after laparoscopic cholecystectomy. Intensity and localization of pain and analysis of predictors in preoperative symptoms and intraoperative events. *Surg Endosc* 1994; **8**: 90-96 [PMID: 8165491]
- 4 **Bisgaard T**, Kehlet H, Rosenberg J. Pain and convalescence after laparoscopic cholecystectomy. *Eur J Surg* 2001; **167**: 84-96 [PMID: 11266262 DOI: 10.1080/110241501750070510]
- 5 **Boddy AP**, Mehta S, Rhodes M. The effect of intraperitoneal local anesthesia in laparoscopic cholecystectomy: a systematic review and meta-analysis. *Anesth Analg* 2006; **103**: 682-688 [PMID: 16931681 DOI: 10.1213/01.ane.0000226268.06279.5a]
- 6 **Gupta A**. Local anaesthesia for pain relief after laparoscopic cholecystectomy--a systematic review. *Best Pract Res Clin Anaesthesiol* 2005; **19**: 275-292 [PMID: 15966498]
- 7 **Gurusamy KS**, Nagendran M, Guerrini GP, Toon CD, Zinnuroglu M, Davidson BR. Intraperitoneal local anaesthetic instillation versus no intraperitoneal local anaesthetic instillation for laparoscopic cholecystectomy. *Cochrane Database Syst Rev* 2014; **3**: CD007337 [PMID: 24627292 DOI: 10.1002/14651858.CD007337.pub3]
- 8 **Kahokehr A**, Sammour T, Soop M, Hill AG. Intraperitoneal use of local anesthetic in laparoscopic cholecystectomy: systematic review and metaanalysis of randomized controlled trials. *J Hepatobiliary Pancreat Sci* 2010; **17**: 637-656 [PMID: 20393755 DOI: 10.1007/s00534-010-0271-7]
- 9 **Higgins JP**, Green S. Cochrane handbook for systematic reviews of interventions. Version 5.1.0. The Cochrane Collaboration, 2011
- 10 **Altman DG**, Bland JM. Statistics notes. Units of analysis. *BMJ* 1997; **314**: 1874 [PMID: 9224131]
- 11 **Higgins JP**, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. *BMJ* 2003; **327**: 557-560 [PMID: 12958120 DOI: 10.1136/bmj.327.7414.557]
- 12 **Ahmed BH**, Ahmed A, Tan D, Awad ZT, Al-Aali AY, Kilkenny J, Orlando FA, Al-Chalabi A, Crass R, Alrawi SJ. Post-laparoscopic cholecystectomy pain: effects of intraperitoneal local anesthetics on pain control--a randomized prospective double-blinded placebo-controlled trial. *Am Surg* 2008; **74**: 201-209 [PMID: 18376682]
- 13 **Alkhamesi NA**, Peck DH, Lomax D, Darzi AW. Intraperitoneal aerosolization of bupivacaine reduces postoperative pain in laparoscopic surgery: a randomized prospective controlled double-blinded clinical trial. *Surg Endosc* 2007; **21**: 602-606 [PMID: 17180268 DOI: 10.1007/s00464-006-9087-6]
- 14 **Barczyński M**, Konturek A, Herman RM. Superiority of preemptive analgesia with intraperitoneal instillation of bupivacaine before rather than after the creation of pneumoperitoneum for laparoscopic cholecystectomy: a randomized, double-blind, placebo-controlled study. *Surg Endosc* 2006; **20**: 1088-1093 [PMID: 16703434 DOI: 10.1007/s00464-005-0458-1]
- 15 **Ingelmo PM**, Bucciero M, Somaini M, Sahillioglu E, Garbagnati A, Charton A, Rossini V, Sacchi V, Scardilli M, Lometti A, Joshi GP, Fumagalli R, Diemunsch P. Intraperitoneal nebulization of ropivacaine for pain control after laparoscopic cholecystectomy: a double-blind, randomized, placebo-controlled trial. *Br J Anaesth* 2013; **110**: 800-806 [PMID: 23293276 DOI: 10.1093/bja/aes495]
- 16 **Karaaslan D**, Sivaci RG, Akbulut G, Dilek ON. Preemptive analgesia in laparoscopic cholecystectomy: a randomized controlled study. *Pain Pract* 2006; **6**: 237-241 [PMID: 17129304 DOI: 10.1111/j.1533-2500.2006.00092.x]
