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EDITORIAL

Adjuvant treatment in biliary tract cancer: To treat or not to treat?

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Abstract

Biliary tract cancer is a rare malignant tumor. There is limited knowledge about biology and natural history of this disease and considerable uncertainty remains regarding its optimal diagnostic and therapeutic management. The role of adjuvant therapy is object of debate and controversy. Although resection is identified as the most effective and the only potentially curative treatment, there is no consensus on the impact of adjuvant chemotherapy and/or radiotherapy on the high incidence of disease recurrence and on survival. This is mainly due to the rarity of this disease and the consequent difficulty in performing randomized trials. The only two prospectively controlled trials concluded that adjuvant chemotherapy did not improve survival. Most of the retrospective trials, which had limited sample size and included heterogeneous patients population and non-standardized therapies, suggested a marginal benefit of chemoradiotherapy in reducing locoregional recurrence and an uncertain impact on survival. Well-designed multi-institutional randomized trials are necessary to clarify the role of adjuvant therapy. Two ongoing phase Ⅲ trials may provide relevant information.

Key words: Biliary tract cancer; Adjuvant therapy; Chemotherapy; Chemoradiation; Surgery

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INTRODUCTION

Biliary tract cancer (BTC) is a rare tumor accounting for approximately 4% of all malignant neoplasms of the gastrointestinal tract. Marked gender, ethnical and geographical variations exist and, in certain regions of the world, like Chile or North India, BTC is one of the most common causes of cancer mortality. The median age at presentation is the seventh decade of life with a male to female ratio of 1.5[1-5].

Surgical resection is the only potentially curative treatment for BTC. However, the resectability rate has been reported to range between 30% and 70%, with large variability based on tumor location (70% for ampullary cancer, 40%-50% for gallbladder, intrahepatic and distal extrahepatic cancer and 30% for hilar BTC)[6,7]. The type of resection and prognosis vary with anatomical location with a 5-year overall survival (OS) rate of 20%-40% for intrahepatic adenocarcinoma, 50%-70% for ampullary cancer, 25%-50% for distal cholangiocarcinoma and for gallbladder cancer and 15%-35% for hilar BTC[8].

Due to the rarity of this disease, in which patients with curatively resected tumors are in the minority, prospective trials have been rarely performed and, sometimes, eligibility criteria allowed the enrolment of both patients with pancreatic cancer and BTC, thus hampering

the interpretation of results. Accordingly, information with a high level of evidence is lacking and wide areas of debate and controversy on optimal adjuvant therapeutic management exist.

PROGNOSTIC FACTORS

The 5-year OS rate with surgery alone is disappointing, ranging from 15% to 70%[6]. Complete surgical resection with histologically negative (R0) surgical margins has been reported to be the most important prognostic factor[9]. Since prognosis varies also with anatomical location, the heterogeneity of patient population of the reported studies may affect the interpretation of the data[8]. Other prognostic factors such as tumor stage, nodal status, vascular and perineural invasion, elevated baseline CA 19-9 level and histologic grade have been identified in many reports[10-13]. Among those, the prognostic relevance of tumor stage, nodal status, histologic grade and resection margin is almost universally accepted and should be taken into account for the stratification of the patients in prospective trials and for the interpretation of the results in non-randomized series; while the prognostic role of elevated baseline CA 19-9 level and vascular and perineural invasion is still controversial[14-45].

THERAPEUTIC MANAGEMENT

Most patients with BTC are not suitable for curative surgery at diagnosis, and the rate of microscopically positive resection margins (R1) has been reported to be up to 74%[14]. In addition, locoregional failure occurs in more than half of the patients, even after R0 resection[9,10]. Isolated locoregional disease was reported in approximately 15% of patients with gallbladder cancer, 20% with periampullary cancer and 60% with hilar cholangiocarcinoma. In contrast, systemic disease, with or without concomitant locoregional recurrence, occurred in 85% of patients with gallbladder cancer, 75% with periampullary cancer and 41% with hilar cholangiocarcinoma[10,11]. Because of poor survival after curative resection due to frequent local relapse and distant metastases[9-11,36], the role of adjuvant therapy has been widely explored[16,32,38,41-56].

Chemoradiation

Previous studies, mainly focusing on adjuvant chemoradiation therapy (CRT) with a variety of regimens, led to conflicting results and the role of this therapeutic strategy remains controversial. No large randomized trial of adjuvant CRT has ever been performed. A small phase Ⅲ European Organization for Research and Treatment of Cancer trial, including 92 eligible patients with periampullary adenocarcinoma, demonstrated no statistically significant difference in survival between adjuvant CRT following resection *vs* observation[37]. However, since this trial included a limited number of patients and an outdated chemoradiation in terms of imaging, techniques, planning, dose and concomitant radiosensitizing chemotherapy, definitive conclusions on the role of modern chemoradiation are impossible to draw.

Table 1 Survival outcome for adjuvant chemoradiation

Ref.	No. of patients	Site	Therapy		OS (median)	
			Yes	No	CRT	Obs
[38]	73	GB	25	48	58	50
[39]	96	PV	54	42	35.2	16.5
[41]	49	All	34	15	16.4	6.7[1]
[42]	48	EHBD	48		44%[2]	NA
[43]	84	All	34	50	42	29
[44]	125	PV	29	96	67	42
[45]	34	EHBD	34	0	35%[2]	NA

[1]Statistically significant; [2]5-year overall survival (OS). CRT: Chemoradiation; Obs: Observation; PV: Papilla of vater; GB: Gallbladder; EHBD: Extrahepatic bile duct; All: PV + GB + EHBD + intrahepatic bile duct; NA: Not applicable.

Conversely, a retrospective series of 73 patients with gallbladder cancer treated between 1985 and 2004 at Mayo Clinic[38] suggested that adjuvant CRT may obtain a statistically significant improvement in OS only for patients with lymph node involvement. Similarly, two retrospective series of fluoropyrimidine-based post-operative chemoradiation from MD Anderson Cancer Center[39] and from South Korea[40], including 96 patients affected by ampullary adenocarcinoma and 91 patients with extrahepatic bile duct cancer, respectively, suggested an improved OS only in patients with locally advanced tumor (T3/T4)[39] or with R1 resection[40]. A few other smaller retrospective series also reported a modest potential OS benefit with adjuvant CRT (Table 1)[38,39,41-45].

Apart from the controversial impact on OS, CRT may have a role in improving local control, especially in patients at higher risk of local failure, such as those with R1 margins and positive lymph nodes. In fact, 5-year local control rate raised from 40%-50% in patients with ampullary cancer treated with surgery alone to 65%-80% in those who received post-operative CRT[1,39-41,57].

Unfortunately, the retrospective nature of most of these studies, the small sample size, the lack of correction for multiple comparisons, patient selection bias; heterogeneity in terms of patients' characteristics, treatment regimens, tumor site and stage; long-lasting accrual periods, different surgical, radiotherapy and radiological techniques in different historical periods and other confounding factors do not allow to draw any firm conclusion on the role of CRT. In fact, younger and healthier patients were more likely to be offered adjuvant CRT. On the other hand, patients with high risk features were more likely to receive adjuvant therapy than those with favorable features.

Chemotherapy

A few studies evaluated the role of adjuvant chemotherapy in BTC. A retrospective single centre analysis on 42 patients suggested that postoperative gemcitabine-based chemotherapy may be a promising strategy to improve OS after surgical resection for hilar cholangiocarcinoma[55].

Consistently, the addition of fluorouracil-based chemotherapy to adjuvant CRT seemed to improve disease free survival (3-year DFS 45% *vs* 27%) and OS (3-year OS 63% *vs* 31%) compared to CRT alone in another retrospective series of 120 patients with radically resected extrahepatic BTC.

A phase Ⅲ trial addressed the role of adjuvant chemotherapy with 5-fluorouracil and mitomycin-C in a series of 508 patients with surgically treated pancreaticobiliary malignancies including 335 patients with BTC[58]. OS was significantly increased when compared to observation arm only in the unplanned subset analysis of 61 resected patients with macroscopically positive resection margins (R2) affected by gallbladder cancer. Similarly, a more recent phase Ⅲ trial exploring the role of single agent adjuvant chemotherapy with either gemcitabine or 5-fluorouracil, in 304 patients with ampullary adenocarcinoma submitted to curative resection did not demonstrate a survival benefit for any of the adjuvant therapy arms when compared to surgery alone[59].

GUIDELINES AND CURRENT CLINICAL PRACTICE

The National Comprehensive Cancer Network (NCCN) guidelines[60] recommend only observation or adjuvant CRT with concomitant fluoropyrimidine for patients with R0 margins or negative lymph-nodes and adjuvant therapy with concurrent 5-fluorouracil-based CRT followed or not by additional fluoropyrimidine- or gemcitabine-based regimens in patients with R1 margins or metastatic lymph nodes. The use of chemotherapy is recommended due to the high incidence of systemic relapse and to the results observed in the therapeutic management of advanced disease[61,62]. The European Clinical Practice guidelines[63] are more vague, only suggesting CRT as a possible therapeutic option after surgery for BTC. More restrictive indications derive from a modified and implemented version of NCCN guidelines proposed by a committee of specialists of the Middle East and North Africa Region, which recommend only observation or enrolment into a clinical trial after an R0 and/or a negative regional nodes resection, because of conflicting data regarding adjuvant CRT[64].

Given the lack of guidelines based on high level of evidence, it is not surprising that patients submitted to curative surgery for biliary tract tumors receive heterogeneous management around the world. A survey of therapeutic strategies recommended in the clinical practice in 2001-2002, reported that adjuvant CRT was widely adopted in the majority of American centers (71%), followed by Asian/Pacific centers (55%), but only by 29% of European institutions[65]. This scenario may have changed in more recent years with a trend towards possibly increasing use of adjuvant treatment due to the numerous positive experiences reported in the literature in the last decade[9-11,38-42,44,45,55,57-59,66,67]. However, eighty-eight percent of the interviewed physicians recognized the unmet need for achieving higher levels of evidence from large prospective trials to support the routine use of adjuvant treatment[65].

FUTURE DIRECTIONS

Altogether, the available data do not allow to answer the question whether patients submitted to curative resection for BTC should receive an adjuvant therapy and which treatment strategy may provide the largest benefit.

In fact, neither adjuvant CRT nor adjuvant chemotherapy with either single agent or a 5-fluorouracil-mitomycin doublet improved OS when compared to observation alone in phase Ⅲ trials[14,58,59], while only a modest benefit in loco-regional control rather than on OS was suggested with CRT by retrospective series that, in any case, suffer from previously mentioned methodological limitations[1,38,41-45,65-68].

The causes of this disappointing scenario and of the lack of a convincing answer are manifold. First, when compared to trials on advanced disease, trials on adjuvant therapy are more demanding, also due to the involvement of different specialists (surgeon, radiologist, oncologist and radiotherapist); more resource- and time-consuming, due to the longer patient's life expectancy and to the inferior number of patients; and require a more selective choice of centers to be involved. Second, evidence-based information on the most active and effective chemotherapy regimen against unresectable or metastatic disease is limited as well. Accordingly, the selection of promising regimens that may have a relevant impact on disease natural history is challenging. Only recently, cisplatin and gemcitabine regimen became the new standard of treatment in advanced BTC[61] providing a rational for investigating the role of this combination in the adjuvant setting. Additionally, two ongoing phase Ⅲ trials are currently exploring the role of capecitabine (NCT00363584) and of GEMOX regimen (NCT01313377) in the adjuvant setting and may provide further information in the next future. Third, the rarity of disease limits the interest of pharmaceutical companies while investigator initiated trials are hindered by the restricted availability of agents already registered and authorized for the treatment of the disease. Fourth, the choice of the most promising therapeutic strategy is crucial in this disease that has a very high rate of both local and systemic recurrence. Systemic chemotherapy and CRT, rather than being taken as alternative therapies, should be combined taken into account in the design of post-operative management. However, the role of sequential therapy with CRT followed by systemic chemotherapy or the inverse sequence was rarely addressed in prospective trials[55]. Fifth, the knowledge on tumor biology is limited and, at the moment, does not allow to identify new agents that may contribute to improve the outcome of the disease. Last but not least, the interpretation of trials result is often challenging due to the heterogeneity of enrolled patients population. Stratification based on tumor location, extent of resection, lymph node

status and resection margin status will be crucial to the success of future studies.

CONCLUSION

A multi-institutional worldwide effort to conduct well designed phase Ⅲ trial and to expand biological knowledge of the disease is necessary to clarify the role of adjuvant therapy in BTC.

REFERENCES

1 **Aljiffry M**, Walsh MJ, Molinari M. Advances in diagnosis, treatment and palliation of cholangiocarcinoma: 1990-2009. *World J Gastroenterol* 2009; **15**: 4240-4262

2 **de Groen PC**, Gores GJ, LaRusso NF, Gunderson LL, Nagorney DM. Biliary tract cancers. *N Engl J Med* 1999; **341**: 1368-1378

3 **Andreotti G**, Liu E, Gao YT, Safaeian M, Rashid A, Shen MC, Wang BS, Deng J, Han TQ, Zhang BH, Hsing AW. Medical history and the risk of biliary tract cancers in Shanghai, China: implications for a role of inflammation. *Cancer Causes Control* 2011; **22**: 1289-1296

4 **von Hahn T**, Ciesek S, Wegener G, Plentz RR, Weismüller TJ, Wedemeyer H, Manns MP, Greten TF, Malek NP. Epidemiological trends in incidence and mortality of hepatobiliary cancers in Germany. *Scand J Gastroenterol* 2011; **46**: 1092-1098

5 **Farges O**, Fuks D, Le Treut YP, Azoulay D, Laurent A, Bachellier P, Nuzzo G, Belghiti J, Pruvot FR, Regimbeau JM. AJCC 7th edition of TNM staging accurately discriminates outcomes of patients with resectable intrahepatic cholangiocarcinoma: By the AFC-IHCC-2009 study group. *Cancer* 2011; **117**: 2170-2177

6 **Talamini MA**, Moesinger RC, Pitt HA, Sohn TA, Hruban RH, Lillemoe KD, Yeo CJ, Cameron JL. Adenocarcinoma of the ampulla of Vater. A 28-year experience. *Ann Surg* 1997; **225**: 590-599; discussion 590-599

7 **Nagakawa T**, Kayahara M, Ikeda S, Futakawa S, Kakita A, Kawarada H, Matsuno M, Takada T, Takasaki K, Tanimura H, Tashiro S, Yamaoka Y. Biliary tract cancer treatment: results from the Biliary Tract Cancer Statistics Registry in Japan. *J Hepatobiliary Pancreat Surg* 2002; **9**: 569-575

8 **Heron DE**, Stein DE, Eschelman DJ, Topham AK, Waterman FM, Rosato EL, Alden M, Anne PR. Cholangiocarcinoma: the impact of tumor location and treatment strategy on outcome. *Am J Clin Oncol* 2003; **26**: 422-428

9 **Jan YY**, Yeh CN, Yeh TS, Chen TC. Prognostic analysis of surgical treatment of peripheral cholangiocarcinoma: two decades of experience at Chang Gung Memorial Hospital. *World J Gastroenterol* 2005; **11**: 1779-1784

10 **Jarnagin WR**, Ruo L, Little SA, Klimstra D, D'Angelica M, DeMatteo RP, Wagman R, Blumgart LH, Fong Y. Patterns of initial disease recurrence after resection of gallbladder carcinoma and hilar cholangiocarcinoma: implications for adjuvant therapeutic strategies. *Cancer* 2003; **98**: 1689-1700

11 **Smeenk HG**, van Eijck CH, Hop WC, Erdmann J, Tran KC, Debois M, van Cutsem E, van Dekken H, Klinkenbijl JH, Jeekel J. Long-term survival and metastatic pattern of pancreatic and periampullary cancer after adjuvant chemoradiation or observation: long-term results of EORTC trial 40891. *Ann Surg* 2007; **246**: 734-740

12 **Iacono C**, Verlato G, Zamboni G, Scarpa A, Montresor E, Capelli P, Bortolasi L, Serio G. Adenocarcinoma of the ampulla of Vater: T-stage, chromosome 17p allelic loss, and extended pancreaticoduodenectomy are relevant prognostic factors. *J Gastrointest Surg* 2007; **11**: 578-588

13 **Balachandran P**, Agarwal S, Krishnani N, Pandey CM, Kumar A, Sikora SS, Saxena R, Kapoor VK. Predictors of long-term survival in patients with gallbladder cancer. *J Gastrointest Surg* 2006; **10**: 848-854

14 **Qiao QL**, Zhang TP, Guo JC, Zhan HX, Zhao JX, Liu YC, Wan YL, Leng XS, Zhao YP. Prognostic factors after pancreatoduodenectomy for distal bile duct cancer. *Am Surg* 2011; **77**: 1445-1448

15 **Ruys AT**, Kate FJ, Busch OR, Engelbrecht MR, Gouma DJ, van Gulik TM. Metastatic lymph nodes in hilar cholangiocarcinoma: does size matter? *HPB* (Oxford) 2011; **13**: 881-886

16 **Tugba Kos F**, Aksoy S, Odabas H, Ozdemir N, Oksuzoglu B, Uncu D, Zengin N. Adjuvant therapy for gallbladder and bile duct cancers: retrospective comparative study. *J BUON* 2011; **16**: 464-468

17 **Yao X**, Zhou L, Han S, Chen Y. High expression of CXCR4 and CXCR7 predicts poor survival in gallbladder cancer. *J Int Med Res* 2011; **39**: 1253-1264

18 **Sun XN**, Cao WG, Wang X, Wang Q, Gu BX, Yang QC, Hu JB, Liu H, Zheng S. Prognostic impact of vascular endothelial growth factor-A expression in resected gallbladder carcinoma. *Tumour Biol* 2011; **32**: 1183-1190

19 **Clark CJ**, Wood-Wentz CM, Reid-Lombardo KM, Kendrick ML, Huebner M, Que FG. Lymphadenectomy in the staging and treatment of intrahepatic cholangiocarcinoma: a population-based study using the National Cancer Institute SEER database. *HPB* (Oxford) 2011; **13**: 612-620

20 **Patel SH**, Kooby DA, Staley CA, Sarmiento JM, Maithel SK. The prognostic importance of lymphovascular invasion in cholangiocarcinoma above the cystic duct: a new selection criterion for adjuvant therapy? *HPB* (Oxford) 2011; **13**: 605-611

21 **Du X**, Wang S, Lu J, Cao Y, Song N, Yang T, Dong R, Zang L, Yang Y, Wu T, Li J. Correlation between MMP1-PAR1 axis and clinical outcome of primary gallbladder carcinoma. *Jpn J Clin Oncol* 2011; **41**: 1086-1093

22 **Qureshi A**, Hassan U, Azam M. Morphology, TNM staging and survival with Pancreatico-duodenectomy specimens received at Shaukat Khanum Memorial Cancer Hospital and Research Centre, Pakistan. *Asian Pac J Cancer Prev* 2011; **12**: 953-956

23 **Kai K**, Kohya N, Kitahara K, Masuda M, Miyoshi A, Ide T, Tokunaga O, Miyazaki K, Noshiro H. Tumor budding and dedifferentiation in gallbladder carcinoma: potential for the prognostic factors in T2 lesions. *Virchows Arch* 2011; **459**: 449-456

24 **Choi SB**, Kim WB, Song TJ, Suh SO, Kim YC, Choi SY. Surgical outcomes and prognostic factors for ampulla of Vater cancer. *Scand J Surg* 2011; **100**: 92-98

25 **de Jong MC**, Nathan H, Sotiropoulos GC, Paul A, Alexandrescu S, Marques H, Pulitano C, Barroso E, Clary BM, Aldrighetti L, Ferrone CR, Zhu AX, Bauer TW, Walters DM, Gamblin TC, Nguyen KT, Turley R, Popescu I, Hubert C, Meyer S, Schulick RD, Choti MA, Gigot JF, Mentha G, Pawlik TM. Intrahepatic cholangiocarcinoma: an international multi-institutional analysis of prognostic factors and lymph node assessment. *J Clin Oncol* 2011; **29**: 3140-3145

26 **Sakata J**, Shirai Y, Wakai T, Ajioka Y, Akazawa K, Hatakeyama K. Assessment of the nodal status in ampullary carcinoma: the number of positive lymph nodes versus the lymph node ratio. *World J Surg* 2011; **35**: 2118-2124

27 **Miyamoto M**, Ojima H, Iwasaki M, Shimizu H, Kokubu A, Hiraoka N, Kosuge T, Yoshikawa D, Kono T, Furukawa H, Shibata T. Prognostic significance of overexpression of c-Met oncoprotein in cholangiocarcinoma. *Br J Cancer* 2011; **105**: 131-138

28 **Wakai T**, Shirai Y, Sakata J, Matsuda Y, Korita PV, Takamura M, Ajioka Y, Hatakeyama K. Prognostic significance of NQO1 expression in intrahepatic cholangiocarcinoma. *Int J Clin Exp Pathol* 2011; **4**: 363-370

29 **Ito H**, Ito K, D'Angelica M, Gonen M, Klimstra D, Allen P, DeMatteo RP, Fong Y, Blumgart LH, Jarnagin WR. Accurate

staging for gallbladder cancer: implications for surgical therapy and pathological assessment. *Ann Surg* 2011; **254**: 320-325
30 **Murakami Y**, Uemura K, Sudo T, Hashimoto Y, Nakashima A, Kondo N, Sakabe R, Kobayashi H, Sueda T. Prognostic factors of patients with advanced gallbladder carcinoma following aggressive surgical resection. *J Gastrointest Surg* 2011; **15**: 1007-1016
31 **Li H**, Qin Y, Cui Y, Chen H, Hao X, Li Q. Analysis of the surgical outcome and prognostic factors for hilar cholangiocarcinoma: a Chinese experience. *Dig Surg* 2011; **28**: 226-231
32 **Showalter TN**, Zhan T, Anne PR, Chervoneva I, Mitchell EP, Yeo CJ, Rosato EL, Kennedy EP, Berger AC. The influence of prognostic factors and adjuvant chemoradiation on survival after pancreaticoduodenectomy for ampullary carcinoma. *J Gastrointest Surg* 2011; **15**: 1411-1416
33 **Kawaguchi T**, Ochiai T, Ikoma H, Inoue K, Morimura R, Murayama Y, Komatsu S, Shiozaki A, Kuriu Y, Nakanishi M, Ichikawa D, Okamoto K, Fujiwara H, Kokuba Y, Sonoyama T, Otsuji E. Prognostic impact of histological blood vessel invasion in patients with ampullary adenocarcinoma. *Hepatogastroenterology* 2010; **57**: 1347-1350
34 **Guglielmi A**, Ruzzenente A, Campagnaro T, Pachera S, Conci S, Valdegamberi A, Sandri M, Iacono C. Prognostic significance of lymph node ratio after resection of peri-hilar cholangiocarcinoma. *HPB* (Oxford) 2011; **13**: 240-245
35 **Anderson C**, Kim R. Adjuvant therapy for resected extrahepatic cholangiocarcinoma: a review of the literature and future directions. *Cancer Treat Rev* 2009; **35**: 322-327
36 **Murakami Y**, Uemura K, Hayasidani Y, Sudo T, Hashimoto Y, Ohge H, Sueda T. Indication for postoperative adjuvant therapy in biliary carcinoma based on analysis of recurrence and survival after surgical resection. *Dig Dis Sci* 2009; **54**: 1360-1364
37 **Klinkenbijl JH**, Jeekel J, Sahmoud T, van Pel R, Couvreur ML, Veenhof CH, Arnaud JP, Gonzalez DG, de Wit LT, Hennipman A, Wils J. Adjuvant radiotherapy and 5-fluorouracil after curative resection of cancer of the pancreas and periampullary region: phase III trial of the EORTC gastrointestinal tract cancer cooperative group. *Ann Surg* 1999; **230**: 776-782; discussion 782-784
38 **Gold DG**, Miller RC, Haddock MG, Gunderson LL, Quevedo F, Donohue JH, Bhatia S, Nagorney DM. Adjuvant therapy for gallbladder carcinoma: the Mayo Clinic Experience. *Int J Radiat Oncol Biol Phys* 2009; **75**: 150-155
39 **Krishnan S**, Rana V, Evans DB, Varadhachary G, Das P, Bhatia S, Delclos ME, Janjan NA, Wolff RA, Crane CH, Pisters PW. Role of adjuvant chemoradiation therapy in adenocarcinomas of the ampulla of vater. *Int J Radiat Oncol Biol Phys* 2008; **70**: 735-743
40 **Kim S**, Kim SW, Bang YJ, Heo DS, Ha SW. Role of postoperative radiotherapy in the management of extrahepatic bile duct cancer. *Int J Radiat Oncol Biol Phys* 2002; **54**: 414-419
41 **Nakeeb A**, Tran KQ, Black MJ, Erickson BA, Ritch PS, Quebbeman EJ, Wilson SD, Demeure MJ, Rilling WS, Dua KS, Pitt HA. Improved survival in resected biliary malignancies. *Surgery* 2002; **132**: 555-563; discission 563-564
42 **Kim K**, Chie EK, Jang JY, Kim SW, Han SW, Oh DY, Im SA, Kim TY, Bang YJ, Ha SW. Adjuvant Chemoradiotherapy After Curative Resection for Extrahepatic Bile Duct Cancer: A Long-term Single Center Experience. *Am J Clin Oncol* 2012; **35**: 136-140
43 **Serafini FM**, Sachs D, Bloomston M, Carey LC, Karl RC, Murr MM, Rosemurgy AS. Location, not staging, of cholangiocarcinoma determines the role for adjuvant chemoradiation therapy. *Am Surg* 2001; **67**: 839-843; discussion 843-844
44 **Bhatia S**, Miller RC, Haddock MG, Donohue JH, Krishnan S. Adjuvant therapy for ampullary carcinomas: the Mayo Clinic experience. *Int J Radiat Oncol Biol Phys* 2006; **66**: 514-519
45 **Hughes MA**, Frassica DA, Yeo CJ, Riall TS, Lillemoe KD, Cameron JL, Donehower RC, Laheru DA, Hruban RH, Abrams RA. Adjuvant concurrent chemoradiation for adenocarcinoma of the distal common bile duct. *Int J Radiat Oncol Biol Phys* 2007; **68**: 178-182
46 **Konishi M.** Adjuvant chemotherapy for resectable biliary tract cancer: current status and future direction. *J Hepatobiliary Pancreat Sci* 2012
47 **Narang AK**, Miller RC, Hsu CC, Bhatia S, Pawlik TM, Laheru D, Hruban RH, Zhou J, Winter JM, Haddock MG, Donohue JH, Schulick RD, Wolfgang CL, Cameron JL, Herman JM. Evaluation of adjuvant chemoradiation therapy for ampullary adenocarcinoma: the Johns Hopkins Hospital-Mayo Clinic collaborative study. *Radiat Oncol* 2011; **6**: 126
48 **González ME**, Giannini OH, González P, Saldaña B. Adjuvant radio-chemotherapy after extended or simple cholecystectomy in gallbladder cancer. *Clin Transl Oncol* 2011; **13**: 480-484
49 **Bonet Beltrán M**, Roth AD, Mentha G, Allal AS. Adjuvant radio-chemotherapy for extrahepatic biliary tract cancers. *BMC Cancer* 2011; **11**: 267
50 **Park HS**, Lim JY, Yoon DS, Park JS, Lee DK, Lee SJ, Choi HJ, Song SY, Lee WJ, Cho JY. Outcome of adjuvant therapy for gallbladder cancer. *Oncology* 2010; **79**: 168-173
51 **Vern-Gross TZ**, Shivnani AT, Chen K, Lee CM, Tward JD, MacDonald OK, Crane CH, Talamonti MS, Munoz LL, Small W. Survival outcomes in resected extrahepatic cholangiocarcinoma: effect of adjuvant radiotherapy in a surveillance, epidemiology, and end results analysis. *Int J Radiat Oncol Biol Phys* 2011; **81**: 189-198
52 **Cho SY**, Kim SH, Park SJ, Han SS, Kim YK, Lee KW, Lee WJ, Woo SM, Kim TH. Adjuvant chemoradiation therapy in gallbladder cancer. *J Surg Oncol* 2010; **102**: 87-93
53 **Park JH**, Choi EK, Ahn SD, Lee SW, Song SY, Yoon SM, Kim YS, Lee YS, Lee SG, Hwang S, Lee YJ, Park KM, Kim TW, Chang HM, Lee JL, Kim JH. Postoperative chemoradiotherapy for extrahepatic bile duct cancer. *Int J Radiat Oncol Biol Phys* 2011; **79**: 696-704
54 **Murakami Y**, Uemura K, Sudo T, Hayashidani Y, Hashimoto Y, Nakamura H, Nakashima A, Sueda T. Adjuvant gemcitabine plus S-1 chemotherapy improves survival after aggressive surgical resection for advanced biliary carcinoma. *Ann Surg* 2009; **250**: 950-956
55 **Lim KH**, Oh DY, Chie EK, Jang JY, Im SA, Kim TY, Kim SW, Ha SW, Bang YJ. Adjuvant concurrent chemoradiation therapy (CCRT) alone versus CCRT followed by adjuvant chemotherapy: which is better in patients with radically resected extrahepatic biliary tract cancer?: a non-randomized, single center study. *BMC Cancer* 2009; **9**: 345
56 **Gwak HK**, Kim WC, Kim HJ, Park JH. Extrahepatic bile duct cancers: surgery alone versus surgery plus postoperative radiation therapy. *Int J Radiat Oncol Biol Phys* 2010; **78**: 194-198
57 **Murakami Y**, Uemura K, Sudo T, Hayashidani Y, Hashimoto Y, Nakamura H, Nakashima A, Sueda T. Gemcitabine-based adjuvant chemotherapy improves survival after aggressive surgery for hilar cholangiocarcinoma. *J Gastrointest Surg* 2009; **13**: 1470-1479
58 **Takada T**, Amano H, Yasuda H, Nimura Y, Matsushiro T, Kato H, Nagakawa T, Nakayama T. Is postoperative adjuvant chemotherapy useful for gallbladder carcinoma? A phase III multicenter prospective randomized controlled trial in patients with resected pancreaticobiliary carcinoma. *Cancer* 2002; **95**: 1685-1695
59 **Neoptolemos JP,** Moore MJ, Cox TF, Valle JW, Palmer DH, Mcdonald A, Carter R, Tebbutt NC, Dervenis C, Smith D, Glimelius B, Coxon FY, Lacaine F, Middleton MR, Ghaneh P, Bassi C, Halloran C, Olah A, Rawcliffe CL, Buchler MW. Ampullary cancer ESPAC-3 (v2) trial: A multicenter, international, open-label, randomized controlled phase III trial of adjuvant chemotherapy versus observation in patients with

adenocarcinoma of the ampulla of vater. *J Clin Oncol* 2011; **29:** abstr LBA4006
60 Available from: URL: http: //www.nccn.org/professionals/physician_gls/f_guidelines.asp
61 **Valle J**, Wasan H, Palmer DH, Cunningham D, Anthoney A, Maraveyas A, Madhusudan S, Iveson T, Hughes S, Pereira SP, Roughton M, Bridgewater J. Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. *N Engl J Med* 2010; **362**: 1273-1281
62 **Glimelius B**, Hoffman K, Sjödén PO, Jacobsson G, Sellström H, Enander LK, Linné T, Svensson C. Chemotherapy improves survival and quality of life in advanced pancreatic and biliary cancer. *Ann Oncol* 1996; **7**: 593-600
63 **Eckel F**, Brunner T, Jelic S. Biliary cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2010; **21** Suppl 5: v65-v69
64 **Yusuf MA**, Kapoor VK, Kamel RR, Kazmi A, Uddin N, Masood N, Al-Abdulkareem A. Modification and implementation of NCCN guidelines on hepatobiliary cancers in the Middle East and North Africa region. *J Natl Compr Canc Netw* 2010; **8** Suppl 3: S36-S40
65 **Nakeeb A**, Pitt HA. Radiation therapy, chemotherapy and chemoradiation in hilar cholangiocarcinoma. *HPB* (Oxford) 2005; **7**: 278-282
66 **Borghero Y**, Crane CH, Szklaruk J, Oyarzo M, Curley S, Pisters PW, Evans D, Abdalla EK, Thomas MB, Das P, Wistuba II, Krishnan S, Vauthey JN. Extrahepatic bile duct adenocarcinoma: patients at high-risk for local recurrence treated with surgery and adjuvant chemoradiation have an equivalent overall survival to patients with standard-risk treated with surgery alone. *Ann Surg Oncol* 2008; **15**: 3147-3156
67 **Wang SJ**, Fuller CD, Kim JS, Sittig DF, Thomas CR, Ravdin PM. Prediction model for estimating the survival benefit of adjuvant radiotherapy for gallbladder cancer. *J Clin Oncol* 2008; **26**: 2112-2117
68 **Yang J**, Yan LN. Current status of intrahepatic cholangiocarcinoma. *World J Gastroenterol* 2008; **14**: 6289-6297

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FIELD OF VISION

Motor vehicle accidents: How should cirrhotic patients be managed?

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Abstract

Motor vehicle accidents (MVAs) are serious social issues worldwide and driver illness is an important cause of MVAs. Minimal hepatic encephalopathy (MHE) is a complex cognitive dysfunction with attention deficit, which frequently occurs in cirrhotic patients independent of severity of liver disease. Although MHE is known as a risk factor for MVAs, the impact of diagnosis and treatment of MHE on MVA-related societal costs is largely unknown. Recently, Bajaj *et al* demonstrated valuable findings that the diagnosis of MHE by rapid screening using the inhibitory control test (ICT), and subsequent treatment with lactulose could substantially reduce the societal costs by preventing MVAs. Besides the ICT and lactulose, there are various diagnostic tools and therapeutic strategies for MHE. In this commentary, we discussed a current issue of diagnostic tools for MHE, including neuropsychological tests. We also discussed the advantages of the other therapeutic strategies for MHE, such as intake of a regular breakfast and coffee, and supplementation with zinc and branched chain amino acids, on the MVA-related societal costs.

INVITED COMMENTARY ON HOT ARTICLES

We have read with great interest the recent article by Bajaj *et al*[1] describing the diagnosis and treatment of minimal hepatic encephalopathy (MHE) to prevent motor vehicle accidents (MVAs), and would strongly recommend it to the readers.

MVAs are serious social issues worldwide[2]. Various factors are intricately involved in the occurrence of MVAs, and driver illness is an important cause[2]. Besides acute myocardial infarction, epileptic seizure, and hypoglycemia related to the use of anti-diabetic agents, liver cirrhosis with MHE has been reported to increase the risk of MVAs[3-6]. Although MHE occurs in up to 80% of patients with chronic liver disease[3] and diagnostic tools and therapeutic strategies for MHE exist[7-11], little information is available about the management of patients with liver cirrhosis with regard to preventing MVAs and subsequently reducing the associated societal costs.

In their study, Bajaj *et al*[1] performed a cost-effectiveness analysis to identify management strategies for the diagnosis and treatment of MHE in patients with liver cirrhosis to reduce MVA-related societal costs. They found that the diagnosis of MHE by rapid screening using the inhibitory control test (ICT), and subsequent treatment with lactulose could substantially reduce societal costs by preventing MVAs[1]. This is a significant study, and we agree with the authors about the benefits of the use of the diagnostic test and therapeutic management. However, we suggest that the management strategy should be modified to some extent for use in general medical institutions to prevent MVAs on a larger scale.

The ICT is a computerized test of attention and response inhibition that has been used to characterize attention deficit disorders[12]. The ICT consists of the presentation of several letters at 0.5 s intervals, while the subject is instructed to respond or inhibit their response to the specific letter[13]. The ICT is considered reliable and sensitive for the diagnosis of MHE[5,13]. In addition, unlike standard neuropsychological tests, ICT results are significantly associated with the future occurrence of MVAs[3]. However, the test takes approximately 30 min to complete and patients need to be familiar with computer operation. Furthermore, validation and standardization are required for each population, and therefore, so far, the ICT is not universally accessible. Similarly, other diagnostic tools for MHE also require trained personnel and specialized equipment[11]. In fact, an American Association for the Study of Liver Disease (AASLD) survey showed that the majority of AASLD members are not able to test for MHE because of a lack of time, resources, and suitable personnel[14]. Along with Bajaj *et al*[14], we propose that rapid and simple tools for the diagnosis MHE should be developed urgently, such as biochemical tests or virtual reality driving simulations.

Lactulose has been used to treat hepatic encephalopathy since 1966[15]. Lactulose reduces blood ammonia levels and improves overt hepatic encephalopathy as well as MHE[7]. In their study, Bajaj *et al*[1] have demonstrated the benefits of lactulose therapy on the occurrence of MVAs and MVA-related societal costs. However, compliance with lactulose treatment is generally poor, primarily because of its side-effects such as abdominal discomfort[16,17]. Recently, other therapeutic strategies for MHE have been reported. First, as prolonged periods of fasting are linked to the development of MHE, having a regular breakfast improves the attention and executive functions of cirrhotic patients with MHE[18]. Second, coffee intake improves cognitive function in elderly people as well as in patients with type 2 diabetes mellitus[19,20]. Although the beneficial effects of coffee on cognitive function have never been investigated in cirrhotic patients, insulin resistance is frequently seen in patients with chronic liver disease[21-23]. In addition, coffee consumption is known to improve hepatic inflammation and fibrosis in patients with chronic liver disease[24]. Third, the blood ammonia level is regulated by the activity of ornithine transcarbamoylase and zinc is a coenzyme required for its up-regulation[25].

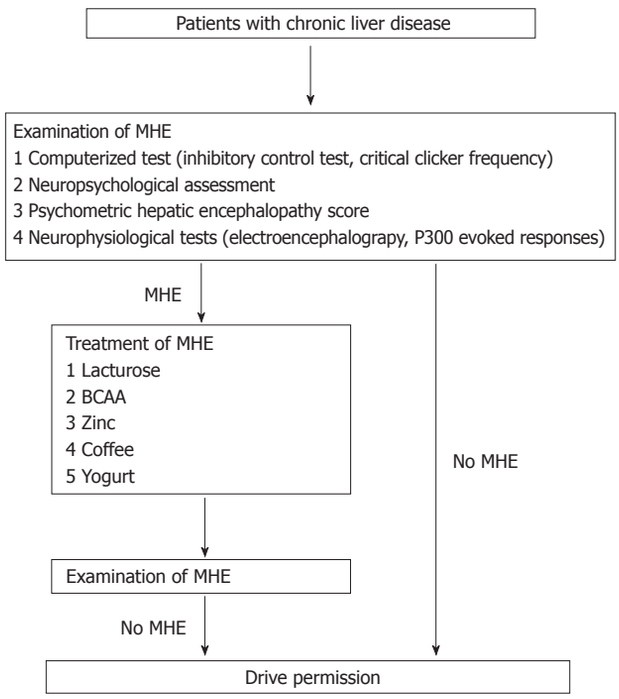

Figure 1 A proposed flow chart of drive permission for patients with chronic liver disease. MHE: Minimal hepatic encephalopathy; BCAA: Branched chain amino acids.

Oral zinc supplementation improves hyperammonemia as well as hepatic encephalopathy, as seen in a double-blind randomized controlled trial[26]. Finally, a decrease in serum branched chain amino acids (BCAA) levels is a feature of chronic liver disease[27]. BCAA is a source of glutamate, which detoxifies ammonia by glutamine synthesis in the skeletal muscle and brain[28]. Therefore, BCAA enhances the detoxification of blood ammonia by incorporating ammonia in the process of glutamine production and is currently used for treating patients with hepatic encephalopathy[29]. Thus, a therapeutic approach comprising the intake of a regular breakfast and coffee, and supplementation with zinc and BCAA may improve the cost-effectiveness of MVA-related events in cirrhotic patients with MHE (Figure 1).

Prevention of MVA by the diagnosis and treatment of MHE is an important component in the management of patients with liver cirrhosis. Collaborative researches among medical institutions, automobile companies, and governmental sectors may help further prevent MVAs and subsequently reduce MVA-related societal costs.