- 17 **Kucuk C**, Kadiogullari N, Canoler O, Savli S. A placebo-controlled comparison of bupivacaine and ropivacaine instillation for preventing postoperative pain after laparoscopic cholecystectomy. *Surg Today* 2007; **37**: 396-400 [PMID: 17468821 DOI: 10.1007/s00595-006-3408-1]
- 18 **Lee IO**, Kim SH, Kong MH, Lee MK, Kim NS, Choi YS, Lim SH. Pain after laparoscopic cholecystectomy: the effect and timing of incisional and intraperitoneal bupivacaine. *Can J Anaesth* 2001; **48**: 545-550 [PMID: 11444448 DOI: 10.1007/bf03016830]
- 19 **Louizos AA**, Hadzilia SJ, Leandros E, Kouroukli IK, Georgiou LG, Bramis JP. Postoperative pain relief after laparoscopic cholecystectomy: a placebo-controlled double-blind randomized trial of preincisional infiltration and intraperitoneal instillation of levobupivacaine 0.25%. *Surg Endosc* 2005; **19**: 1503-1506 [PMID: 16328673 DOI: 10.1007/s00464-005-3002-4]
- 20 **Pappas-Gogos G**, Tsimogiannis KE, Zikos N, Nikas K, Manataki A, Tsimoyiannis EC. Preincisional and intraperitoneal ropivacaine plus normal saline infusion for postoperative pain relief after laparoscopic cholecystectomy: a randomized double-blind controlled trial. *Surg Endosc* 2008; **22**: 2036-2045 [PMID: 18270769 DOI: 10.1007/s00464-008-9762-x]
- 21 **Pasqualucci A**, Contardo R, Da Broi U, Colo F, Terrosu G, Donini A, Sorrentino M, Pasetto A, Bresadola F. The effects of intraperitoneal local anesthetic on analgesic requirements and endocrine response after laparoscopic cholecystectomy: a randomized double-blind controlled study. *J Laparoendosc Surg* 1994; **4**: 405-412 [PMID: 7881144]
- 22 **Pasqualucci A**, de Angelis V, Contardo R, Colò F, Terrosu G, Donini A, Pasetto A, Bresadola F. Preemptive analgesia: intraperitoneal local anesthetic in laparoscopic cholecystectomy. A randomized, double-blind, placebo-controlled study. *Anesthesiology* 1996; **85**: 11-20 [PMID: 8694355]
- 23 **Rademaker BM**, Kalkman CJ, Odoom JA, de Wit L, Ringers J. Intraperitoneal local anaesthetics after laparoscopic cholecystectomy: effects on postoperative pain, metabolic responses and lung function. *Br J Anaesth* 1994; **72**: 263-266 [PMID: 8130042]
- 24 **Raetzell M**, Maier C, Schröder D, Wulf H. Intraperitoneal application of bupivacaine during laparoscopic cholecystectomy--risk or benefit? *Anesth Analg* 1995; **81**: 967-972 [PMID: 7486086]
- 25 **Scheinin B**, Kellokumpu I, Lindgren L, Haglund C, Rosenberg PH. Effect of intraperitoneal bupivacaine on pain after laparoscopic cholecystectomy. *Acta Anaesthesiol Scand* 1995; **39**: 195-198 [PMID: 7793186]
- 26 **Golubović S**, Golubović V, Cindrić-Stancin M, Tokmadžić VS. Intraperitoneal analgesia for laparoscopic cholecystectomy: bupivacaine versus bupivacaine with tramadol. *Coll Antropol* 2009; **33**: 299-302 [PMID: 19408641]
- 27 **Jabbour-Khoury SI**, Dabbous AS, Gerges FJ, Azar MS, Ayoub CM, Khoury GS. Intraperitoneal and intravenous routes for pain relief in laparoscopic cholecystectomy. *JSLS* 2005; **9**: 316-321 [PMID: 16121879]
- 28 **Lepner U**, Goroshina J, Samarütel J. Postoperative pain relief after laparoscopic cholecystectomy: a randomised prospective double-blind clinical trial. *Scand J Surg* 2003; **92**: 121-124 [PMID: 12841551]
- 29 **Tsimoyiannis EC**, Glantzounis G, Lekkas ET, Siakas P, Jabarin M, Tzourou H. Intraperitoneal normal saline and bupivacaine infusion for reduction of postoperative pain after laparoscopic cholecystectomy. *Surg Laparosc Endosc* 1998; **8**: 416-420 [PMID: 9864106]
- 30 **Yang SY**, Kang H, Choi GJ, Shin HY, Baek CW, Jung YH, Choi YS. Efficacy of intraperitoneal and intravenous lidocaine on pain