REFERENCES

1 **Bajaj JS**, Pinkerton SD, Sanyal AJ, Heuman DM. Diagnosis and treatment of minimal hepatic encephalopathy to prevent motor vehicle accidents: a cost-effectiveness analysis. *Hepatology* 2012; **55**: 1164-1171

2 **Huebner WW**, Wojcik NC, Jorgensen G, Marcella SP, Nicolich MJ. Mortality patterns and trends among 49,705 U.S.-based women in a petroleum company: update 1979-2000. *J Occup Environ Med* 2010; **52**: 99-108

3 **Bajaj JS**, Saeian K, Schubert CM, Hafeezullah M, Franco J, Varma RR, Gibson DP, Hoffmann RG, Stravitz RT, Heuman

DM, Sterling RK, Shiffman M, Topaz A, Boyett S, Bell D, Sanyal AJ. Minimal hepatic encephalopathy is associated with motor vehicle crashes: the reality beyond the driving test. *Hepatology* 2009; **50**: 1175-1183
4 **Bajaj JS**. Minimal hepatic encephalopathy matters in daily life. *World J Gastroenterol* 2008; **14**: 3609-3615
5 **Bajaj JS**, Hafeezullah M, Hoffmann RG, Saeian K. Minimal hepatic encephalopathy: a vehicle for accidents and traffic violations. *Am J Gastroenterol* 2007; **102**: 1903-1909
6 **Wein C**, Koch H, Popp B, Oehler G, Schauder P. Minimal hepatic encephalopathy impairs fitness to drive. *Hepatology* 2004; **39**: 739-745
7 **Watanabe A**, Sakai T, Sato S, Imai F, Ohto M, Arakawa Y, Toda G, Kobayashi K, Muto Y, Tsujii T, Kawasaki H, Okita K, Tanikawa K, Fujiyama S, Shimada S. Clinical efficacy of lactulose in cirrhotic patients with and without subclinical hepatic encephalopathy. *Hepatology* 1997; **26**: 1410-1414
8 **Kato A**, Suzuki K, Kaneta H, Obara H, Fujishima Y, Sato S. Regional differences in cerebral glucose metabolism in cirrhotic patients with subclinical hepatic encephalopathy using positron emission tomography. *Hepatol Res* 2000; **17**: 237-245
9 **Kato A**, Kato M, Ishii H, Ichimiya Y, Suzuki K, Kawasaki H, Yamamoto SI, Kumashiro R, Yamamoto K, Kawamura N, Hayashi N, Matsuzaki S, Terano A, Okita K, Watanabe A. Development of quantitative neuropsychological tests for diagnosis of subclinical hepatic encephalopathy in liver cirrhosis patients and establishment of diagnostic criteria-multicenter collaborative study in Japanese. *Hepatol Res* 2004; **30**: 71-78
10 **Sugimoto R**, Iwasa M, Maeda M, Urawa N, Tanaka H, Fujita N, Kobayashi Y, Takeda K, Kaito M, Takei Y. Value of the apparent diffusion coefficient for quantification of low-grade hepatic encephalopathy. *Am J Gastroenterol* 2008; **103**: 1413-1420
11 **Dhiman RK**, Saraswat VA, Sharma BK, Sarin SK, Chawla YK, Butterworth R, Duseja A, Aggarwal R, Amarapurkar D, Sharma P, Madan K, Shah S, Seth AK, Gupta RK, Koshy A, Rai RR, Dilawari JB, Mishra SP, Acharya SK. Minimal hepatic encephalopathy: consensus statement of a working party of the Indian National Association for Study of the Liver. *J Gastroenterol Hepatol* 2010; **25**: 1029-1041
12 **Crosbie J**, Pérusse D, Barr CL, Schachar RJ. Validating psychiatric endophenotypes: inhibitory control and attention deficit hyperactivity disorder. *Neurosci Biobehav Rev* 2008; **32**: 40-55
13 **Bajaj JS**, Hafeezullah M, Franco J, Varma RR, Hoffmann RG, Knox JF, Hischke D, Hammeke TA, Pinkerton SD, Saeian K. Inhibitory control test for the diagnosis of minimal hepatic encephalopathy. *Gastroenterology* 2008; **135**: 1591-1600.e1
14 **Bajaj JS**, Etemadian A, Hafeezullah M, Saeian K. Testing for minimal hepatic encephalopathy in the United States: An AASLD survey. *Hepatology* 2007; **45**: 833-834
15 **Bircher J**, Müller J, Guggenheim P, Haemmerli UP. Treatment of chronic portal-systemic encephalopathy with lactulose. *Lancet* 1966; **1**: 890-892
16 **Kalaitzakis E**, Björnsson E. Lactulose treatment for hepatic encephalopathy, gastrointestinal symptoms, and health-related quality of life. *Hepatology* 2007; **46**: 949-50; author reply 951
17 **Horsmans Y**, Solbreux PM, Daenens C, Desager JP, Geubel AP. Lactulose improves psychometric testing in cirrhotic patients with subclinical encephalopathy. *Aliment Pharmacol Ther* 1997; **11**: 165-170
18 **Vaisman N**, Katzman H, Carmiel-Haggai M, Lusthaus M, Niv E. Breakfast improves cognitive function in cirrhotic patients with cognitive impairment. *Am J Clin Nutr* 2010; **92**: 137-140
19 **Cropley V**, Croft R, Silber B, Neale C, Scholey A, Stough C, Schmitt J. Does coffee enriched with chlorogenic acids improve mood and cognition after acute administration in healthy elderly? A pilot study. *Psychopharmacology* (Berl) 2012; **219**: 737-749
20 **Biessels GJ**. Caffeine, diabetes, cognition, and dementia. *J Alzheimers Dis* 2010; **20** Suppl 1: S143-S150
21 **Kawaguchi T**, Yoshida T, Harada M, Hisamoto T, Nagao Y, Ide T, Taniguchi E, Kumemura H, Hanada S, Maeyama M, Baba S, Koga H, Kumashiro R, Ueno T, Ogata H, Yoshimura A, Sata M. Hepatitis C virus down-regulates insulin receptor substrates 1 and 2 through up-regulation of suppressor of cytokine signaling 3. *Am J Pathol* 2004; **165**: 1499-1508
22 **Kawaguchi T**, Ide T, Taniguchi E, Hirano E, Itou M, Sumie S, Nagao Y, Yanagimoto C, Hanada S, Koga H, Sata M. Clearance of HCV improves insulin resistance, beta-cell function, and hepatic expression of insulin receptor substrate 1 and 2. *Am J Gastroenterol* 2007; **102**: 570-576
23 **Eslam M**, Aparcero R, Kawaguchi T, Del Campo JA, Sata M, Khattab MA, Romero-Gomez M. Meta-analysis: insulin resistance and sustained virological response in hepatitis C. *Aliment Pharmacol Ther* 2011; **34**: 297-305
24 **Kawaguchi T**, Sata M. Importance of hepatitis C virus-associated insulin resistance: therapeutic strategies for insulin sensitization. *World J Gastroenterol* 2010; **16**: 1943-1952
25 **Reding P**, Duchateau J, Bataille C. Oral zinc supplementation improves hepatic encephalopathy. Results of a randomised controlled trial. *Lancet* 1984; **2**: 493-495
26 **Katayama K**. Ammonia metabolism and hepatic encephalopathy. *Hepatol Res* 2004; **30S**: 73-80
27 **Reding P**, Duchateau J, Bataille C. Oral zinc supplementation improves hepatic encephalopathy. Results of a randomised controlled trial. *Lancet* 1984; **2**: 493-495
28 **Platell C**, Kong SE, McCauley R, Hall JC. Branched-chain amino acids. *J Gastroenterol Hepatol* 2000; **15**: 706-717
29 **Moriwaki H,** Shiraki M, Fukushima H, Shimizu M, Iwasa J, Naiki T, Nagaki M. Long-term outcome of branched-chain amino acid treatment in patients with liver cirrhosis. *Hepatol Res* 2008; **38**: S102-S106

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TOPIC HIGHLIGHT

Yeng Ang, Dr., *Series Editor*

Pregnancy related issues in inflammatory bowel disease: Evidence base and patients' perspective

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Abstract

Inflammatory bowel disease (IBD) affects women of childbearing age and can influence fertility, pregnancy and decisions regarding breastfeeding. Women with IBD need to consider the possible course of disease during pregnancy, the benefits and risks associated with medications required for disease management during pregnancy and breastfeeding and the effects of mode of delivery on their disease. When indicated, aminosalicylates and thiopurines can be safely used during pregnancy. Infliximab and Adalimumab are considered probably safe during the first two trimesters. During the third trimester the placenta can be crossed and caution should be applied. Methotrexate is associated with severe teratogenicity due to its folate antagonism and is strictly contraindicated. Women with IBD tend to deliver earlier than healthy women, but can have a vaginal delivery in most cases. Caesarean sections are generally recommended for women with active perianal disease or after ileo-anal pouch surgery. While the impact of disease activity and medication has been addressed in several studies, there are minimal studies evaluating patients' perspective on these issues. Women's attitudes may influence their decision to have children and can positively or negatively influence the chance of conceiving, and their beliefs regarding therapies may impact on the course of their disease during pregnancy and/or breastfeeding. This review article outlines the impact of IBD and its treatment on pregnancy, and examines the available data on patients' views on this subject.

Key words: Pregnancy; Breast-feeding; Nursing; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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INTRODUCTION

Inflammatory bowel disease (IBD) predominantly affects the younger-aged population and therefore is prominent in women of child-bearing age. It often requires medication for maintenance of remission[1]. Literature on pregnancy in IBD has mainly utilized tertiary hospital cohorts and focused on pregnancy outcomes. Current guidelines emphasize the importance of inducing and maintaining disease remission prior to conception and during pregnancy[1,2]. Studies of patients' perspective of the influence of IBD on fertility, pregnancy and breastfeeding

are highly relevant and beginning to emerge. This review article summarizes this evidence and examines the currently available data describing the patients' views.

LITERATURE SEARCH

A literature search was conducted using Pubmed from 1980 to 2010 with the search terms "IBD", "Crohn's disease (CD)", "ulcerative colitis (UC)", "pregnancy", "breast feeding" and "nursing". Further relevant articles were identified from the reference lists of identified articles.

Fertility and fecundity

Factors determining fecundity and fertility in IBD include disease systemic effects (e.g., fatigue and anaemia) as well as libido and sexual inactivity, which, in turn, may be influenced by body image issues and dyspareunia[3]. Furthermore, concerns over the influence of disease activity or medications on a successful pregnancy may also influence fecundability. In UC, overall fertility is comparable to that of the healthy population[3-5], but women with ileo-anal pouches (IPAA) have reduced fecundity[6,7]. Fertility in CD in remission is equivalent to that of healthy women[4,8,9] but may be reduced in patients with active disease[9-12]. As in UC, women with CD also have reduced fecundity following extensive surgery involving the pelvis[5-7,13-18].

Disease behaviour during and after pregnancy

Disease activity during pregnancy is similar to that in non-pregnant women as reported by two Danish cohort studies. In 97 women with UC, flare rates were 34% per year during pregnancy and 32% per year outside pregnancy[19], while in 68 women with CD there was also no increased risk of a flare during pregnancy[20]. The vast majority of women have quiescent disease during pregnancy as shown in a population based cohort of 461 women from the Northern California Kaiser Permanente population[21].

Women with IBD might experience a more benign course of disease in the post-partum period. A significantly reduced number of flares in comparison to pre-pregnancy in a small cohort of 18 CD and 19 UC patients was reported by Castiglione *et al*[22]. A larger pan-European study of 93 women with CD and 173 women with UC confirmed this finding[23]. The reason for this phenomenon is unknown, but it has been proposed that disparity between maternal and foetal human leukocyte antigen class Ⅱ antigens leads to a state of immuno-tolerance or suppression that in turn leads to a more benign course of IBD[24].

Effects of IBD and IBD disease activity on pregnancy

Active disease during pregnancy has been linked to adverse pregnancy outcomes of low birth weight, pre-term birth and foetal loss[19,25-27]. Population-based studies have, however, reported conflicting results. In 461 women (300 with UC) of the Kaiser Permanente population in North America, no correlation between adverse pregnancy outcomes and disease activity was found[21], while a study of 157 Danish women with CD revealed that active disease increased the risk of pre-term birth[28]. The higher proportion of patients with active disease in the Danish study (45% *vs* 20% in the Kaiser Permanente population) may explain this difference.

Some studies, including a meta-analysis by Cornish *et al*[29], reported an increase in pre-term births in IBD patients[21]. Other studies, however, that differentiated UC from CD found an increase in pre-term birth rates for patients with CD only[30,31]. CD women also delivered lower birth weight babies than healthy controls and UC women[30,31]. It is important to recognise that all studies examining pregnancy outcomes in IBD used healthy women controls rather than those with other chronic diseases.

Congenital abnormalities in offspring of mothers with IBD

Congenital abnormalities of variable severities occur in 3%-7% of babies of healthy mothers[32]. Studies of congenital malformations in the offspring of mothers with IBD have reported conflicting results. In a population based cohort of 262 women from Washington state, North America, an increased risk of congenital malformations was found in the offspring of UC patients (7.9% *vs* 1.7% in healthy controls; $P < 0.001$) but not in those of CD patients (3.4%; P = NS)[30]. The study did not control for medication use[30]. In contrast, the Hungarian Case Control Surveillance of Congenital Anomalies (HCCSCA) (1980-1996) found no increase in the risk for "any" malformation [odd ratio (OR): 1.2, 95% (confidence interval) CI: 0.9-1.8] in the offspring of 79 UC mothers compared to 95 control mothers after adjusting for parity, age and medication use[33]. Specific malformations of the limb (OR: 6.2, 95% CI: 2.9-13.1), urinary tract (OR: 3.3, 95% CI: 1.1-9.5) and multiple malformations (OR: 2.6, 95% CI: 1.3-5.4), however, were significantly increased[33].

An Italian case-control study reported a significantly increased risk of congenital anomalies in the offspring of 90 women with IBD (5.5% *vs* 0% in 240 healthy controls) and that CD and UC had equivalent risks[31]. The reported 0% incidence of congenital abnormality rate in healthy controls, however, was unusually low given this rate can approach 7%[32].

The risk of congenital malformations in the offspring of 461 women with IBD from the Northern California Kaiser Permanente population was not increased in comparison to 493 healthy controls[21]. Cornish *et al*[29] found in a meta-analysis of four studies an increased risk (OR: 2.37, 95% CI: 1.47-3.82) of reporting congenital abnormalities for UC, while for CD the risk increase was not statistically significant (OR: 2.14, 95% CI: 0.97-4.74; P = 0.06).

Data on congenital malformations may be contradictory due to differences in study design, the inclusion of cohort *vs* population subjects, and sample sizes. The majority of studies, however, report an increased risk for malformations in UC, but not in CD. Further prospective

Table 1 Food and Drug Administration categories of drug safety during pregnancy

FDA category	Definition
A	Controlled studies in animals and women have shown no risk in the first trimester, and possible foetal harm is remote
B	Either animal studies have not demonstrated a foetal risk but there are no controlled studies in pregnant women, or animal studies have shown an adverse effect that was not confirmed in controlled studies in women in the first trimester
C	No controlled studies in humans have been performed, and animal studies have shown adverse events, or studies in humans and animals not available; give if potential benefit outweighs the risk
D	Positive evidence of foetal risk is available, but the benefits may outweigh the risk if life-threatening or serious disease
X	Studies in animals or humans show foetal abnormalities; drug contraindicated

FDA: Food and Drug Administration.

studies adjusting for disease activity, medication and age of the mother are needed.

Medication during pregnancy

The risks and benefits of medication need to be considered on an individual patient level, since active disease poses a risk to the pregnancy[26-28]. The European Crohn's and Colitis Organisation (ECCO) guidelines state the risk of adverse pregnancy outcomes from active disease to be higher than the risk of using most IBD medications[2]. The United States Food and Drug Administration issued a categorisation of drugs safety in pregnancy (Table 1)[34].

Aminosalicylates: Sulfasalazine, mesalazine and balsalazide are category B medications, while olsalazine is category C. Initial case reports[35-37] suggesting sulphasalazine teratogenicity was refuted by a Danish cohort study demonstrating no adverse outcomes in 17 pregnant CD patients exposed to sulfasalazine[28]. Another study of 181 pregnant patients with IBD exposed to sulfasalazine reported lower rates of adverse outcomes than those expected in the general population[38]. Sulfasalazine impairs folate absorption, which is vital for neural tube development. Therefore folate supplementation is mandated[2].

Several studies have demonstrated the safety of mesalazine[39-41]. Robust data from a Danish population-based cohort and a prospective controlled trial of 165 pregnant women with mesalazine exposure reported no increased risk of congenital abnormalities[42,43]. It is unclear whether an excess of stillbirth and preterm birth in 88 pregnancies of women with CD on mesalazine reported in the population based study[43], occured as a result of IBD itself rather than the medication since this study incorporated 19 418 pregnancies of healthy women without any chronic illnesses and medication exposure as the control group. Thus, sulfasalazine and mesalazine are considered safe in pregnancy.

Antibiotics: Antibiotics play an important role in the management of perianal CD[2]. Metronidazole is a category B medication. In a population based case-control study using HCCSCA data (1980-1991), metronidazole exposure during the second or third trimester was associated with cleft defects[44]. Other studies including two meta-analyses and a prospective controlled study, however, found no increased risk of congenital abnormalities relating to metronidazole use[45,46]. Quinolones can induce congenital abnormalities in animals due to their accumulation in bone and cartilage[47]. In 2 human studies totalling over 250 patients without IBD, no increased risk of adverse pregnancy outcomes or foetal malformations was found[48,49].

Short-term antibiotic use in pregnancy appears safe in largely non-IBD cohorts. In IBD, however, exposure to antibiotics can be prolonged and the associated risks may therefore be higher[2]. A small series of IBD patients found no adverse pregnancy outcomes in 27 patients exposed to metronidazole and 18 to ciprofloxacin[50].

Tetracyclines (retardation of foetal skeletal development) and sulphonamides (interferes with folic acid metabolism) should both be avoided in pregnancy[2].

Corticosteroids: Prednisone and budesonide have category C ratings. Corticosteroids cross the placenta and some adverse data exist in humans. A meta-analysis of ten cohort and case-control studies totalling 50 845 patients without IBD found no increase in overall risk of major congenital abnormalities, but there was a significant risk of oral clefts[51]. In contrast a prospective study of 262 women found that neither the overall risk for malformations nor the risk for clefts was increased[52]. No human data exist on orally administered budesonide, but studies looking at women exposed to inhaled or intra-nasal budesonide have found it to be safe during pregnancy[53,54]. Due to oral budesonide's high first pass hepatic metabolism, significant foetal exposure is less likely.

Thiopurines: Mercaptopurine and its pro-drug azathioprine are category D due to previous links with spontaneous abortions[55,56]. Animal studies, using doses of 1.5-2.5 mg/kg, have not reported any adverse outcomes apart from low birth weights[57,58]. Transplant and rheumatology cohorts demonstrated the safety of thiopurines[59-61]. Most studies in pregnant IBD patients report no adverse outcomes[62,63], but a Danish study reported a increased pre-term birth, low birth weight and foetal abnormalities in a cohort of only 10 patients[64]. That study compared pregnancy outcomes in thiopurine exposed patients to those of the general population rather than non-exposed IBD patients. It is therefore unclear whether the increased risk detected in the study was due to medication or IBD itself. In contrast, a prospective Austrian abstract of 33 women[65] and a French study on 86 women show no increase in adverse outcomes[66]. Paternal thiopurine exposure within 3 mo prior to conception led to a higher

rate of pregnancy related complications in one study[67], but another study reported no increased risk of adverse outcomes[68].

Thus, studies comparing outcomes of thiopurine exposed and unexposed IBD patients demonstrate no association with adverse outcomes. The single study showing a possibly increased risk is small and compares exposed patients to the general population[64]. Based on the overall evidence and despite the category D classification, the ECCO guidelines therefore consider thiopurines safe and well tolerated in pregnancy[2].

Methotrexate: Methotrexate is rated category X as it is clearly teratogenic and an abortifacient due its biological action as a folate antagonist. It is associated with numerous foetal abnormalities and high risk of foetal mortality and absolutely contraindicated for women attempting pregnancy[69,70].

Other immunosuppressants: Data on cyclosporine and tacrolimus mainly stems from the transplant and rheumatology literature and both are rated category C[2].

Biological agents: Infliximab has been classed as category B. It does not cross the placenta during the first trimester and hence there is no exposure in this critical phase of development[71]. Infliximab crosses the placenta in later stages of pregnancy and may be present in the newborn for several weeks[71]. Safety data for infliximab are provided by three large scale studies. The Crohn's Therapy Resource, Evaluation and Assessment Tool registry-a prospective, North American observational multi-centre study-enrolled infliximab-exposed and unexposed CD patients from 1999 to 2004[72]. No differences in miscarriages and neonatal complications were found between infliximab-exposed (117 pregnancies) and unexposed (49 pregnancies) women[72]. The retrospective Infliximab Safety Database (maintained by Centocor) found no differences in adverse outcomes between 96 pregnancies in women with infliximab exposure compared to the general population[73]. The Leuven group in Belgium treated 29 women treated with infliximab during 35 pregnancies and six women with adalimumab during seven pregnancies. In comparison to IBD patients without infliximab or adalimumab exposure, no increased risk of adverse events was found[74].

Adalimumab is also classed as a category B drug and expected to have the same placental transfer as infliximab. Apart from the Leuven experience[74] there are few reports on IBD patients exposed to adalimumab during pregnancy[75,76]. The organisation for teratology specialists' registry of women with rheumatoid arthritis compared 34 adalimumab-exposed pregnancies to 52 pregnancies of healthy women and found no increased risk of adverse pregnancy outcomes[77]. Certolizumab and natalizumab are category B and C drugs respectively, but there is currently little data on their effects in pregnancy[2].

Infliximab use in pregnancy resulted in the death of a 3 mo old child with disseminated Bacillus Calmette-Guérin (BCG) after receiving the live BCG vaccination[78]. Live vaccinations are contra-indicated for immuno-compromised patients. Newborns to mothers exposed to infliximab and adalimumab mothers should have their vaccination postponed[78].

Mode of delivery

Patients with IBD (especially CD) were more likely to have a caesarean section in Cornish's meta-analysis of six studies (OR: 1.5, 95% CI: 1.26-1.79; $P < 0.001$) compared to the general population[29], but there was no difference in caesarean rates between UC and controls[29]. Concerns regarding the preservation of the anal sphincter function and the development of perianal disease after traumatic injuries occurring during vaginal delivery may partially explain this phenomenon. However, the chance of developing perianal disease in women with CD without prior perianal involvement is low; in a population based cohort study from Mannitoba, Canada only one of 27 women without prior perianal disease developed perianal disease after vaginal delivery and episiotomy[79]. Conversely, in a self-report survey of 179 women without perianal disease, 18% reported perianal involvement after delivery[80], but it is possible that selection bias, that is, more women with than without perianal disease may have responded, and recall bias may have occurred[80].

A population based cohort study from Mannitoba of 11 patients with inactive perianal disease and two single case reports from France and United States, revealed that inactive perianal disease may tolerate a vaginal delivery with episiotomy if needed without risking a flare[79,81,82]. However, women with active perianal disease should be advised to have a caesarean section as a high risk of deterioration is anticipated. Delivery trauma can lead to poor-healing in the perineum. ECCO guidelines advise that elective caesarean section is indicated for all women with perianal involvement[2] even though there is little evidence suggesting harm from a vaginal delivery in cases of inactive disease.

ECCO guidelines recommend mandatory caesarean sections after IPAA[2]. Changes in anal sphincter function are temporary and long-term disturbances seem to be independent of the mode of delivery in patients with IPAA[14,16,83]. The largest study of 232 females with pregnancies after IPAA found no difference in pouch-related complications between women undergoing vaginal delivery or caesarean section[84]. In another survey, functional pouch outcomes of 85 women with vaginal deliveries after IPAA were no different to those of 343 age matched women who did not have children after IPAA[85]. A Finish survey study of 39 women with IPAA found no differences in pouch function after 19 vaginal deliveries in comparison to 21 caesarean sections and the rate of 5 tears after vaginal delivery was similar to a healthy control group[86]. In a cohort of women investigated four years after IPAA at the Cleveland clinic, United States no clinical differences were demonstrated between 20 women

Table 2 Medication recommendation for pregnancy and breast feeding

Class	Drugs	FDA category	Pregnancy advice	Detection in breast milk
Aminosalicylates	Sulfasalazine, balsalazide, mesalazine	B	Safe to use (folate supplementation for sulfasalazine)	Low levels detectable
Aminosalicylates	Olsalazine	C	Safe to use	
Antibiotics	Metronidazole	B	Safe to use	
Antibiotics	Ciprofloxacin		Limited data; probably safe	
Corticosteroids	Prednisolone, budesonide	C	Safe to use	Detectable
Thiopurines	Azathioprine, 6-mercaptopurine	D	Safe to use	Very low levels detectable
Folate antagonist	Methotrexate	X	Absolutely contraindicated	
Biological agent	Infliximab	B	Probably safe (avoid during 3rd trimester)	Not detectable
Biological agent	Adalimumab	B	Probably safe (avoid during 3rd trimester)	Very low levels detectable

FDA: Food and Drug Administration.

with at least one vaginal delivery compared to 62 women who only had caesarean sections[87]. Subclinical differences in anorectal physiology were however demonstrated as women with vaginal deliveries had significantly lower squeeze pressure on anorectal manometry and significantly more anal sphincter defects detected by anorectal sonography than those women with caesarean sections[87].

The ECCO guidelines state that women are at borderline incontinence after IPAA surgery and that any further disruption by a vaginal delivery might compromise this[2]. This theoretical concern is supported by sonographic and manometric evidence of sphincter dysfunction, but this does not translate to clinical differences in pouch function or quality of life. Precautionary caesarean sections are however recommended[2].

Thus, mandatory recommendation for caesarean sections in IBD is only for very specific indications and decisions should be made on a case by case basis.

Breast feeding

Breast milk provides ideal nutrition and has positive effects for the immune system of the newborn[88]. Data on the protective effect of breastfeeding against the development of IBD report are conflicting. Study design, recall bias, definition of breast feeding (especially duration) and the design bias often inherent in retrospective case-control studies may explain some of the differences[89]. A meta-analysis of 17 studies found reduced ORs of 0.67 (95% CI: 0.52-0.86) for CD and 0.77 (95% CI: 0.61-0.96) for UC. The relevance of a French case-control study reporting an increased risk (OR: 2.1, 95% CI: 1.3-3.4) of developing CD[90] remains unclear, in light of previous studies showing no effect or a protective effect[89,91].

Fewer patients with IBD breastfeed compared to the general population, and this may relate to fears about adverse effects of maternal medication[92]. Most maternal medications can be detected in breast milk, but this does not always lead to biological effects in the infant.

Based on two case reports of bloody diarrhoea in newborns breastfed by mothers taking sulfasalazine and 5-aminosalicylates respectively, the American Academy of Pediatrics advises against breastfeeding by mothers taking these medications[93-95]. In contrast, several studies demonstrate the safety of sulfasalazine and 5-aminosalicylates while breastfeeding by detecting low levels of drug in breast milk or the infant's serum[39,96,97]. Based on above evidence mothers on sulfasalazine and 5-aminosalicylates should not be discouraged from breastfeeding unless the infant develops diarrhoea.

Since corticosteroids are found in human breast milk in low concentrations, women are advised to avoid feeding within 4 h of taking an oral dose to reduce exposure[2,98]. Thiopurine and associated metabolite levels are either at undetectable, or extremely low levels, in human breast milk[99-101], while infants' levels were undetectable[102,103]. Infliximab can not be detected in infants of breastfeeding mothers[104], but adalimumab has been found in miniscule concentrations in breast milk in a single case[105]. Methotrexate should be avoided as it is found in breast milk[106]. Sulfasalazine, 5-aminosalicylates, steroids and thiopurines are all considered advisable during breast feeding[2] (Table 2).

Women's beliefs and attitudes

Few studies have evaluated the perspectives of patients with IBD on fertility, pregnancy, breast feeding and pregnancy outcomes. Women with IBD may have fewer children as more stay "voluntarily childless"[107]. A survey of Crohn's and Colitis Foundation of America members reported that 18% of females with CD and 14% of females with UC decided to stay childless compared to 6% of the general population ($P = 0.001$ for CD, $P = 0.08$ for UC). Notably, the decision to stay childless amongst IBD patients contrasts to the much lower "involuntary" childlessness rates (inability to conceive) (5% in CD, 1.7% in UC and 2.5% in the general population, P = NS)[107]. Concerns about the effect of pregnancy on their disease, about passing on IBD to their children and about their ability to look after children were given as the main reasons for voluntarily childlessness[107]. IBD patients have expressed a high interest in genetic testing to determine their future health or that of their family members[108], but there are no data whether women with IBD would base decisions to have children on the results of genetic testing should a reliable test become available.

Data from an Australian IBD cohort were recently described by Mountifield *et al*[109,110] in two studies. The first postal questionnaire study evaluated the experience and views regarding fertility in 255 women with IBD and

found live birth rates of 1.0 for CD, 1.2 for UC, which are considerably lower than those of the general population of 1.8[109]. This coincided with a fear of infertility in 42.7% of patients, which was particularly apparent in women with CD or previous surgery[109]. Unfortunately, the study did not report whether women fearing infertility were indeed experiencing problems conceiving. Conversely, women sought fertility advice only as often as the general population[109]. The study highlights a difference between the medical evidence and patients' perception of infertility as patients overestimate their risk of infertility largely. The second study examined the experiences and views of 219 women after pregnancy[110]. The patients' main concern related to potential harm towards their pregnancy from IBD medication (84%) rather than the need to control active disease[110]. "Free text" responses suggested that women would rather "put up with the disease than harm my baby with medications". The main concerns regarding side effects of IBD medication related to congenital abnormalities rather than more common adverse outcomes of pregnancy such as pre-mature delivery or low birth weight[110]. Of note, some patients considered steroid "rescue" therapy safer than continuation of IBD maintenance medication[110]. The authors suggested their findings reflected a lack of patients' knowledge in the field of IBD and pregnancy'[110]. The study provides insight into patients' views: many opinions were contrary to current medical evidence, and women may make decisions based on incorrect perceptions.

Two population based Danish studies have examined adherence to IBD medication prior and during pregnancy in 58 women with CD and 63 women with UC. Adherence, measured by retrospective self-reporting, was relatively high in both CD (72%)[111] and UC (60%)[112] as adherence in the non-pregnant IBD population ranges from 55%-70%[113]. It is, however, difficult to interpret the study findings since there was no direct control group of non-pregnant patients included and selection bias (responders *vs* non-responders) and recall bias (the questionnaires were sent years after the pregnancies) may be likely. Furthermore, adherence was assessed using a simple question rather than a validated tool. Reasons for non-adherence were quiescent disease (59%) and a fear of negative effects on the unborn child (50%), while forgetfulness was uncommon (5%)[112]. These studies highlight that unwanted effects of medication play an important part in women's views and influence decision making.

CONCLUSION

IBD affects women of childbearing age and may have an effect on their offspring. Fertility is reduced in active CD and after surgery. The risk of active disease during pregnancy carries a significant risk to baby and mother. The limited data available on patients' beliefs and attitudes suggest that many women hold views contrary to medical evidence. Women with IBD are therefore at risk of making uninformed choices that could in turn lead to adverse outcomes. There is a need for further studies examining women's views in more detail and to investigate whether these beliefs are driven by a lack of knowledge. In the meantime, women with IBD should receive advice and counselling by their physician prior and during pregnancy.

REFERENCES

1 **Carter MJ**, Lobo AJ, Travis SP. Guidelines for the management of inflammatory bowel disease in adults. *Gut* 2004; **53** Suppl 5: V1-16

2 **Van Assche G**, Dignass A, Reinisch W, van der Woude CJ, Sturm A, De Vos M, Guslandi M, Oldenburg B, Dotan I, Marteau P, Ardizzone A, Baumgart DC, D'Haens G, Gionchetti P, Portela F, Vucelic B, Söderholm J, Escher J, Koletzko S, Kolho KL, Lukas M, Mottet C, Tilg H, Vermeire S, Carbonnel F, Cole A, Novacek G, Reinshagen M, Tsianos E, Herrlinger K, Oldenburg B, Bouhnik Y, Kiesslich R, Stange E, Travis S, Lindsay J. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Special situations. *J Crohns Colitis* 2010; **4**: 63-101

3 **Dubinsky M**, Abraham B, Mahadevan U. Management of the pregnant IBD patient. *Inflamm Bowel Dis* 2008; **14**: 1736-1750

4 **Baird DD**, Narendranathan M, Sandler RS. Increased risk of preterm birth for women with inflammatory bowel disease. *Gastroenterology* 1990; **99**: 987-994

5 **Hudson M**, Flett G, Sinclair TS, Brunt PW, Templeton A, Mowat NA. Fertility and pregnancy in inflammatory bowel disease. *Int J Gynaecol Obstet* 1997; **58**: 229-237

6 **Olsen KO**, Joelsson M, Laurberg S, Oresland T. Fertility after ileal pouch-anal anastomosis in women with ulcerative colitis. *Br J Surg* 1999; **86**: 493-495

7 **Ørding Olsen K**, Juul S, Berndtsson I, Oresland T, Laurberg S. Ulcerative colitis: female fecundity before diagnosis, during disease, and after surgery compared with a population sample. *Gastroenterology* 2002; **122**: 15-19

8 **Khosla R**, Willoughby CP, Jewell DP. Crohn's disease and pregnancy. *Gut* 1984; **25**: 52-56

9 **Woolfson K**, Cohen Z, McLeod RS. Crohn's disease and pregnancy. *Dis Colon Rectum* 1990; **33**: 869-873

10 **Fonager K**, Sørensen HT, Olsen J, Dahlerup JF, Rasmussen SN. Pregnancy outcome for women with Crohn's disease: a follow-up study based on linkage between national registries. *Am J Gastroenterol* 1998; **93**: 2426-2430

11 **Baiocco PJ**, Korelitz BI. The influence of inflammatory bowel disease and its treatment on pregnancy and fetal outcome. *J Clin Gastroenterol* 1984; **6**: 211-216

12 **Mayberry JF**, Weterman IT. European survey of fertility and pregnancy in women with Crohn's disease: a case control study by European collaborative group. *Gut* 1986; **27**: 821-825

13 **Oresland T**, Palmblad S, Ellström M, Berndtsson I, Crona N, Hultén L. Gynaecological and sexual function related to anatomical changes in the female pelvis after restorative proctocolectomy. *Int J Colorectal Dis* 1994; **9**: 77-81

14 **Ravid A**, Richard CS, Spencer LM, O'Connor BI, Kennedy ED, MacRae HM, Cohen Z, McLeod RS. Pregnancy, delivery, and pouch function after ileal pouch-anal anastomosis for ulcerative colitis. *Dis Colon Rectum* 2002; **45**: 1283-1288

15 **Tiainen J**, Matikainen M, Hiltunen KM. Ileal J-pouch--anal anastomosis, sexual dysfunction, and fertility. *Scand J Gastroenterol* 1999; **34**: 185-188

16 **Juhasz ES**, Fozard B, Dozois RR, Ilstrup DM, Nelson H. Ileal pouch-anal anastomosis function following childbirth. An extended evaluation. *Dis Colon Rectum* 1995; **38**: 159-165

17 **Damgaard B**, Wettergren A, Kirkegaard P. Social and sexual function following ileal pouch-anal anastomosis. *Dis Colon Rectum* 1995; **38**: 286-289

18 **Johnson E**, Carlsen E, Nazir M, Nygaard K. Morbidity and functional outcome after restorative proctocolectomy for ulcerative colitis. *Eur J Surg* 2001; **167**: 40-45

19 **Nielsen OH**, Andreasson B, Bondesen S, Jarnum S. Pregnancy in ulcerative colitis. *Scand J Gastroenterol* 1983; **18**: 735-742
20 **Nielsen OH**, Andreasson B, Bondesen S, Jacobsen O, Jarnum S. Pregnancy in Crohn's disease. *Scand J Gastroenterol* 1984; **19**: 724-732
21 **Mahadevan U**, Sandborn WJ, Li DK, Hakimian S, Kane S, Corley DA. Pregnancy outcomes in women with inflammatory bowel disease: a large community-based study from Northern California. *Gastroenterology* 2007; **133**: 1106-1112
22 **Castiglione F**, Pignata S, Morace F, Sarubbi A, Baratta MA, D'Agostino L, D'Arienzo A, Mazzacca G. Effect of pregnancy on the clinical course of a cohort of women with inflammatory bowel disease. *Ital J Gastroenterol* 1996; **28**: 199-204
23 **Riis L**, Vind I, Politi P, Wolters F, Vermeire S, Tsianos E, Freitas J, Mouzas I, Ruiz Ochoa V, O'Morain C, Odes S, Binder V, Moum B, Stockbrügger R, Langholz E, Munkholm P. Does pregnancy change the disease course? A study in a European cohort of patients with inflammatory bowel disease. *Am J Gastroenterol* 2006; **101**: 1539-1545
24 **Kane S**, Kisiel J, Shih L, Hanauer S. HLA disparity determines disease activity through pregnancy in women with inflammatory bowel disease. *Am J Gastroenterol* 2004; **99**: 1523-1526
25 **Morales M**, Berney T, Jenny A, Morel P, Extermann P. Crohn's disease as a risk factor for the outcome of pregnancy. *Hepatogastroenterology* 2000; **47**: 1595-1598
26 **Bush MC**, Patel S, Lapinski RH, Stone JL. Perinatal outcomes in inflammatory bowel disease. *J Matern Fetal Neonatal Med* 2004; **15**: 237-241
27 **Fedorkow DM**, Persaud D, Nimrod CA. Inflammatory bowel disease: a controlled study of late pregnancy outcome. *Am J Obstet Gynecol* 1989; **160**: 998-1001
28 **Nørgård B**, Hundborg HH, Jacobsen BA, Nielsen GL, Fonager K. Disease activity in pregnant women with Crohn's disease and birth outcomes: a regional Danish cohort study. *Am J Gastroenterol* 2007; **102**: 1947-1954
29 **Cornish J**, Tan E, Teare J, Teoh TG, Rai R, Clark SK, Tekkis PP. A meta-analysis on the influence of inflammatory bowel disease on pregnancy. *Gut* 2007; **56**: 830-837
30 **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
31 **Bortoli A**, Saibeni S, Tatarella M, Prada A, Beretta L, Rivolta R, Politi P, Ravelli P, Imperiali G, Colombo E, Pera A, Daperno M, Carnovali M, de Franchis R, Vecchi M. Pregnancy before and after the diagnosis of inflammatory bowel diseases: retrospective case-control study. *J Gastroenterol Hepatol* 2007; **22**: 542-549
32 **Arbour LT**, Beking K, Le ND, Ratner PA, Spinelli JJ, Teschke K, Gallagher RP, Abanto ZU, Dimich-Ward H. Rates of congenital anomalies and other adverse birth outcomes in an offspring cohort of registered nurses from British Columbia, Canada. *Can J Public Health* 2010; **101**: 230-234
33 **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
34 Administration FDA. *Regulations* 1980; **44:** 37434-37467
35 **Craxi A**, Pagliarello F. Possible embryotoxicity of sulfasalazine. *Arch Intern Med* 1980; **140**: 1674
36 **Hoo JJ**, Hadro TA, Von Behren P. Possible teratogenicity of sulfasalazine. *N Engl J Med* 1988; **318**: 1128
37 **Newman NM**, Correy JF. Possible teratogenicity of sulphasalazine. *Med J Aust* 1983; **1**: 528-529
38 **Mogadam M**, Dobbins WO, Korelitz BI, Ahmed SW. Pregnancy in inflammatory bowel disease: effect of sulfasalazine and corticosteroids on fetal outcome. *Gastroenterology* 1981; **80**: 72-76
39 **Habal FM**, Hui G, Greenberg GR. Oral 5-aminosalicylic acid for inflammatory bowel disease in pregnancy: safety and clinical course. *Gastroenterology* 1993; **105**: 1057-1060
40 **Marteau P**, Tennenbaum R, Elefant E, Lémann M, Cosnes J. Foetal outcome in women with inflammatory bowel disease treated during pregnancy with oral mesalazine microgranules. *Aliment Pharmacol Ther* 1998; **12**: 1101-1108
41 **Trallori G**, d'Albasio G, Bardazzi G, Bonanomi AG, Amorosi A, Del Carlo P, Palli D, Galli M, Pacini F. 5-Aminosalicylic acid in pregnancy: clinical report. *Ital J Gastroenterol* 1994; **26**: 75-78
42 **Diav-Citrin O**, Park YH, Veerasuntharam G, Polachek H, Bologa M, Pastuszak A, Koren G. The safety of mesalamine in human pregnancy: a prospective controlled cohort study. *Gastroenterology* 1998; **114**: 23-28
43 **Nørgård B**, Fonager K, Pedersen L, Jacobsen BA, Sørensen HT. Birth outcome in women exposed to 5-aminosalicylic acid during pregnancy: a Danish cohort study. *Gut* 2003; **52**: 243-247
44 **Czeizel AE**, Rockenbauer M. A population based case-control teratologic study of oral metronidazole treatment during pregnancy. *Br J Obstet Gynaecol* 1998; **105**: 322-327
45 **Burtin P**, Taddio A, Ariburnu O, Einarson TR, Koren G. Safety of metronidazole in pregnancy: a meta-analysis. *Am J Obstet Gynecol* 1995; **172**: 525-529
46 **Caro-Patón T**, Carvajal A, Martin de Diego I, Martin-Arias LH, Alvarez Requejo A, Rodríguez Pinilla E. Is metronidazole teratogenic? A meta-analysis. *Br J Clin Pharmacol* 1997; **44**: 179-182
47 **Niebyl JR**. Antibiotics and other anti-infective agents in pregnancy and lactation. *Am J Perinatol* 2003; **20**: 405-414
48 **Loebstein R**, Addis A, Ho E, Andreou R, Sage S, Donnenfeld AE, Schick B, Bonati M, Moretti M, Lalkin A, Pastuszak A, Koren G. Pregnancy outcome following gestational exposure to fluoroquinolones: a multicenter prospective controlled study. *Antimicrob Agents Chemother* 1998; **42**: 1336-1339
49 **Larsen H**, Nielsen GL, Schønheyder HC, Olesen C, Sørensen HT. Birth outcome following maternal use of fluoroquinolones. *Int J Antimicrob Agents* 2001; **18**: 259-262
50 **Moskovitz DN**, Bodian C, Chapman ML, Marion JF, Rubin PH, Scherl E, Present DH. The effect on the fetus of medications used to treat pregnant inflammatory bowel-disease patients. *Am J Gastroenterol* 2004; **99**: 656-661
51 **Park-Wyllie L**, Mazzotta P, Pastuszak A, Moretti ME, Beique L, Hunnisett L, Friesen MH, Jacobson S, Kasapinovic S, Chang D, Diav-Citrin O, Chitayat D, Nulman I, Einarson TR, Koren G. Birth defects after maternal exposure to corticosteroids: prospective cohort study and meta-analysis of epidemiological studies. *Teratology* 2000; **62**: 385-392
52 **Gur C**, Diav-Citrin O, Shechtman S, Arnon J, Ornoy A. Pregnancy outcome after first trimester exposure to corticosteroids: a prospective controlled study. *Reprod Toxicol* 2004; **18**: 93-101
53 **Gluck PA**, Gluck JC. A review of pregnancy outcomes after exposure to orally inhaled or intranasal budesonide. *Curr Med Res Opin* 2005; **21**: 1075-1084
54 **Norjavaara E**, de Verdier MG. Normal pregnancy outcomes in a population-based study including 2,968 pregnant women exposed to budesonide. *J Allergy Clin Immunol* 2003; **111**: 736-742
55 **Blatt J**, Mulvihill JJ, Ziegler JL, Young RC, Poplack DG. Pregnancy outcome following cancer chemotherapy. *Am J Med* 1980; **69**: 828-832
56 **Nicholson HO**. Cytotoxic drugs in pregnancy. Review of reported cases. *J Obstet Gynaecol Br Commonw* 1968; **75**: 307-312
57 **Platzek T**, Bochert G. Dose-response relationship of teratogenicity and prenatal-toxic risk estimation of 6-mercaptopurine riboside in mice. *Teratog Carcinog Mutagen* 1996; **16**: 169-181
58 **Mosesso P**, Palitti F. The genetic toxicology of 6-mercaptopurine. *Mutat Res* 1993; **296**: 279-294
59 **Bermas BL**, Hill JA. Effects of immunosuppressive drugs during pregnancy. *Arthritis Rheum* 1995; **38**: 1722-1732