- relief after laparoscopic cholecystectomy. *J Int Med Res* 2014; **42**: 307-319 [PMID: 24648482 DOI: 10.1177/0300060513505493]
- 31 **Cha SM**, Kang H, Baek CW, Jung YH, Koo GH, Kim BG, Choi YS, Cha SJ, Cha YJ. Peritrocal and intraperitoneal ropivacaine for laparoscopic cholecystectomy: a prospective, randomized, double-blind controlled trial. *J Surg Res* 2012; **175**: 251-258 [PMID: 21658722 DOI: 10.1016/j.jss.2011.04.033]
 - 32 **Yeh CN**, Tsai CY, Cheng CT, Wang SY, Liu YY, Chiang KC, Hsieh FJ, Lin CC, Jan YY, Chen MF. Pain relief from combined wound and intraperitoneal local anesthesia for patients who undergo laparoscopic cholecystectomy. *BMC Surg* 2014; **14**: 28 [PMID: 24886449 DOI: 10.1186/1471-2482-14-28]
 - 33 **Garcia JB**, Alencar Júnior AM, Santos CE. [Intraperitoneal administration of 50% enantiomeric excess (S75-R25) bupivacaine in postoperative analgesia of laparoscopic cholecystectomy]. *Rev Bras Anesthesiol* 2007; **57**: 344-355 [PMID: 19462110]
 - 34 **Joris J**, Thiry E, Paris P, Weerts J, Lamy M. Pain after laparoscopic cholecystectomy: characteristics and effect of intraperitoneal bupivacaine. *Anesth Analg* 1995; **81**: 379-384 [PMID: 7618731]
 - 35 **Labaille T**, Mazoit JX, Paqueron X, Franco D, Benhamou D. The clinical efficacy and pharmacokinetics of intraperitoneal ropivacaine for laparoscopic cholecystectomy. *Anesth Analg* 2002; **94**: 100-115, table of contents [PMID: 11772809]
 - 36 **Ng A**, Swami A, Smith G, Robertson G, Lloyd DM. Is intraperitoneal levobupivacaine with epinephrine useful for analgesia following laparoscopic cholecystectomy? A randomized controlled trial. *Eur J Anaesthesiol* 2004; **21**: 653-657 [PMID: 15473621]
 - 37 **Alper I**, Ulukaya S, Ertugrul V, Makay O, Uyar M, Balcioglu T. Effects of intraperitoneal levobupivacaine on pain after laparoscopic cholecystectomy: a prospective, randomized, double-blinded study. *Agri* 2009; **21**: 141-145 [PMID: 20127533]
 - 38 **Alper I**, Ulukaya S, Yüksel G, Uyar M, Balcioglu T. Laparoscopic cholecystectomy pain: effects of the combination of incisional and intraperitoneal levobupivacaine before or after surgery. *Agri* 2014; **26**: 107-112 [PMID: 25205408 DOI: 10.5505/agri.2014.42650]
 - 39 **Chundrigar T**, Hedges AR, Morris R, Stamatakis JD. Intraperitoneal bupivacaine for effective pain relief after laparoscopic cholecystectomy. *Ann R Coll Surg Engl* 1993; **75**: 437-439 [PMID: 8285548]
 - 40 **Elfberg BA**, Sjövall-Mjöberg S. Intraperitoneal bupivacaine does not effectively reduce pain after laparoscopic cholecystectomy: a randomized, placebo-controlled and double-blind study. *Surg Laparosc Endosc Percutan Tech* 2000; **10**: 357-359 [PMID: 11147908]
 - 41 **Elhakim M**, Elkott M, Ali NM, Tahoun HM. Intraperitoneal lidocaine for postoperative pain after laparoscopy. *Acta Anaesthesiol Scand* 2000; **44**: 280-284 [PMID: 10714840]
 - 42 **El-Labban GM**, Hokkam EN, El-Labban MA, Morsy K, Saadl S, Heissam KS. Intra-incisional vs intraperitoneal infiltration of local anaesthetic for controlling early post-laparoscopic cholecystectomy pain. *J Minim Access Surg* 2011; **7**: 173-177 [PMID: 22022099 DOI: 10.4103/0972-9941.83508]
 - 43 **Hilivering B**, Draaisma WA, van der Bilt JD, Valk RM, Kofman KE, Consten EC. Randomized clinical trial of combined preincisional infiltration and intraperitoneal instillation of levobupivacaine for postoperative pain after laparoscopic cholecystectomy. *Br J Surg* 2011; **98**: 784-789 [PMID: 21412996 DOI: 10.1002/bjs.7435]
 - 44 **Maestroni U**, Sortini D, Devito C, Pour Morad Kohan Brunaldi F, Anania G, Pavanelli L, Pasqualucci A, Donini A. A new method of preemptive analgesia in laparoscopic cholecystectomy. *Surg Endosc* 2002; **16**: 1336-1340 [PMID: 11988800 DOI: 10.1007/s00464-001-9181-8]
 - 45 **Mraović B**, Jurišić T, Kogler-Majerić V, Sustić A. Intraperitoneal bupivacaine for analgesia after laparoscopic cholecystectomy. *Acta Anaesthesiol Scand* 1997; **41**: 193-196 [PMID: 9062598]
 - 46 **Szem JW**, Hydo L, Barie PS. A double-blinded evaluation of intraperitoneal bupivacaine vs saline for the reduction of postoperative pain and nausea after laparoscopic cholecystectomy. *Surg Endosc* 1996; **10**: 44-48 [PMID: 8711605]
 - 47 **Zimmer PW**, McCann MJ, O'Brien MM. Bupivacaine use in the Insuflow device during laparoscopic cholecystectomy: results of a prospective randomized double-blind controlled trial. *Surg Endosc* 2010; **24**: 1524-1527 [PMID: 20108156 DOI: 10.1007/s00464-009-0804-9]
 - 48 **Niknam F**, Saxena A, Niles N, Budak UU, Mekisic A. Does irrigation of the subdiaphragmatic region with ropivacaine reduce the incidence of right shoulder tip pain after laparoscopic cholecystectomy? A prospective randomized, double-blind, controlled study. *Am Surg* 2014; **80**: E17-E18 [PMID: 24401503]
 - 49 **Cunniffe MG**, McAnena OJ, Dar MA, Callearly J, Flynn N. A prospective randomized trial of intraoperative bupivacaine irrigation for management of shoulder-tip pain following laparoscopy. *Am J Surg* 1998; **176**: 258-261 [PMID: 9776154]
 - 50 **Gharaibeh KI**, Al-Jaberi TM. Bupivacaine instillation into gallbladder bed after laparoscopic cholecystectomy: does it decrease shoulder pain? *J Laparoendosc Adv Surg Tech A* 2000; **10**: 137-141 [PMID: 10883990]
 - 51 **Paulson J**, Mellinger J, Baguley W. The use of intraperitoneal bupivacaine to decrease the length of stay in elective laparoscopic cholecystectomy patients. *Am Surg* 2003; **69**: 275-278; discussion 278-279 [PMID: 12716083]
 - 52 **Draper K**, Jefson R, Jongeward R, McLeod M. Duration of postlaparoscopic pneumoperitoneum. *Surg Endosc* 1997; **11**: 809-811 [PMID: 9266640]
 - 53 **Kahokehr A**, Sammour T, Vather R, Taylor M, Stapelberg F, Hill AG. Systemic levels of local anaesthetic after intra-peritoneal application—a systematic review. *Anaesth Intensive Care* 2010; **38**: 623-638 [PMID: 20715724]
 - 54 **Loizides S**, Gurusamy KS, Nagendran M, Rossi M, Guerrini GP, Davidson BR. Wound infiltration with local anaesthetic agents for laparoscopic cholecystectomy. *Cochrane Database Syst Rev* 2014; **3**: CD007049 [PMID: 24619479 DOI: 10.1002/14651858.CD007049.pub2]

P- Reviewer: Gonzalez-Ojeda A, Hu H **S- Editor:** Yu J
L- Editor: A **E- Editor:** Zhang DN



Over-the-scope clip to close a gastrocutaneous fistula after esophagectomy

Shan-Shan Shen, Xiao-Qi Zhang, Zhen-Lei Li, Xiao-Ping Zou, Ting-Sheng Ling

Shan-Shan Shen, Xiao-Qi Zhang, Zhen-Lei Li, Xiao-Ping Zou, Ting-Sheng Ling, Department of Gastroenterology, Affiliated Drum Tower Hospital of Nanjing University, Medical School, Nanjing 210008, Jiangsu Province, China

Author contributions: Shen SS wrote the paper and collected some of the patient's clinical data; Li ZL administrated and followed the patient; Zhang XQ made critical revisions of the paper; Zou XP designed the operation; Ling TS performed the operation and approved the publication of the paper.

Supported by National Natural Science Foundation of China, No. 81201908.

Institutional review board statement: The case report was approved by the Ethics Committee of Nanjing Drum Tower Hospital.

Informed consent statement: Informed consent was obtained from the patient.

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

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Ting-Sheng Ling, MD, PhD, Department of Gastroenterology, Affiliated Drum Tower Hospital of Nanjing University, Medical School, No. 321 Zhongshan Road, Nanjing 210008, Jiangsu Province, China. chinalts@126.com
Telephone: +86-25-83304616

Received: April 19, 2015

Peer-review started: April 20, 2015

First decision: May 18, 2015

Revised: June 15, 2015

Accepted: September 13, 2015

Article in press: September 14, 2015

Published online: December 21, 2015

Abstract

Over-the-scope clip (OTSC) system is becoming a new reliable technique which is available for the endoscopic closure of fistulas, bleeding, perforations and so on. We describe the case of a patient with a non-healing gastrocutaneous fistula after esophagectomy for esophageal squamous cell carcinoma which was successfully closed using an OTSC system. This is the first report of the use of OTSC to treat a non-healing gastrocutaneous fistula successfully after esophagectomy. We believe our experience will give such patients an ideal way to cure the fistula without suffering too much and also explore new application of OTSC.

Key words: Over-the-scope clip; Gastrocutaneous fistula; Digestive endoscopy; Esophageal squamous cell carcinoma

© **The Author(s) 2015.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Gastrocutaneous fistula after esophagectomy is infrequent, but it is notorious for the difficulty of achieving closure. We describe the case of a patient with a non-healing gastrocutaneous fistula after esophagectomy for esophageal squamous cell carcinoma which was successfully closed using an over-the-scope clip (OTSC) system. This is the first report of the use of OTSC to treat this type of gastrocutaneous fistula.

Shen SS, Zhang XQ, Li ZL, Zou XP, Ling TS. Over-the-scope clip to close a gastrocutaneous fistula after esophagectomy. *World J Gastroenterol* 2015; 21(47): 13396-13399 Available from:

URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13396.htm>
DOI: <http://dx.doi.org/10.3748/wjg.v21.i47.13396>

INTRODUCTION

Radical surgery for esophageal squamous cell carcinoma is the first-line option for most patients, however, refractory gastrocutaneous fistula is a recognized complication. Here we describe the case of a patient with a non-healing gastrocutaneous fistula after esophagectomy for esophageal squamous cell carcinoma which was successfully closed using an over-the-scope clip (OTSC) system.

CASE REPORT

The patient was a 51-year-old man who was diagnosed with esophageal squamous cell carcinoma which was located 20-24 cm from the incisor teeth in September 2011 and subsequently underwent radical esophagectomy using the Ivor-Lewis method. The pathological type was esophageal squamous cell carcinoma with moderate differentiation and the TNM stage was T2N1M0. Two months after surgery, he developed a fever, systemic inflammatory response syndrome and a gastrocutaneous fistula with purulent secretion appearing on the left wall of his chest. The fistula was located in the 6th intercostal space of the left anterior axillary line on the chest. Endoscopy was applied and found that the esophagogastric anastomosis was located 18 cm from the incisor teeth and the gastrocutaneous fistula was situated 37 cm from the incisor teeth. The gastrocutaneous fistula in the stomach was approximately 1 cm × 1 cm in size and there was abscess in the fistula. Since the complication occurred so promptly after surgery that he did not accept any other therapies for the tumor itself such as radiotherapy from then on. Subsequently, he underwent drainage of the abscess with a tube placed in the chest. Meanwhile, the patient had been fasted and received gastrointestinal decompression therapy and nose-jejunum nutrition support for 28 mo before coming to our hospital in February 2014. Endoscopic titanium clip closure was attempted to close the fistula in the stomach in January 2014 but failed. The patient lost 40 pounds of weight before admission to our hospital and had a low quality of life. The reason why the patient suffered such long time nose-jejunum nutrition may be that the fistula size was big and could not be sealed after local drainage and systemic antibiotic administration. Prior to any therapeutic procedure, an endoscopy was performed in our hospital and revealed a purulent secretion flowing from the fistula (Figure 1). Closing the fistula with pus may aggravate the infection, therefore no step was taken to seal the fistula. Instead, a drainage tube was placed in the chest. The depth of the tube in