60 **Roubenoff R**, Hoyt J, Petri M, Hochberg MC, Hellmann DB. Effects of antiinflammatory and immunosuppressive drugs on pregnancy and fertility. *Semin Arthritis Rheum* 1988; **18**: 88-110

61 **Willis FR**, Findlay CA, Gorrie MJ, Watson MA, Wilkinson AG, Beattie TJ. Children of renal transplant recipient mothers. *J Paediatr Child Health* 2000; **36**: 230-235

62 **Alstead EM**, Ritchie JK, Lennard-Jones JE, Farthing MJ, Clark ML. Safety of azathioprine in pregnancy in inflammatory bowel disease. *Gastroenterology* 1990; **99**: 443-446

63 **Francella A**, Dyan A, Bodian C, Rubin P, Chapman M, Present DH. The safety of 6-mercaptopurine for childbearing patients with inflammatory bowel disease: a retrospective cohort study. *Gastroenterology* 2003; **124**: 9-17

64 **Nørgård B**, Pedersen L, Fonager K, Rasmussen SN, Sørensen HT. Azathioprine, mercaptopurine and birth outcome: a population-based cohort study. *Aliment Pharmacol Ther* 2003; **17**: 827-834

65 **Dejaco C,** Angelberger S, Waldhoer T. Pregnancy and birth outcomes under thiopurine therapy for inflammatory bowel disease. *Gastroenterology* 2005; **128** (Suppl 2): A-12

66 **Coelho J**, Beaugerie L, Colombel JF, Hébuterne X, Lerebours E, Lémann M, Baumer P, Cosnes J, Bourreille A, Gendre JP, Seksik P, Blain A, Metman EH, Nisard A, Cadiot G, Veyrac M, Coffin B, Dray X, Carrat F, Marteau P. Pregnancy outcome in patients with inflammatory bowel disease treated with thiopurines: cohort from the CESAME Study. *Gut* 2011; **60**: 198-203

67 **Rajapakse RO**, Korelitz BI, Zlatanic J, Baiocco PJ, Gleim GW. Outcome of pregnancies when fathers are treated with 6-mercaptopurine for inflammatory bowel disease. *Am J Gastroenterol* 2000; **95**: 684-688

68 **Teruel C**, López-San Román A, Bermejo F, Taxonera C, Pérez-Calle JL, Gisbert JP, Martín-Arranz M, Ponferrada A, Van Domselaar M, Algaba A, Estellés J, López-Serrano P, Linares PM, Muriel A. Outcomes of pregnancies fathered by inflammatory bowel disease patients exposed to thiopurines. *Am J Gastroenterol* 2010; **105**: 2003-2008

69 **Briggs GG,** Freeman RK, Yaffe SJ. Drugs in pregnancy and lactation for PDA: A reference guide to fetal and neonatal risk. Philadelphia: Lippincott, Williams & Wilkins, 2005

70 **Del Campo M**, Kosaki K, Bennett FC, Jones KL. Developmental delay in fetal aminopterin/methotrexate syndrome. *Teratology* 1999; **60**: 10-12

71 **Simister NE**. Placental transport of immunoglobulin G. *Vaccine* 2003; **21**: 3365-3369

72 **Lichtenstein GR**, Feagan BG, Cohen RD, Salzberg BA, Diamond RH, Chen DM, Pritchard ML, Sandborn WJ. Serious infections and mortality in association with therapies for Crohn 's disease: TREAT registry. *Clin Gastroenterol Hepatol* 2006; **4**: 621-630

73 **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

74 **Schnitzler F,** Fidder 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

75 **Coburn LA,** Schwartz DA. The successful use of Adalimumab to treat active Crohn's disease of an ioleanal pouch during pregnancy. *Dig Dis Scis* 2005; **51:** 2045-2047

76 **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

77 Johnson DL, Jones KL, Chambers CD, Salas E. Pregnancy outcoms in women exposed to adalimumab: the OTIS autoimmune diseases in pregnancy project. *Gastroenterology* 2009; **136** (Suppl 1): A-27

78 **Cheent K**, Nolan J, Shariq S, Kiho L, Pal A, Arnold J. Case Report: Fatal case of disseminated BCG infection in an infant born to a mother taking infliximab for Crohn's disease. *J Crohns Colitis* 2010; **4**: 603-605

79 **Ilnyckyji A**, Blanchard JF, Rawsthorne P, Bernstein CN. Perianal Crohn's disease and pregnancy: role of the mode of delivery. *Am J Gastroenterol* 1999; **94**: 3274-3278

80 **Brandt LJ**, Estabrook SG, Reinus JF. Results of a survey to evaluate whether vaginal delivery and episiotomy lead to perineal involvement in women with Crohn's disease. *Am J Gastroenterol* 1995; **90**: 1918-1922

81 **Beniada A**, Benoist G, Maurel J, Dreyfus M. [Inflammatory bowel disease and pregnancy: report of 76 cases and review of the literature]. *J Gynecol Obstet Biol Reprod* (Paris) 2005; **34**: 581-588

82 **Rogers RG**, Katz VL. Course of Crohn's disease during pregnancy and its effect on pregnancy outcome: a retrospective review. *Am J Perinatol* 1995; **12**: 262-264

83 **Kitayama T**, Funayama Y, Fukushima K, Shibata C, Takahashi K, Ogawa H, Ueno T, Hashimoto A, Sasaki I. Anal function during pregnancy and postpartum after ileal pouch anal anastomosis for ulcerative colitis. *Surg Today* 2005; **35**: 211-215

84 **Hahnloser D**, Pemberton JH, Wolff BG, Larson D, Harrington J, Farouk R, Dozois RR. Pregnancy and delivery before and after ileal pouch-anal anastomosis for inflammatory bowel disease: immediate and long-term consequences and outcomes. *Dis Colon Rectum* 2004; **47**: 1127-1135

85 **Farouk R**, Pemberton JH, Wolff BG, Dozois RR, Browning S, Larson D. Functional outcomes after ileal pouch-anal anastomosis for chronic ulcerative colitis. *Ann Surg* 2000; **231**: 919-926

86 **Lepistö A**, Sarna S, Tiitinen A, Järvinen HJ. Female fertility and childbirth after ileal pouch-anal anastomosis for ulcerative colitis. *Br J Surg* 2007; **94**: 478-482

87 **Remzi FH**, Gorgun E, Bast J, Schroeder T, Hammel J, Philipson E, Hull TL, Church JM, Fazio VW. Vaginal delivery after ileal pouch-anal anastomosis: a word of caution. *Dis Colon Rectum* 2005; **48**: 1691-1699

88 **Jackson KM**, Nazar AM. Breastfeeding, the immune response, and long-term health. *J Am Osteopath Assoc* 2006; **106**: 203-207

89 **Klement E**, Cohen RV, Boxman J, Joseph A, Reif S. Breastfeeding and risk of inflammatory bowel disease: a systematic review with meta-analysis. *Am J Clin Nutr* 2004; **80**: 1342-1352

90 **Jantchou P**, Turck D, Baldé M, Gower-Rousseau C. Breastfeeding and risk of inflammatory bowel disease: results of a pediatric, population-based, case-control study. *Am J Clin Nutr* 2005; **82**: 485-486

91 **Gearry RB**, Richardson AK, Frampton CM, Dodgshun AJ, Barclay ML. Population-based cases control study of inflammatory bowel disease risk factors. *J Gastroenterol Hepatol* 2010; **25**: 325-333

92 **Kane S**, Lemieux N. The role of breastfeeding in postpartum disease activity in women with inflammatory bowel disease. *Am J Gastroenterol* 2005; **100**: 102-105

93 **Branski D**, Kerem E, Gross-Kieselstein E, Hurvitz H, Litt R, Abrahamov A. Bloody diarrhea--a possible complication of sulfasalazine transferred through human breast milk. *J Pediatr Gastroenterol Nutr* 1986; **5**: 316-317

94 **Nelis GF**. Diarrhoea due to 5-aminosalicylic acid in breast milk. *Lancet* 1989; **1**: 383

95 **American Academy of Pediatrics Committee on Drugs**. Transfer of drugs and other chemicals into human milk. *Pediatrics* 2001; **108**: 776-789

96 **Esbjörner E**, Järnerot G, Wranne L. Sulphasalazine and sulphapyridine serum levels in children to mothers treated with sulphasalazine during pregnancy and lactation. *Acta Paediatr Scand* 1987; **76**: 137-142

97 **Silverman DA**, Ford J, Shaw I, Probert CS. Is mesalazine real-

ly safe for use in breastfeeding mothers? *Gut* 2005; **54**: 170-171
98 **Ost L**, Wettrell G, Björkhem I, Rane A. Prednisolone excretion in human milk. *J Pediatr* 1985; **106**: 1008-1011
99 **Coulam CB**, Moyer TP, Jiang NS, Zincke H. Breast-feeding after renal transplantation. *Transplant Proc* 1982; **14**: 605-609
100 **Christensen LA**, Dahlerup JF, Nielsen MJ, Fallingborg JF, Schmiegelow K. Azathioprine treatment during lactation. *Aliment Pharmacol Ther* 2008; **28**: 1209-1213
101 **Moretti ME**, Verjee Z, Ito S, Koren G. Breast-feeding during maternal use of azathioprine. *Ann Pharmacother* 2006; **40**: 2269-2272
102 **Gardiner SJ**, Gearry RB, Roberts RL, Zhang M, Barclay ML, Begg EJ. Exposure to thiopurine drugs through breast milk is low based on metabolite concentrations in mother-infant pairs. *Br J Clin Pharmacol* 2006; **62**: 453-456
103 **Sau A**, Clarke S, Bass J, Kaiser A, Marinaki A, Nelson-Piercy C. Azathioprine and breastfeeding: is it safe? *BJOG* 2007; **114**: 498-501
104 **Kane S**, Ford J, Cohen R, Wagner C. Absence of infliximab in infants and breast milk from nursing mothers receiving therapy for Crohn's disease before and after delivery. *J Clin Gastroenterol* 2009; **43**: 613-616
105 **Ben-Horin S**, Yavzori M, Katz L, Picard O, Fudim E, Chowers Y, Lang A. Adalimumab level in breast milk of a nursing mother. *Clin Gastroenterol Hepatol* 2010; **8**: 475-476
106 **Johns DG**, Rutherford LD, Leighton PC, Vogel CL. Secretion of methotrexate into human milk. *Am J Obstet Gynecol* 1972; **112**: 978-980
107 **Marri SR**, Ahn C, Buchman AL. Voluntary childlessness is increased in women with inflammatory bowel disease. *Inflamm Bowel Dis* 2007; **13**: 591-599
108 **Lal S**, Appelton J, Mascarenhas J, Stempak JM, Esplen MJ, Silverberg MS. Attitudes toward genetic testing in patients with inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 2007; **19**: 321-327
109 **Mountifield RE**, Bampton P, Prosser R, Muller K, Andrews JM. Fear and fertility in inflammatory bowel disease: a mismatch of perception and reality affects family planning decisions. *Inflamm Bowel Dis* 2009; **15**: 720-725
110 **Mountifield RE**, Prosser R, Bampton P, Muller K, Andrews JM. Pregnancy and IBD treatment: this challenging interplay from a patients' perspective. *J Crohns Colitis* 2010; **4**: 176-182
111 **Nielsen MJ**, Nørgaard M, Holland-Fisher P, Christensen LA. Self-reported antenatal adherence to medical treatment among pregnant women with Crohn's disease. *Aliment Pharmacol Ther* 2010; **32**: 49-58
112 **Julsgaard M,** Nørgaard M, Hvas CL, Buck D, Christensen LA. Self-reported adherence to medical treatment prior to and during pregnancy among women with ulcerative colitis. *Inflamm Bowel Dis* 2010; **17:** 1573-1580
113 **Jackson CA**, Clatworthy J, Robinson A, Horne R. Factors associated with non-adherence to oral medication for inflammatory bowel disease: a systematic review. *Am J Gastroenterol* 2010; **105**: 525-539

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REVIEW

Leaky gut and the liver: A role for bacterial translocation in nonalcoholic steatohepatitis

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Abstract

Gut flora and bacterial translocation (BT) play important roles in the pathogenesis of chronic liver disease, including cirrhosis and its complications. Intestinal bacterial overgrowth and increased bacterial translocation of gut flora from the intestinal lumen predispose patients to bacterial infections, major complications and also play a role in the pathogenesis of chronic liver disorders. Levels of bacterial lipopolysaccharide, a component of gram-negative bacteria, are increased in the portal and/or systemic circulation in several types of chronic liver disease. Impaired gut epithelial integrity due to alterations in tight junction proteins may be the pathological mechanism underlying bacterial translocation. Preclinical and clinical studies over the last decade have suggested a role for BT in the pathogenesis of nonalcoholic steatohepatitis (NASH). Bacterial overgrowth, immune dysfunction, alteration of the luminal factors, and altered intestinal permeability are all involved in the pathogenesis of NASH and its complications. A better understanding of the cell-specific recognition and intracellular signaling events involved in sensing gut-derived microbes will help in the development of means to achieve an optimal balance in the gut-liver axis and ameliorate liver diseases. These may suggest new targets for potential therapeutic interventions for the treatment of NASH. Here, we review some of the mechanisms connecting BT and NASH and potential therapeutic developments.

Key words: Nonalcoholic steatohepatitis; Bacterial translocation; Insulin resistance; Leaky gut; Lipopolysaccharide

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INTRODUCTION

Bacterial translocation (BT) and the derangement of gut flora are of substantial clinical relevance to patients with chronic liver disease and cirrhosis[1,2]. Intestinal bacterial overgrowth and increased bacterial translocation of gut flora from the intestinal lumen predispose patients to bacterial infections and major complications[3,4]. Furthermore, levels of bacterial lipopolysaccharide (LPS), a component of gram-negative bacteria, are increased in the portal and/or systemic circulation in several types of chronic liver disease. Bauer *et al*[5-7] have demonstrated this phenomenon in cirrhosis. Impaired gut epithelial integrity due to alterations in tight junction proteins may be the pathological mechanism underlying bacterial trans-

location. Over the last decade, increased gut permeability and increased LPS levels have been described in patients with alcoholic and nonalcoholic steatohepatitis (NASH)[8,9]. Increased serum LPS levels and activation of proinflammatory signaling cascades have been suggested to be important for disease progression in these settings[10]. Some potential mechanisms to explain the association between BT and liver disease associated with lipid accumulation and the development of NASH are reviewed here. These mechanisms may suggest new targets for potential therapeutic interventions for the treatment of NASH.

BACTERIAL TRANSLOCATION, THE INNATE IMMUNE SYSTEM AND TOLL-LIKE RECEPTORS PLAY A ROLE IN THE PATHOGENESIS OF LIVER DAMAGE

Inflammation is involved in the pathogenesis of chronic liver diseases and plays a role in the development of progressive hepatic damage and fibrosis[11]. Liver inflammation and chronic damage are mediated by innate immune responses that are regulated by toll-like receptors (TLRs)[12]. Innate immune cells can both initiate and maintain inflammation in the liver. Bacteria translocated from the gut activate lymphocytes after interacting at the mesenteric lymph nodes (MLNs)[13].

Innate immune cells, particularly dendritic cells, play a pivotal role in sensing pathogens and initiating adaptive immune responses through the activation and regulation of T lymphocyte responses[11]. The immune system is abnormally activated in patients and experimental models with cirrhosis and ascites[14-16]. In an animal model of cirrhosis, systemic activation of the immune system occurs before ascites develop and is driven by the recirculation of cells activated in hepatic lymph nodes (HLNs)[13]. In compensated cirrhosis, bacterial DNA fragments reach the MLNs, where they elicit a local inflammatory response. Bacterial DNA fragments were present in the MLNs of 54% of rats with cirrhosis, indicating their potential role in systemic inflammation[13]. BT initiates a Th1 immune response in MLNs, leading to T-helper 1 (Th1) polarization and the production of tumor necrosis factor (TNF)-α by monocytes. The recirculation of these activated effector immune cells into the blood promotes systemic inflammation[16]. A systemic inflammatory state with increased circulating TNF-α has been linked to increased susceptibility to bacterial infections and hemodynamic dysfunctionin patients with cirrhosis[15-18].

The liver provides a tolerogenic immune environment for antigen-specific T cells. The liver is a source for activated immune cells present in the blood. The activation of Kupffer cells, recruited macrophages, and inflammatory cells results in the production of cytokines and chemokines that lead to prolonged inflammation and hepatocyte damage[11]. A direct correlation between activated cells in the blood and HLNs, but not in MLNs, supports this concept, as does the fact that the changes in activated cells in the MLNs, but not in the blood or HLNs, can be reversed by gut decontamination with antibiotics[13]. Th1 cells and monocytes were expanded and activated to produce intracellular interferon (IFN)-γ and TNF-α in the MLNs of cirrhotic rats[18]. Abrogation of bacterial translocation by bowel decontamination reduced the number of activated Th1 cells and monocytes and normalized IFN-γ and TNF-α production by monocytes in the MLNs and blood[16,19].

TLRs and TLR ligands play roles in the pathophysiology of liver fibrosis[20,21] and cirrhosis, viral hepatitis, ALD[22,23], nonalcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma[24]. TLRs recognize pathogen-associated molecular patterns (PAMPs) to detect the presence of pathogens[24]. TLRs are expressed on immune cells, Kupffer cells, endothelial cells, dendritic cells, biliary epithelial cells, HSCs, and hepatocytes. TLR signaling induces potent innate immune responses in these cells[25]. The liver is constantly exposed to PAMPs, such as LPS and bacterial DNA, through bacterial translocation *via* the portal vein system connecting it to the intestine[25].

TLRs also play a role in the regulation of inflammation based on their ability to recognize endogenous TLR ligands, termed damage-associated molecular patterns (DAMPs)[24]. The liver not only represents a major target of bacterial PAMPs in many disease states but also upregulates several DAMPs following injury[11,24]. The activation of inflammatory cells, including Kupffer cells, is a crucial step in the activation of hepatic stellate cells (HSCs)[25]. Intestinal bacterial microflora and functional TLR4, but not TLR2, are required for hepatic fibrogenesis[20]. Crosstalk between TLR4 signaling and transforming growth factor beta (TGF-β) signaling in HSCs has been reported[25]. Quiescent HSCs have been shown to be the target through which TLR4 ligands promote fibrogenesis. TGF-β signaling through the TLR4-MyD88-NFkappaB axis provides a novel link between proinflammatory and profibrogenic signals[20].

The activation of innate immune responses involving TLR4 and complement play important roles in initiating alcoholic steatohepatitis and fibrosis[26]. Activation of the TLR4-mediated myeloid differentiation factor 88 (MyD88)-independent [TRIF/interferon regulatory factor (IRF)-3] signaling pathway in Kupffer cells contributes to alcoholic steatohepatitis, whereas activation of TLR4 signaling in HSCs promotes liver fibrosis[26].

Activation of the innate immune system and increased release of proinflammatory cytokines and other mediators play an important role in the development of alcoholic liver disease (ALD)[27,28]. Alcohol-induced hepatocellular damage may occur as a result of bacterial or endotoxin translocation due to a reduction in reticuloendothelial system function in ALD. The recognition of gut-derived endotoxin by TLR4 contributes to the development of ALD through the activation of TLR-induced intracellular signaling pathways, cytokine production, and ROS[29]. TLR-dependent, ethanol-induced oxidative stress is important for the regulation of NFkappaB activation and

cytokine production by Kupffer cells[28]. Kupffer cells are stimulated by gut-derived endotoxin *via* mechanisms dependent on increased gut permeability and alcohol-induced liver injury[28].

TLR9 and TLR2 mediate *Propionibacterium acnes*-induced sensitization to LPS-triggered acute liver injury in mice[12]. Ligand-specific activation of TLR2 and TLR9 is dependent on the common TLR adaptor MyD88. MyD88 in immune cells, but not in liver parenchymal cells, plays important roles in inflammatory cell recruitment and liver injury[12].

Activation of TLR9 induces type I interferons *via* IRF-7. Type I IFNs were upregulated during TLR9-associated liver injury in WT mice. Type I IFN signaling is therefore required for protection from immune-mediated liver injury[30]. Type I IFNs have anti-inflammatory effects mediated by endogenous interleukin (IL)-1ra, which regulates the extent of TLR9-induced liver damage[30,31]. These data support the notion that bacterial translocation, the innate immune system and TLRs play an important role in the pathogenesis of liver damage.

BACTERIAL TRANSLOCATION IS ASSOCIATED WITH FAT ACCUMULATION IN THE LIVER

Several mechanisms have been proposed to explain the association between fat accumulation in the liver and bacterial translocation. A link between inflammation and hepatic steatosis was shown both in alcoholic and non-ALD[32,33]. The consumption of refined carbohydrates in soft drinks has been postulated to be a key factor in the development of NAFLD.

Results of several studies have shown that an increased consumption of fructose may result in an increased lipid accumulation in the liver which was accompanied by insulin resistance and elevated plasma triglycerides (Ackerman, 2005 No. 260; Jurgens, 2005 No. 261; Faeh, 2005 No. 262; Lewis, 2004 No. 264). Consumption of high levels of fructose lead to liver damage through overfeeding and also may induce a proinflammatory response by increasing intestinal translocation of endotoxin[34]. In a mouse model, hepatic lipid accumulation was higher in mice consuming fructose; these mice also showed high endotoxin levels in portal blood, lipid peroxidation and TNF-α expression[34].

Macrophages facilitate the clearance of cholesterol from the body *via* reverse cholesterol transport[35]. LPS has been shown to suppress PPARgamma1 and its downstream target genes in macrophages, inducing foam cell formation. This was proposed as a mechanism underlying the development of bacterial infection-induced atherosclerosis[35]. LPS induces the expression of adipocyte enhancer-binding protein 1 (AEBP1) during monocyte differentiation. LPS-induced down-regulation of pivotal macrophage cholesterol efflux mediators, leading to foam cell formation, is mediated by AEBP1. AEBP1-independent pathways contribute to the delayed effects of LPS on macrophage cholesterol efflux and the development of foam cells[35].

The published data support the hypothesis that bacterial translocation may underlie some of the mechanisms associated with fat accumulation in the liver.

BACTERIAL TRANSLOCATION IS ASSOCIATED WITH MITOCHONDRIAL DYSFUNCTION

Mitochondrial dysfunction is a pathogenic feature of NASH[29,36] and there is evidence that mitochondrial damage contributes to apoptotic/necrotic cellular damage in NASH[37]. NASH is associated with an increase in reactive oxygen species (ROS) production in Kupffer cells and hepatocytes[38]. The greater the decrease in cytochrome and oxidase activity seen, the more significant is the increase in ROS production. Mitochondrial dysfunction and overproduction of ROS play key roles in the progression of chronic hepatitis C and ethanol-induced liver injury. Ethanol also causes bacterial translocation in the intestine, and the resulting LPS activates Kupffer cells to produce pro-inflammatory cytokines[38]. It has been suggested that NASH may also result from increased ROS production in Kupffer cells and hepatocytes that may be dependent on bacterial translocation[38]. Therefore, in addition to its effects which are directly or indirectly associated with fat delivery, BT may also be associated with mitochondrial dysfunction that further contributes to fat accumulation in NAFLD.

BACTERIAL TRANSLOCATION IS ASSOCIATED WITH THE DEVELOPMENT OF NONALCOHOLIC STEATOHEPATITIS

Evidence supporting a role for the liver-gut axis in the pathogenesis of NAFLD has been slowly accumulating over the past 7 years[39-41]. Both preclinical and clinical data suggest an association between BT, small intestinal bacterial overgrowth (SIBO) and NASH[42,43]. Recently, the presence of SIBO has been associated with the severity of liver steatosis[44]. Exposure to bacterial products of intestinal origin, most notably endotoxin, including LPS, leads to liver inflammation, hepatocyte injury and hepatic fibrosis[43].

TLR4[45] and its coreceptor, myeloid differentiation factor-2 (MD-2), recognize LPS and activate proinflammatory signaling pathways. TLR4 can specifically recognize LPS as a danger signal and induce activation of inflammation-associated genes[46,47]. A 4-wk high-fat diet increased plasma LPS concentration two to three times[48] and the LPS recognition complex (TLR4 and MD-2) activates NADPH in liver steatosis and induces fibrosis in a NASH model in mice. These data support the role of these receptors in the development of steatosis, inflammation and fibrosis in NASH[49].

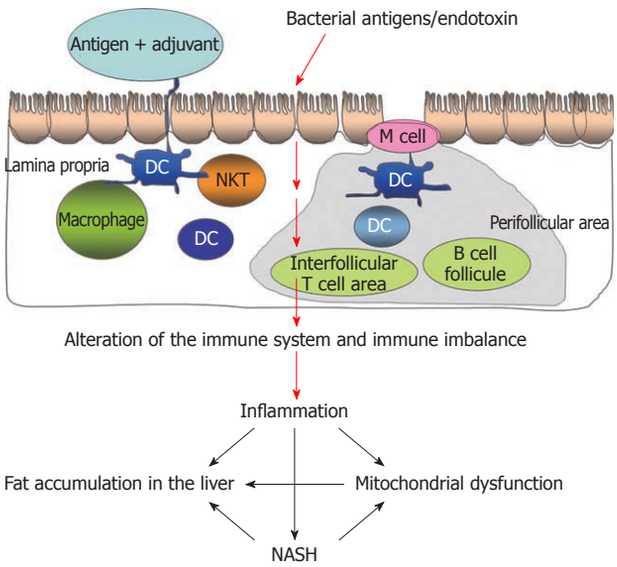

Figure 1 Bacterial translocation is associated with the development of nonalcoholic steatohepatitis. NASH: Nonalcoholic steatohepatitis.

The suppression of inflammation and immune tolerance are known to occur in normal livers. Suppressed inflammation has been shown despite bacterial colonization in normal human livers maintaining liver immune homeostasis[50]. In spite of increased bacterial colonization of liver tissues, lower levels of TLR2/4 mRNA and TLR4 and pIKKalpha, a marker for nuclear factor-kappa B (NFkappaB) activation, proteins were found in liver tissues from healthy subjects compared with samples from patients with primary biliary cirrhosis and NASH. Although these data raise the question of whether BT initiates the inflammatory process in the liver or whether it is instead a result of the inflammatory process, these results further support a role for BT in the pathogenesis of the disease.

SIBO also plays a role in NASH *via* interactions with TLR-4 and the induction of IL-8[43]. SIBO has been reported to coexist with NASH[51-56]. Patients with NASH show higher levels of expression of TLR-4/MD-2 on CD14-positive cells[43]. Serum levels of the proinflammatory cytokine IL-8 were higher in samples from NASH patients than in those from control subjects and were correlated positively with TLR-4 expression.

Leptin is a proinflammatory cytokine associated with the progression of NASH. Leptin enhanced TNF-α production and caused a dose-dependent increase in MAPK activity in LPS-stimulated KCs[57]. KCs isolated from the leptin receptor-deficient Zucker rat (fa/fa) showed reduced production of TNF-α upon stimulation with LPS[57]. Furthermore, treatment of normal rats with leptin increased LPS-induced hepatic TNF-α production in vivo, and leptin receptor-deficient Zucker rats showed reduced hepatic TNF-α production upon addition of LPS *in vivo*[57].

Following BT, LPS activates inflammasomes[58-60]. Inflammasomes respond to endogenous and exogenous danger signals by inducing the processing of pre-IL-1ss into secreted IL-1ss[36]. In the methionine-choline-deficient (MCD) or high fat diet-induced models, saturated fatty palmitic acid activates the inflammasome and sensitizes hepatocytes to LPS-induced IL-1ss release. Hepatocytes exposed to saturated fatty acid release danger signals that trigger inflammasome activation in immune cells[36]. LPS treatment significantly increased hepatic TNF-α production in MCD mice. LPS also induced a significant increase in TUNEL-positive cells[61]. This increase in apoptosis was inhibited by treatment with a neutralizing anti-mouse TNF receptor antibody or pentoxifylline.

In humans, dietary fructose intake has been associated with increased intestinal permeability and translocation of bacterial endotoxin; plasminogen activator inhibitor (PAI-1) may also contribute to the development of NAFLD in humans[62]. Plasma concentrations of endotoxin and PAI-1 and hepatic mRNA expression levels of TLR4 and PAI-1 were higher in NAFLD patients than in control subjects. Serum levels of LPS-binding protein (LBP) were increased in obese patients with NAFLD[63]. Plasma levels of LBP were further increased in patients with steatohepatitis when compared with patients with simple steatosis[63]. TNF-α mRNA expression in liver tissue was significantly higher in patients with NASH than in control subjects and was correlated with the increase in plasma levels of LBP.

BT is also involved in nitric oxide synthase (NOS) upregulation through the activation of both endothelial NOS and inducible NOS[64-66]. The prevention of intestinal gram-negative bacterial translocation by norfloxacin corrects circulatory changes by decreasing nitric oxide (NO) production in cirrhosis. Norfloxacin administration significantly decreased the incidence of gram-negative bacterial translocation and production of proinflammatory TNF-α, IFN-γ and IL-6[66,67].

The published data support the hypothesis that BT is associated with the development and maintenance of continued lipid accumulation, inflammation and fibrosis in patients with NASH (Figure 1).

BACTERIAL TRANSLOCATION IS CLINICALLY RELEVANT IN CHRONIC LIVER DISEASE

BT was shown to affect the development of chronic liver disease and the associated complications. It is also associated with an impaired prognosis[68]. Bacterial DNA is a marker of bacterial translocation and can be detected in uninfected patients with cirrhosis and ascites[69,70]. It is associated with a marked inflammatory response and with the activation of the inducible form of NOS and the release of NO. A similar effect is observed in patients with SBP[68].

The induction of cirrhosis in rats by CCl_4 led to prolonged oxidative stress in the intestine, accompanied by increased sugar content in both the intestinal brush border and the surfactant layers[71]. This was accompanied by changes in bacterial flora in the gut, and these bacteria

showed increased hydrophobicity and adherence to the mucosa. These data support the notion that oxidative stress in the intestine during cirrhosis alters mucosal glycosylation and increases the hydrophobicity of the luminal bacteria, enabling increased bacterial adherence to epithelial cells, facilitating BT[71].

In a human trial, the presence of bacterial DNA was associated with aggravation of peripheral vasodilation and with a worsening of intrahepatic endothelial dysfunction[68]. Patients exposed to bacterial DNA had a significantly lower mean arterial pressure and systemic vascular resistance. In response to increased blood flow caused by postprandial hyperemia, these patients had greater increases in hepatic vein pressure gradient and impaired hepatic vasorelaxation[68]. In contrast, a prospective trial of 151 patients with cirrhosis and ascites found no evidence that the detection of bacterial DNA in the ascites of cirrhotics is of clinical or diagnostic relevance to the detection of SBP[72]. This discrepancy in the published data remains to be resolved.

Increased intestinal permeability and abnormal motility were frequently observed in cirrhotics without ascites, even in the absence of evidence of BT. It has been suggested that these factors facilitate BT and thus precede it[73]. Systemic reactivity to microbial components as measured by the development of antibodies was suggested to reflect the compromised mucosal immunity in cirrhotic patients[74]. The presence of bacterial DNA in blood and ascites correlates with BT and is frequent in patients with advanced cirrhosis without overt infection; BT can also precede the occurrence of overt bacterial infection in patients with cirrhosis[73]. Altered permeability of the mucosa and deficiencies in host immune defenses that allow bacterial translocation from the intestine due to intestinal bacterial overgrowth have been implicated in the development of SBP[71]. Altered intestinal permeability was observed in 45% of patients with cirrhosis and was associated with Child-Pugh status, with the presence of ascites, and with a history of SBP[75,76]. SIBO is much more frequent in patients with cirrhosis and was highly correlated with BT, especially in ascitic patients[77].

Higher levels of *Enterobacteriaceae* were identified in cirrhotic rats than in healthy rats, and *Bifidobacteria* treatment resulted in lower levels of *Enterobacteriaceae*[61]. These results suggest the existence of an imbalance in the gut flora in cirrhotic rats, which may further result in BT and altered liver function[61].

BT to MLNs in cirrhosis has been linked to impaired host defense in these patients[78]. BT and endotoxemia are contributing factors in the expansion of specific subsets of lymphocyte populations[79]. In a clinical trial of 40 cirrhotics, the percentage of activated monocytes and T lymphocytes was increased in patients, and the proportions of effector cells and of those expressing CD95+ were higher. LBP modulates the biologic activity of circulating endotoxin, and its levels have been shown to rise in response to LPS[80-82]. Patients with elevated levels of LBP showed higher frequencies of regulatory T cells (CD4+CD25+FoxP3+) than those with normal levels of LBP[79]. In a rat model, BT was associated with an increase in the phagocytic capacity of polymorphonuclear leukocytes[78]. Both TNF-α and IL-6 were increased in patients with translocation of bacterial DNA from gram-positive microorganisms regardless of endotoxin and LBP levels[83].

These data suggest that BT is of clinical relevance in patients with chronic liver disease and may be a contributing factor in the development of liver disease and the degree of severity of the associated complications.

BACTERIAL TRANSLOCATION: IMPLICATIONS FOR THERAPY

Detoxification of gut-derived toxins and microbial products from gut-derived microbes is one function of the liver. Levels of bacterial LPS are increased in the portal and/or systemic circulation in several types of chronic liver diseases. Increased gut permeability and LPS also play roles in several liver disorders. NASH is associated with increased serum LPS levels and the activation of proinflammatory signaling[8,11], both of which suggest BT as a potential therapeutic target in these disorders.

Probiotics have been suggested as a treatment for different types of chronic liver damage because of their abilities to augment intestinal barrier function and to prevent BT[84,85]. The administration of probiotics reduced BT in a rat model[86]. Both viable and heat-killed yeast cells prevented BT. This effect was suggested to be the result of an immune modulatory effect and the maintenance of gut barrier integrity[87,88]. Oral treatment with viable or heat-killed *Saccharomyces cerevisiae* strain UFMG 905 prevented BT in a murine model of intestinal obstruction. Treatment with either viable or heat-killed yeast reduced intestinal permeability and increased IL-10 levels. Orally administered probiotics, nonpathogenic *Escherichia coli*, and gentamicin decreased BT and attenuated liver damage, decreasing levels of TNF-α, IL-6, IL-10 and IL-12[89]. An enteral diet supplemented with *Chlorella* sp. microalgae had significant protective effects on the intestinal mucosal barrier in a rat model of obstructive jaundice and reduced BT[90].

Oral treatment with resveratrol, curcumin or simvastatin ameliorated small intestinal inflammation by maintaining gut barrier function, preventing BT, and decreasing Th1-type immune responses[91]. Oral administration of these compounds increased regulatory T cell numbers and augmented intestinal epithelial cell regeneration in the ileum. Levels of the anti-inflammatory cytokine IL-10 in the ileum, MLNs and spleen were increased, whereas the proinflammatory cytokines IL-23p19, IFN-γ and TNF-α were decreased[91]. Treated animals displayed fewer proinflammatory enterobacteria and enterococci and higher anti-inflammatory lactobacilli and Bifidobacteria loads.

Glutamine decreased intestinal permeability and BT to physiologic levels in treated animals and preserved intesti-

nal barrier integrity[92]. Arginine supplementation reduced intestinal permeability and BT, leading to mucosal ileum preservation[93]. A specific nutritional combination rich in protein, L-leucine, fish oil and specific oligosaccharides resulted in reduced BT along with reduced production of proinflammatory cytokines[94], and supplementation with honey in the presence of obstructive jaundice ameliorated BT[95].

Treatment with an anti-TNF-α mAb in a model of CCl_4-induced cirrhosis decreased the incidence of BT in a TNF-α- and TNF-α receptor-independent manner without increasing the risk of systemic infection[96].