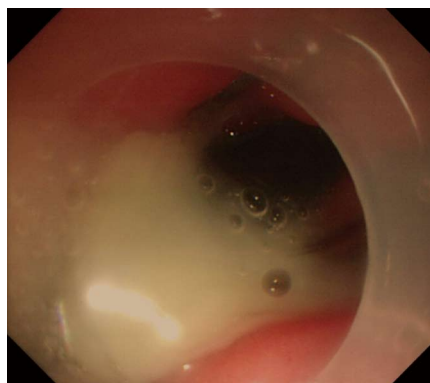


Figure 1 Endoscopic view of purulent secretion flowing out from gastrocutaneous fistula in a 51-year-old man after esophagectomy for esophageal squamous cell carcinoma.

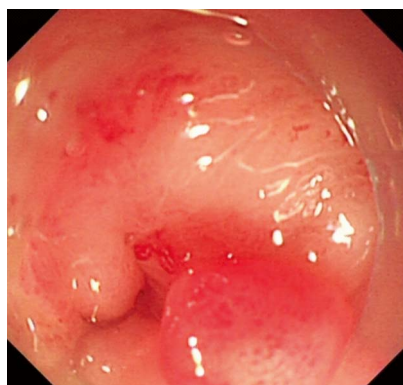


Figure 2 After 42-d drainage with a tube in the wall of the chest together with gastrointestinal decompression, antibiotic application and parenteral nutrition, there was no obvious purulent secretion from the gastric aspect of the fistula and the diameter of the fistula was about 1 cm × 1 cm.

the fistula was about 7 cm. One side of the tube was placed in the remnant stomach and the other side was on the chest. Antibiotics were administered to control the infection. After 42 d of comprehensive treatments and when the drainage liquid turned to clear, a second endoscopy was performed, which revealed no obvious purulent secretion in the fistula and the diameter of the fistula was about 1 cm × 1 cm (Figure 2). The gastric aspect of the fistula was successfully closed with the 11/6t OTSC system (Ovesco Endoscopy GmbH, Tübingen, Germany). The OTSC system was placed on the tip of the endoscope, the margins of the fistula were apposed with graspers, and the clip was then deployed by using the external hand wheel and "anchor" to allow precise alignment between the target tissue and the applicator cap. Immediate closure of the fistula was confirmed by the inability to flush water through the stomach into the fistula (Figures 3 and 4A). The patient was discharged 3 d later with semiliquid food by mouth and returned to our hospital 21 d later to check the closure of the fistula without any symptoms. Upper gastrointestinal endoscopy showed that the OTSC was still in place and the fistula



Figure 3 Appearance of the over-the-scope clip device and it placed on the tip of the endoscope.



Figure 5 Closure of the fistula was confirmed again by the inability to leak Omnipaque through the fistula in chest wall to the stomach.

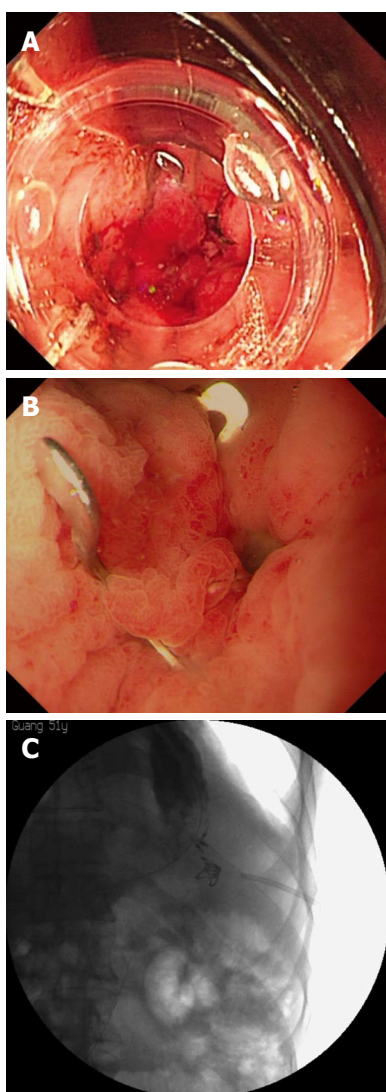


Figure 4 Gastric fistula was successfully closed with the 11/6t OTSC system. A: The gastric aspect of the fistula was successfully closed using over-the-scope clip; B: A follow-up endoscopy on 21 d after over-the-scope clip placement found the over-the-scope clip was still in place and the fistula was sealed successfully; C: Closure of the fistula was confirmed by the inability to leak Omnipaque through the stomach to fistula.

disappeared (Figure 4B). Omnipaque injected through the endoscope verified the watertight closure of the fistula (Figure 4C). Closure of the fistula was also confirmed by the inability to leak Omnipaque from the chest to the stomach (Figure 5).

DISCUSSION

Due to the chronic inflammation and infection in the patient, repeat surgery would have been technically difficult and endoscopic intervention was required in such situation. Several methods have been described for closing non-healing gastrocutaneous fistulas, including endoscopic clips, fibrin glue, sutures, stents and a combination of these techniques^[1]. Because of the failure of the titanium clip and other traditional methods not being suitable for this patient with a persistent fistula, we decided to close the fistula using the OTSC system. Its capacity of grasping more visceral tissue and applying a greater compressive force is thought to achieve a sturdy closure of the gastrocutaneous fistula^[2]. There have been some case reports about gastrocutaneous fistulas after sleeve gastrectomy, percutaneous endoscopic gastrostomy removal and gastric bypass sealed with the OTSC device^[3-5]. This is the first report of the use of OTSC to treat a non-healing gastrocutaneous fistula after esophagectomy. The short-term closure was confirmed in our case. However, the long-term outcome needs to be further explored.

COMMENTS

Case characteristics

The middle-aged male patient presented with a non-healing gastrocutaneous fistula which was located in the 6th intercostal space of the left anterior axillary line on the chest after esophagectomy for esophageal squamous cell carcinoma.

Clinical diagnosis

A fever, systemic inflammatory response syndrome and a gastrocutaneous fistula with purulent secretion appeared on the left wall of the chest which was

located in the 6th intercostal space of the left anterior axillary line.

Differential diagnosis

Fistula caused by inflammatory bowel disease and subcutaneous infection.

Laboratory diagnosis

WBC $12 \times 10^9/L$; N 90%; HGB 90 g/L; liver and kidney function tests were within normal limits.

Imaging diagnosis

Gastrocutaneous fistula was situated 37 cm from the incisor teeth with approximately 1 cm \times 1 cm in size and there was abscess in the fistula by endoscopy.

Treatment

After systemic antibiotic treatments and sufficient drainage on the chest, the gastric aspect of the fistula was successfully closed with the 11/6t over-the-scope clip (OTSC) system (Ovesco Endoscopy GmbH, Tübingen, Germany).

Related reports

There have been some case reports about gastrocutaneous fistulas after sleeve gastrectomy, percutaneous endoscopic gastrostomy removal and gastric bypass sealed with the OTSC device.

Term explanation

OTSC system designed by Ovesco Endoscopy GmbH, Tübingen, Germany, represents a new class of endoscopic clips providing significantly more strength and better tissue capture compared to conventional clips delivered through the working channel of the flexible endoscope.

Experiences and lessons

This is the first report of the use of OTSC to treat a non-healing gastro-

cutaneous fistula after esophagectomy and the short-term closure was confirmed in our case, however, the long-term outcome needs to be further explored.

Peer-review

The notorious non-healing gastrocutaneous fistula after esophagectomy for esophageal squamous cell carcinoma can be successfully closed using an OTSC system.

REFERENCES

- 1 **Kumar N**, Thompson CC. Endoscopic therapy for postoperative leaks and fistulae. *Gastrointest Endosc Clin N Am* 2013; **23**: 123-136 [PMID: 23168123 DOI: 10.1016/j.giec.2012.10.002]
- 2 **Nishiyama N**, Mori H, Kobara H, Rafiq K, Fujihara S, Kobayashi M, Oryu M, Masaki T. Efficacy and safety of over-the-scope clip: including complications after endoscopic submucosal dissection. *World J Gastroenterol* 2013; **19**: 2752-2760 [PMID: 23687412 DOI: 10.3748/wjg.v19.i18.2752]
- 3 **Aly A**, Lim HK. The use of over the scope clip (OTSC) device for sleeve gastrectomy leak. *J Gastrointest Surg* 2013; **17**: 606-608 [PMID: 23090281 DOI: 10.1007/s11605-012-2062-8]
- 4 **Sandmann M**, Heike M, Faehndrich M. Application of the OTSC system for the closure of fistulas, anastomosal leakages and perforations within the gastrointestinal tract. *Z Gastroenterol* 2011; **49**: 981-985 [PMID: 21811949 DOI: 10.1055/s-0029-1245972]
- 5 **Donatelli G**, Vergeau BM, Dumont JL, Tuszyński T, Dritsas S, Dhumane P, Meduri B. Late presentation of a giant gastrogastroic fistula following gastric bypass, treated with a colic over-the-scope clip after unsuccessful surgical repair. *Endoscopy* 2014; **46** Suppl 1 UCTN: E128-E129 [PMID: 24676824 DOI: 10.1055/s-0034-1364889]

P- Reviewer: Mathisen DJ, Shiryajev YN

S- Editor: Yu J **L- Editor:** Wang TQ **E- Editor:** Liu XM





Current status of superparamagnetic iron oxide contrast agents for liver magnetic resonance imaging

Yi-Xiang J Wang

Yi-Xiang J Wang, Department of Imaging and Interventional Radiology, Faculty of Medicine, the Chinese University of Hong Kong, Hong Kong, China

Author contributions: Wang YXJ designed research, analyzed data, and wrote the paper.