Desferrioxamine attenuated mucosal injury from post-hepatectomy liver dysfunction, and this was associated with decreased BT in the portal circulation, decreased portal endotoxin levels, and decreased systemic endotoxin levels[97]. Low concentrations of histamine inhibited bacteria from entering epithelial cells and inhibited intestinal BT[98]. In histamine-treated rats, the average numbers of bacteria in the liver and lymph nodes were much lower than those in control rats.

The sympathetic nervous system is activated in advanced cirrhosis, particularly in the splanchnic circulation, and exerts potent immunosuppressive actions. Splanchnic sympathectomy reduced bacterial translocation to MLNs in cirrhotic rats[99].

TREATMENT OF BACTERIAL TRANSLOCATION AS A MEANS OF TREATING NONALCOHOLIC STEATOHEPATITIS

In light of the potential role of BT in the development of steatosis, steatohepatitis and fibrosis, several studies have evaluated the potential effects of treatments aimed at BT.

Probiotics exhibit immunoregulatory and anti-inflammatory activity. Administration of the probiotic VSL#3 modulated liver fibrosis *via* the modulation of collagen expression and impairment of TGF-β signaling in a NASH model[100]; however, this treatment did not prevent inflammation and steatosis.

Oxidative stress contributes to the development of NASH, suggesting that antioxidants, which decrease oxidative stress, may ameliorate the disease. Increasing hepatic α- or γ-tocopherol protected against LPS-induced NASH by decreasing liver damage, lipid peroxidation, and inflammation without affecting body mass or hepatic steatosis[101]. Resveratrol decreased NAFLD severity in rats *via* TNF-α inhibition and antioxidant activity[102]. Specifically, it decreased fat deposition, increased levels of activity of superoxide dismutase, glutathione peroxidase and catalase and decreased NOS in the liver.

LPS-induced liver injury was prevented by 8A8, a synthetic triglyceride containing an arachidonic acid branch, through the inhibition of TNF-α and NO production by hepatic macrophages[103]. Stimulation of peripheral blood mononuclear cells from NASH patients with LPS resulted in a strong increase in TNF-α production. Pentoxifylline caused a dose-dependent suppression of TNF-α secretion, suggesting that it may be able to serve as a potential treatment for NASH[104].

In an open-label trial, subjects with biopsy-proven NASH and insulin resistance were orally treated for 30 days with an IgG-enhanced fraction of enterotoxigenic *Escherichia coli* colostrum[105] (Imm-124©, Immuron, Australia). An alleviation of insulin resistance was detected by a decrease in fasting glucose levels, an elevation in the early peak of insulin secretion following glucose administration, improved OGTT, improved insulin secretion during the OGTT, and improvements in the HOMA score and HBA1C levels. Treated patients showed a decrease in serum levels of triglycerides, total cholesterol, and LDL cholesterol. A decrease in liver enzymes was noted in most treated patients. These effects were associated with increased serum levels of GLP-1 and adiponectin. An increase in CD25+ and CD4+CD25+Foxp3+ regulatory T cells was also noted. An anti-LPS effect along with the promotion of regulatory T cells suggests that a combined mechanism is responsible for these effects.

CONCLUSION

Alterations to intestinal microbiota seem to play important roles in the induction and promotion of liver damage progression and in the development and severity of NASH. Bacterial overgrowth, immune dysfunction, alteration of the luminal factors, and altered intestinal permeability are all involved in the pathogenesis of NASH and its complications, including progression to cirrhosis, infections, hepatic encephalopathy, SBP and renal failure[84]. A better understanding of the cell-specific recognition and intracellular signaling events involved in sensing gut-derived microbes will help in the development of means to achieve an optimal balance in the gut-liver axis and ameliorate liver diseases[36]. The data described here support the notion that BT may serve as a new therapeutic target for NASH.

BT induces an immune imbalance leading to a state of chronic inflammation, fat accumulation in the liver, mitochondrial dysfunction and NASH.

REFERENCES

1 **Almeida J**, Galhenage S, Yu J, Kurtovic J, Riordan SM. Gut flora and bacterial translocation in chronic liver disease. *World J Gastroenterol* 2006; **12**: 1493-1502

2 **Floch MH**, Katz J, Conn HO. **Qualitative and quantitative** relationships of the fecal flora in cirrhotic patients with portal systemic encephalopathy and following portacaval anastomosis. *Gastroenterology* 1970; **59**: 70-75

3 **Pardo A**, Bartolí R, Lorenzo-Zúñiga V, Planas R, Viñado B, Riba J, Cabré E, Santos J, Luque T, Ausina V, Gassull MA. Effect of cisapride on intestinal bacterial overgrowth and bacterial translocation in cirrhosis. *Hepatology* 2000; **31**: 858-863

4 **Wiest R**, Garcia-Tsao G. Bacterial translocation (BT) in cirrhosis. *Hepatology* 2005; **41**: 422-433

5 **Bauer TM**, Schwacha H, Steinbrückner B, Brinkmann FE,

Ditzen AK, Aponte JJ, Pelz K, Berger D, Kist M, Blum HE. Small intestinal bacterial overgrowth in human cirrhosis is associated with systemic endotoxemia. *Am J Gastroenterol* 2002; **97**: 2364-2370

6 **Bauer TM**, Steinbrückner B, Brinkmann FE, Ditzen AK, Schwacha H, Aponte JJ, Pelz K, Kist M, Blum HE. Small intestinal bacterial overgrowth in patients with cirrhosis: prevalence and relation with spontaneous bacterial peritonitis. *Am J Gastroenterol* 2001; **96**: 2962-2967

7 **Bauer TM**, Schwacha H, Steinbrückner B, Brinkmann FE, Ditzen AK, Kist M, Blum HE. Diagnosis of small intestinal bacterial overgrowth in patients with cirrhosis of the liver: poor performance of the glucose breath hydrogen test. *J Hepatol* 2000; **33**: 382-386

8 **Szabo G**, Bala S, Petrasek J, Gattu A. Gut-liver axis and sensing microbes. *Dig Dis* 2010; **28**: 737-744

9 **Wang HJ**, Zakhari S, Jung MK. Alcohol, inflammation, and gut-liver-brain interactions in tissue damage and disease development. *World J Gastroenterol* 2010; **16**: 1304-1313

10 **Szabo G**, Mandrekar P, Dolganiuc A, Catalano D, Kodys K. Reduced alloreactive T-cell activation after alcohol intake is due to impaired monocyte accessory cell function and correlates with elevated IL-10, IL-13, and decreased IFNgamma levels. *Alcohol Clin Exp Res* 2001; **25**: 1766-1772

11 **Szabo G**, Mandrekar P, Dolganiuc A. Innate immune response and hepatic inflammation. *Semin Liver Dis* 2007; **27**: 339-350

12 **Hritz I**, Velayudham A, Dolganiuc A, Kodys K, Mandrekar P, Kurt-Jones E, Szabo G. Bone marrow-derived immune cells mediate sensitization to liver injury in a myeloid differentiation factor 88-dependent fashion. *Hepatology* 2008; **48**: 1342-1347

13 **Úbeda M**, Muñoz L, Borrero MJ, Díaz D, Francés R, Monserrat J, Lario M, Lledó L, Such J, Álvarez-Mon M, Albillos A. Critical role of the liver in the induction of systemic inflammation in rats with preascitic cirrhosis. *Hepatology* 2010; **52**: 2086-2095

14 **Albillos A**, de la Hera A, González M, Moya JL, Calleja JL, Monserrat J, Ruiz-del-Arbol L, Alvarez-Mon M. Increased lipopolysaccharide binding protein in cirrhotic patients with marked immune and hemodynamic derangement. *Hepatology* 2003; **37**: 208-217

15 **Albillos A**, Hera Ad Ade L, Reyes E, Monserrat J, Muñoz L, Nieto M, Prieto A, Sanz E, Alvarez-Mon M. Tumour necrosis factor-α expression by activated monocytes and altered T-cell homeostasis in ascitic alcoholic cirrhosis: amelioration with norfloxacin. *J Hepatol* 2004; **40**: 624-631

16 **Muñoz L**, Albillos A, Nieto M, Reyes E, Lledó L, Monserrat J, Sanz E, de la Hera A, Alvarez-Mon M. Mesenteric Th1 polarization and monocyte TNF-α production: first steps to systemic inflammation in rats with cirrhosis. *Hepatology* 2005; **42**: 411-419

17 **Chedid A**, Mendenhall CL, Moritz TE, French SW, Chen TS, Morgan TR. Expression of the beta 1 chain (CD29) of integrins and CD45 in alcoholic liver disease. The VA Cooperative Study Group No. 275. *Am J Gastroenterol* 1993; **88**: 1920-1927

18 **Girón JA**, Alvarez-Mon M, Menéndez-Caro JL, Abreu L, Albillos A, Manzano L, Durántez A. Increased spontaneous and lymphokine-conditioned IgA and IgG synthesis by B cells from alcoholic cirrhotic patients. *Hepatology* 1992; **16**: 664-670

19 **Heumann D**, Barras C, Severin A, Glauser MP, Tomasz A. Gram-positive cell walls stimulate synthesis of tumor necrosis factor α and interleukin-6 by human monocytes. *Infect Immun* 1994; **62**: 2715-2721

20 **Seki E**, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, Schwabe RF. TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med* 2007; **13**: 1324-1332

21 **Isayama F**, Hines IN, Kremer M, Milton RJ, Byrd CL, Perry AW, McKim SE, Parsons C, Rippe RA, Wheeler MD. LPS signaling enhances hepatic fibrogenesis caused by experimental cholestasis in mice. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G1318-G1328

22 **Uesugi T**, Froh M, Arteel GE, Bradford BU, Thurman RG. Toll-like receptor 4 is involved in the mechanism of early alcohol-induced liver injury in mice. *Hepatology* 2001; **34**: 101-108

23 **Hritz I**, Mandrekar P, Velayudham A, Catalano D, Dolganiuc A, Kodys K, Kurt-Jones E, Szabo G. The critical role of toll-like receptor (TLR) 4 in alcoholic liver disease is independent of the common TLR adapter MyD88. *Hepatology* 2008; **48**: 1224-1231

24 **Mencin A**, Kluwe J, Schwabe RF. Toll-like receptors as targets in chronic liver diseases. *Gut* 2009; **58**: 704-720

25 **Aoyama T**, Paik YH, Seki E. Toll-like receptor signaling and liver fibrosis. *Gastroenterol Res Pract* 2010; **2010**

26 **Gao B**, Seki E, Brenner DA, Friedman S, Cohen JI, Nagy L, Szabo G, Zakhari S. Innate immunity in alcoholic liver disease. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G516-G525

27 **Tsukamoto H**, Takei Y, McClain CJ, Joshi-Barve S, Hill D, Schmidt J, Deaciuc I, Barve S, Colell A, Garcia-Ruiz C, Kaplowitz N, Fernandez-Checa JC, Yokoyama H, Okamura Y, Nakamura Y, Ishii H, Chawla RK, Barve S, Joshi-Barve S, Watson W, Nelson W, Lin M, Ohata M, Motomura K, Enomoto N, Ikejima K, Kitamura T, Oide H, Hirose M, Bradford BU, Rivera CA, Kono H, Peter S, Yamashina S, Konno A, Ishikawa M, Shimizu H, Sato N, Thurman R. How is the liver primed or sensitized for alcoholic liver disease? *Alcohol Clin Exp Res* 2001; **25**: 171S-181S

28 **Nagata K**, Suzuki H, Sakaguchi S. Common pathogenic mechanism in development progression of liver injury caused by non-alcoholic or alcoholic steatohepatitis. *J Toxicol Sci* 2007; **32**: 453-468

29 **Csak T**, Dolganiuc A, Kodys K, Nath B, Petrasek J, Bala S, Lippai D, Szabo G. Mitochondrial antiviral signaling protein defect links impaired antiviral response and liver injury in steatohepatitis in mice. *Hepatology* 2011; **53**: 1917-1931

30 **Petrasek J**, Dolganiuc A, Csak T, Kurt-Jones EA, Szabo G. Type I interferons protect from Toll-like receptor 9-associated liver injury and regulate IL-1 receptor antagonist in mice. *Gastroenterology* 2011; **140**: 697-708.e4

31 **Petrasek J**, Dolganiuc A, Csak T, Nath B, Hritz I, Kodys K, Catalano D, Kurt-Jones E, Mandrekar P, Szabo G. Interferon regulatory factor 3 and type I interferons are protective in alcoholic liver injury in mice by way of crosstalk of parenchymal and myeloid cells. *Hepatology* 2011; **53**: 649-660

32 **Hotamisligil GS**. Inflammation and metabolic disorders. *Nature* 2006; **444**: 860-867

33 **Nath B**, Levin I, Csak T, Petrasek J, Mueller C, Kodys K, Catalano D, Mandrekar P, Szabo G. Hepatocyte-specific hypoxia-inducible factor-1α is a determinant of lipid accumulation and liver injury in alcohol-induced steatosis in mice. *Hepatology* 2011; **53**: 1526-1537

34 **Bergheim I**, Weber S, Vos M, Krämer S, Volynets V, Kaserouni S, McClain CJ, Bischoff SC. Antibiotics protect against fructose-induced hepatic lipid accumulation in mice: role of endotoxin. *J Hepatol* 2008; **48**: 983-992

35 **Majdalawieh A**, Ro HS. LPS-induced suppression of macrophage cholesterol efflux is mediated by adipocyte enhancer-binding protein 1. *Int J Biochem Cell Biol* 2009; **41**: 1518-1525

36 **Csak T**, Ganz M, Pespisa J, Kodys K, Dolganiuc A, Szabo G. Fatty acid and endotoxin activate inflammasomes in mouse hepatocytes that release danger signals to stimulate immune cells. *Hepatology* 2011; **54**: 133-144

37 **Begriche K**, Igoudjil A, Pessayre D, Fromenty B. Mitochondrial dysfunction in NASH: causes, consequences and pos-

sible means to prevent it. *Mitochondrion* 2006; **6**: 1-28
38 **Sato N**. Central role of mitochondria in metabolic regulation of liver pathophysiology. *J Gastroenterol Hepatol* 2007; **22** Suppl 1: S1-S6
39 **Dumas ME**, Barton RH, Toye A, Cloarec O, Blancher C, Rothwell A, Fearnside J, Tatoud R, Blanc V, Lindon JC, Mitchell SC, Holmes E, McCarthy MI, Scott J, Gauguier D, Nicholson JK. Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. *Proc Natl Acad Sci USA* 2006; **103**: 12511-12516
40 **Solga SF**, Diehl AM. Non-alcoholic fatty liver disease: lumen-liver interactions and possible role for probiotics. *J Hepatol* 2003; **38**: 681-687
41 **Farhadi A**, Gundlapalli S, Shaikh M, Frantzides C, Harrell L, Kwasny MM, Keshavarzian A. Susceptibility to gut leakiness: a possible mechanism for endotoxaemia in non-alcoholic steatohepatitis. *Liver Int* 2008; **28**: 1026-1033
42 **Miele L**, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, Mascianà R, Forgione A, Gabrieli ML, Perotti G, Vecchio FM, Rapaccini G, Gasbarrini G, Day CP, Grieco A. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. *Hepatology* 2009; **49**: 1877-1887
43 **Shanab AA**, Scully P, Crosbie O, Buckley M, O'Mahony L, Shanahan F, Gazareen S, Murphy E, Quigley EM. Small intestinal bacterial overgrowth in nonalcoholic steatohepatitis: association with toll-like receptor 4 expression and plasma levels of interleukin 8. *Dig Dis Sci* 2011; **56**: 1524-1534
44 **Sabaté JM**, Jouët P, Harnois F, Mechler C, Msika S, Grossin M, Coffin B. High prevalence of small intestinal bacterial overgrowth in patients with morbid obesity: a contributor to severe hepatic steatosis. *Obes Surg* 2008; **18**: 371-377
45 **Beutler B**. Tlr4: central component of the sole mammalian LPS sensor. *Curr Opin Immunol* 2000; **12**: 20-26
46 **Lu YC**, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. *Cytokine* 2008; **42**: 145-151
47 **Pålsson-McDermott EM**, O'Neill LA. **Signal transduction** by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* 2004; **113**: 153-162
48 **Cani PD**, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmée E, Cousin B, Sulpice T, Chamontin B, Ferrières J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, Burcelin R. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 2007; **56**: 1761-1772
49 **Csak T**, Velayudham A, Hritz I, Petrasek J, Levin I, Lippai D, Catalano D, Mandrekar P, Dolganiuc A, Kurt-Jones E, Szabo G. Deficiency in myeloid differentiation factor-2 and toll-like receptor 4 expression attenuates nonalcoholic steatohepatitis and fibrosis in mice. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G433-G441
50 **Singh R**, Bullard J, Kalra M, Assefa S, Kaul AK, Vonfeldt K, Strom SC, Conrad RS, Sharp HL, Kaul R. Status of bacterial colonization, Toll-like receptor expression and nuclear factor-kappa B activation in normal and diseased human livers. *Clin Immunol* 2011; **138**: 41-49
51 **Nazim M**, Stamp G, Hodgson HJ. Non-alcoholic steatohepatitis associated with small intestinal diverticulosis and bacterial overgrowth. *Hepatogastroenterology* 1989; **36**: 349-351
52 **Riordan SM**, McIver CJ, Williams R. Liver damage in human small intestinal bacterial overgrowth. *Am J Gastroenterol* 1998; **93**: 234-237
53 **Zhao LF**, Jia JM, Han DW. [The role of enterogenous endotoxemia in the pathogenesis of non-alcoholic steatohepatitis]. *Zhonghua Ganzangbing Zazhi* 2004; **12**: 632
54 **Fu JF**, Fang YL, Liang L, Wang CL, Hong F, Dong GP. A rabbit model of pediatric nonalcoholic steatohepatitis: the role of adiponectin. *World J Gastroenterol* 2009; **15**: 912-918
55 **Soza A**, Riquelme A, González R, Alvarez M, Pérez-Ayuso RM, Glasinovic JC, Arrese M. Increased orocecal transit time in patients with nonalcoholic fatty liver disease. *Dig Dis Sci* 2005; **50**: 1136-1140
56 **Wigg AJ**, Roberts-Thomson IC, Dymock RB, McCarthy PJ, Grose RH, Cummins AG. The role of small intestinal bacterial overgrowth, intestinal permeability, endotoxaemia, and tumour necrosis factor α in the pathogenesis of non-alcoholic steatohepatitis. *Gut* 2001; **48**: 206-211
57 **Shen J**, Sakaida I, Uchida K, Terai S, Okita K. Leptin enhances TNF-α production via p38 and JNK MAPK in LPS-stimulated Kupffer cells. *Life Sci* 2005; **77**: 1502-1515
58 **Schroder K**, Tschopp J. The inflammasomes. *Cell* 2010; **140**: 821-832
59 **Tschopp J**, Schroder K. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? *Nat Rev Immunol* 2010; **10**: 210-215
60 **Schroder K**, Zhou R, Tschopp J. The NLRP3 inflammasome: a sensor for metabolic danger? *Science* 2010; **327**: 296-300
61 **Kudo H**, Takahara T, Yata Y, Kawai K, Zhang W, Sugiyama T. Lipopolysaccharide triggered TNF-α-induced hepatocyte apoptosis in a murine non-alcoholic steatohepatitis model. *J Hepatol* 2009; **51**: 168-175
62 **Thuy S**, Ladurner R, Volynets V, Wagner S, Strahl S, Königsrainer A, Maier KP, Bischoff SC, Bergheim I. Nonalcoholic fatty liver disease in humans is associated with increased plasma endotoxin and plasminogen activator inhibitor 1 concentrations and with fructose intake. *J Nutr* 2008; **138**: 1452-1455
63 **Ruiz AG**, Casafont F, Crespo J, Cayón A, Mayorga M, Estebanez A, Fernadez-Escalante JC, Pons-Romero F. Lipopolysaccharide-binding protein plasma levels and liver TNF-α gene expression in obese patients: evidence for the potential role of endotoxin in the pathogenesis of non-alcoholic steatohepatitis. *Obes Surg* 2007; **17**: 1374-1380
64 **Li H**, Förstermann U. Nitric oxide in the pathogenesis of vascular disease. *J Pathol* 2000; **190**: 244-254
65 **Pateron D**, Tazi KA, Sogni P, Heller J, Chagneau C, Poirel O, Philippe M, Moreau R, Lebrec D. Role of aortic nitric oxide synthase 3 (eNOS) in the systemic vasodilation of portal hypertension. *Gastroenterology* 2000; **119**: 196-200
66 **Tazi KA**, Moreau R, Hervé P, Dauvergne A, Cazals-Hatem D, Bert F, Poirel O, Rabiller A, Lebrec D. Norfloxacin reduces aortic NO synthases and proinflammatory cytokine up-regulation in cirrhotic rats: role of Akt signaling. *Gastroenterology* 2005; **129**: 303-314
67 **Heller J**, Sogni P, Barrière E, Tazi KA, Chauvelot-Moachon L, Guimont MC, Bories PN, Poirel O, Moreau R, Lebrec D. Effects of lipopolysaccharide on TNF-α production, hepatic NOS2 activity, and hepatic toxicity in rats with cirrhosis. *J Hepatol* 2000; **33**: 376-381
68 **Bellot P**, García-Pagán JC, Francés R, Abraldes JG, Navasa M, Pérez-Mateo M, Such J, Bosch J. Bacterial DNA translocation is associated with systemic circulatory abnormalities and intrahepatic endothelial dysfunction in patients with cirrhosis. *Hepatology* 2010; **52**: 2044-2052
69 **Such J**, Francés R, Muñoz C, Zapater P, Casellas JA, Cifuentes A, Rodríguez-Valera F, Pascual S, Sola-Vera J, Carnicer F, Uceda F, Palazón JM, Pérez-Mateo M. Detection and identification of bacterial DNA in patients with cirrhosis and culture-negative, nonneutrocytic ascites. *Hepatology* 2002; **36**: 135-141
70 **Cirera I**, Bauer TM, Navasa M, Vila J, Grande L, Taurá P, Fuster J, García-Valdecasas JC, Lacy A, Suárez MJ, Rimola A, Rodés J. Bacterial translocation of enteric organisms in patients with cirrhosis. *J Hepatol* 2001; **34**: 32-37
71 **Natarajan SK**, Ramamoorthy P, Thomas S, Basivireddy J, Kang G, Ramachandran A, Pulimood AB, Balasubramanian KA. Intestinal mucosal alterations in rats with carbon tetrachloride-induced cirrhosis: changes in glycosylation and

luminal bacteria. *Hepatology* 2006; **43**: 837-846
72 **Appenrodt B**, Lehmann LE, Thyssen L, Gentemann M, Rabe C, Molitor E, Trebicka J, Stüber F, Sauerbruch T. Is detection of bacterial DNA in ascitic fluid of clinical relevance? *Eur J Gastroenterol Hepatol* 2010; **22**: 1487-1494
73 **Thalheimer U**, De Iorio F, Capra F, del Mar Lleo M, Zuliani V, Ghidini V, Tafi MC, Caburlotto G, Gennari M, Burroughs AK, Vantini I. Altered intestinal function precedes the appearance of bacterial DNA in serum and ascites in patients with cirrhosis: a pilot study. *Eur J Gastroenterol Hepatol* 2010; **22**: 1228-1234
74 **Papp M**, Norman GL, Vitalis Z, Tornai I, Altorjay I, Foldi I, Udvardy M, Shums Z, Dinya T, Orosz P, Lombay B, Par G, Par A, Veres G, Csak T, Osztovits J, Szalay F, Lakatos PL. Presence of anti-microbial antibodies in liver cirrhosis--a tell-tale sign of compromised immunity? *PLoS One* 2010; **5**: e12957
75 **Lauritano EC**, Valenza V, Sparano L, Scarpellini E, Gabrielli M, Cazzato A, Ferraro PM, Gasbarrini A. Small intestinal bacterial overgrowth and intestinal permeability. *Scand J Gastroenterol* 2010; **45**: 1131-1132
76 **Scarpellini E**, Valenza V, Gabrielli M, Lauritano EC, Perotti G, Merra G, Dal Lago A, Ojetti V, Ainora ME, Santoro M, Ghirlanda G, Gasbarrini A. Intestinal permeability in cirrhotic patients with and without spontaneous bacterial peritonitis: is the ring closed? *Am J Gastroenterol* 2010; **105**: 323-327
77 **Jun DW**, Kim KT, Lee OY, Chae JD, Son BK, Kim SH, Jo YJ, Park YS. Association between small intestinal bacterial overgrowth and peripheral bacterial DNA in cirrhotic patients. *Dig Dis Sci* 2010; **55**: 1465-1471
78 **Neugebauer H**, Hartmann P, Krenn S, Glück T, Schölmerich J, Straub R, Wiest R. Bacterial translocation increases phagocytic activity of polymorphonuclear leucocytes in portal hypertension: priming independent of liver cirrhosis. *Liver Int* 2008; **28**: 1149-1157
79 **Márquez M**, Fernández-Gutiérrez C, Montes-de-Oca M, Blanco MJ, Brun F, Rodríguez-Ramos C, Girón-González JA. Chronic antigenic stimuli as a possible explanation for the immunodepression caused by liver cirrhosis. *Clin Exp Immunol* 2009; **158**: 219-229
80 **Calvano SE**, Thompson WA, Marra MN, Coyle SM, de Riesthal HF, Trousdale RK, Barie PS, Scott RW, Moldawer LL, Lowry SF. Changes in polymorphonuclear leukocyte surface and plasma bactericidal/permeability-increasing protein and plasma lipopolysaccharide binding protein during endotoxemia or sepsis. *Arch Surg* 1994; **129**: 220-226
81 **Schumann RR**, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, Tobias PS, Ulevitch RJ. Structure and function of lipopolysaccharide binding protein. *Science* 1990; **249**: 1429-1431
82 **Zweigner J**, Schumann RR, Weber JR. The role of lipopolysaccharide-binding protein in modulating the innate immune response. *Microbes Infect* 2006; **8**: 946-952
83 **González-Navajas JM**, Bellot P, Francés R, Zapater P, Muñoz C, García-Pagán JC, Pascual S, Pérez-Mateo M, Bosch J, Such J. Presence of bacterial-DNA in cirrhosis identifies a subgroup of patients with marked inflammatory response not related to endotoxin. *J Hepatol* 2008; **48**: 61-67
84 **Cesaro C**, Tiso A, Del Prete A, Cariello R, Tuccillo C, Cotticelli G, Del Vecchio Blanco C, Loguercio C. Gut microbiota and probiotics in chronic liver diseases. *Dig Liver Dis* 2011; **43**: 431-438
85 **Frazier TH**, DiBaise JK, McClain CJ. Gut microbiota, intestinal permeability, obesity-induced inflammation, and liver injury. *JPEN J Parenter Enteral Nutr* 2011; **35**: 14S-20S
86 **Zhou HJ**, Yin L, Chen CQ, Shi MM, Zhang MJ. Administration of probiotics reduces bacterial translocation after intestinal transplantation in rats. *Transplant Proc* 2010; **42**: 4643-4647
87 **Generoso SV**, Viana M, Santos R, Martins FS, Machado JA, Arantes RM, Nicoli JR, Correia MI, Cardoso VN. Saccharomyces cerevisiae strain UFMG 905 protects against bacterial translocation, preserves gut barrier integrity and stimulates the immune system in a murine intestinal obstruction model. *Arch Microbiol* 2010; **192**: 477-484
88 **Li Z**, Yang S, Lin H, Huang J, Watkins PA, Moser AB, Desimone C, Song XY, Diehl AM. Probiotics and antibodies to TNF inhibit inflammatory activity and improve nonalcoholic fatty liver disease. *Hepatology* 2003; **37**: 343-350
89 **Li YT**, Wang L, Chen Y, Chen YB, Wang HY, Wu ZW, Li LJ. Effects of gut microflora on hepatic damage after acute liver injury in rats. *J Trauma* 2010; **68**: 76-83
90 **Bedirli A**, Kerem M, Ofluoglu E, Salman B, Katircioglu H, Bedirli N, Yilmazer D, Alper M, Pasaoglu H. Administration of Chlorella sp. microalgae reduces endotoxemia, intestinal oxidative stress and bacterial translocation in experimental biliary obstruction. *Clin Nutr* 2009; **28**: 674-678
91 **Bereswill S**, Muñoz M, Fischer A, Plickert R, Haag LM, Otto B, Kühl AA, Loddenkemper C, Göbel UB, Heimesaat MM. Anti-inflammatory effects of resveratrol, curcumin and simvastatin in acute small intestinal inflammation. *PLoS One* 2010; **5**: e15099
92 **dos Santos RG**, Viana ML, Generoso SV, Arantes RE, Davisson Correia MI, Cardoso VN. Glutamine supplementation decreases intestinal permeability and preserves gut mucosa integrity in an experimental mouse model. *JPEN J Parenter Enteral Nutr* 2010; **34**: 408-413
93 **Viana ML**, Santos RG, Generoso SV, Arantes RM, Correia MI, Cardoso VN. Pretreatment with arginine preserves intestinal barrier integrity and reduces bacterial translocation in mice. *Nutrition* 2010; **26**: 218-223
94 **Faber J**, van Limpt K, Kegler D, Luiking Y, Garssen J, van Helvoort A, Vos AP, Knol J. Bacterial translocation is reduced by a specific nutritional combination in mice with chemotherapy-induced neutropenia. *J Nutr* 2011; **141**: 1292-1298
95 **Gencay C**, Kilicoglu SS, Kismet K, Kilicoglu B, Erel S, Muratoglu S, Sunay AE, Erdemli E, Akkus MA. Effect of honey on bacterial translocation and intestinal morphology in obstructive jaundice. *World J Gastroenterol* 2008; **14**: 3410-3415
96 **Francés R**, Chiva M, Sánchez E, González-Navajas JM, Lluovet T, Zapater P, Soriano G, Muñoz C, Balanzó J, Pérez-Mateo M, Song XY, Guarner C, Such J. Bacterial translocation is downregulated by anti-TNF-α monoclonal antibody administration in rats with cirrhosis and ascites. *J Hepatol* 2007; **46**: 797-803
97 **Nastos C**, Kalimeris K, Papoutsidakis N, Defterevos G, Pafiti A, Kalogeropoulou H, Zerva L, Nomikos T, Kostopanagiotou G, Smyrniotis V, Arkadopoulos N. Antioxidant treatment attenuates intestinal mucosal damage and gut barrier dysfunction after major hepatectomy. Study in a porcine model. *J Gastrointest Surg* 2011; **15**: 809-817
98 **Duan L**, Chen X, Alexander JW. Regulatory effect of histamine on the barrier function of intestinal mucosal. *J Gastrointest Surg* 2010; **14**: 1180-1185
99 **Worlicek M**, Knebel K, Linde HJ, Moleda L, Schölmerich J, Straub RH, Wiest R. Splanchnic sympathectomy prevents translocation and spreading of E coli but not S aureus in liver cirrhosis. *Gut* 2010; **59**: 1127-1134
100 **Velayudham A**, Dolganiuc A, Ellis M, Petrasek J, Kodys K, Mandrekar P, Szabo G. VSL#3 probiotic treatment attenuates fibrosis without changes in steatohepatitis in a diet-induced nonalcoholic steatohepatitis model in mice. *Hepatology* 2009; **49**: 989-997
101 **Chung MY**, Yeung SF, Park HJ, Volek JS, Bruno RS. Dietary α- and γ-tocopherol supplementation attenuates lipopolysaccharide-induced oxidative stress and inflammatory-

related responses in an obese mouse model of nonalcoholic steatohepatitis. *J Nutr Biochem* 2010; **21**: 1200-1206
102 **Bujanda L**, Hijona E, Larzabal M, Beraza M, Aldazabal P, García-Urkia N, Sarasqueta C, Cosme A, Irastorza B, González A, Arenas JI. Resveratrol inhibits nonalcoholic fatty liver disease in rats. *BMC Gastroenterol* 2008; **8**: 40
103 **Piao N**, Ikejima K, Kon K, Aoyama T, Osada T, Takei Y, Sato N, Watanabe S. Synthetic triglyceride containing an arachidonic acid branch (8A8) prevents lipopolysaccharide-induced liver injury. *Life Sci* 2009; **85**: 617-624
104 **Duman DG**, Ozdemir F, Birben E, Keskin O, Ekşioğlu-Demiralp E, Celikel C, Kalayci O, Kalayci C. Effects of pentoxifylline on TNF-α production by peripheral blood mononuclear cells in patients with nonalcoholic steatohepatitis. *Dig Dis Sci* 2007; **52**: 2520-2524
105 **Mizrahi M**, Shabat Y, Adar T, Ben Ya'acov A, Ilan Y. Alleviation of insulin resistance and liver damage by oral administration of etec colostrums is mediated by increased GLP-1, adiponectin serum levels and tregs: Results of a phase I/II clinical trial in NASH. *Hepatology* 2010; **52**: 163A

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ORIGINAL ARTICLE

Juvenile ferric iron prevents microbiota dysbiosis and colitis in adult rodents

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Abstract

AIM: To assess whether juvenile chronic ferric iron ingestion limit colitis and dysbiosis at adulthood in rats and mice.

METHODS: Two sets of experiments were designed. In the first set, recently weaned mice were either orally administered ferrous (Fe^{2+}) iron salt or ferric (Fe^{3+}) microencapsulated iron for 6 wk. The last week of experiments trinitrobenzene sulfonic acid (TNBS) colitis was induced. In the second set, juvenile rats received the microencapsulated ferric iron for 6 wk and were also submitted to TNBS colitis during the last week of experiments. In both sets of experiments, animals were sacrificed 7 d after TNBS instillation. Severity of the inflammation was assessed by scoring macroscopic lesions and quantifying colonic myeloperoxidase (MPO) activity. Alteration of the microflora profile was estimated using quantitative polymerase chain reaction (qPCR) by measuring the evolution of total caecal microflora, Bacteroidetes, Firmicutes and enterobacteria.

RESULTS: Neither ferrous nor ferric iron daily exposures at the juvenile period result in any effect in control animals at adulthood although ferrous iron repeated administration in infancy limited weight gain. Ferrous iron was unable to limit the experimental colitis (1.71 ± 0.27 MPO U/mg protein *vs* 2.47 ± 0.22 MPO U/mg protein in colitic mice). In contrast, ferric iron significantly prevented the increase of MPO activity (1.64 ± 0.14 MPO U/mg protein) in TNBS-induced colitis. Moreover, this positive effect was observed at both the doses of ferric iron used (75 and 150 mg/kg per day po - 6 wk). In the study we also compared, in both rats and mice, the consequences of chronic repeated low level exposure to ferric iron (75 mg/kg per day po - 6 wk) on TNBS-induced colitis and its related dysbiosis. We confirmed that ferric iron limited the TNBS-induced increase of MPO activity in both the rodent species. Furthermore, we assessed the ferric iron incidence on TNBS-induced intestinal microbiota dysbiosis. At first, we needed to optimize the isolation and quantify DNA copy numbers using standard curves to perform by qPCR this interspecies comparison. Using this approach, we determined that total microflora was similar in control rats and mice and was mainly composed of Firmicutes and Bacteroidetes at a ratio of 10/1. Ferric juvenile administration did not modify the microflora profile in control animals. Total microflora numbers remained unchanged whichever experimental conditions studied. Following TNBS-induced colitis, the Firmicutes/Bacteroidetes ratio was altered resulting in a decrease of the Firmicutes numbers and an increase of the Bacteroidetes numbers typical of a gut inflammatory reaction. In parallel, the subdominant population, the enterobacteria was also increased. However, ferric iron supplementation for the juvenile period prevented the increase of Bacteroidetes and of enterobacteria numbers consecutive to the colitis in both the studied species at adulthood.

CONCLUSION: Rats and mice juvenile chronic ferric iron ingestion prevents colitis and dysbiosis at adulthood as assessed by the first interspecies comparison.

Key words: Chronic ferric iron supplementation; Experimental colitis; Microflora dysbiosis; Rat; Mice

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INTRODUCTION

In humans neonates never carry iron deficiency at birth and needs remain low during the first trimester of life because of a slowdown of red blood cells production as well as physiological hemolysis[1]. From the fourth month of life, iron requirements increase and appropriate diet supply, generally corresponding to food diversification, becomes necessary. Western countries recommendations leaded to iron fortification of food for infants to prevent any risk of anemia. Classical forms of supplementation are ferrous (Fe^{2+}) salts but are associated with frequent gastrointestinal side effects leading to poor compliance. In addition to this, presence of ferrous iron in the colon could select gut microbiota for humans that are unfavorable to the host because iron is known to be essential for the growth and virulence of many pathogenic enterobacteria[2]. To limit the unfavorable effects linked to ferrous iron absorption, food industry manufacturers have the possibility of using ferric (Fe^{3+}) forms mainly as of pyrophosphate. However, this form displays not only a poor solubility in the food matrix but also a low bioavailability. To bypass these constraints, ferric pyrophosphate may be microencapsulated in lecithin beads (Lipofer®). The incidence of the daily ingestion of this form in juvenile individuals has not yet been investigated.

At the gut level, and particularly during the perinatal period, the crosstalk between bacteria, epithelium and lymphoid tissue is establishing. This crosstalk is involved in modelling the memory mechanisms of systemic immunity response[3], when those systems mature to reach the full functionality of the intestinal barrier. In humans, postnatal dominant colonisation by facultative anaerobic bacteria, among which we find enterobacteria, quickly gives way to dominant anaerobic bacteria development within a couple of weeks, in order to reach an equilibrium during the second year of life[4]. Intestinal microbiota is a large and extremely complex ecosystem, constituted by dynamic and diverse bacterial communities. The adult human tract environment harbours different bacterial phylotypes superior to 400 different species[5,6]. In permanent contact with the mucosal surface through symbiotic interactions, it takes a prominent part on the maintenance of health of the host[7]. A 10/1 Firmicutes/Bacteroidetes ratio is considered to be representative of an health status[8] which reflects a stable equilibrium between bacteria. However, some groups, that are generally subdominant, such as enterobacteria may become potentially pathogenic and can affect host homeostasis[9]. Several studies have described a higher level of Bacteroidetes and enterobacteria associated to a reduction of Firmicutes in inflammatory bowel disease (IBD) patients[9-14]. IBD are a group of chronic disorders of the gastrointestinal tract and include Crohn's disease (CD) and ulcerative colitis. The pathogenesis is not known but involves, at least in part a loss of tolerance towards the commensal colonic microbiota. Therefore, disruption of this crosstalk results in a deregulation of the immune response to the gut microbiota and may lead to development of chronic inflammation such as IBD[9].

Since ferrous oral iron may be at the origin of enterobacteria proliferation[2] and since this development may contribute to intestinal mucosal barrier function deregulation leading to intestinal inflammation, we hypothesized that ferric iron supplementation may modulate microbiota profile settlement when given to juvenile animals and could modify the course of an experimental colitis in young adults. However, as individuals vary in their resistance to pathogenic stimuli and as interspecies comparisons remain difficult to address, particularly when analysing microbiota profile, this study aimed at: (1) comparing the incidence of microencapsulated ferric iron with the ferrous iron on experimental colitis in mice; (2) evaluating the dose-response effect of ferric iron in this model of inflammation in mice; and (3) analyzing the consequences of repeated exposure to ferric iron on a moderate colitis and microbiota dysbiosis on two models of rodents (mice and rats) to allow interspecies comparisons.

MATERIALS AND METHODS

Chemicals

Ferric pyrophosphate microencapsulated in lecithin beads (Lipofer®) used in this study was kindly provided as a stable solution by Lipofoods SA (Barcelona, Spain). Anesthetics were obtained from Centravet (Nancy, France). All other chemical molecules were purchased from Sigma-Aldrich SA (St Quentin-Fallavier, France) except if specified.

Animals

Experiments were conducted using male BalbC mice (15-17 g) and male Wistar rats (100-125 g) obtained from HARLAN Laboratories, Ganat (France). All animals were housed in stainless steel cages under controlled temperature (21 ± 1 ℃) and a 12 h light-dark cycles. They had free access to food (A04, SAFE, Epinay sur Orge, France) and water throughout the study. This study was performed at the Animal House Unit of the Institut Polytechnique LaSalle Beauvais (policy agreement No. A60) and received prior approval from both the animal protocol review committee and of the Picardie Council veterinary office.