Conflict-of-interest statement: The author declares no conflict-of-interest.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Yi-Xiang J Wang, PhD, MMed, Department of Imaging and Interventional Radiology, Faculty of Medicine, the Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China. yixiang_wang@cuhk.edu.hk
Telephone: +852-26322289

Received: May 28, 2015

Peer-review started: May 31, 2015

First decision: August 26, 2015

Revised: September 15, 2015

Accepted: November 9, 2015

Article in press: November 9, 2015

Published online: December 21, 2015

Abstract

Five types of superparamagnetic iron oxide (SPIO), *i.e.* Ferumoxides (Feridex[®] IV, Berlex Laboratories), Ferucarbotran (Resovist[®], Bayer Healthcare), Ferumoxtran-10 (AMI-227 or Code-7227, Combidex[®], AMAG Pharma; Sinerem[®], Guerbet), NC100150 (Clariscan[®], Nycomed,) and (VSOP C184, Ferropharm)

have been designed and clinically tested as magnetic resonance contrast agents. However, until now Resovist[®] is current available in only a few countries. The other four agents have been stopped for further development or withdrawn from the market. Another SPIO agent Ferumoxytol (Feraheme[®]) is approved for the treatment of iron deficiency in adult chronic kidney disease patients. Ferumoxytol is comprised of iron oxide particles surrounded by a carbohydrate coat, and it is being explored as a potential imaging approach for evaluating lymph nodes and certain liver tumors.

Key words: Superparamagnetic iron oxide; Liver; Hepatocellular carcinoma; Magnetic resonance imaging; Resovist; Gd-EOB-DTPA; Primovist; Eovist

© **The Author(s) 2015.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Superparamagnetic iron oxide nanoparticle for liver imaging was conceptualized when the speed of both single-slice computed tomography (CT) scan and multiple-slice magnetic resonance imaging (MRI) was slow. It was difficult to accurately observe the "wash-in" and "wash-out" of liver lesion blood flow dynamics. However, recently spiral CT and later multi-slice CT revolutionized liver imaging. MRI scan is also currently much faster due to the improved gradient technology and fast data acquisition sequences. These techniques increased the sensitivity and specificity of dynamic imaging using small molecular agents such as iodinated CT contrast agents and Gadolinium based MRI contrast agents. Gd-EOB-DTPA-enhanced liver MRI is currently emerging as the leading method for diagnosis and staging of hepatocellular carcinoma.

Wang YXJ. Current status of superparamagnetic iron oxide contrast agents for liver magnetic resonance imaging. *World J Gastroenterol* 2015; 21(47): 13400-13402 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i47/13400.htm> DOI:

<http://dx.doi.org/10.3748/wjg.v21.i47.13400>

TO THE EDITOR

I read with interest the recent systemic review on superparamagnetic iron oxide (SPIO) for magnetic resonance imaging of focal hepatic lesions by Li *et al*^[1]. This paper further confirmed the value of SPIO in liver magnetic resonance imaging (MRI). I hope to make some additional comments. Five types of SPIO, *i.e.* Ferumoxides (Feridex® IV, Berlex Laboratories), Ferucarbotran (Resovist®, Bayer Healthcare), Ferumoxtran-10 (AMI-227 or Code-7227, Combidx®, AMAG Pharma; Sinerem®, Guerbet), NC100150 (Clariscan®, Nycomed,) and (VSOP C184, Ferropharm) have been designed and clinically tested as MR contrast agent. They all have a core composed of SPIO crystals, but have different coating materials and different hydrodynamic size and therefore different *in-vivo* pharmacokinetic profiles^[1-5]. Clariscan® and VSOP C184 were designed for MR angiography and blood pool imaging^[5,6], but did not receive regulatory approval. Combidx® and Sinerem® were primarily designed for lymph node imaging. Despite initial promising data^[7], the pivotal study failed to demonstrate a consistent and statistically significant benefit for sensitivity and failed to confirm non-inferiority with regard to specificity^[8]. Therefore their clinical development was stopped^[4]. Feridex® and Resovist® were primarily designed for liver imaging, and received regulatory approval in the United States and Europe respectively, as well as number of other countries such as Japan. Feridex® cannot be administered as an intravenous bolus as it may be associated with severe back pain, while Resovist® can be administered by fast bolus injection, and therefore imaging of the arterial phase is feasible. However, it has been shown that there is no significant difference in the number of Kupffer cells between well-differentiated hepatocellular carcinoma (HCC) and the surrounding healthy liver tissue^[9]. Another study showed how Resovist®-enhanced MRI is less efficient than Gd-BOPTA-enhanced dynamic MRI in the detection and characterization of HCC^[10]. This can be explained by SPIO's inability to evaluate the pathological vascularity of liver nodules. Due to lack of clinical users, Feridex® has been withdrawn from the market, and Resovist® is current available in only limited countries^[11].

SPIO for liver imaging was conceptualized when the speed of both single-slice CT scan and multiple-slice MRI was slow. It was difficult to accurately observe the "wash-in" and "wash-out" of liver lesion blood flow dynamics. However, recently spiral CT and later multi-slice CT revolutionized liver imaging. MRI scanning is also currently much faster due to the improved gradient technology and fast data acquisition sequences. These techniques increase the sensitivity

and specificity of dynamic imaging using small molecular agents such as iodinated CT contrast agents and Gadolinium based MRI contrast agents.