Treatments

Two sets of experiments have been designed. In the first series of experiments, 4 groups of 16 male BalbC mice (10-12 g) were used. Three groups received either a solution of ferrous iron (150 mg/kg per day po) or ferric iron (75 or 150 mg/kg per day po - Lipofer®) daily for 6 wk. The 4th group received water. 2,4,6 Trinitrobenzene sulfonic acid (TNBS) colitis was induced the last week of the experiment on half of each group of animals (n = 8/subgroup). The other half served as control (sham colitis). In the second series of experiments, 16 male Wistar rats (75-100 g) were used. The animals were separated in two groups receiving either ferric iron (Lipofer®) at the dose of 75 mg/kg per day po or water under the same conditions for 6 wk. Colitis was also induced during the last week of the experiment. Half of each group of animals were submitted to TNBS colitis (n = 8), the other half (n = 8) served as a control (sham colitis). Body weight was monitored throughout the experiments.

Experimental colitis

Among the chemically induced experimental colitis[15], TNBS in 50% ethanol is one of the classical models because this mix induces a barrier break resulting in severe colitis with penetrating ulcers, a reduced colon length and thickening of the colon wall as observed in IBD patients[16]. Mice were fasted overnight prior to induction of colitis but were allowed free access to water. They were anesthetized with a mixture (50% v/v) of ketamine and xylazine (100 mg/mL) diluted in saline (NaCl 0.9%-w/v) at a dose 1 mL/kg ip. TNBS (Sigma Aldrich, France) diluted in 50% of ethanol (v/v) was injected *via* a polyethylene catheter inserted at 4 cm from the anus at the dose of 100 mg/kg in 25 μL to induce an experimental colitis. Control mice were also anesthetized and received an equal volume of saline. Following instillation mice were maintained in a head-down position for 2 min and received 0.2 mL sc of saline to prevent dehydration. Their awakening was closely monitored. Mice were sacrificed 7 d later. Rats were anesthetized with the same mixture of anesthetics at the dose of 5 mL/kg ip and the solution of TNBS was administered at 7 cm from the anus at the dose of 40 mg/kg in 100 μL. Saline (0.5 mL sc) was administered to prevent dehydration. Rats were sacrificed 7 d later. At sacrifice, macroscopic lesions were evaluated and pieces of proximal colon (1 cm from the caecocolonic junction) and caecal content were collected, snap frozen and stored at -80 ℃ until further evaluation in both rat and mice experiments.

Assessment of the inflammatory reaction

Macroscopic damage scores: After sacrifice, the colon was removed immediately and severity of colonic mucosal alteration was determined according to a modified scale of Wallace *et al*[17]. Briefly, determination of the inflammatory damage was based on the presence of mucosal hyperaemia and bowel wall thickening, presence and extent of ulceration and necrosis, and the event of adhesions and diarrhea. Final quotation was ranging from 0 (normal appearance) to 10 (severe damage).

Myeloperoxidase assay: Myeloperoxidase (MPO) activity, a marker of polynuclear neutrophils, was measured in pieces of colon adjacent to the instillation point as described previously[18]. Briefly, frozen pieces of the proximal colon were homogenised in a phosphate buffer (50 mmol/L, pH = 6) containing hexadecyl trimethyl ammonium bromide (0.5% w/v) with a tissue lyser Ⅱ (Qiagen, France). The homogenates were submitted to 3 cycles of freezing/thawing (Liquid N2, 1 min/37 ℃, 10 min) and then further disrupted with a sonicator (Bioblock scientific, France) and then centrifuged (6000 *g* at 4 ℃ for 15 min). Supernatants were collected for measuring MPO activity and total protein contents. Samples were diluted into a reaction buffer containing O- dianisidine dihydrochloride (1 mg/mL) and hydrogen peroxide (3×10^{-4} % v/v). Human MPO from purified neutrophils was used as a standard. The absorbance was measured after 10 min of incubation at 450 nm. Total protein content was assessed from the supernatants according to Lowry's method (Bio Rad DC Protein Assay, France).

Real time quantitative polymerase chain reaction for microflora quantification

As we planned to assess microflora DNA from caecal content as well as comparing two species to validate the repeatability, we aimed at optimizing the bacterial DNA extraction by improving the extraction procedure of Gram positive bacteria and by determining the appropriate amount of the initial sample to treat.

DNA extraction: Total DNA from caecal samples (25, 50, 100 and 200 mg content) was extracted using the Qiamp DNA stool Mini Kit (Qiagen, France). Before proceeding according to the manufacturer's recommendations, frozen samples were lysed in an ASL lysis buffer and incubated for 3 consecutive cycles of freezing/thawing (Liquid N2, 1 min/37 ℃, 10 min). The lysates were clarified by centrifugation (14 000 *g* at 4 ℃ for 3 min). Polymerase chain reaction (PCR) inhibitors and impurities were absorbed by action of an inhibitex tablet (Qiagen). The supernatants were collected following a second centrifugation and DNA was automatically purified in the Qiacube automat (Qiagen,

Table 1 Primers used for real-time polymerase chain reaction amplification of *16S rRNA* gene

PCR assay	Primers	Primers Sequences 5'→3'	Accession number (NCBI)	Linear regression curves with coefficient of correlation	Sources of reference
Total Bacteria	UnivF	TCCTACGGGAGGCAGCAGTG	-	Y = -2.941 x + 36.580	Watanabe *et al*[21], 2001
	UnivR	TTACCGCGGCTGCTGGCACG		r^2 = 0.9997	Nadkarni *et al*[22], 2002
Bacteroidetes	BacF	CCTWCGATGGATAGGGGTT	-	Y = -2.908 x + 35.839	Firmesse *et al*[23], 2008
	BactR	TCCCCAGGTGGAATACTTAACG		r^2 = 0.9995	
Enterobacteria	EntF	CATTGACGTTACCCGCAGAAGAA	AX110239/AX109631	Y = -3.192 x + 36.762	This study
	EntR	CGCTTGCACCCTCCGTATTA	AF293850/U26176	r^2 = 0.9747	
Firmicutes	FirmF	ACCCGCGTCTGATTAGCTAGTT	M59090/L34627	Y = -3.298 x + 39.136	This study
	FirmR	CCTCTCAGGCCGGCTACTG	Y10584/FJ345661	r^2 = 0.9924	

Primers sequences were designed using the following website: www.ncbi.nlm.nih.gov/BLAST/. PCR: Polymerase chain reaction; NCBI: National Center for Biotechnology Information.

France) using Qiamp Minispin columns. Concentrations were determined on a Hellma TrayCel using a Biophotometer (Eppendorf, France). Absorbance ratios at 260/280 and at 260/230 were determined to quantify and assess the purity of DNA samples.

Plasmids for standard curves: Competent DH10b cells were used for the cloning experiments described previously[19]. The recombinant plasmids containing specific *16S rRNA* gene inserts either from Firmicutes, or Bacteroidetes, or enterobacteria or a consensus sequence for total microflora, were purified using a HiSpeed Plasmid Maxi kit (Qiagen, France). DNA concentration was determined following measurement of optical densities both at 260 nm and 280 nm before converting it into *16S rRNA* gene copy numbers as described previously[19]. Standard curves were established from serial dilutions of recombinants plasmids performed using real-time quantitative PCR. Copy numbers of the plasmid were calculated following a previously established equation [Copy numbers = 6.02 × 10^{23} (copy/mol) × DNA amount (g)/DNA length (dp) × 660 (g/mol per dp)][20].

Real time polymerase chain reaction: Two pairs of primers (enterobacteria and Firmicutes) corresponding to specific bacterial regions targets within *16S rRNA* gene were designed (Table 1). The two other pairs used (Total Flora and Bacteroidetes) had already been designed[21-23]. qPCR was performed using an ABI Prism 7300 sequence detector system (Applied Biosystems, France) on both plasmids and samples extracted DNA. Reactions were performed in duplicate using the Sybr Green PCR master mix (Qiagen, France) in a final volume of 25 μL with 0.3 μmol/L final concentration of each primer and appropriate dilutions of DNA samples. Amplification was initiated at 95 ℃ for 3 min to activate Taq plus DNA polymerase followed by 40 cycles at 95 ℃ for 3 s and 61 ℃ for 30 s. Two consecutive tenfold series of dilutions were realized to verify the linearity (slope of -3.32). A melting step was added and curves were analysed to look for any unspecific amplification. Standard curves were obtained following amplification under similar conditions of different samples containing different numbers of copies from the respective specific clones of the targeted gene. PCR efficiency (E) was calculated according to the equation from the standard curve: E = 10 ˆ(1/ slope) -1 according to Ibekwe and Grieve[24]. qPCR was realised using the cycle number threshold (Ct) and was based on the calculated standard curves. Each qPCR assay systematically included control reactions performed in parallel to the samples.

Statistical analysis

Results were expressed in mean ± SD error to the mean. Macroscopic lesions scores were compared using the Wilcoxon test for non parametric data followed by the Dunn post-test. For all the others parameters, data were submitted to an ANOVA followed by the Tuckey post-test. A value of $P < 0.05$ was considered to be significant.

RESULTS

Optimization of DNA isolation and quantification of microflora for realising interspecies comparison

Because of high inter- and intra-species variations, we aimed at improving the DNA extraction steps by determining the best amount of caecal content to use and by adding the preliminary step to improve the extraction of Gram positive bacteria. The best yield of DNA extraction was obtained when using 100 mg of caecal content and by adding a preliminary thermic lysis step (data not shown). Specificity of the *16S rRNA* gene targeted primers used for qPCR was tested both in silico and using pure DNA extracts from specific target strains (positive controls) and by confronting it to no target species (negative controls). Selected primers (Table 1) were run and the specificities of the amplification products were confirmed by the analysis of the dissociation curve in both caecal samples and DNA controls. We thus determined that a unique target gene was amplified for each species. Analysis of the regression curve for Ct values obtained from serial dilution of DNA samples showed a linear correlation for all target DNA regions; for all the microbiota species studied, the coefficient of correlation (r^2) was higher than 0.97 and amplification efficiencies were between 100 % and 120 % which involves curve slopes ranging from -3.29 to -2.9 (Table 1). All below mentioned analyses have been performed under those conditions.

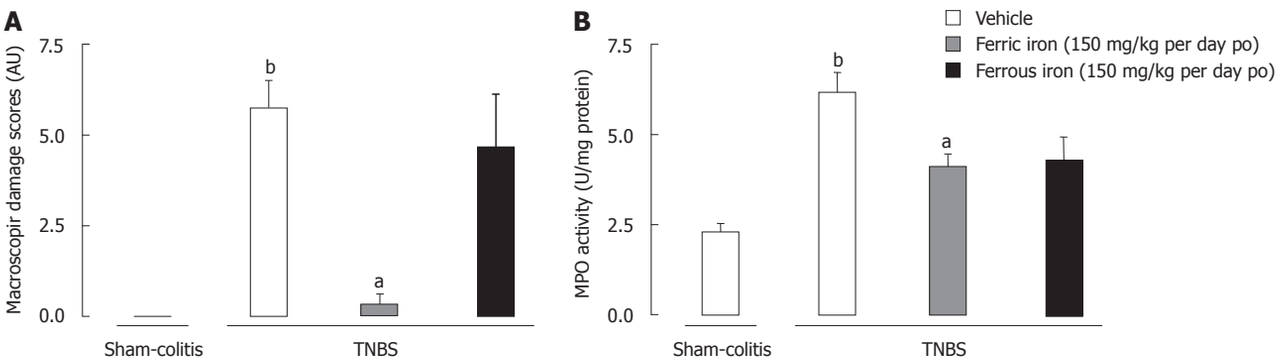

Figure 1 Effect of a 6 wk juvenile ingestion of ferrous (150 mg/kg per day po) *vs* ferric (150 mg/kg per day po) iron on the inflammatory response at adulthood to a trinitrobenzene sulfonic acid-induced colitis in mice. A: Macroscopic damage scores in non-inflamed (sham-colitis) and inflamed (trinitrobenzene sulfonic acid, TNBS) mice; B: Colonic myeloperoxidase (MPO) activity in non-inflamed (sham-colitis) and inflamed (TNBS) mice. Data are expressed as mean ± SE (n = 8 per group). [a]$P < 0.05$ *vs* TNBS-treated group, [b]$P < 0.01$ *vs* Sham-colitis.

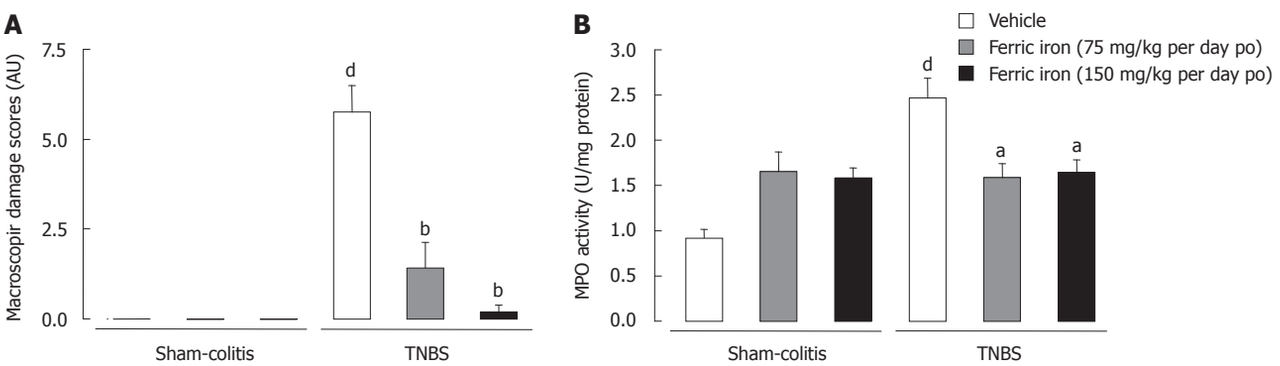

Figure 2 Effect of a 6 wk juvenile ferric iron administration (75 and 150 mg/kg per day po) on the inflammatory response at adulthood to a trinitrobenzene sulfonic acid-induced colitis in mice. A: Macroscopic damage scores in non-inflamed (sham-colitis) and inflamed trinitrobenzene sulfonic acid (TNBS) mice; B: Colonic myeloperoxidase (MPO) activity in non-inflamed (sham-colitis) and inflamed (TNBS) mice. Data are expressed as mean ± SE (n = 8 per group). [a]$P < 0.05$, [b]$P < 0.01$ *vs* TNBS-treated group, [d]$P < 0.01$ *vs* Sham-colitis.

Effects of ferric iron on TNBS colitis

Repeated daily ingestion of ferrous iron (150 mg/kg per day po - 6 wk) by juvenile mice did not prevent the induction of a TNBS-induced moderate colitis. In fact, juvenile ferrous iron daily exposure failed to limit macroscopic lesions (Figure 1A) in TNBS treated mice. In contrast, juvenile ferric iron prevented these lesions (Figure 1A) in inflamed mice. Furthermore, while TNBS enema resulted in an increased MPO activity in controls (2.47 ± 0.22 MPO U/mg protein *vs* 0.91 ± 0.09 MPO U/mg protein), we did not observe any significant reduction of MPO activity in mice exposed to a repeated ingestion of ferrous iron (1.71 ± 0.27 MPO U/mg protein *vs* 2.47 ± 0.22 MPO U/mg protein in the TNBS group) (Figure 1B). In contrast, the daily exposure of juvenile mice to the same dose of ferric iron (150 mg/kg per day po - 6 wk) was able to limit the inflammatory response since MPO activity was significantly lower ($P < 0.05$) (1.64 ± 0.14 MPO U/mg protein *vs* 2.47 ± 0.22 MPO U/mg protein in the TNBS group) (Figure 1B). Furthermore, we observed that before inducing TNBS colitis (5 wk of treatment), mice treated with ferrous iron put on significantly ($P < 0.05$) less weight than mice treated with ferric iron and controls (124.6% ± 1.12% and 136.6% ± 1.33 % *vs* 143.7% ± 2.29 % in control, respectively).

Ferric iron juvenile supplementation and dysbiosis

We also aimed at determining the dose-response effect of ferric iron supplementation on the same model of colitis in mice.

In control animals, the repeated administration of both 75 and 150 mg/kg per day po (6 wk) of ferric iron did not result in any alteration of growth, nor did it result in colonic inflammation or an alteration of the microflora profile. In fact, weight gain under iron treatment remained similar whichever species considered (142.7% ± 2.66% for 75 mg/kg per day and 136.6% ± 1.33 % for 150 mg/kg per day *vs* 143.7% ± 2.29 % in controls). Similarly, no macroscopic alterations of the gut mucosa have been observed on mice following either the low or the high chronic ferric supplementation (Figure 2A). Those results were correlated to low levels of MPO activity in the colonic mucosa following exposure to both doses of ferric iron (1.65 ± 0.21 MPO U/mg protein and 1.58 ± 0.11 MPO U/mg protein for respectively 75 and 150 mg/kg per day ferric iron po *vs* 0.91 ± 0.09 MPO U/mg protein in controls) (Figure 2B). Daily consumption of ferric iron by juvenile rodents did not modify the bacte-

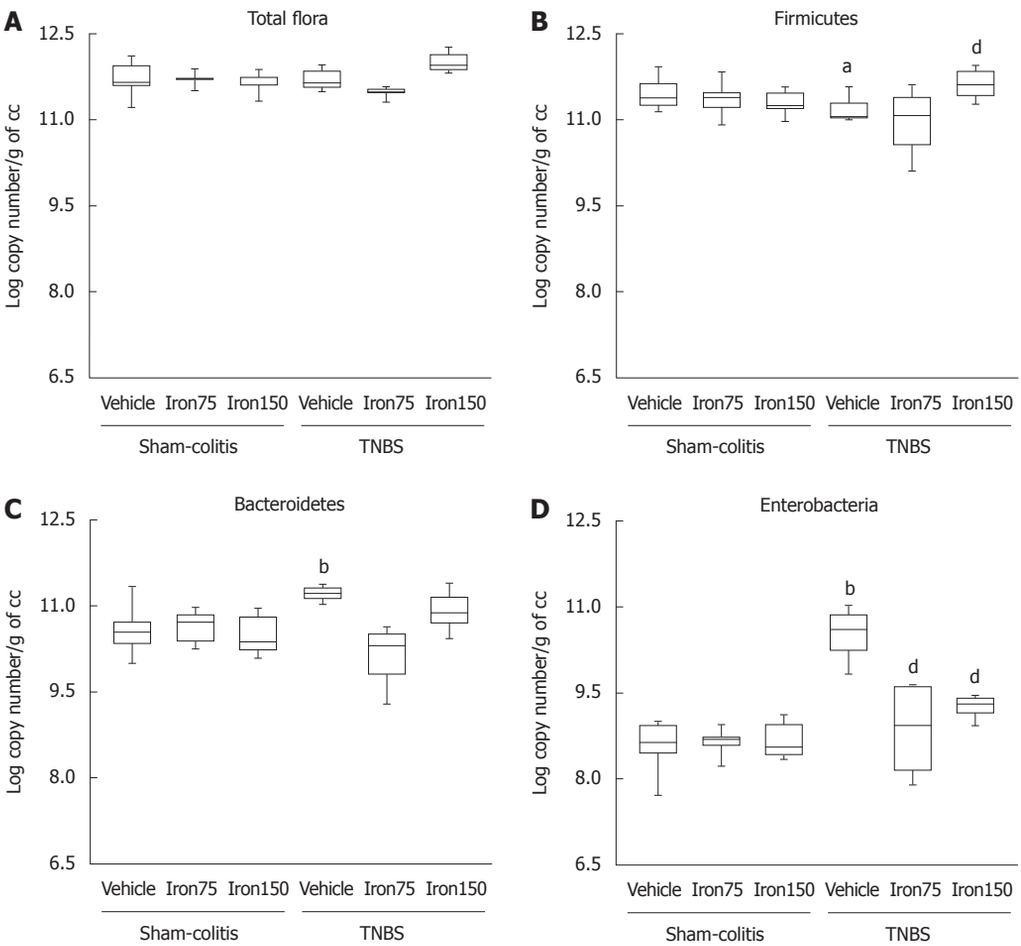

Figure 3 Effect of a juvenile ferric iron administration for 6 wk on the microflora profile of mice and its evolution consecutively to a trinitrobenzene sulfonic acid-induced colitis. A: Total Flora; B: Firmicutes; C: Bacteroidetes; D: Enterobacteria. Iron75: Ferric iron 75 mg/kg/d po - 6 wk; Iron150: Ferric iron 150 mg/kg per day po - 6 wk. Data are expressed as mean ± SE (n = 8 per group). [a]$P < 0.05$, [b]$P < 0.01$ *vs* Sham-colitis; [d]$P < 0.01$ *vs* TNBS-treated group.

ria profile since the total flora number remained at circa 11.8 log copy number/g of caecal content (Figure 3A). The Firmicutes number was estimated to be around 11.4 log copy number/g of caecal content (Figure 3B); The Bacteroidetes number remained around 10.5 log copy number/g caecal content (Figure 3C); and enterobacteria levels at 8.7 ± 0.1 log copy number/g of caecal content (Figure 3D).

Daily administration of both the doses of ferric iron on juvenile rodents prevented the TNBS-induced colitis at adulthood. In fact, exposure to ferric iron supplementation before inducing an experimental colitis limited the onset of macroscopic lesions (1.42 ± 0.72 AU and 0.2 ± 0.2 AU *vs* 5.75 ± 0.75 AU in TNBS-treated mice) (Figure 2A) and the increase of colonic MPO activity (1.58 ± 0.15 MPO U/mg protein and 1.64 ± 0.14 MPO U/mg protein *vs* 2.47 ± 0.22 MPO U/mg protein in TNBS-treated mice) (Figure 2B). The limitation of the inflammatory lesions was correlated to the maintenance of a healthy microflora profile. This supplementation also prevented the decrease of the Firmicutes (Figure 3B) and increase of the Bacteroidetes and enterobacteria populations (Figure 3C and D). Since efficiency was observed with the dose of 75 mg/kg per day po for 6 wk, we chose to continue with this dose of ferric iron.

Juvenile exposure to ferric iron comparably prevents a moderate TNBS-induced colitis in both rats and mice

Good comparisons need to be performed under the same conditions necessitating the induction of a comparable inflammatory reaction that is representative of pathophysiological conditions observed in humans. Using the optimised technique of extraction and quantification of DNA, total flora in control rats and mice was estimated to be around 11.8 log copy number/g of caecal content in control animals (Figures 3A and 4A). We also noticed that it is mainly composed of Firmicutes (around 11.3 log copy number/g of caecal content in both species) (Figures 3B and 4B) and Bacteroidetes (around 10.5 ± log copy number/g caecal content) (Figures 3C and 4C) followed by an enterobacteria population ranging from 8.6 ± 0.1 log copy number/g of caecal content in mice to 9.4 ± 0.1 log copy number/g of caecal content in rats (Figures 3D and 4D).

Instillation of TNBS resulted in a significant ($P <$ 0.05) increase of macroscopic damage scores in rats (8.33 ± 0.35 AU) and mice (5.75 ± 0.75 AU) (Figures 2A and 5A) as well as a significant ($P < 0.05$) increase of MPO activity in rats (773.7 ± 36.62 MPO U/mg protein *vs* 496.1 ± 63.94 MPO U/mg protein) and mice (2.47 ± 0.22 MPO U/mg protein *vs* 0.91 ± 0.09 MPO U/mg protein)

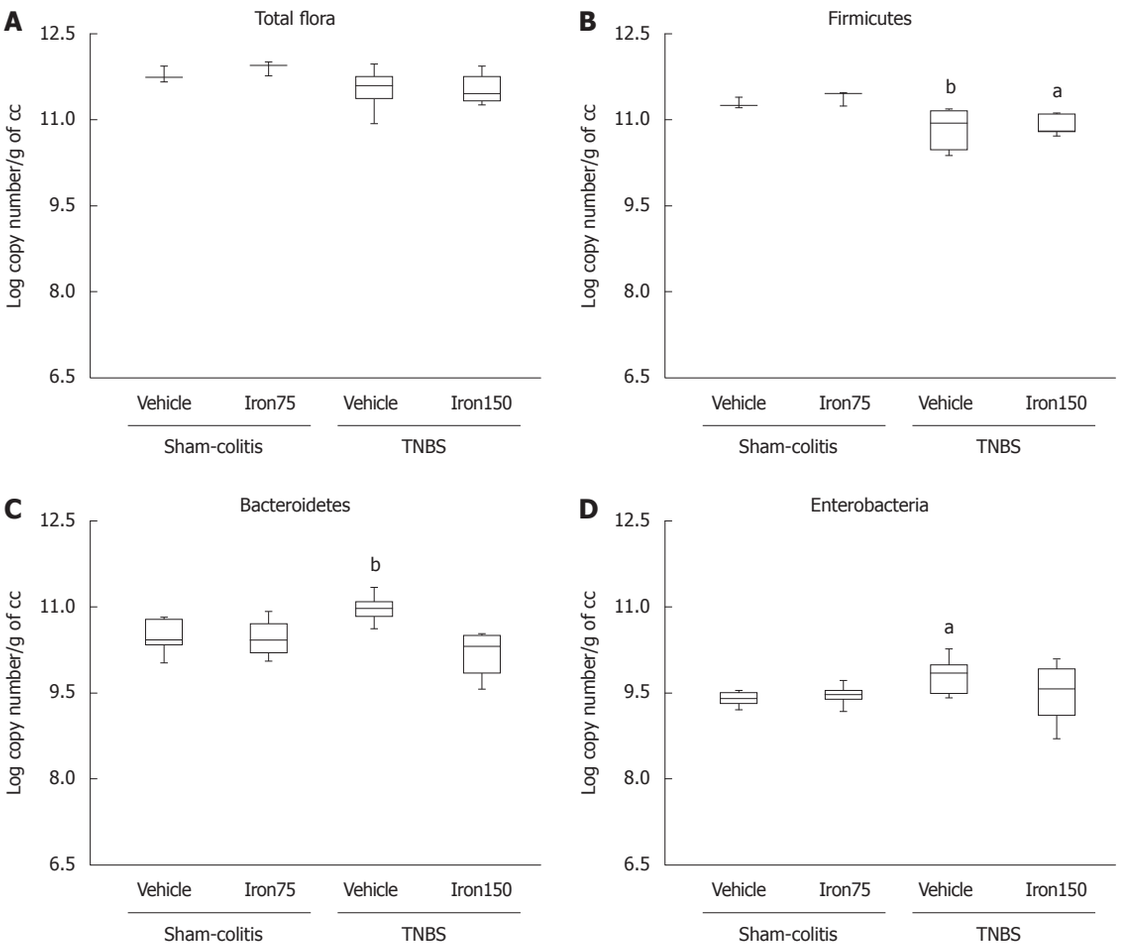

Figure 4 Effect of a 6 wk juvenile ferric iron administration (75 mg/kg per day po) on the microflora profile of rats and its evolution consecutively to a trinitrobenzene sulfonic acid colitis. A: Total Flora; B: Firmicutes; C: Bacteroidetes; D: Enterobacteria; Iron75: Ferric iron 75 mg/kg per day po - 6 wk; Iron150: Ferric iron 150 mg/kg per day po - 6wk. Data are expressed as mean ± SE (n = 8 per group). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Sham-colitis.

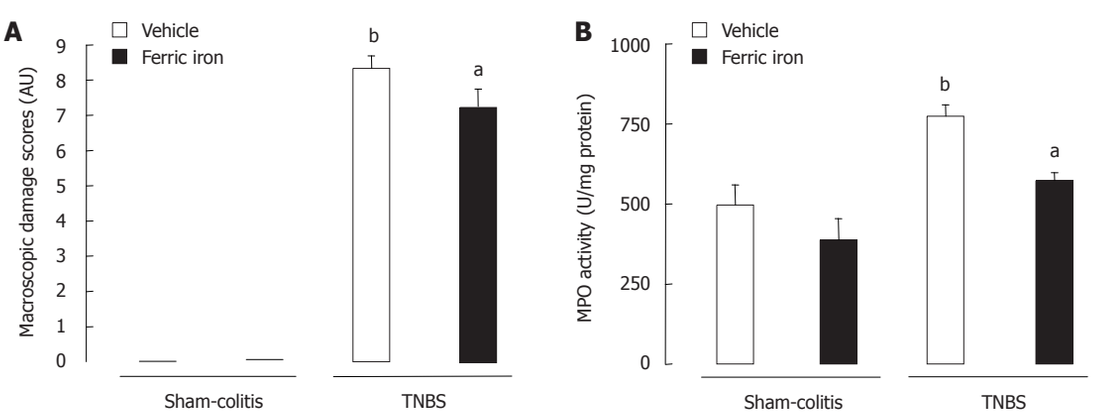

Figure 5 Effect of a 6 wk juvenile ferric iron supplementation (75 mg/kg per day po) on the inflammatory response at adulthood of rats submitted or not (sham-colitis) to a trinitrobenzene sulfonic acid-induced colitis. A: Macroscopic damage scores (MDS); B: Myeloperoxidase (MPO) activity Data are expressed as mean ± SE (n = 8 per group). $^{a}P < 0.05$ *vs* trinitrobenzene sulfonic acid (TNBS)-treated group, $^{b}P < 0.01$ *vs* Sham-colitis.

(Figures 2B and 5B). However, we did not observe any significant modification of total microflora neither in rats nor in mice 7 d after TNBS instillation (circa 11.7 log copy number/g of caecal content in both rodent species) (Figures 3A and 4A). However, the balance between the two major phyla of the bacterial population observed was altered. A significant reduction ($P < 0.05$) of Firmicutes was registered in both colitic rats (-0.4 Δ log copy number) and mice (-0.3 Δ log copy number) (Figures 3B and 4B) as compared to controls. Conversely, significant higher levels of Bacteroidetes ($P < 0.05$) were detected in both the colitic groups; it increased by 0.5 Δ log copy

number in rats and by 0.7 Δ log copy number in mice (Figures 3C and 4C). Enterobacteria numbers were also found to be significantly higher ($P < 0.05$) in colitic rats and mice with increases of respectively 0.4 and 2 Δ log copy number/g of caecal content as compared to controls (Figures 3D and 4D).

Juvenile ferric iron supplementation for 6 wk, before inducing an experimental colitis, significantly limited the onset of macroscopic lesions not only in TNBS-treated mice (1.42 ± 0.72 AU *vs* 5.75 ± 0.75 AU) but also in TNBS-treated rats (7.25 ± 0.49 AU *vs* 8.33 ± 0.35 AU). Juvenile iron administration also significantly ($P < 0.05$) prevented the increase of colonic MPO activity in TNBS-treated mice (1.59 ± 0.5 MPO U/mg protein *vs* 2.47 ± 0.22 MPO U/mg protein) as well as in TNBS-treated rats (572.5 ± 26.39 MPO U/mg protein *vs* 773.7 ± 36.63 MPO U/mg protein) (Figures 2B and 5B). This juvenile ferric administration (75 mg/kg per day po - 6 wk) did not modify total bacteria number even when inducing colitis (Figure 3A and 4A). Juvenile ferric iron repeated administration before inducing the colitis not only prevented the increase of the Bacteroidetes population (Figures 3B and 4B) but also the enterobacteria population (Figures 3D and 4D) in both mice and rats.

DISCUSSION

When using a microencapsulated ferric pyrophosphate form (Lipofer®), we were the first to evidence a beneficial effect of ferric iron oral supplementation in juvenile animals to prevent the induction of colitis at adulthood. Ferric pyrophosphate is a water insoluble compound, marketed as a food additive in Europe to fortify infant cereals and chocolate powder drinks[25]. Because of its chemical composition, it has been microencapsulated to improve both its bioavailability and its incorporation in food to be fortified[26], rendering it a good alternative to the ferrous forms without having any of the side effects.

First, we provided evidence of the benefits of ferric iron supplementation during the juvenile period. Iron oral administration is recommended in young infants to prevent anemia and its consecutive neurologic and developmental deficits. Iron also has benefits, as a trace element, in supporting immune function by strengthening not only epithelial barriers but also cellular and humoral immune responses. However, ferrous iron may be at the origin of deleterious effects such as lipid peroxydation[27] or enterobacteria growth facilitation[2]. Here, with ferric iron, we did not observe any negative effect of a repeated administration for 6 wk. In fact, in non-inflamed mice, juvenile ferric iron ingestion did not result in either an inflammatory reaction or did it modify the microflora profile since both total bacteria and enterobacteria numbers remained unchanged as compared to non supplemented sham-colitic mice. This absence of action on gut microbiota profile is in favour of the ferric form instead of the ferrous form for infant food fortification.

Secondly, we aimed at evaluating the dose response effect of ferric iron supplementation in the juvenile period the induction of an experimental colitis at adulthood. Environmental factors such as smoking, luminal enteric bacteria and trace elements such as iron are involved in the pathogenesis of IBD in a genetically susceptible host[28]. In fact, anemia is often described during chronic gut inflammatory disorders such as IBD[29]. This anemia is caused by two factors, the first being impaired proliferation and differentiation of erythroid progenitor cells, and the second being consecutive to iron retention within monocytes and macrophages both activated under the inflammatory conditions. Classically, patients receive either oral or systemic iron supplementation generally as ferrous sulfate or fumarate. However, ferrous iron oral administration often aggravates the inflammatory reaction because of its accumulation in the intestinal lumen and its participation in the Haber-Weiss reaction[27]. Compared to non supplemented mice, ferric iron juvenile supplementation proved to be efficient against this experimental colitis, even at the lowest dose used since their MPO activity levels were similar to those of control mice. The lowest dose of iron used corresponded to an overall daily intake of 1.2 mg iron per day, in other words to 15 μg iron/g mouse/day which is two times lower than the dose used by Werner *et al*[30] but it is however efficient in our model too.

The positive effect of iron supplementation in mice was corroborated by the results obtained in rats. In our study, we were able to demonstrate that ferric iron oral administration did not induce any alteration but rather prevented the course of an experimental colitis in both rodent species. In fact, both rats and mice submitted to iron supplementation did not develop any sign of inflammation. In both those rodent species, we also induced an experimental inflammatory reaction of comparative intensity. In fact, in comparison to the literature[31], they developed a moderate but homogenous colitis characterised by tissular lesions, ulcerations of the distal colon and neutrophil infiltration. These results are in agreement with a previous report indicating that the TNBS-induced colitis model is associated to an increase of inflammatory response including granulomas and tissular MPO activity[31-33].

Finally, we aimed at evaluating the reproducibility of colonic microflora evaluation under control conditions and its alteration during a moderate experimental colitis in rats and mice. This study is the first to evidence a comparative alteration of gut microbiota during an experimental colitis in two rodent species. Following the qPCR optimisation process described above, we observed no difference in total microbiota numbers between control rats and mice groups. In this work, we also found that caecal microflora of rodents is composed predominantly of Firmicutes followed by Bacteroidetes which profile correlates to the observations realised on humans. In fact, human Firmicutes population is 10 times higher than their Bacteroidetes population; which is considered to be a good indicator of health status[34,35]. In this study, we observed the same ratio profile not only in mice but also in rats. In non supplemented animals and under

inflammatory conditions, we did not observe any alteration of total bacteria numbers in both the rodent species. This is in agreement with literature which describes no principal differences in the composition of the total mucosal flora in IBD patients compared to controls[36]. Furthermore, while tissular healing might have already started in the animals, in both murine models we observed a net increase of Bacteroidetes and a reduction of the Firmicutes population indicating a clear reversion of this profile. Similar trends were also observed in clinical studies, showing a lower representation of Firmicutes phyla during inflammatory acute phases[34,37]. As already evidenced, the diminution of the Firmicutes phylum promotes the development and the invasion of tissues by opportunistic bacteria species and the gut becomes very susceptible to invading pathogens among which enterobacteria[38,39]. We also noted, under colitic conditions, a higher level of enterobacteria which is concordant with literature reporting a higher incidence of *Escherichia coli* in IBD patient compared to healthy subjects[40,41]. We chose to work in comparing rat and mice microbiota alteration during this moderate experimental colitis to ensure that not only could we obtain comparative results to human dysbiosis but also to make sure that the results obtained were linked to the intensity of the inflammatory response rather that to species sensitivity. Such comparisons are of great interest in testing the reproducibility of the method set up. Overall, our results are correlated to clinical observations realised on IBD patients such as fever, diarrhea, weight loss, rectal bleeding[42], and severe alteration of the microbiota equilibrium, especially with Bacteroidetes and enterobacteria increases in opposition to Firmicutes reduction[9,10]. In both rats and mice receiving ferric iron in the juvenile period for 6 wk the microbiota profile was not altered. In addition, rats and mice submitted to an experimental colitis during the last week of iron treatment did not display any modification of either total number bacteria or enterobacteria numbers as compared to non supplemented animals. This is in favour of a positive role of ferric iron onto gut microbiota equilibrium which will limit the onset of the inflammatory reaction as compared to classical ferrous forms. It reinforces the idea that iron is one of the key markers for limiting the onset of the inflammatory response in genetically susceptible patients[30]. Furthermore, we may suggest that ferric iron in contrast to ferrous iron contributes to the settlement of an appropriate microflora during the post natal period and that this profile is less sensitive to inflammatory stimuli. One cannot ascertain the mechanisms producing such results, but we may suggest that the lower incidence of ferric iron on colitis might be linked to, either a lower susceptibility to stimulate oxidative stress reactions as compared to ferrous iron, or to its interactions with the immune system which is partly driving an appropriate microbiota implantation during the perinatal period. Rather than working with animals whose microflora profile had been modified because they display a modified immune pattern[43,44], we decided to work on two rodent species of the same age, gender and submitted to a similar level of an inflammatory stimulus Since this microbiota/immune system interaction is said to be species, gender and age specific.

In conclusion, this study shows comparative rodent dysbiosis to human IBD dysbiosis following a moderate TNBS colitis. It also shows the benefits of ferric iron oral ingestion during the juvenile period in the prevention of an experimental colitis induced at adulthood in healthy animals. These interesting results would necessitate checking how anemic juvenile animals would react to such a treatment and especially to an induced colitis at adulthood. To further understand this incidence of ferric iron on overall intestinal functionalisation, one point we did not address is the role of the immune system and particularly its fine orientation modulation by iron fortification during the perinatal period in regards to the observed effects. We may hypothesize that iron could participate in reinforcing the immune system orientation, thus contributing to the limitation of the inflammatory response due to TNBS. This question will be addressed in another set of experiments. If these results are confirmed, this form of microencapsulated ferric iron could thus be clinically assessed as an interesting alternative to iron sulfate in young individuals at risk of anemia but also in subjects at risk of chronic gut inflammation such as CD.

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COMMENTS

Background

Juvenile iron supplementation is now widely accepted to limit the risk of anemia. However, oral iron ingestion may have side effects especially at this period when the crosstalk between bacteria, epithelium and the immune system is being set up. In fact, an impaired settlement of this crosstalk may predispose to chronic diseases such as inflammatory bowel disease (IBD). No one has so far evaluated the consequences of food matrix composition during the postnatal period on the predisposition to develop chronic diseases at adulthood. This study was thus aimed at evaluating the consequences of juvenile chronic iron exposure on the onset of experimental colitis and its related dysbiosis in both rats and mice at adulthood.

Research frontiers

In these recent years, research on the role of commensal microbiota has evidenced its strong impact on digestive physiology and particularly its modulating role in IBD. Moreover, recent data evidenced that microbiota implantation in the perinatal period conditions, the maturation of the gut mucosa and the immune system renders the host tolerant to commensal bacteria. Since oral ferrous iron may promote potential pathogenic bacteria growth, which may condition the development of chronic gut inflammation, the authors evaluated in this study the modulation by repeated juvenile administration of ferric iron on the onset of an experimental colitis at adulthood in two rodent species.

Innovations and breakthroughs

Literature largely describes the necessity to maintain a good equilibrium of the microbiota to stay healthy. Food matrix also conditions the diversity and stability of the microbiota especially during the postnatal period crucial for these aspects. This study is the first to evidence the preventive effects of oral ferric iron administration at the juvenile period on the inflammatory response and dysbiosis related to an experimental colitis at adulthood in two rodent species.

Applications

This study proposes to evaluate the consequences of oral supplementation at

infancy on risk of developing gut inflammation at adulthood. These results necessitate being completed by the evaluation of the consequences of ferric iron on the immune system maturation and to be clinically proven but they could represent a good opportunity for families at risk of developing IBD.

Terminology

IBD comprises chronic inflammations of the gut and more especially Crohn's Diseases and Ulcerative Colitis. Microbiota designates the pool of microorganisms harboured in our gut under physiological conditions. They divide into dominant and subdominant and control each other's growth to reach dynamic equilibrium. Dysmicrobism is an alteration of the microbiota equilibrium. Iron is a metal. In aqueous solution, it exists in two oxidation states: ferrous (Fe^{2+}) and ferric (Fe^{3+}).

Peer review

The study aimed to study rodent dysbiosis and compare it to human IBD dysbiosis following a moderate trinitrobenzene sulfonic acid colitis. It shows the benefits of ferric iron oral ingestion during the juvenile period in the prevention of an experimental colitis induced at adulthood. The paper is well written and has potential application in food supplemental.

REFERENCES

1 **Ziegler EE**, Nelson SE, Jeter JM. Iron supplementation of breastfed infants from an early age. *Am J Clin Nutr* 2009; **89**: 525-532

2 **Zimmermann MB**, Chassard C, Rohner F, N'goran EK, Nindjin C, Dostal A, Utzinger J, Ghattas H, Lacroix C, Hurrell RF. The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d' Ivoire. *Am J Clin Nutr* 2010; **92**: 1406-1415

3 **Choi YJ**, Im E, Chung HK, Pothoulakis C, Rhee SH. TRIF mediates Toll-like receptor 5-induced signaling in intestinal epithelial cells. *J Biol Chem* 2010; **285**: 37570-37578

4 **Sekirov I**, Russell SL, Antunes LC, Finlay BB. Gut microbiota in health and disease. *Physiol Rev* 2010; **90**: 859-904

5 **Eckburg PB**, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. *Science* 2005; **308**: 1635-1638

6 **Rajilić-Stojanović M**, Smidt H, de Vos WM. Diversity of the human gastrointestinal tract microbiota revisited. *Environ Microbiol* 2007; **9**: 2125-2136

7 **Cani PD**, Delzenne NM, Amar J, Burcelin R. Role of gut microflora in the development of obesity and insulin resistance following high-fat diet feeding. *Pathol Biol* (Paris) 2008; **56**: 305-309

8 **Blaut M**, Collins MD, Welling GW, Doré J, van Loo J, de Vos W. Molecular biological methods for studying the gut microbiota: the EU human gut flora project. *Br J Nutr* 2002; **87** Suppl 2: S203-S211

9 **Seksik P**, Rigottier-Gois L, Gramet G, Sutren M, Pochart P, Marteau P, Jian R, Doré J. Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon. *Gut* 2003; **52**: 237-242

10 **Rehman A**, Lepage P, Nolte A, Hellmig S, Schreiber S, Ott SJ. Transcriptional activity of the dominant gut mucosal microbiota in chronic inflammatory bowel disease patients. *J Med Microbiol* 2010; **59**: 1114-1122

11 **Willing B,** Halfvarson J, Dicksved J, Rosenquist M, Järnerot G, Engstrand L, Tysk C, Jansson JK. Twin studies reveal specific imbalances in the mucosa-associated microbiota of patients with ileal Crohn's disease. *Inflamm Bowel* 2009; **15:** 653-660

12 **Scarpa M**, Grillo A, Faggian D, Ruffolo C, Bonello E, D'Incà R, Scarpa M, Castagliuolo I, Angriman I. Relationship between mucosa-associated microbiota and inflammatory parameters in the ileal pouch after restorative proctocolectomy for ulcerative colitis. *Surgery* 2011; **150**: 56-67

13 **Manichanh C**, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, Nalin R, Jarrin C, Chardon P, Marteau P, Roca J, Dore J. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* 2006; **55**: 205-211

14 **Conte MP**, Schippa S, Zamboni I, Penta M, Chiarini F, Seganti L, Osborn J, Falconieri P, Borrelli O, Cucchiara S. Gut-associated bacterial microbiota in paediatric patients with inflammatory bowel disease. *Gut* 2006; **55**: 1760-1767

15 **Jurjus AR**, Khoury NN, Reimund JM. Animal models of inflammatory bowel disease. *J Pharmacol Toxicol Methods* 2004; **50**: 81-92

16 **Morris GP**, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL. Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology* 1989; **96**: 795-803

17 **Wallace JL**, Braquet P, Ibbotson GC, MacNaughton WK, Cirino G. Assessment of the role of platelet-activating factor in an animal model of inflammatory bowel disease. *J Lipid Mediat* 1989; **1**: 13-23

18 **Bradley PP**, Christensen RD, Rothstein G. Cellular and extracellular myeloperoxidase in pyogenic inflammation. *Blood* 1982; **60**: 618-622

19 **Vasquez N**, Suau A, Magne F, Pochart P, Pélissier MA. Differential effects of Bifidobacterium pseudolongum strain Patronus and metronidazole in the rat gut. *Appl Environ Microbiol* 2009; **75**: 381-386

20 **Whelan JA**, Russell NB, Whelan MA. A method for the absolute quantification of cDNA using real-time PCR. *J Immunol Methods* 2003; **278**: 261-269

21 **Watanabe K**, Kodama Y, Harayama S. Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. *J Microbiol Methods* 2001; **44**: 253-262

22 **Nadkarni MA**, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* 2002; **148**: 257-266

23 **Firmesse O**, Mogenet A, Bresson JL, Corthier G, Furet JP. Lactobacillus rhamnosus R11 consumed in a food supplement survived human digestive transit without modifying microbiota equilibrium as assessed by real-time polymerase chain reaction. *J Mol Microbiol Biotechnol* 2008; **14**: 90-99

24 **Ibekwe AM**, Grieve CM. Detection and quantification of Escherichia coli O157: H7 in environmental samples by real-time PCR. *J Appl Microbiol* 2003; **94**: 421-431

25 **Blanco-Rojo R**, Pérez-Granados AM, Toxqui L, González-Vizcayno C, Delgado MA, Vaquero MP. Efficacy of a microencapsulated iron pyrophosphate-fortified fruit juice: a randomised, double-blind, placebo-controlled study in Spanish iron-deficient women. *Br J Nutr* 2011; **105**: 1652-1659

26 **Fidler MC**, Walczyk T, Davidsson L, Zeder C, Sakaguchi N, Juneja LR, Hurrell RF. A micronised, dispersible ferric pyrophosphate with high relative bioavailability in man. *Br J Nutr* 2004; **91**: 107-112

27 **Buffinton GD**, Doe WF. Depleted mucosal antioxidant defences in inflammatory bowel disease. *Free Radic Biol Med* 1995; **19**: 911-918

28 **Perl DP**, Fogarty U, Harpaz N, Sachar DB. Bacterial-metal interactions: the potential role of aluminum and other trace elements in the etiology of Crohn's disease. *Inflamm Bowel Dis* 2004; **10**: 881-883

29 **Kulnigg S**, Gasche C. Systematic review: managing anaemia in Crohn's disease. *Aliment Pharmacol Ther* 2006; **24**: 1507-1523

30 **Werner T**, Wagner SJ, Martínez I, Walter J, Chang JS, Clavel T, Kisling S, Schuemann K, Haller D. Depletion of luminal iron alters the gut microbiota and prevents Crohn's disease-like ileitis. *Gut* 2011; **60**: 325-333

31 **Punkkinen J**, Konkka I, Punkkinen O, Korppi-Tommola T, Färkkilä M, Koskenpato J. Measuring gastric emptying: comparison of 13C-octanoic acid breath test and scintigraphy. *Dig Dis Sci* 2006; **51**: 262-267

32 **Elson CO**, Sartor RB, Tennyson GS, Riddell RH. Experimental models of inflammatory bowel disease. *Gastroenterology* 1995; **109**: 1344-1367

33 **Qiu W**, Wu B, Wang X, Buchanan ME, Regueiro MD, Hartman DJ, Schoen RE, Yu J, Zhang L. PUMA-mediated intestinal epithelial apoptosis contributes to ulcerative colitis in humans and mice. *J Clin Invest* 2011; **121**: 1722-1732

34 **Sokol H**, Seksik P, Furet JP, Firmesse O, Nion-Larmurier I, Beaugerie L, Cosnes J, Corthier G, Marteau P, Doré J. Low counts of Faecalibacterium prausnitzii in colitis microbiota. *Inflamm Bowel Dis* 2009; **15**: 1183-1189

35 **Mariat D**, Firmesse O, Levenez F, Guimarães V, Sokol H, Doré J, Corthier G, Furet JP. The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol* 2009; **9**: 123

36 **Swidsinski A**, Ladhoff A, Pernthaler A, Swidsinski S, Loening-Baucke V, Ortner M, Weber J, Hoffmann U, Schreiber S, Dietel M, Lochs H. Mucosal flora in inflammatory bowel disease. *Gastroenterology* 2002; **122**: 44-54

37 **Sokol H**, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux JJ, Blugeon S, Bridonneau C, Furet JP, Corthier G, Grangette C, Vasquez N, Pochart P, Trugnan G, Thomas G, Blottière HM, Doré J, Marteau P, Seksik P, Langella P. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci USA* 2008; **105**: 16731-16736

38 **Tlaskalová-Hogenová H**, Stepánková R, Hudcovic T, Tuckova L, Cukrowska B, Lodinová-Zádníková R, Kozáková H, Rossmann P, Bártová J, Sokol D, Funda DP, Borovská D, Reháková Z, Sinkora J, Hofman J, Drastich P, Kokesová A. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. *Immunol Lett* 2004; **93**: 97-108

39 **Stelter C**, Käppeli R, König C, Krah A, Hardt WD, Stecher B, Bumann D. Salmonella-induced mucosal lectin RegIIIβ kills competing gut microbiota. *PLoS One* 2011; **6**: e20749

40 **Curová K**, Kmetová M, Sabol M, Gombosová L, Lazúrová I, Siegfried L. Enterovirulent E. coli in inflammatory and non-inflammatory bowel diseases. *Folia Microbiol* (Praha) 2009; **54**: 81-86

41 **Thomazini CM**, Samegima DA, Rodrigues MA, Victoria CR, Rodrigues J. High prevalence of aggregative adherent Escherichia coli strains in the mucosa-associated microbiota of patients with inflammatory bowel diseases. *Int J Med Microbiol* 2011; **301**: 475-479

42 **Strober W**, Fuss I, Mannon P. The fundamental basis of inflammatory bowel disease. *J Clin Invest* 2007; **117**: 514-521

43 **Tlaskalova-Hogenova H,** Sterzl J, Stepankova R, Dlabac V, Veticka V, Rossmann P, Mandel L, Rejnek J. Development of immunological capacity under germfree and conventional conditions. *Ann N Y Acad Sci* 1983; **409:** 96-113

44 **Tlaskalová-Hogenová H**, Stěpánková R, Farré M, Funda DP, Reháková Z, Sinkora J, Tucková L, Horak I, Horáková D, Cukrowska B, Kozáková H, Kolínská J. Autoimmune reactions induced by gliadin feeding in germ-free AVN rats and athymic nude mice. Animal models for celiac disease. *Ann N Y Acad Sci* 1997; **815**: 503-505

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ORIGINAL ARTICLE

High dose glargine alters the expression profiles of microRNAs in pancreatic cancer cells

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Abstract

AIM: To investigate the effect of high dose glargine on the expression profiles of microRNAs in human pancreatic cancer cells.

METHODS: Real-time polymerase chain reaction array (RT-PCR) was applied to investigate miRNAs differentially expressed in Sw1990 cells treated with or without 100 IU/L glargine. Stem-loop RT-PCR was used to confirm the results of the array assay in Sw1990 and Panc-1 cells. The effects of miR-95 on cell growth, apoptosis, invasion and migration abilities were respectively examined by CCK8 assay, apoptosis assay, Matrigel invasion and migration assay in Sw1990 and Panc-1 cells. Nude mice xenograft models with Sw1990 cells were built to investigate pancreatic cancer growth *in vivo* after transfection by the lentivirus pGLV3-GFP- miR-95.

RESULTS: Ten miRNAs were significantly up-regulated and 2 miRNAs down-regulated in glargine treated Sw1990 cells when compared with non-treated cells (2.48-fold changes on average, $P < 0.01$). miR-95, miR-134 and miR-34c-3p are the top three miRNAs regulated by glargine (3.65-fold, 2.67-fold and 2.60-fold changes respectively, $P < 0.01$) in Sw1990 cells. Stem-loop RT-PCR confirmed that high dose glargine up-regulated the expression of miR-95 and miR-134 in both Sw1990 and Panc-1 cells. The most obvious change is the apparent increase of miR-95. Forced expression of miR-95 significantly increased cell proliferation (Sw1990: 2.510 ± 0.129 *vs* 2.305 ± 0.187, $P < 0.05$; Panc-1: 2.439 ± 0.211 *vs* 2.264 ± 0.117, $P < 0.05$), invasion (Sw1990: 67.90 ± 12.33 *vs* 47.30 ± 5.89, $P < 0.01$; Panc-1: 37.80 ± 8.93 *vs* 30.20 ± 5.14, $P < 0.01$), migration (Sw1990: 101 ± 6.00 *vs* 51.20 ± 8.34, $P < 0.01$; Panc-1: 91.80 ± 9.22 *vs* 81.50 ± 7.47, $P < 0.01$) and inhibited cell apoptosis (Sw1990: 22.05% ± 1.92% *vs* 40.32% ± 1.93%, $P < 0.05$; Panc-1: 20.17% ± 0.85% *vs* 45.60% ± 1.43%, $P < 0.05$) when compared with paired negative controls, whereas knockdown of miR-95 obtained the opposite effect. Nude mice xenograft models confirmed that miR-95 promoted the growth of pancreatic cancer *in vivo* when compared with negative control (tumor volume: 373.82 ± 23.67 mL *vs* 219.69 ± 17.82 mL, $P < 0.05$).

CONCLUSION: These observations suggested that modulation of miRNA expression may be an important mechanism underlying the biological effects of glargine.

Key words: Glargine; MicroRNAs; Pancreatic cancer; Lentivirus; Cancer growth

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INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related deaths in Western countries and has the poorest survival rate (< 5%) among the common malignancies[1,2]. Recently, antidiabetic therapies have been shown to affect the risk of pancreatic cancer. Some observational studies in humans have linked glargine with a putative increased cancer risk, including pancreatic cancer[3,4].

Glargine (A21Gly, B31Arg, B32Arg human insulin) is a widely used insulin analog in which a 24-h action profile is achieved by altering the amino acid sequence of the alpha (α) and beta (β) chains of the C terminus[5]. It has been shown that glargine increases resistance to apoptosis in several tumor cell lines[6]. Given the increased affinity to the insulin-like growth factor- I receptor (IGF-IR) *in vitro*[7], glargine may increase the bioavailability of IGF- I by altering the levels of IGF-binding proteins[8,9]. IGF- I is a more potent growth factor than insulin, promoting proliferation and inhibiting apoptosis, and plays an important role in facilitating malignant cell survival and metastasis[10,11]. This may be the theoretical basis for the potential carcinogenicity of glargine. However, data regarding the effect of glargine on pancreatic cancer are inconsistent. Administration of glargine didn't alter proliferation of Colo-357 pancreatic carcinoma cells and survival of patients with pancreatic carcinoma[12]. Thus, the role of glargine in pancreatic carcinogenesis deserves further investigation.

MicroRNAs (miRNAs) are endogenous, non-coding small RNAs, 19-25 nucleotides in length, which are now recognized as crucial post-transcriptional regulators of gene expression[13-15]. It has been demonstrated that miRNAs play important roles in biological processes that affect tumor progression including migration, invasion, epithelial to mesenchymal transition (EMT) and metastasis[16-18]. miRNAs are promising as early biomarkers, prognostic indicators and therapeutic targets for anticancer treatments[19-21]. Aberrant miRNA expression has also been frequently reported in pancreatic cancer[22]. However, very few compounds, not to mention glargine, which affect cell growth and/or development, have been shown to affect miRNA expression.

In this present study, we elucidated the miRNAs signature in response to glargine treatment in human pancreatic cancer cells. Our results indicated that glargine alters specific miRNA expression in human pancreatic cells, especially miR-95. The effect of miR-95 on apoptosis, proliferation, migration and invasion ability of pancreatic cancer cells were further investigated. Moreover, nude mice xenograft models were built to investigate pancreatic cancer growth *in vivo* after transfection by the lentivirus pGLV3-GFP-miR-95. It therefore appeared that miR-95-related changes were important effects of glargine.

MATERIALS AND METHODS

Cell lines and cultures

Pancreatic ductal cancer cell lines Sw1990 and Panc-1 were conserved in our own laboratory and were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO) with 10% fetal bovine serum (FBS; GIBCO) in a humidified incubator at 37 ℃ with an atmosphere of 5% CO_2.

miRNAs real time polymerase chain reaction array

Sw1990 cells (3×10^5 per well) were plated on 6-well plates in DMEM with 10% FBS. After 24 h of incubation at 37 ℃, the cells were treated with or without 100 IU/L glargine. Glargine was replenished every 24 h. The cultures were incubated for 2 d, then the total RNA was isolated from cell samples using Trizol reagent (Invitrogen) following the manufacturer's protocol. Then, cDNA synthesis was performed using Universal cDNA synthesis kit (Exiqon). The expression levels of 372 human mature miRNAs were examined using the miRCURY LNA™ Universal real time microRNA polymerase chain reaction system, Ready-to-use human panel I (Exiqon, kangchen, China).

Briefly, total RNA containing miRNA was polyadenylated, and cDNA was synthesized using a poly (T) primer with a 3'degenerate anchor and a 5'universal tag. Then, cDNA served as a template for microRNA quantitative real-time polymerase chain reaction (qPCR) using miRCURY LNA Universal RT miRNA PCR kit (Exiqon). The miRNA Ready-to-use human panel I is a 384-well PCR plate containing dried down LNA™ primer sets for one real-time PCR reaction per well. Three small RNA (U6snRNA, SNORD38B, SNORD49A) and three miRNA (miR-103, miR-191 and miR-423-5p) reference genes are included on the panel. The amplification profile was denatured at 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 10 s and 60 ℃ for 60 s. At the end of the PCR cycles, melting curve analyses were performed. All reactions were conducted three times. Expression levels of mature miRNAs were evaluated using comparative CT method ($2^{-\Delta CT}$).

Stem-loop real-time reverse transcription-PCR

The miRNAs (miR-95, miR-134 and miR-34c-3p) were quantitated by stem-loop real time reverse transcription (RT)-PCR to confirm the reliability of the miRNA array assay. In brief, Sw1990 and Panc-1 cells (3×10^5 per well) were seeded on 6-well plates in DMEM with 10% FBS. After 24 h of incubation, the cells were treated with different concentrations of glargine (0-150 IU/L) for 48 h or treated with 100 IU/L glargine for different periods (24-72 h). Glargine was replenished every 24 h. Then the total RNA was isolated. 0.2-0.5 μg of total RNA was reverse transcribed to cDNA using a target-specific stem-loop primer indicated in Table 1. cDNA in water was added to 5 μL of the 2 × SYBR green master mix (Applied Biosystems Inc, Foster City, United States), 400 nmol/L of gene-specific primer and water used to make the solution up to 10 μL. The reactions were amplified at 95 ℃ for 10 min followed by 40 cycles at 95 ℃ for 15 s and 60 ℃ for 60 s. U6 small nuclear RNA (U6) served as the endogenous control. The relative amount of each miRNA

Table 1 Primers used for reverse transcription or polymerase chain reaction of microRNAs

Gene name	Primer sequences (5'-3')	
miRNA-95	Stem-loop primer	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGTGCTCAAT
	Sense	CGGGTATTTATTGAGCA
	Antisense	AACTGGTGTCGTGGAG
miRNA-134	Stem-loop primer	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGCCCCTCTG
	Sense	TGTGACTGGTTGACCAGAG
	Antisense	AACTGGTGTCGTGGAG
miRNA-34c-3p	Stem-loop primer	CTCAACTGGTGTCGTGGAGTCGG-CAATTCAGTTGAGCCTGGCCGTG
	Sense	AATCACTAACCACACGG
	Antisense	AACTGGTGTCGTGGAG
U6	Stem-loop primer	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGAAAAATAT
	Sense	CAAGGATGACACGCAAAT
	Antisense	TGGTGTCGTGGAGTCG

to U6 was described using the formula $2^{-\Delta Ct}$ where ΔCt = (Ct miRNA - CtU6). Each sample was run in triplicate.

Vector constructs and lentivirus production

The miR-95 sequence was constructed as follows: (forward) hsa-miR-95-*Bam*H I: GATCCGTTCAACGGGTATTTATTGAGCATTCAAGAGATGCTCAATATACCCGTTGAACTTTTTTG; (reverse) hsa-miR-95-*Eco*R I: AATTCAAAAAAGTTCAACGGGTATATTGAGCATCTCTTGAATGCTCAATAAATACCCGTTGAACG. The sequence was amplified and cloned into the pGLV3-GFP vector (GenePhama) to generate pGLV3-GFP- miR-95. The negative control was pGLV3-shRNA-NC. Virus packaging was performed in HEK 293T cells after the co-transfection of 20 mg pGLV3-GFP-miR-95 vector with 15 mg of the packaging plasmid pHelper 1.0 Vector and 10 mg of the envelope plasmid pHelper 2.0 Vector using Lipofectamine 2000 (Invitrogen).Viruses were harvested 48 h after transfection, and viral titers were determined.

Oligonucleotide construction

After glargine treatment, the expression of miR-95 was increased most obviously in Sw1990 cells and Panc-1 cells, so we further investigated the functional roles of miR-95 in pancreatic cancer cells. miR-95 mimics, miR-95 inhibitor and negative control siRNA oligonucleotides were chemosynthesized (Shanghai GenePhama Co. Ltd). The oligonucleotides used in these studies were has-miR-95 mimics: 5'-UUCAACGGGUAU UUAUUGAGCA-3' and 5'-CUCAAUAAAUACCCGUUGAAUU-3'. Mimics negative control: 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3', hsa-miR-95 inhibitor: 5'-UGCUCAAUAAAUACCCGUUGAA-3'. MicroRNA inhibitor negative control: 5'-CAGUACUUUUGUGUAGUACA A-3'.

Cell transfection

Cells were cultured to 80% to 90% confluence after being seeded into 6-well plates and were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For transient transfection, Sw1990 or Panc-1 cells in each well of a 6-well plate were transfected with 12.5 μL miRNA inhibitor or 7.5 μL miRNA mimic oligonucleotides. Transfection efficiency was evaluated by FAM in control vector or real-time PCR. For stably transfected cells, cells were transfected with lentivirus at 80%-90% confluency. Sw1990 cells (1×10^5) were infected with recombinant lentivirus in the presence of 5 μg/mL polybrene (GenePhama).

Cell proliferation assay

A total of 10^4 Sw1990 or Panc-1 cells per well were plated in 96-well plates before transfection and cultured for 24 h in normal conditions. They were then transfected with hsa-miR-95 mimics or hsa-miR-95 inhibitor along with paired negative controls. The cells were incubated at 37 ℃ for 48 h. Cell proliferation was assessed using Cell Counting Kit 8 (Dojindo, Tokyo, Japan) according to manufacturer's protocol.

Apoptosis assay

At 72 h after transfection, apoptosis was detected using Annexin V-FITC Apoptosis Detection Kit (Biovision, United States). Results were calculated by the percentage of apoptotic cells in all cells counted.

Matrigel invasion assay

At 48 h after transfection, the invasive ability of the cells was assayed using Transwells (8 mm pore size, Corning Costar Corp). The Transwells were put into the 24-well plates. First, 0.1 mL Matrigel (50 mg/mL, BD Biosciences) was added onto the plate surface and incubated for 2 h, and then the supernatant was removed. Freshly trypsinized and washed Panc-1 or Sw1990 cells were suspended in DMEM containing 1% FBS. Then 0.1 mL of the cell suspension (1×10^5 cells) was added to the upper chamber of each insert that was coated with Matrigel. Next, 0.6 mL of DMEM containing 10% FBS was added into the lower compartment, and the cells were allowed to invade for 24 h at 37 ℃ in a 5% CO_2 humidified incubator. After incubation, the cells were fixed with 95% absolute alcohol and followed by crystal violet stain. The number of migrated cells on the lower surface of the membrane was counted under a microscope in 10 fields with magnification of × 200. Each experiment was performed in triplicate.

Cell migration

At 48 h after transfection, the ability of Panc-1 or Sw1990 cells to migrate was detected using Transwells (8 mm pore size, Corning Costar Corp). The Transwells were put into the 24-well plates. Freshly trypsinized and washed cells were suspended in DMEM containing 1% FBS. 5×10^4 cells/well were placed in the top chamber of each insert (BD Biosciences, NJ), with the non-coated membrane. 0.6 mL of DMEM containing 10% FBS was added into the lower chambers. After incubating for 24 h at 37 ℃ in

a 5% CO_2 humidified incubator, the cells were fixed with 95% absolute alcohol and stained with crystal violet stain. The number of migrated cells on the lower surface of the membrane was counted under a microscope in 10 fields with magnification of × 200. Each experiment was performed in triplicate.

Mice xenografts

The stable cell line Sw1990 was harvested from tissue culture flasks after transfection with the pGLV3-GFP-miR-95 and control pGLV3-GFP vector using trypsin and washed three times with PBS. About 1×10^7 cells were implanted into the right flanks of female nu/nu mice (five in each group). Tumor volume (V) was measured with an external caliper every 4 d and it was calculated as V = 0.52 (length × width^2). After four weeks, all the animals were sacrificed and tumors were removed.

Statistical analysis

Data were expressed as the mean ± SD unless otherwise noted. The differences between groups were analyzed using a two-tailed Student's *t*-test when only two groups were present and the null hypothesis was rejected at the 0.05 level.

RESULTS

Glargine treatment alters miRNAs expression profiles

To study the responses of miRNAs to glargine, miRNA real time PCR array analysis of miRNA expression was conducted with total RNAs extracted from Sw1990 pancreatic cells treated with or without 100 IU/L glargine. Differential expression between glargine-treated and non-treated cells was defined using a cut off value of 2-fold change. We observed that 10 miRNAs were significantly up-regulated and 2 miRNAs were significantly down-regulated (2.48-fold on average, $P < 0.01$) in glargine treated Sw1990 cells when compared with non-treated cells. miR-95, miR-134 and miR-34c-3p are the top three miRNAs regulated by glargine (3.65-fold, 2.67-fold and 2.60-fold changes respectively, $P < 0.01$) in Sw1990 cells (Figure 1A).

Confirmatory studies with differentially expressed miRNAs by stem-loop real-time PCR

After treatment with increasing concentrations of glargine (50, 100, 150 IU/L) for 48 h, miR-95 was up-regulated by 1.18 fold ($P > 0.05$), 3.41 fold ($P < 0.01$) and 2.92 fold ($P < 0.01$) on average respectively in Sw1990 cells and 1.45 fold ($P < 0.01$), 3.41 fold ($P < 0.01$) and 2.92 fold ($P < 0.01$) on average respectively in Panc-1 cells, when compared with non-treated cells (0 IU/L). No obvious dose dependent responses were observed; miR-134 was up-regulated in a dose dependent manner (Sw1990: 1.69, 2.10 and 2.93 fold on average respectively, $P < 0.01$; Panc-1: 1.56, 1.99 and 2.88 fold on average respectively, $P < 0.01$) in both Sw1990 and Panc-1 cells; miR-34c-3p showed no significant changes (Sw1990: 1.03, 1.05 and 1.06 fold on average respectively, $P > 0.05$; Panc-1: 1.25, 1.25 and 1.19 fold on average respectively, $P > 0.05$) in both Sw1990 and Panc-1 cells (Figure 1B-1,2).

After treatment with 100 IU/L glargine for different periods (24, 48 and 72 h), miR-95 was up-regulated by 2.50 fold ($P < 0.01$), 3.10 fold ($P < 0.01$) and 2.99 fold ($P < 0.01$) on average, respectively, in Sw1990 cells and 2.31 fold ($P < 0.01$), 2.46 fold ($P < 0.01$) and 2.16 fold ($P < 0.01$) on average, respectively, in Panc-1 cells, when compared with the cells at 0 h; miR-134 was up-regulated by 2.22 fold ($P < 0.01$), 2.37 fold ($P < 0.01$), 2.17 fold ($P < 0.01$) on average, respectively, in Sw1990 cells and 2.10 fold ($P < 0.01$), 2.31 fold ($P < 0.01$) and 2.37 fold ($P < 0.01$) on average, respectively, in Panc-1 cells; miR-34c-3p showed no significant changes (Sw1990: 1.16, 1.14 and 1.13 fold on average respectively, $P > 0.05$; Panc-1: 1.17,1.24 and 1.13 fold on average, respectively, $P > 0.05$) in both Sw1990 and Panc-1 cells (Figure 1B-3,4).

miR-95 increases cell proliferation and inhibits cell apoptosis

We investigated the potential oncogenic role of miR-95 in Sw1990 and Panc-1 cells. First, we tested miR-95 expression using stem-loop real-time PCR. It increased or decreased after transfected with miR-95 mimics or anti-miR-95 inhibitor. We observed a significant increase in proliferation (Sw1990: 2.51 ± 0.13 *vs* 2.31 ± 0.19, $P < 0.05$; Panc-1: 2.44 ± 0.21 *vs* 2.26 ± 0.12, $P < 0.05$) after transfection of miR-95 mimics (Figure 2A-1). In contrast, anti-miR-95 inhibitor significantly decreased cell proliferation (Sw1990: 2.11 ± 0.07 *vs* 2.23 ± 0.13, $P < 0.05$; Panc-1: 2.09 ± 0.09 *vs* 2.31 ± 0.13, $P < 0.05$) (Figure 2A-2). These data indicate that cell proliferation can be significantly promoted by increase of miR-95 expression.

We further investigated the effect of miR-95 on apoptosis and found that apoptosis decreased dramatically (Sw1990: 22.05% ± 1.92% *vs* 40.32% ± 1.93%, $P < 0.05$; Panc-1: 20.17% ± 0.85% *vs* 45.60% ± 1.43%, $P < 0.05$) in Sw1990 and Panc-1 cells 72 h after transfection with miR-95 mimics (Figure 2B). It suggested that miR-95 may function as a strong apoptotic suppressor in human pancreatic cancer cells.

miR-95 regulates pancreatic cancer cell invasion and migration in vitro

In the cell invasion and migration assay, we observed that depletion of miR-95 significantly impaired the ability of Sw1990 cells to migrate and invade through the matrigel-coated membranes or the non-matrigel-coated membranes towards serum-containing medium (invasion: 49.40 ± 6.59 *vs* 65.80 ± 5.09; migration: 52.30 ± 10.87 *vs* 88.90 ± 10.46, $P < 0.01$), when compared with a paired negative control (Figure 3B); Increased expression of miR-95 significantly promoted the ability of Sw1990 cells to migrate and invade through matrigel-coated membranes or non-matrigel-coated membranes towards serum-containing medium (invasion: 67.90 ± 12.33 *vs* 47.30 ± 5.89; migration: 101.00 ± 6.00 *vs* 51.20 ± 8.34, $P < 0.01$), when compared with the paired negative control (Figure 3A). Similar results were found in Panc-1 cells (depletion of miR-95,

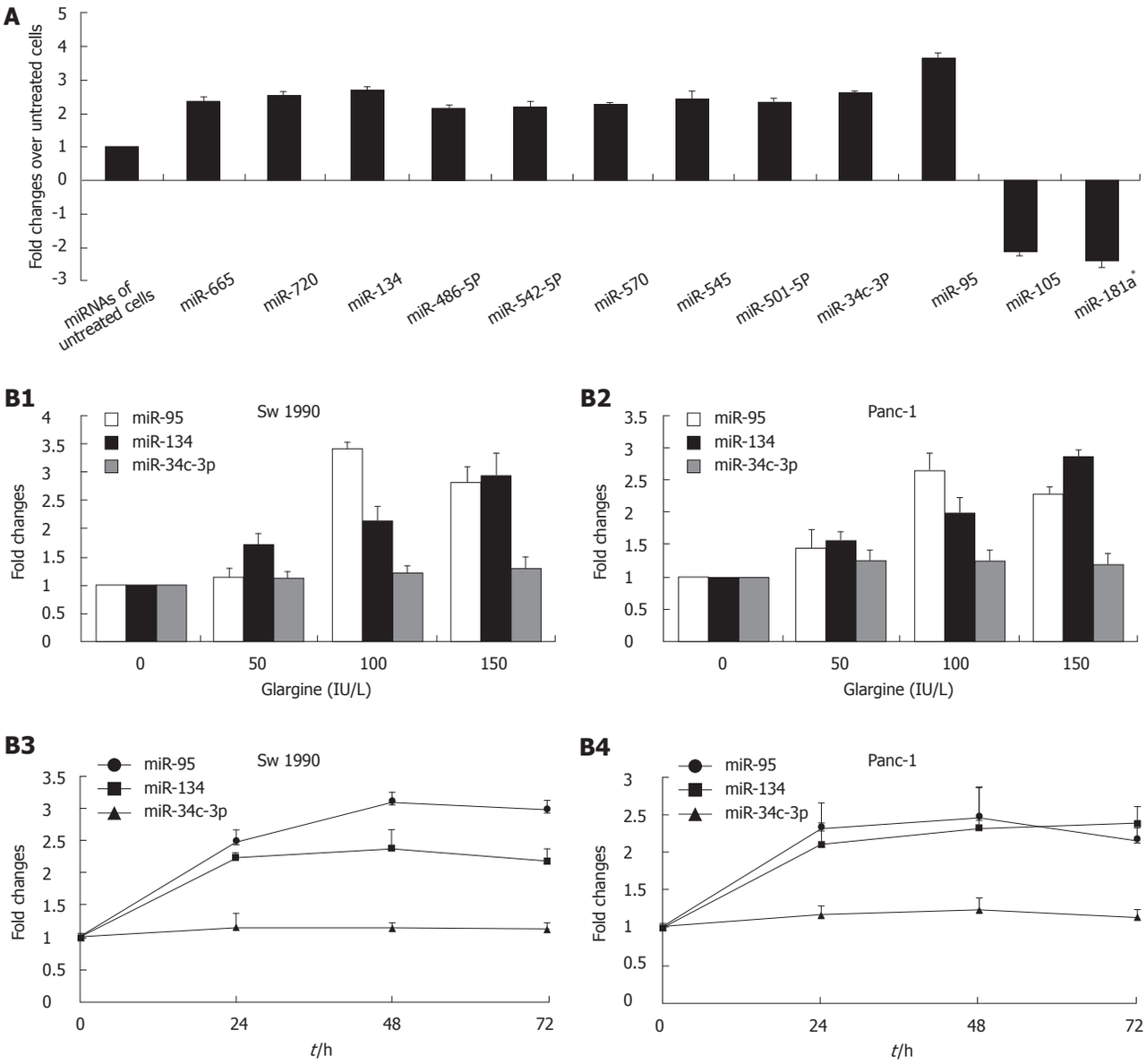

Figure 1 Effect of insulin glargine on miRNAs expression in Sw1990 cell line (A), insulin glargine up-regulated the expression of miR-95 and miR-134, miR-34c-3p showed no obvious changes in Sw1990 and Panc-1 cells (B). Ten miRNAs were significantly up-regulated and two miRNAs were down-regulated after 100 IU/L insulin glargine treatment for 48 h compared with non-treated cells. The cut off line for miRNA expression change was 2-fold. Data were presented as mean ± SD. Sw1990 and Panc-1 cells were incubated with increasing concentrations of insulin glargine (50-150 IU/L) for 48 h or treated with 100 IU/L insulin glargine for different periods (24-72 h). Then the expression of the three miRNAs were detected by stem-loop real-time reverse transcription-polymerase chain reaction array. miR-95 was significantly up-regulated, but no dose or time dependent changes were observed; miR-134 was up-regulated in a dose-dependent manner. miR-181a*: The miRNA of lower abundance.

invasion: 57.90 ± 10.55 *vs* 73.80 ± 11.95, migration: 66.40 ± 10.1 *vs* 99.50 ± 8.85, $P < 0.01$; forced expression of miR-95, invasion: 37.80 ± 8.93 *vs* 30.20 ± 5.14; migration: 91.80 ± 9.22 *vs* 81.50 ± 7.47, $P < 0.01$) (Figure 4A and B). These results indicated that miR-95 may be important in the progression of pancreatic cancer through increasing cell invasion and migration.

miR-95 promotes the growth of Sw1990 xenografts

Sw1990 cells transfected with pGLV3-GFP-miR-95 or negative control pGLV3-GFP were injected into the right flank of nude mice. Four weeks later, the tumor volumes of xenografts were 373.82 ± 23.67 mm^3 in the miR-95 transfected group and 219.69 ± 17.82 mm^3 in the negative control group. The weight of xenografts was 0.40 ± 0.08 g in the miR-95 transfected group and 0.23 ± 0.05 g in the negative control group. miR-95 significantly increased the growth of the Sw1990 xenografts (Figure 5, $P < 0.05$).

DISCUSSION

In the present study we demonstrated that glargine altered specific miRNA expression in human pancreatic cells at 50-150 IU/L, which is equivalent to 300-900 nmol and is much higher than the physiological concentration of

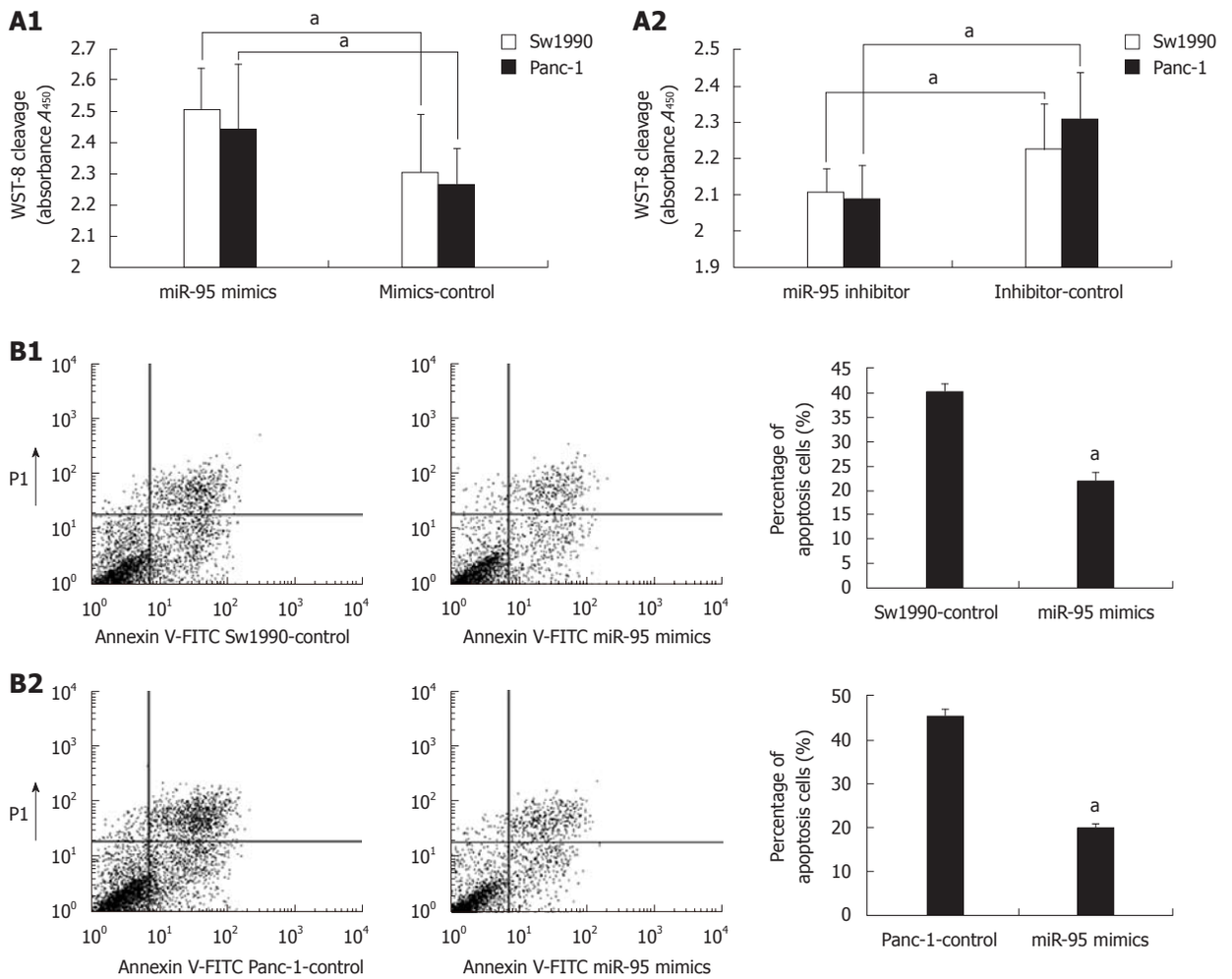

Figure 2 miR-95 promotes cell proliferation (A) and enhancement of miR-95 inhibit apoptosis (B). A total of 10^4 Sw1990 or Panc-1 cells per well were plated in 96-well plates for 24 h and then were transfected with hsa-miR-95 mimics or hsa-miR-95 inhibitor along with paired negative controls. The cells were incubated at 37 ℃ for 48 h and then cell proliferation was assessed using Cell Counting Kit 8. Data were shown as mean ± SD. $^aP < 0.05$ *vs* pairing negative control.

insulin (0.1-1 nmol). Our miRNA real time PCR array showed that high dose glargine (100 IU/L) up-regulated the expression of 10 miRNAs and down-regulated 2 miRNAs in Sw1990 pancreatic cancer cells. The most obvious change was the apparent increase of miR-95. Stem-loop real-time PCR confirmed the aberrant changes of miR-95 after treatment of high dose glargine in Sw1990 and Panc-1 pancreatic cancer cells. Then miR-95 showed significant anti-apoptotic and growth-promoting effects *in vitro* and *in vivo*. Ectopic expression and siRNA knockdown of miR-95 confirmed its invasion-promoting activity *in vitro*. Therefore, these results highlighted the miR-95-related changes as important effects of glargine.