Another SPIO agent Ferumoxytol (Feraheme®, AMAG Pharmaceuticals, United States; Rienso®, Europe) has been approved for the treatment of iron deficiency in adult chronic kidney disease patients. Ferumoxytol is comprised of iron oxide particles surrounded by a carbohydrate coat. The agent is taken up by macrophages and ultimately the reticuloendothelial system, opening the door for a potential imaging approaches to evaluate lymph nodes and certain liver tumors^[12].

Several recent studies demonstrated that MRI using hepatocyte-specific contrast agent (Gd-EOB-DTPA, Primovist®, Europe; Eovist®, United States; Bayer HealthCare) can provide better diagnostic performance for the detection and characterization of HCCs in cirrhotic livers than dynamic CT or dynamic MRI using extracellular agents^[13]. Gd-EOB-DTPA-enhanced liver MRI is currently emerging as the leading method for diagnosis and staging of HCC^[13].

REFERENCES

- 1 Li YW, Chen ZG, Wang JC, Zhang ZM. Superparamagnetic iron oxide-enhanced magnetic resonance imaging for focal hepatic lesions: systematic review and meta-analysis. *World J Gastroenterol* 2015; **21**: 4334-4344 [PMID: 25892885 DOI: 10.3748/wjg.v21.i14.4334]
- 2 Corot C, Robert P, Idée JM, Port M. Recent advances in iron oxide nanocrystal technology for medical imaging. *Adv Drug Deliv Rev* 2006; **58**: 1471-1504 [PMID: 17116343 DOI: 10.1016/j.addr.2006.09.013]
- 3 Wang YX, Hussain SM, Krestin GP. Superparamagnetic iron oxide contrast agents: physicochemical characteristics and applications in MR imaging. *Eur Radiol* 2001; **11**: 2319-2331 [PMID: 11702180 DOI: 10.1007/s003300100908]
- 4 Wang YX. Superparamagnetic iron oxide based MRI contrast agents: Current status of clinical application. *Quant Imaging Med Surg* 2011; **1**: 35-40 [PMID: 23256052]
- 5 Klein C, Nagel E, Schnackenburg B, Bornstedt A, Schalla S, Hoffmann V, Lehning A, Fleck E. The intravascular contrast agent Clariscan (NC 100150 injection) for 3D MR coronary angiography in patients with coronary artery disease. *MAGMA* 2000; **11**: 65-67 [PMID: 11186991 DOI: 10.1007/BF02678498]
- 6 Wagner M, Wagner S, Schnorr J, Schellenberger E, Kivelitz D, Krug L, Dewey M, Laule M, Hamm B, Taupitz M. Coronary MR angiography using citrate-coated very small superparamagnetic iron oxide particles as blood-pool contrast agent: initial experience in humans. *J Magn Reson Imaging* 2011; **34**: 816-823 [PMID: 21769977 DOI: 10.1002/jmri.22683]
- 7 Harisinghani MG, Barentsz J, Hahn PF, Deserno WM, Tabatabaei S, van de Kaa CH, de la Rosette J, Weissleder R. Noninvasive detection of clinically occult lymph-node metastases in prostate cancer. *N Engl J Med* 2003; **348**: 2491-2499 [PMID: 12815134 DOI: 10.1056/NEJMoa022749]
- 8 European Medicines Agency. International Nonproprietary Name (INN): superparamagnetic iron oxide nanoparticles stabilised with dextran and sodium citrate. 2008. Available from: URL: http://www.ema.europa.eu/docs/en_GB/document_library/Application_withdrawal_assessment_report/2010/01/WC500067463.pdf
- 9 Tanaka M, Nakashima O, Wada Y, Kage M, Kojiro M. Pathomorphological study of Kupffer cells in hepatocellular carcinoma and hyperplastic nodular lesions in the liver. *Hepatology*

- 1996; **24**: 807-812 [PMID: 8855180 DOI: 10.1053/jhep.1996.v24.pm0008855180]
- 10 **Kim YK**, Kim CS, Kwak HS, Lee JM. Three-dimensional dynamic liver MR imaging using sensitivity encoding for detection of hepatocellular carcinomas: comparison with superparamagnetic iron oxide-enhanced mr imaging. *J Magn Reson Imaging* 2004; **20**: 826-837 [PMID: 15503325 DOI: 10.1002/jmri.20188]
- 11 **Maurea S**, Mainenti PP, Tambasco A, Imbriaco M, Mollica C, Laccetti E, Camera L, Liuzzi R, Salvatore M. Diagnostic accuracy of MR imaging to identify and characterize focal liver lesions: comparison between gadolinium and superparamagnetic iron oxide contrast media. *Quant Imaging Med Surg* 2014; **4**: 181-189 [PMID: 24914419]
- 12 **Bashir MR**, Bhatti L, Marin D, Nelson RC. Emerging applications for ferumoxytol as a contrast agent in MRI. *J Magn Reson Imaging* 2015; **41**: 884-898 [PMID: 24974785 DOI: 10.1002/jmri.24691]
- 13 **Lee JM**, Park JW, Choi BI. 2014 KLCSCG-NCC Korea Practice Guidelines for the management of hepatocellular carcinoma: HCC diagnostic algorithm. *Dig Dis* 2014; **32**: 764-777 [PMID: 25376295 DOI: 10.1159/000368020]

P- Reviewer: Pierre P, Radmard AR

S- Editor: Gong ZM **L- Editor:** A **E- Editor:** Liu XM





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



9 771007 932045