Recent studies linked the use of glargine with increased risk of cancer. Hemkens *et al*[4] published a registry study that demonstrated a significantly increased risk of cancer diagnosis associated with high dosages of glargine. However, the Scottish study found a non-significant increased risk for specifically breast cancers[23]. The UK study found no link between glargine and cancer[3]. In addition, although glargine has been shown to increase resistance to apoptosis in several tumor cell lines, administration of glargine didn't alter proliferation of Colo-357 pancreatic carcinoma cells *in vitro*[12]. Therefore, all epidemiological and laboratory evidence remains inconclusive and new indicators are needed to determine the role of glargine in carcinogenesis.

miRNAs have been recognized as promising diagnostic and prognostic markers for cancer diagnosis or treatment. For example , miR-34a family members were found to be directly regulated by TP53 and act as tumor suppressors[24]; miR-217 inhibited pancreatic cancer cell growth through targeting KRAS[25]; miR-10b promoted pancreatic cancer invasiveness and correlates with a poor prognosis[26]; The miRNA-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) were found to inhibit tumor invasion and metastasis by regulating EMT[27]; miR-126 can inhibit cell adhesion, migration and invasion through the suppression of CRK[28]. Our study confirmed, for the first time, that miR-95 and miR-134 were primary glargine-responsive miRNAs.

miR-95 has been shown to be involved in carcinogenesis. A highly characterized example is colorectal carcinoma, in which miR-95 can promote cell proliferation by

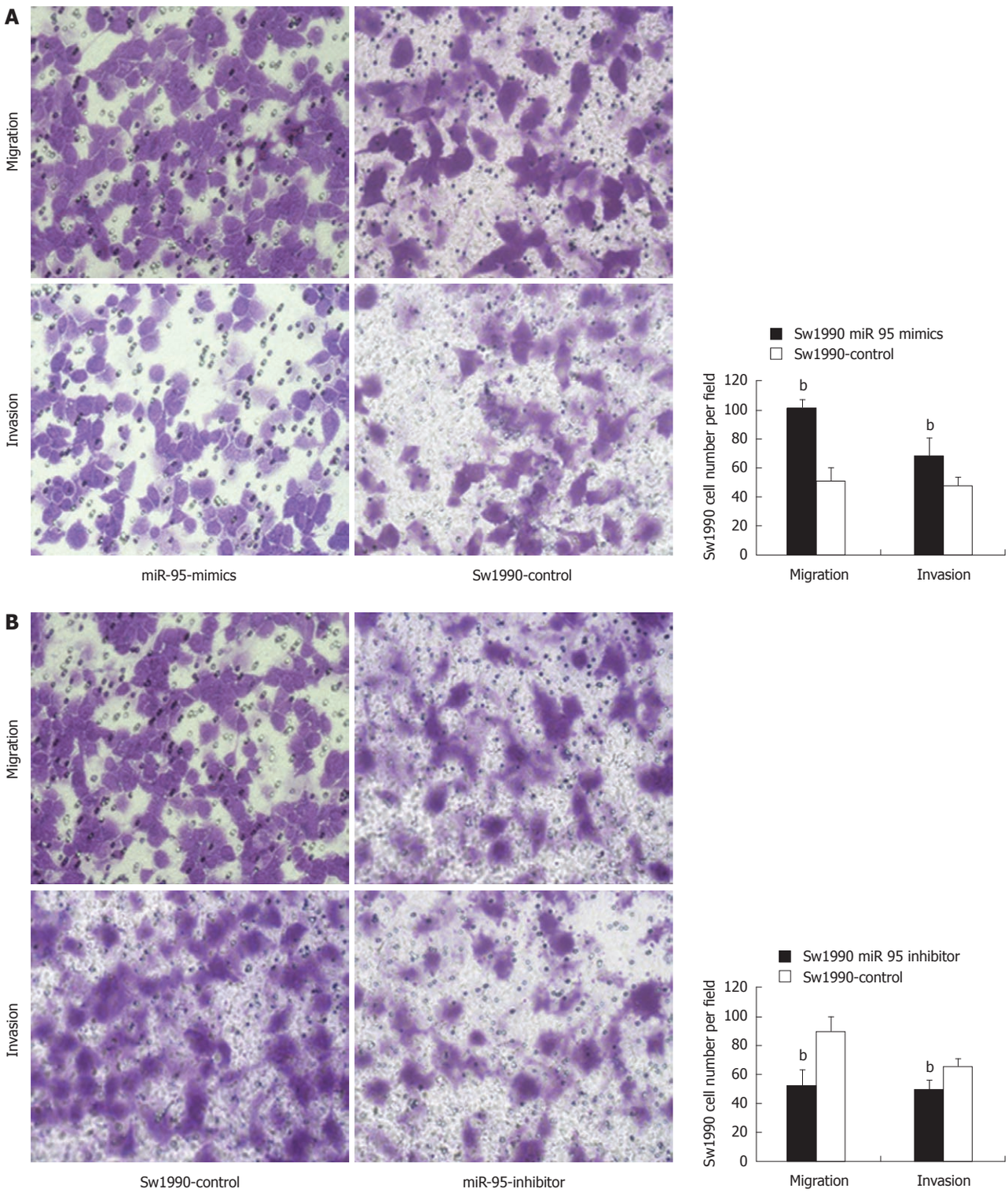

Figure 3 Effect of miR-95 on tumor cell migration, invasion of Sw1990 cells. A: Invasion and migration assay. Representative fields of invasion (down) or migration (up) cells on the membrane (left, magnification of × 200). Average invasion or migration cell number per field (right). The invasion or migration cell number of Sw1990 transfected with miR-95-mimics drastically increased than that transfected with pairing negative control; B: The invasion or migration cell number of Sw1990 cells transfected with miR-95-inhibitor dramatically decreased than that transfected with pairing negative control. $^{b}P < 0.01$ *vs* Sw1990-control, $n = 10$.

regulating sorting Nexin 1[29]. In pancreatic cancer, miR-95 is significantly upregulated in most tissues and cell lines[30]. In HeLa cells, inhibition of miR-95 caused a decrease in cell growth[31]. *miR-134* gene is located at 14q32, and is involved in several physiological and pathological processes. For example, miR-134 plays an important role in translation-dependent guidance of nerve growth cones[32]; miR-134 is regarded as a potential plasma biomarker for the diagnosis of acute pulmonary embolism[33]; plasma miR-134 in bipolar disorder serves as a potential periph-

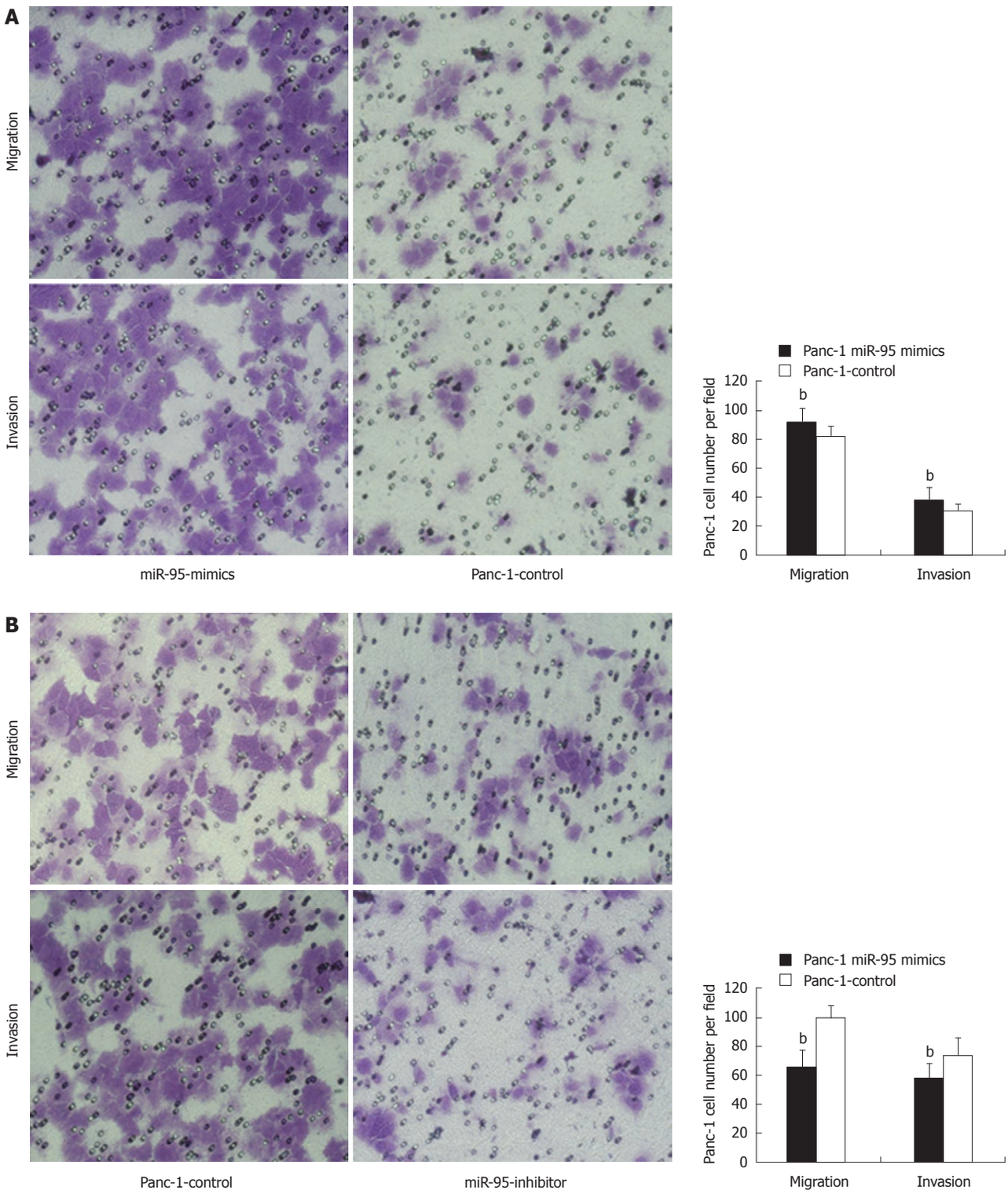

Figure 4 Effect of miR-95 on tumor cell migration, invasion of Panc-1 cells. A: Invasion and migration assay. Representative fields of invasion (down) or migration (up) cells on the membrane (left, magnification of × 200). Average invasion or migration cell number per field (right). The invasion or migration cell number of Panc-1 transfected with miR-95-mimics drastically increased compared with that when transfected with the pairing negative control. B: The invasion or migration cell number of Panc-1 cells transfected with miR-26a-inhibitor dramatically decreased compared with that when transfected with the pairing negative control. $^{b}P < 0.01$ *vs* Panc-1-control, $n = 10$.

eral marker that can respond to acute manic episodes and is associated with effective mood stabilizer treatment[34]. Interestingly, recent studies indicated that miR-134 may also be involved in carcinogenesis. p53/p63/p73, the tumor suppressors, were believed to be regulators of the miR-134 processing complex[35]. Our study showed that high dose glargine can significantly upregulate the expression of miR-95 (no time or dose dependent manner) and

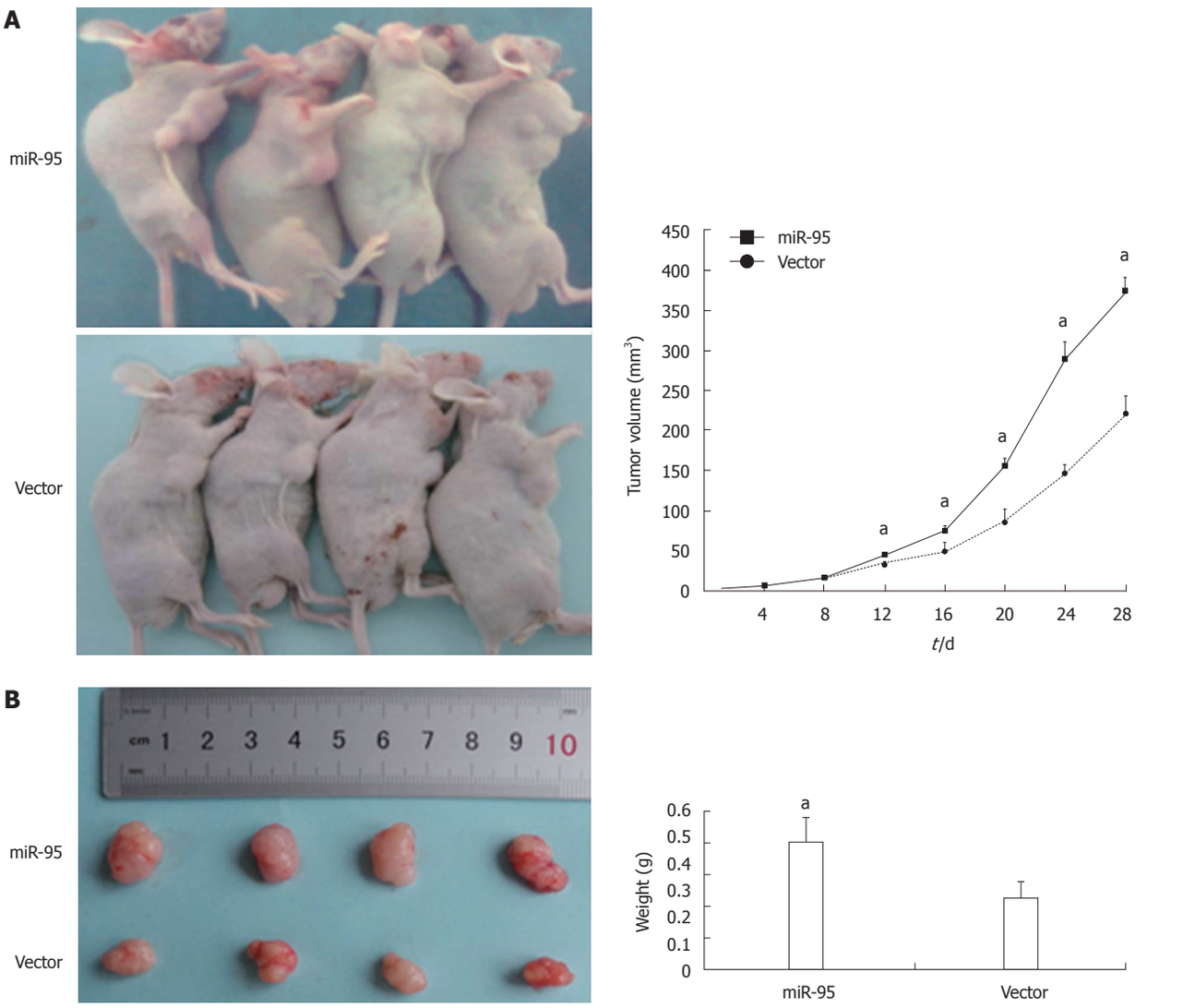

Figure 5 miR-95 promotes the growth of Sw1990 xenografts. A: miR-95 strikingly increased the growth of Sw1990 cells xenografted in nude mice; B: At the end of the experiment, all animals were sacrificed and the tumors were removed. The tumors were much heavier in miR-95 group than in the vector group. Data were shown as mean ± SD ($^aP < 0.05$ *vs* vector).

miR-134 (dose dependent manner) *in vitro*, and for the first time investigated the role of miR-95 in pancreatic cancer.

In conclusion, our study demonstrated that alterations of specific miRNAs and miRNA-related changes were important effects of glargine, suggesting an important and novel new mechanism by which glargine mediates its potent effects on cell growth and apoptosis.

COMMENTS

Background

Glargine is widely used in the treatment of type 1 and type 2 diabetes mellitus. Recently, this insulin analogue has been suspected to be associated with an increased risk of cancer, including pancreatic cancer, but available evidence remains inconclusive.

Research frontiers

Anti-diabetic therapies have been shown to affect the risk of pancreatic cancer. Metformin use was associated with reduced risk, and insulin or insulin analogue use was suspected to be associated with increased risk of pancreatic cancer in diabetic patients. However, data regarding the effect of glargine, one of the long-acting insulin analogues, on pancreatic cancer are inconsistent. Several researchers believed glargine to be a motigen, not a carcinogen. New biomarkers are needed to determine the role of glargine in the carcinogenesis of pancreatic cancer.

Innovations and breakthroughs

Recent reports have highlighted that miRNAs play important roles in biological processes that affect tumor progression and are promising biomarkers for cancer diagnosis or treatment. This is the first study to show the effects of high dose glargine on miRNA expression in pancreatic cancer cells. miR-95 was proved to be affected by high dose glargine and to be involoved in the carcinogenesis of pancreatic cancer.

Applications

By understanding the effects of glargine on pancreatic cancer cells, this study may help to clarify the role of glargine in the progress of pancreatic cancer.

Terminology

Glargine is a widely used insulin analog in which a 24 h action profile is achieved. MicroRNAs (miRNAs) are endogenous, non-coding small RNAs, 19-25 nucleotides in length. It has been demonstrated that miRNAs play important roles in biological processes that affect tumor progression including migration, invasion and metastasis.

Peer review

The authors examined the effects of high dose glargine on pancreatic cancer cells *in vitro*; miR-95 is up-regulated significantly by glargine. miR-95 can significantly increase pancreatic cancer cell proliferation, invasion and migration and inhibit cell apoptosis. Moreover, miR-95 is proved to inhibit pancreatic

cancer growth *in vivo*. The results are interesting and may represent the role of glargine in pancreatic carcinogenesis.

REFERENCES

1 **Jemal A**, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. *CA Cancer J Clin* 2008; **58**: 71-96
2 **Warshaw AL**, Fernández-del Castillo C. Pancreatic carcinoma. *N Engl J Med* 1992; **326**: 455-465
3 **Currie CJ**, Poole CD, Gale EA. The influence of glucose-lowering therapies on cancer risk in type 2 diabetes. *Diabetologia* 2009; **52**: 1766-1777
4 **Hemkens LG**, Grouven U, Bender R, Günster C, Gutschmidt S, Selke GW, Sawicki PT. Risk of malignancies in patients with diabetes treated with human insulin or insulin analogues: a cohort study. *Diabetologia* 2009; **52**: 1732-1744
5 **Gerich JE**. Insulin glargine: long-acting basal insulin analog for improved metabolic control. *Curr Med Res Opin* 2004; **20**: 31-37
6 **Weinstein D**, Simon M, Yehezkel E, Laron Z, Werner H. Insulin analogues display IGF-I-like mitogenic and anti-apoptotic activities in cultured cancer cells. *Diabetes Metab Res Rev* 2009; **25**: 41-49
7 **Kurtzhals P**, Schäffer L, Sørensen A, Kristensen C, Jonassen I, Schmid C, Trüb T. Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. *Diabetes* 2000; **49**: 999-1005
8 **Li G**, Barrett EJ, Wang H, Chai W, Liu Z. Insulin at physiological concentrations selectively activates insulin but not insulin-like growth factor I (IGF-I) or insulin/IGF-I hybrid receptors in endothelial cells. *Endocrinology* 2005; **146**: 4690-4696
9 **Strasser-Vogel B**, Blum WF, Past R, Kessler U, Hoeflich A, Meiler B, Kiess W. Insulin-like growth factor (IGF)-I and -II and IGF-binding proteins-1, -2, and -3 in children and adolescents with diabetes mellitus: correlation with metabolic control and height attainment. *J Clin Endocrinol Metab* 1995; **80**: 1207-1213
10 **Michell NP**, Dent S, Langman MJ, Eggo MC. Insulin-like growth factor binding proteins as mediators of IGF-I effects on colon cancer cell proliferation. *Growth Factors* 1997; **14**: 269-277
11 **Baserga R**. The insulin-like growth factor I receptor: a key to tumor growth? *Cancer Res* 1995; **55**: 249-252
12 **Erbel S**, Reers C, Eckstein VW, Kleeff J, Büchler MW, Nawroth PP, Ritzel RA. Proliferation of colo-357 pancreatic carcinoma cells and survival of patients with pancreatic carcinoma are not altered by insulin glargine. *Diabetes Care* 2008; **31**: 1105-1111
13 **Inui M**, Martello G, Piccolo S. MicroRNA control of signal transduction. *Nat Rev Mol Cell Biol* 2010; **11**: 252-263
14 **Sharma S**, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis* 2010; **31**: 27-36
15 **Schickel R**, Boyerinas B, Park SM, Peter ME. MicroRNAs: key players in the immune system, differentiation, tumorigenesis and cell death. *Oncogene* 2008; **27**: 5959-5974
16 **Bandres E**, Agirre X, Ramirez N, Zarate R, Garcia-Foncillas J. MicroRNAs as cancer players: potential clinical and biological effects. *DNA Cell Biol* 2007; **26**: 273-282
17 **Baranwal S**, Alahari SK. miRNA control of tumor cell invasion and metastasis. *Int J Cancer* 2010; **126**: 1283-1290
18 **Nicoloso MS**, Spizzo R, Shimizu M, Rossi S, Calin GA. MicroRNAs--the micro steering wheel of tumour metastases. *Nat Rev Cancer* 2009; **9**: 293-302
19 **Waldman SA**, Terzic A. Translating MicroRNA discovery into clinical biomarkers in cancer. *JAMA* 2007; **297**: 1923-1925
20 **Bartels CL**, Tsongalis GJ. MicroRNAs: novel biomarkers for human cancer. *Clin Chem* 2009; **55**: 623-631
21 **Wiggins JF**, Ruffino L, Kelnar K, Omotola M, Patrawala L, Brown D, Bader AG. Development of a lung cancer therapeutic based on the tumor suppressor microRNA-34. *Cancer Res* 2010; **70**: 5923-5930
22 **Bloomston M**, Frankel WL, Petrocca F, Volinia S, Alder H, Hagan JP, Liu CG, Bhatt D, Taccioli C, Croce CM. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA* 2007; **297**: 1901-1908
23 **Colhoun HM**. Use of insulin glargine and cancer incidence in Scotland: a study from the Scottish Diabetes Research Network Epidemiology Group. *Diabetologia* 2009; **52**: 1755-1765
24 **Chang TC**, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, Feldmann G, Yamakuchi M, Ferlito M, Lowenstein CJ, Arking DE, Beer MA, Maitra A, Mendell JT. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell* 2007; **26**: 745-752
25 **Zhao WG**, Yu SN, Lu ZH, Ma YH, Gu YM, Chen J. The miR-217 microRNA functions as a potential tumor suppressor in pancreatic ductal adenocarcinoma by targeting KRAS. *Carcinogenesis* 2010; **31**: 1726-1733
26 **Nakata K**, Ohuchida K, Mizumoto K, Kayashima T, Ikenaga N, Sakai H, Lin C, Fujita H, Otsuka T, Aishima S, Nagai E, Oda Y, Tanaka M. MicroRNA-10b is overexpressed in pancreatic cancer, promotes its invasiveness, and correlates with a poor prognosis. *Surgery* 2011; **150**: 916-922
27 **Gregory PA**, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* 2008; **10**: 593-601
28 **Crawford M**, Brawner E, Batte K, Yu L, Hunter MG, Otterson GA, Nuovo G, Marsh CB, Nana-Sinkam SP. MicroRNA-126 inhibits invasion in non-small cell lung carcinoma cell lines. *Biochem Biophys Res Commun* 2008; **373**: 607-612
29 **Huang Z**, Huang S, Wang Q, Liang L, Ni S, Wang L, Sheng W, He X, Du X. MicroRNA-95 promotes cell proliferation and targets sorting Nexin 1 in human colorectal carcinoma. *Cancer Res* 2011; **71**: 2582-2589
30 **Zhang Y**, Li M, Wang H, Fisher WE, Lin PH, Yao Q, Chen C. Profiling of 95 microRNAs in pancreatic cancer cell lines and surgical specimens by real-time PCR analysis. *World J Surg* 2009; **33**: 698-709
31 **Cheng AM**, Byrom MW, Shelton J, Ford LP. Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res* 2005; **33**: 1290-1297
32 **Han L**, Wen Z, Lynn RC, Baudet ML, Holt CE, Sasaki Y, Bassell GJ, Zheng JQ. Regulation of chemotropic guidance of nerve growth cones by microRNA. *Mol Brain* 2011; **4**: 40
33 **Xiao J**, Jing ZC, Ellinor PT, Liang D, Zhang H, Liu Y, Chen X, Pan L, Lyon R, Liu Y, Peng LY, Liang X, Sun Y, Popescu LM, Condorelli G, Chen YH. MicroRNA-134 as a potential plasma biomarker for the diagnosis of acute pulmonary embolism. *J Transl Med* 2011; **9**: 159
34 **Rong H**, Liu TB, Yang KJ, Yang HC, Wu DH, Liao CP, Hong F, Yang HZ, Wan F, Ye XY, Xu D, Zhang X, Chao CA, Shen QJ. MicroRNA-134 plasma levels before and after treatment for bipolar mania. *J Psychiatr Res* 2011; **45**: 92-95
35 **Boominathan L**. The tumor suppressors p53, p63, and p73 are regulators of microRNA processing complex. *PLoS One* 2010; **5**: e10615

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ORIGINAL ARTICLE

Suppression of colorectal cancer metastasis by nigericin through inhibition of epithelial-mesenchymal transition

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Abstract

AIM: To evaluate the effect of nigericin on colorectal cancer and to explore its possible mechanism.

METHODS: The human colorectal cancer (CRC) cell lines HT29 and SW480 were treated with nigericin or oxaliplatin under the conditions specified. Cell viability assay and invasion and metastasis assay were performed to evaluate the effect of nigericin on CRC cells. Sphere-forming assay and soft agar colony-forming assay were implemented to assess the action of nigericin on the cancer stem cell properties of CRC cells undergone epithelial-mesenchymal transition (EMT).

RESULTS: Compared with oxaliplatin, nigericin showed more toxicity for the HT29 cell line (IC50, 12.92 ± 0.25 μmol *vs* 37.68 ± 0.34 μmol). A similar result was also obtained with the SW116 cell line (IC50, 15.86 ± 0.18 μmol *vs* 41.02 ± 0.23 μmol). A Boyden chamber assay indicated that a significant decrease in the number of HT29 cells migrating through polyvinylidene fluoride membrane was observed in the nigericin-treated group, relative to the vehicle-treated group [11 ± 2 cells per high-power field (HPF) *vs* 19.33 ± 1.52 cells per HPF, $P < 0.05$]. Compared to the control group, the numbers of HT29 cells invading through the Matrigel-coated membrane also decreased in the nigericin-treated group (6.66 ± 1.52 cells per HPF *vs* 14.66 ± 1.52 cells per HPF, $P < 0.05$). Nigericin also reduced the proportion of $CD133^+$ cells from 83.57% to 63.93%, relative to the control group ($P < 0.05$). Nigericin decreased the number of spheres relative to the control group (0.14 ± 0.01 *vs* 0.35 ± 0.01, $P < 0.05$), while oxaliplatin increased the number of spheres relative to the control group (0.75 ± 0.02 *vs* 0.35 ± 0.01; $P < 0.05$). Nigericin also showed a decreased ability to form colonies under anchorage-independent conditions in a standard soft agar assay after 14 d in culture, relative to the control group (1.66 ± 0.57 *vs* 7 ± 1.15, $P < 0.05$), whereas the colony numbers were higher in the oxaliplatin group relative to the vehicle-treated controls (14.33 ± 0.57 *vs* 7 ± 1.15, $P < 0.05$). We further detected the expression of E-cadherin and vimentin in cells treated with nigericin and oxaliplatin. The results showed that HT29 cells treated with nigericin induced an increase in E-cadherin expression and a decrease in the vimentin expression relative to vehicle controls. In contrast, oxaliplatin downregulated the expression of E-cadherin and upregulated the expression of vimentin in HT29 cells relative to vehicle controls.

CONCLUSION: This study demonstrated that nigericin could partly reverse the EMT process during cell invasion and metastasis.

Key words: Colorectal cancer; Nigericin; Cancer invasion; Metastasis; Epithelial-mesenchymal transition; CD133; E-cadherin; Vimentin

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INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in men and the second most commonly diagnosed cancer in women[1], with a 5-year survival rate < 10% for patients with metastatic disease[2]. Despite the use of active targeted drugs for treatment of metastatic CRC, the cure rate has remained low in the past decade. Activating invasion and metastasis is the hallmark of cancer[3,4], during which malignant cells spread from the primary tumor to distant organs.

The pathogenesis of metastasis involves a series of steps, often termed the invasion and metastasis cascade, which includes the following: local invasion of the host stroma by tumor cells; detachment and embolization of tumor cell aggregates; extravasation of the tumor embolus; survival of tumor cells that are transported through the circulation and stop in the capillary bed; extravasation of the tumor embolus; proliferation of the tumor cells within the organ parenchyma, resulting in a metastatic focus; and reinitiation of these processes for the development of metastases. The first and decisive step of this process is the local invasion through the epithelial basement membrane, because it requires alteration in cell-cell and cell-matrix interactions, reconstruction of the extracellular matrix, remodeling of the cytoskeleton, and enhancement of cell modulation. Great progress has been made on the capacity for invasion and metastasis over the past decade with powerful novel research tools and refined experimental models becoming available. On the other hand, many critical regulatory genes have been identified.

Epithelial-mesenchymal transition (EMT), a transdifferentiation characterized by decreased epithelial markers such as E-cadherin and increased mesenchymal markers such as fibronectin, has become prominently implicated as a means by which transformed epithelial tumor cells acquire the ability to invade, resist apoptosis, and propagate[5-9]. More importantly, EMT has been shown to result in cancer cells with stem-cell-like characteristics that have a propensity to invade surrounding tissue and display resistance to chemotherapeutic interventions[6,10,11]. Nigericin is a potassium ionophore, which has been reported to be toxic to breast stem cells passing through EMT[12]. Lu *et al*[13] have reported that nigericin, like salinomycin, selectively inhibits Wnt1-mediated signaling in HEK293 cells at nanomolar concentrations.

In this study, we aimed to ascertain the specific activities of nigericin on human CRC cell lines. We selected CD133 as the marker of stem cells of CRC.

MATERIALS AND METHODS

Tumor cell preparation and cell culture

Human CRC cell lines, HT29 and SW116 were used. HT29 cells were cultured in McCoy's 5A medium (Gibco, United States) with 10% fetal bovine serum (FBS). SW116 cells were cultured in RPMI 1640 medium with 10% FBS. The cells were cultured at 37 ℃ in a humidified atmosphere containing 5% CO_2.

Drugs and antibodies

Oxaliplatin and nigericin were both purchased from Sigma-Aldrich (St. Louis, MO, United States). Antibodies used for immunofluorescence staining and Western blotting were as follows: mouse anti-E-cadherin (Abcam Inc., Cambridge, MA, United States), mouse anti-vimentin (Abcam), mouse anti-CD133 (Abcam; used for Western blotting and immunocytochemistry), allophycocyanin-conjugated CD133 antibody (Miltenyi Biotec, Auburn, CA, United States) used for fluorescence-activated cell sorting (FACS), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam).

Analysis of cell viability

For assessment of cell viability in HT29 and SW116 cell lines under different treatments, cells growing at the exponential stage were plated in triplicate, in 96-well plates at a density of 2000 cells/well in a final volume of 100 μL. After incubation for 24 h, oxaliplatin, nigericin and dimethylsulfoxide (DMSO) control were added to each well of the plates. Cell viability was detected after 24 h using Cell Counting Kit 8 (DOJINDO, Japan). Absorbance for each well was read at 570 nm using a microplate reader. Growth inhibition was calculated as a percentage of the untreated controls. Experiments were done three or more times, often in triplicate, for each cell line, and IC_{50} was determined using the four-parameter logistic model.

Cell migration and invasion

Analysis of cell migration was performed using Boyden chambers according to the manufacturer's protocol (Becton Dickinson Labware, Bedford, MA, United States). For cell invasion study, the inserts of the chamber were prepared by coating the upper surfaces with Matrigel (BD Matrigel Matrix, Phenol Red-free). HT29 cells (3×10^3) treated with DMSO control, oxaliplatin and nigericin in McCoy's 5A medium without FBS were plated to the upper chamber. McCoy's 5A with 20% FBS as chemoattractants were plated in the lower chamber of the 24-well pates. After 24 h, nonmigrating or noninvading cells were removed mechanically from the upper chamber using a cotton swab. Cells that migrated or invaded to the lower

surface of the Transwell membrane were fixed in methanol for 30 min at 37 ℃ and stained with 0.05% crystal violet for 1 h. Cells were quantified by counting the number of stained nuclei in five individual fields by fluorescence microscopy, in triplicate.

Immunoblotting

Cell lysates were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis, and the separated proteins were electrophoretically transferred to hydrophobic polyvinylidene fluoride (PVDF) membrane. After blocking in 5% skimmed milk solution for 2 h, the membranes were incubated with the primary antibodies diluted with anti-CD133, anti-E-cadherin, and anti-vimentin. Primary antibodies were detected with mouse secondary antibodies directed against human IgG and visualized with Odyssey Infrared Imaging System.

Real-time polymerase chain reaction

mRNA expression was determined by real-time polymerase chain reaction. RNA was extracted by using the TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and reverse transcription was performed using Superscript Ⅱ (Invitrogen) according to the manufacturer's instructions. TaqMan reactions were done utilizing an ABI 7500 real-time quantitative polymerase chain reaction (PCR) system. For data analysis, raw counts were normalized to housekeeping gene average for the same time point and condition (ΔCt). The following primers were used in this study: CD133 forward CATCCACAGATGCTCCTAAGGC and reverse GCTTTATGGGAGTCTTGGTC; E-cadherin forward CGAGAGCTACACGTTCACGG and reverse GTGTCG AGGGAAAAATAGGCTG; vimentin forward CTCCTCCCCCTGTCACATAC and reverse TGATTGGCATCAGGACCGTTG. GAPDH was used as an internal control. Analysis was performed with the ΔΔCt method.

Flow cytometric analysis

HT29 and SW116 cells, after different treatments, were washed with PBS. Single cell suspensions were incubated with allophycocyanin (APC)-conjugated CD133 antibody (Miltenyi Biotec) for 30 min at 4 ℃. Mouse IgG1-APC was selected as an isotype control body. 7-Aminoactinomycin was used to eliminate the dead cells. The labeled cells were detected by the BD FACSVantage Systems (Becton Dickinson) according to the manufacturer's protocols. Gating was implemented on the basis of negative control staining profiles.

Colonosphere assay

McCoy's 5A with B27 supplement (Invitrogen), 20 μg/mL epidermal growth factor (Invitrogen), 20 μg/mL fibroblast growth factor (Invitrogen), and penicillin-streptomycin served as the stem cell medium (SCM) for this experiment. HT29 cells, after the indicated treatments, were plated at a concentration of 200 cells/100 μL SCM in each of the 20 wells of a 96-well ultralow-attachment plate (Corning Life Sciences, CA, United States). Cells were supplemented with 100 μL SCM after 7 d of incubation and analyzed on day 14, and MTT solution (40 μL) was added to each well, and a colorimetric assessment was done. The average absorbance measurement for each group was used as an index of sphere number.

Soft agar colony-forming unit assays

We mixed 1.2% agar with 2 × McCoy's 5A medium at a ratio of 1:1 to make a 0.6% agar growth medium solution. We pipetted 2 mL of the 0.6% growth medium mixture into each well of the six-well cell culture cluster (Corning Life Sciences). We avoided bubble formation and spread the mixture evenly by slowly rotating the plate. We allowed the 0.6% agar growth medium layer to harden for 30-40 min at room temperature in a sterile laminar flow hood. We determined the concentration of HT29 cells treated with DMSO control, oxaliplatin and salinomycin, and adjust the suspension to 5×10^3 cells/mL in 0.3% agar diluted with PBS. We transferred 2 mL of the cell suspension to the 0.6% agar growth medium plate and cultured at 37 ℃ in the presence of 5% CO_2 for 14-21 d. We counted the number of colonies using a microscope.

Immunocytochemistry

Cells were directly sorted onto a glass slide, fixed with 4% paraformaldehyde, and stained with anti-E-cadherin, anti-CD133 and anti-vimentin monoclonal antibodies. Nuclei were identified by staining with 4', 6-diamidino-2-phenylindole. Subcellular localizations were determined by using confocal microscopy. The fluorescence intensity of each region was analyzed by different people on three occasions.

Statistical analysis

All values were shown as mean ± SD. Statistical significance was calculated by *t* test unless otherwise stated (SPSS 17.0), considering $P < 0.05$ as statistically significant.

RESULTS

Nigericin inhibits tumor growth and invasion

We examined the *in vitro* effect of nigericin on tumor growth and metastasis. Compared with oxaliplatin, nigericin exhibited more toxicity for the HT29 cell line (IC_{50}, 12.92 ± 0.25 μmol *vs* 37.68 ± 0.34 μmol) (Figure 1A). We also obtained similar results with the SW116 cell line (IC_{50}, 15.86 ± 0.18 μmol *vs* 41.02 ± 0.23 μmol) (Figure 1B). We then checked whether nigericin had functional influence on the migratory and invasive capacity of CRC cells. After incubation for 24 h, nigericin induced a conspicuous reduction in the number of cells migrating through the PVDF membrane relative to the vehicle-treated controls [11 ± 2 cells per high-power field (HPF) *vs* 19.33 ± 1.52 cells per HPF, $P < 0.05$] (Figure 1C and D). It was surprising that oxaliplatin promoted the migration of CRC cells through PVDF membrane compared with the vehicle-treated controls (38 ± 2 cells per HPF *vs* 19.33 ± 1.52 cells per HPF, $P < 0.05$) (Figure 1C and D). Compared to

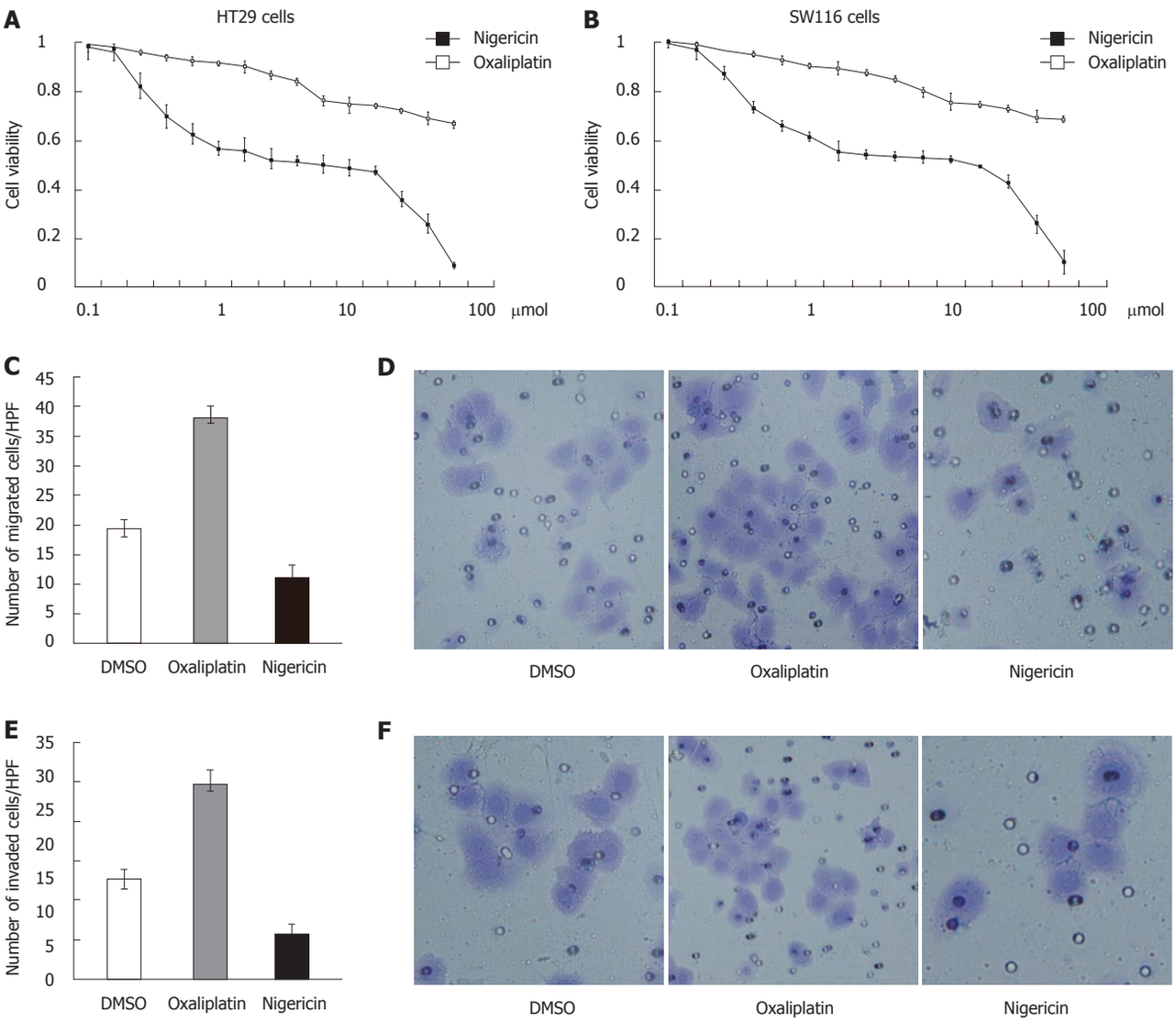

Figure 1 Nigericin inhibits tumor growth and metastasis. A: Dose-response curves of HT29 cells treated with nigericin and oxaliplatin. Bars denote SD (n = 5); B: Dose-response curves of SW116 cells treated with nigericin and oxaliplatin. Bars denote SD (n = 5); C: Boyden chamber assays were done to compare the migratory capacities of HT29 cells treated with oxaliplatin and nigericin. Bars denote SD (n = 5); D: Also shown are phase-contrast images of HT29 cells migrating through the collagen membrane; E: Numbers of cells invading through the Matrigel-coated hydrophobic polyvinylidene fluoride membrane after treatment with the indicated compounds. Bars denote SD (n = 5); F: Images of HT29 cells migrating through the collagen membrane are also shown. DMSO: Dimethylsulfoxide; HPF: High-power field.

the control group, the numbers of HT29 cells invading through the Matrigel-coated membrane also decreased in the nigericin-treated group (6.66 ± 1.52 cells per HPF *vs* 14.66 ± 1.52 cells per HPF, $P < 0.05$) (Figure 1E and F). Correspondingly, oxaliplatin treatment increased the number of HT29 cells invading through the Matrigel-coated membrane (28.66 ± 2.08 cells per HPF *vs* 14.66 ± 1.52 cells per HPF, $P < 0.05$) (Figure 1E and F).

Effects of nigericin and oxaliplatin on expression of cancer stem cell marker

In order to complete subsequent experiments logically, we treated the HT29 cells with nigericin, oxaliplatin, and DMSO vehicle control for 3 d, and then replaced the culture medium containing drugs with normal McCoy's 5A medium with 10% FBS for another 3 d incubation.

The stem cell marker prominin-1 (CD133), a pentaspan membrane protein, may not be the only marker, but it remains the most widely reported marker of cancer stem cells (CSCs) of CRC validated by different groups[14-18].

We further assessed the expression of CD133 on HT29 cells after treatment with nigericin and oxaliplatin using flow cytometry. The results demonstrated that nigericin reduced the positive rate of CD133 from 83.57% to 63.93%, relative to the control group ($P < 0.05$) (Figure 2A and B). In contrast, oxaliplatin treatment increased the expression of CD133 from 79.18% to 97.22%. In order to verify this result, we selected the SW116 cell line to repeat the experiment. Similarly, nigericin decreased the proportion of $CD133^+$ cells from 4.55% to 0.31%; on the contrary, the expression rate of CD133 increased from 4.55% to 36.89% (Figure 2C and D). The data from real-time PCR, Western blotting, and immunocytochemistry indicated analogous results (Figure 2E-G).

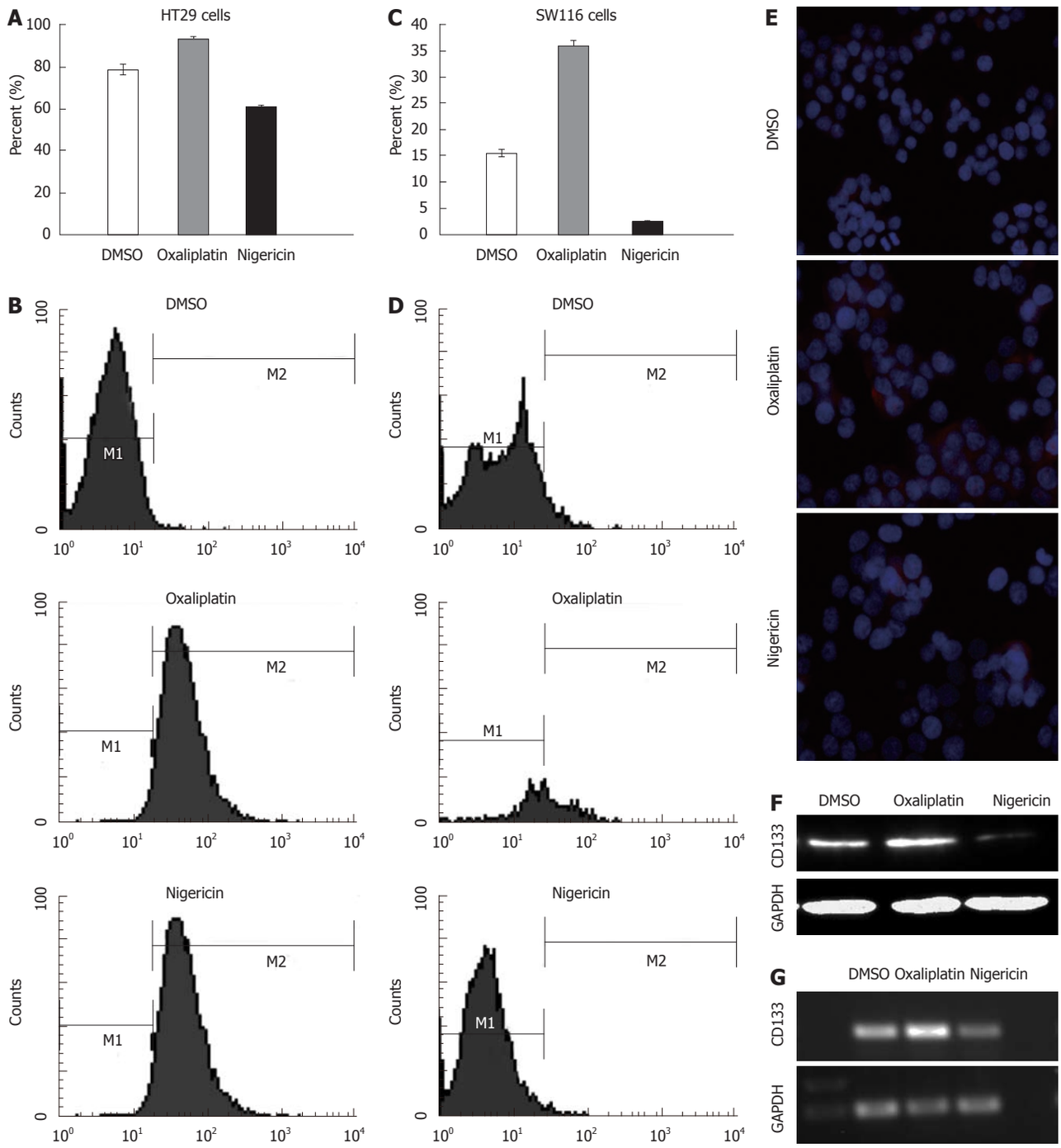

Figure 2 Effects of nigericin and oxaliplatin on expression of cancer stem cell marker. A: Percentages of CD133$^+$ cells after treatment of HT29 cells with nigericin and oxaliplatin. Bars denote SD; B: CD133 fluorescence-activated cell sorting (FACS) profiles are indicated for HT29 cell treatment with nigericin and oxaliplatin; C: Percentages of CD133$^+$ cells after treatment of SW116 cells with nigericin and oxaliplatin. Bars denote SD; D: CD133 expression in SW116 cells after treatment was assayed with FACS; E: Immunofluorescence staining analysis of CD133 expression in HT29 cells after treatment; F: CD133 protein expression in HT29 cells after treatment was assayed with immunoblotting; G: Real-time polymerase chain reaction analysis of CD133 mRNA expression in HT29 cells. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; DMSO: Dimethylsulfoxid.

Effect of nigericin and oxaliplatin on sphere- or colony-forming ability of CRC cells

To evaluate the ability to form colonies or spheres of HT29 cells treated with nigericin and oxaliplatin in the absence of serum and without attachment to culture plates[19]. We performed the sphere-forming assay and soft agar forming assay under serum-free conditions.

Differences between the nigericin and oxaliplatin groups were quantitated by plating a limited number of cells in each well of a low-attachment 96-well plate and evaluating the ability of HT29 cells to form colonospheres. Nigericin decreased the number of spheres relative to the control group (0.14 ± 0.01 *vs* 0.35 ± 0.01, $P < 0.05$), while oxaliplatin increased the number of spheres relative to the control group (0.75 ± 0.02 *vs* 0.35 ± 0.01, $P < 0.05$) (Figure 3A). Nigericin also showed a decreased ability to

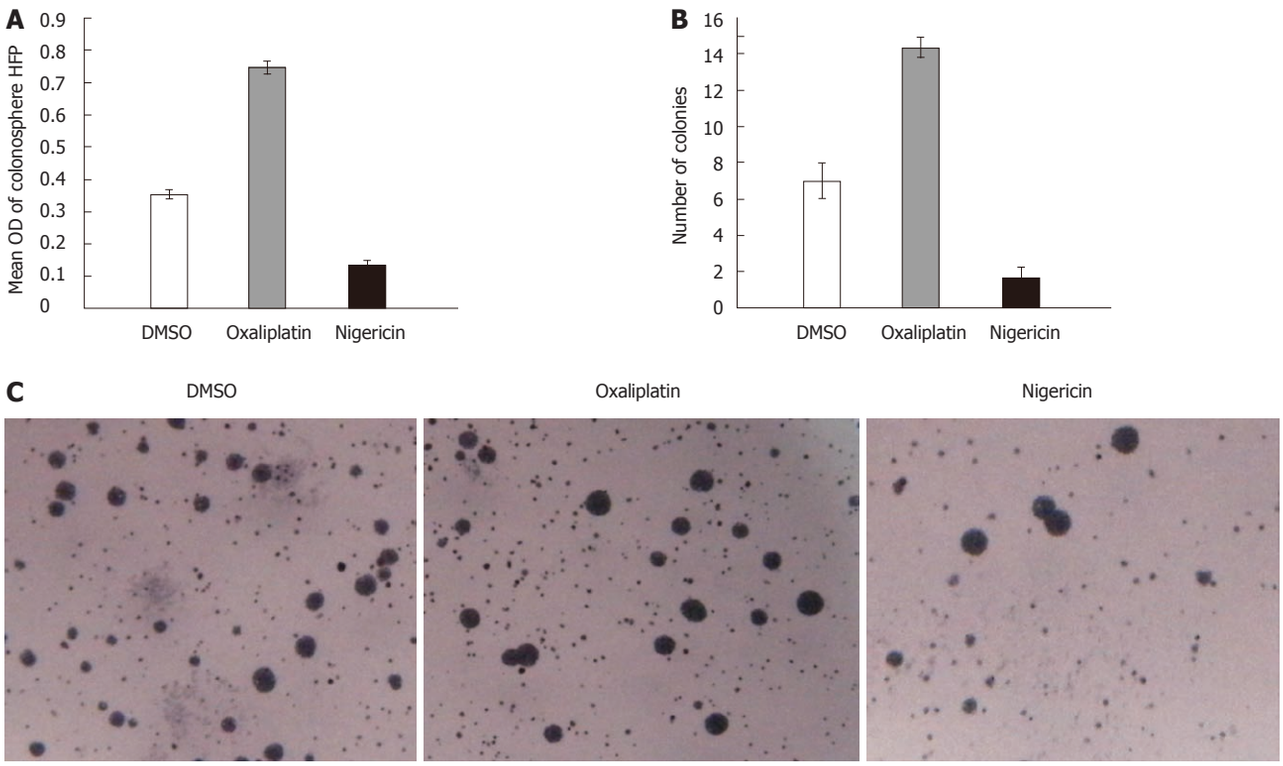

Figure 3 Effects of nigericin and oxaliplatin on sphere- or colony-forming ability of cancer stem cells. A: Mean OD of sphere-forming HT29 cells after different treatments was assayed by methyl thiazolyl tetrazolium assay. Bars denote SD (n = 5); B: Numbers of colonies formed by HT29 cells after treatment in independent experiments. Bars denote SD (n = 5); C: Phase-contrast images of colonies formed in soft agar assays after treatment. DMSO: Dimethylsulfoxide; HPF: High-power field.

form colonies under anchorage-independent conditions in a standard soft agar assay after 14 d in culture, relative to the control group (1.66 ± 0.57 *vs* 7 ± 1.15, $P < 0.05$), whereas the colony numbers were higher in oxaliplatin group relative to the vehicle-treated controls (14.33 ± 0.57 *vs* 7 ± 1.15, $P < 0.05$) (Figure 3B and C).

Up-regulation of E-cadherin and downregulation of vimentin in CRC cells after nigericin treatment

E-cadherin, encoded by the *CDH1* gene, has dual functions in epithelial cells: as a cell-cell adhesion molecule and as a negative regulator of the canonical WNT signaling cascade; in particular, of its central mediator β-catenin. E-cadherin downregulation in mammalian cell systems is sufficient to trigger EMT[20]. Gupta *et al*[12] have reported that nigericin preferentially kills cells that have undergone EMT. In colorectal carcinomas, the embryonic EMT is activated during tumor invasion in disseminating cancer cells[21]. Characteristic of these cells is a loss of E-cadherin expression.

We detected the expression of epithelial marker (E-cadherin) and mesenchymal marker (vimentin) of cells treated with nigericin and oxaliplatin to ascertain the effects of diverse compounds on EMT.

As shown in Figure 4A and B, nigericin induced an increase in expression of E-cadherin and a decrease in expression of vimentin relative to vehicle controls. In contrast, the expression of E-cadherin in the cells treated with oxaliplatin was downregulated in contrast to vehicle controls; correspondingly, oxaliplatin treatment upregulated the expression level of vimentin. The data from real-time PCR showed similar results to immunocytochemistry and Western blotting (Figure 4C).

DISCUSSION

Significant progress has been made in understanding the molecular pathogenesis, diagnosis (hereditary and sporadic), and treatment of CRC. Despite the use of active targeted drugs for treatment of metastatic CRC in the past decade, and improvement of overall survival to nearly 2 years for nonresectable disease, the cure rate remains low[22].

5-Fluorouracil and oxaliplatin formed the mainstay of chemotherapeutic regimens for metastatic CRC. Oxaliplatin covalently binds to DNA, forming platinum-DNA adducts that cause prolonged G2 arrest and inhibition of growth, which lead to apoptotic cell death[23].

There is a large body of evidence that tumor cells that are resistant to chemotherapy represent a subpopulation of cells from the primary tumor, which is molecularly and phenotypically distinct. These cells are referred to by several names, including tumor-initiating cells, tumor-promoting cells, or more commonly, CSCs[16]. EMT is a highly conserved cellular process during embryonic development and a pathogenic feature in tumorigenesis[10,24].

During the process of EMT, epithelial cells lose the expression of E-cadherin and other components of

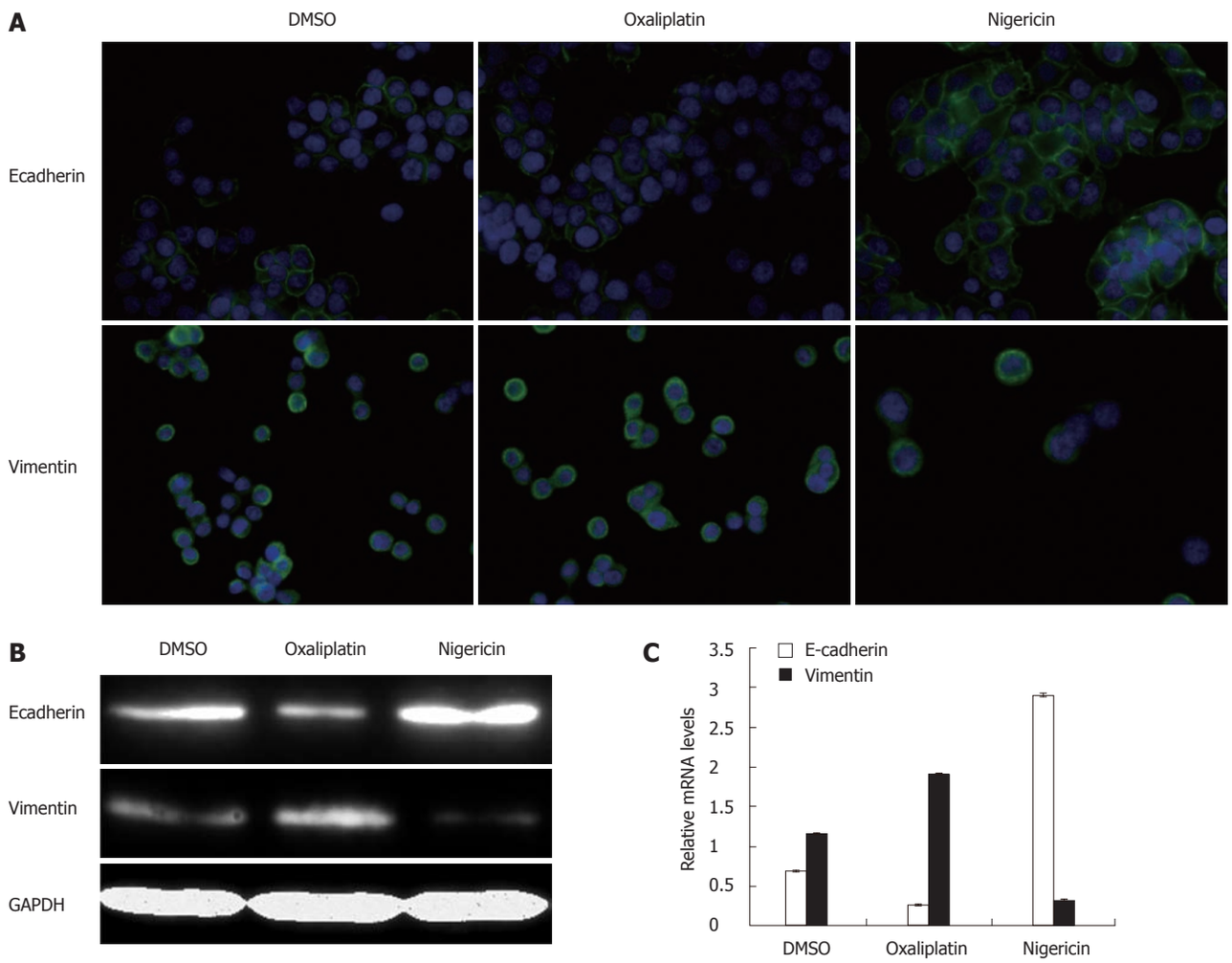

Figure 4 Upregulation of E-cadherin and downregulation of vimentin in cancer stem cells after nigericin treatment. A: Immunofluorescence staining analysis of treated HT29 cells using epithelial E-cadherin and vimentin staining; B: E-cadherin and vimentin expression in HT29 cells after treatment, assayed by Western blotting; C: Real-time polymerase chain reaction analysis of E-cadherin and vimentin mRNA expression in HT29 cells after treatment with dimethylsulfoxide (DMSO) vehicle, oxaliplatin and nigericin.

epithelial cell junctions, adopt a mesenchymal cell phenotype, and acquire motility and invasive ability[25,26]. Furthermore, Mani *et al*[10] have induced EMT in nontumorigenic, immortalized human mammary epithelial cells (HMLEs) by ectopic expression of either the Twist or Snail transcription factors; these cells formed > 30-fold more mammospheres than did HMLEs infected with the corresponding control vector. They have concluded that the cells generated by EMT acquired yet another attribute of mammary stem cells. EMT, which enables cancer cell dissemination, also imparts a self-renewal capability to disseminating cancer cells.

There is no consensus as to the exact criteria that define a CSC, because markers might vary according to the tumor type. In our study, we suggested CD133 as a marker of tumor-initiating cells of CRC[14-18].

We evaluated the effect of nigericin and oxaliplatin on CRC cell lines, including invasion and metastasis, and growth on colon cancer spheres, or colonospheres. From the results of cell viability and flow cytometry assays, we could see that nigericin specifically targets $CD133^+$ cell subpopulations within CRC cell lines. Moreover, nigericin induced inhibition of invasion and metastasis in HT29 cells. These effects may have been due to the fact that nigericin upregulated the expression of E-cadherin, while E-cadherin played an important role in cancer progression and EMT induction[27,28].

In a variety of human cancers, E-cadherin loss was closely related to poor prognosis, tumor progression, and metastasis[29,30]. Therefore, E-cadherin also could be a sign of drug efficiency of nigericin therapy in the future. Through analysis of the expression level of E-cadherin and vimentin, we may conclude that nigericin partly reverses EMT to affect the ability of CRC cells to invade and metastasize.

We further evaluated the effects of nigericin treatment on the characteristics of CSC phenotype. The nigericin treatment group had a decreased number of spheres or colonies relative to the vehicle control group. Our data led us to hypothesize further that nigericin treatment suppresses EMT-generating cells with the properties of stem cells. This hypothesis needs further studies using animal

experiments and preclinical and clinical trials. Nigericin may prove to be the therapeutic strategy that is effective in patients with metastatic disease.

However, the molecular mechanisms involved in the effect of nigericin are poorly understood. Lu *et al*[13] have reported that nigericin, as a potassium ionophore, selectively inhibits Wnt1-mediated signaling in HEK293 cells.

The polyether ionophores like nigericin interfere with transmembrane potassium potential and promote mitochondrial and cell potassium efflux. We hypothesize that nigericin treatment antagonizes the Wnt signaling cascade, while Wnt signaling plays a crucial role in embryonic development and cancer[31-35]. Besides, certain other CSC markers and signaling pathways, including EZH2 and Hedgehog pathways may also play some important roles in the mechanism of nigericin treatment, and thus need further studies[36]. Further studies will focus on the relation between nigericin-induced EMT and Wnt signaling.

We showed for the first time that nigericin not only partly reversed the EMT process during cell invasion and metastasis, but also suppressed some of the CSC phenotypes generated by EMT. EMT plays a pivotal role in tumor invasion and metastasis; therefore, nigericin treatment may be of benefit in the future.

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COMMENTS

Background

Despite therapeutic innovations, metastatic colorectal cancer (CRC) often has a poor prognosis and high mortality.

Research frontiers

Epithelial-mesenchymal transition (EMT) provides a new basis for understanding the progression of carcinoma towards dedifferentiated and more malignant states. The EMT program, which involves dissolution of adherens and tight junctions and a loss of cell polarity, dissociates the cells with epithelial cell sheets into individual cells that exhibit multiple mesenchymal attributes, including heightened invasiveness. EMT also generates cells with properties of stem cells. Nigericin has been reported recently to act as a selective breast cancer stem cell inhibitor. However, the effect of nigericin on CRC is unknown. In this study, the authors evaluated the anticancer effect of nigericin and its possible mechanisms.

Innovations and breakthroughs

Recent reports have highlighted the important role of EMT during the invasion-metastasis cascade. In particular, EMT can be seen at the edges of colon carcinomas that are invading adjacent tissues. This is the first study to report that nigericin could suppress CRC metastasis. Furthermore, our studies indicated the possible mechanisms of action of nigericin.

Applications

Through understanding the effect of nigericin on CRC cells, this study may indicate a future promising therapeutic strategy in the treatment of patients with metastatic colorectal carcinoma.

Terminology

E-cadherin is a hallmark of epithelial cell protein expression; vimentin is an intermediate filament component of the mesenchymal cell cytoskeleton. CD133 protein, a pentaspan cell surface receptor, is a putative CRC stem cell marker.

Peer review

The study investigated the antitumor activity of nigericin on CRC stem cells. The authors found that nigericin selectively targeted cancer stem cells, and inhibited EMT. It is well designed and well presented.

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
2 **Boyle P**, Ferlay J. Cancer incidence and mortality in Europe, 2004. *Ann Oncol* 2005; **16**: 481-488
3 **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674
4 **Talmadge JE**, Fidler IJ. AACR centennial series: the biology of cancer metastasis: historical perspective. *Cancer Res* 2010; **70**: 5649-5669
5 **Klymkowsky MW**, Savagner P. Epithelial-mesenchymal transition: a cancer researcher's conceptual friend and foe. *Am J Pathol* 2009; **174**: 1588-1593
6 **Polyak K**, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* 2009; **9**: 265-273
7 **Thiery JP**. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002; **2**: 442-454
8 **Yilmaz M**, Christofori G. EMT, the cytoskeleton, and cancer cell invasion. *Cancer Metastasis Rev* 2009; **28**: 15-33
9 **Barrallo-Gimeno A**, Nieto MA. The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* 2005; **132**: 3151-3161
10 **Mani SA**, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 2008; **133**: 704-715
11 **Morel AP**, Lièvre M, Thomas C, Hinkal G, Ansieau S, Puisieux A. Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS One* 2008; **3**: e2888
12 **Gupta PB**, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, Lander ES. Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell* 2009; **138**: 645-659
13 **Lu D**, Choi MY, Yu J, Castro JE, Kipps TJ, Carson DA. Salinomycin inhibits Wnt signaling and selectively induces apoptosis in chronic lymphocytic leukemia cells. *Proc Natl Acad Sci U S A* 2011; **108**: 13253-13257
14 **O'Brien CA**, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 2007; **445**: 106-110
15 **Ricci-Vitiani L**, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R. Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007; **445**: 111-115
16 **Dallas NA**, Xia L, Fan F, Gray MJ, Gaur P, van Buren G, Samuel S, Kim MP, Lim SJ, Ellis LM. Chemoresistant colorectal cancer cells, the cancer stem cell phenotype, and increased sensitivity to insulin-like growth factor-I receptor inhibition. *Cancer Res* 2009; **69**: 1951-1957
17 **Ferrand A**, Sandrin MS, Shulkes A, Baldwin GS. Expression of gastrin precursors by CD133-positive colorectal cancer cells is crucial for tumour growth. *Biochim Biophys Acta* 2009; **1793**: 477-488
18 **Haraguchi N**, Ohkuma M, Sakashita H, Matsuzaki S, Tanaka F, Mimori K, Kamohara Y, Inoue H, Mori M. CD133+CD44+ population efficiently enriches colon cancer initiating cells. *Ann Surg Oncol* 2008; **15**: 2927-2933
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
20 **Onder TT**, Gupta PB, Mani SA, Yang J, Lander ES, Wein-

berg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res* 2008; **68**: 3645-3654
21 **Yilmaz M**, Christofori G, Lehembre F. Distinct mechanisms of tumor invasion and metastasis. *Trends Mol Med* 2007; **13**: 535-541
22 **Cunningham D**, Atkin W, Lenz HJ, Lynch HT, Minsky B, Nordlinger B, Starling N. Colorectal cancer. *Lancet* 2010; **375**: 1030-1047
23 **Kelland L**. The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer* 2007; **7**: 573-584
24 **Huber MA**, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 2005; **17**: 548-558
25 **Kalluri R**, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009; **119**: 1420-1428
26 **Acloque H**, Adams MS, Fishwick K, Bronner-Fraser M, Nieto MA. Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *J Clin Invest* 2009; **119**: 1438-1449
27 **Hajra KM**, Fearon ER. Cadherin and catenin alterations in human cancer. *Genes Chromosomes Cancer* 2002; **34**: 255-268
28 **Jeanes A**, Gottardi CJ, Yap AS. Cadherins and cancer: how does cadherin dysfunction promote tumor progression? *Oncogene* 2008; **27**: 6920-6929
29 **Kowalski PJ**, Rubin MA, Kleer CG. E-cadherin expression in primary carcinomas of the breast and its distant metastases. *Breast Cancer Res* 2003; **5**: R217-R222
30 **Dorudi S**, Sheffield JP, Poulsom R, Northover JM, Hart IR. E-cadherin expression in colorectal cancer. An immunocytochemical and in situ hybridization study. *Am J Pathol* 1993; **142**: 981-986
31 **Clevers H**. Wnt/beta-catenin signaling in development and disease. *Cell* 2006; **127**: 469-480
32 **Moon RT**, Kohn AD, De Ferrari GV, Kaykas A. WNT and beta-catenin signalling: diseases and therapies. *Nat Rev Genet* 2004; **5**: 691-701
33 **Willert K**, Jones KA. Wnt signaling: is the party in the nucleus? *Genes Dev* 2006; **20**: 1394-1404
34 **Moon RT**, Bowerman B, Boutros M, Perrimon N. The promise and perils of Wnt signaling through beta-catenin. *Science* 2002; **296**: 1644-1646
35 **Logan CY**, Nusse R. The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 2004; **20**: 781-810
36 **Crea F**, Fornaro L, Paolicchi E, Masi G, Frumento P, Loupakis F, Salvatore L, Cremolini C, Schirripa M, Graziano F, Ronzoni M, Ricci V, Farrar WL, Falcone A, Danesi R. An EZH2 polymorphism is associated with clinical outcome in metastatic colorectal cancer patients. *Ann Oncol* 2012; **23**: 1207-1213

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BRIEF ARTICLE

Association between autoimmune pancreatitis and systemic autoimmune diseases

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Abstract

AIM: To investigate the association between autoimmune pancreatitis (AIP) and systemic autoimmune diseases (SAIDs) by measurement of serum immunoglobulin G4 (IgG4).

METHODS: The serum level of IgG4 was measured in 61 patients with SAIDs of different types who had not yet participated in glucocorticosteroid treatment. Patients with an elevated IgG4 level were examined by abdominal ultrasonography (US) and, in some cases, by computer tomography (CT).

RESULTS: Elevated serum IgG4 levels (919 ± 996 mg/L) were detected in 17 (28%) of the 61 SAID patients. 10 patients had Sjögren's syndrome (SS) (IgG4: 590 ± 232 mg/L), 2 of them in association with Hashimoto's thyroiditis, and 7 patients (IgG4: 1388 ± 985.5 mg/L) had systemic lupus erythematosus (SLE). The IgG4 level in the SLE patients and that in patients with SS were not significantly different from that in AIP patients (783 ± 522 mg/L). Abdominal US and CT did not reveal any characteristic features of AIP among the SAID patients with an elevated IgG4 level.

CONCLUSION: The serum IgG4 level may be elevated in SAIDs without the presence of AIP. The determination of serum IgG4 does not seem to be suitable for the differentiation between IgG4-related diseases and SAIDs.

Key words: Autoimmune pancreatitis; Serum immunoglobulin G4 level; Systemic lupus erythematosus; Sjögren's syndrome; Mikulicz's disease

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INTRODUCTION

Autoimmune pancreatitis (AIP) is an increasingly recognized type of chronic pancreatitis that is clearly distinct from other types of chronic pancreatitis. It is characterized by its morphology, immunologic features, pathology and glucocorticosteroid responsiveness[1-4].

Immunological examinations in AIP patients have demonstrated high incidences of hypergammaglobulinemia (43%), increased serum levels of immunoglobulin G (IgG) (62%-80%) and IgG4 (68%-92%), and the presence of antinuclear antibodies (40%-64%) and rheumatoid factor (25%). Among all the serological diagnostic features, an elevated serum level of IgG4 has the highest individual diagnostic value; however, it is not disease specific. Furthermore, an elevated serum IgG4 level correlates with the activity of AIP[5,6]. Kamisawa *et al*[7] reported an association between serum IgG4 level and extrapancreatic lesions in patients with AIP. AIP patients with a serum IgG4 level ⩾ 2200 mg/L frequently exhibit extrapancreatic lesions.

The immunologic and histologic features of AIP and the glucocorticosteroid responsiveness suggest an autoimmune mechanism for the development of the disease[8]. AIP is accompanied by other autoimmune diseases (sclerosing cholangitis, sclerosing sialadenitis, retroperitoneal fibrosis, enlarged celiac and hilar lymph nodes, chronic thyroiditis and interstitial nephritis, *etc*.) in 50%-63% of cases, suggesting that AIP may be a systemic disorder[1-4]. The occurrence of autoimmune diseases in association with AIP is well documented[9,10], but the incidence of such associations has not been reported.

The aim of the present study was to assess the presence of AIP in different systemic autoimmune diseases (SAIDs) through measurement of the serum IgG4 level and examination of the morphology of the pancreas.

MATERIALS AND METHODS

Patients and diagnosis of diseases

Serum samples were obtained from 61 patients with different SAIDs who had been admitted to our Department of Rheumatology and had not participated in glucocorticosteroid treatment during the past 2 years. One male and 60 females (mean age 54.5 years, range 29-82 years) were recruited.

Autoimmune diseases were diagnosed according to standard diagnostic criteria[11-14]. The diagnosis of AIP was based on the HISORt criteria[15]. The most frequent diagnosis was Sjögren's syndrome (SS), but systemic lupus erythematosus (SLE), Hashimoto thyroiditis, Raynaud's syndrome, polymyositis and systemic sclerosis also occurred (Table 1).

Serum samples were additionally obtained from 7 age- and sex-matched healthy subjects, and 6 patients with AIP. In one AIP patient, the AIP was accompanied by rheumatoid arthritis and ankylosing spondylitis.

All participants provided their written informed consent. The study protocol was approved by the ethics committee at the University of Szeged and was carried out in full accordance with the most recent revisions of the Helsinki Declaration.

IgG4 assay

After collection, serum samples were stored at -70 ℃ until analyzed. The IgG4 subclass was determined by the radial immunodiffusion (RID) method (The Binding Site Limited, Birmingham, United Kingdom). The diameters of precipitation rings were measured after 72 h. The results were read using the RID reference table. The lowest detection limit was 22.4 mg/L. The intra- and inter-assay coefficients of variation were 3.26 and 0.89 CV%, respectively, as stated by the manufacturer. A cutoff value of 400 mg/L was employed.

Patients with a serum IgG4 level of > 400 mg/L were examined by a gastroenterologist. The clinical and laboratory data were reviewed and abdominal ultrasonography (US) and computed tomography (CT) were performed.

Table 1 Distribution of gender and age in groups of patients

	No. of patients	Male/ female	Age mean (range)
Sjögren's syndrome	35	1/34	56.7 (29-82)
Systemic lupus erythematosus	22	0/22	50.2 (31-68)
Systemic sclerosis	4	0/4	59.5 (45-80)
Normal subjects	7	4/3	68 (56-80)
Autoimmune pancreatitis	6	3/3	53.7 (27-75)

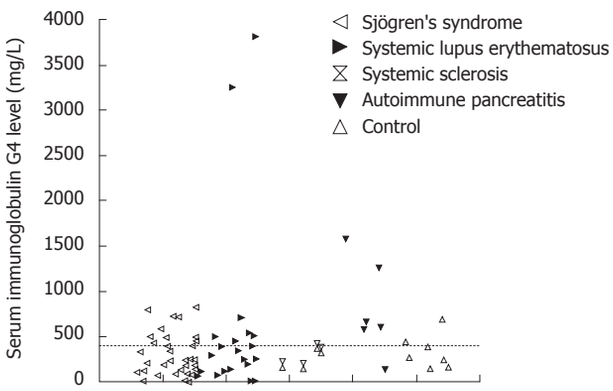

Figure 1 Serum immunoglobulin G4 levels in different systemic autoimmune diseases and autoimmune pancreatitis. Dotted line: Cutoff value (400 mg/L).

Anti-SS-A/SS-B autoantibody determination

The presence of anti-SS-A/SS-B autoantibodies was determined by means of commercial enzyme-linked immunosorbent assays, conducted according to the protocols provided by the manufacturers.

Experimental data were evaluated statistically with the independent-samples *t* test. *P*-values < 0.05 were accepted as being statistically significant. Statistical data is expressed as mean ± SD.

RESULTS

An elevated serum IgG4 level (mean value 919 ± 996 mg/L) was detected in 17 (28%) of the 61 SAID patients (Figure 1). Ten of the 17 patients had SS (mean serum IgG4 590 ± 232 mg/L) (2 cases were associated with Hashimoto's thyroiditis), 7 (mean serum IgG4 1388 ± 985.5 mg/L) were diagnosed with SLE. Two SLE patients showed markedly elevated IgG4 levels (> 3000 mg/L). In

one case, SLE was associated with Raynaud's syndrome, while the other patient suffered from xerophtalmia and bronchial asthma. The serum IgG4 level was elevated (mean serum IgG4 783 ± 522 mg/L) in 5 (83%) of the 6 AIP patients. The patient with a normal level of IgG4 had typical pancreatic histology and his condition improved with steroid therapy. The IgG4 levels in these SLE and SS patients were not significantly different from that in the AIP patients.

US examination revealed a normal pancreas in 11 of the 17 SAID patients with elevated serum IgG4 levels, but raised the suspicion of AIP by demonstrating a gracile pancreas in 2 cases (both suffered from SS), and widening of the body or the tail of pancreas, each in a further one patient (both suffered from SLE). However, in none of these 4 cases was AIP confirmed by an abdominal CT scan. The US examinations indicated pancreatic steatosis in 2 additional cases. None of the SAID patients had pancreatic duct dilatation.

The presence of anti-SS-A/SS-B autoantibodies and the potential relation of this to an elevated IgG4 level were examined in the patients with SS. Both anti-SS-A-positivity and anti-SS-B-positivity was detected in 22 patients; 7 of them exhibited an elevated IgG4 level. The anti-SS-A was positive and the anti-SS-B was negative in 9 cases; 2 of these patients had a high IgG4 level. In 4 patients with SS, neither anti-SS-A-positivity, nor anti-SS-B-positivity was found; an elevated IgG4 level was detected in only one of these cases.

DISCUSSION

The present study has demonstrated that the serum IgG4 level may be elevated in SAIDs, without the presence of AIP.

AIP can be complicated by a variety of extrapancreatic lesions, which appear synchronously or metachronously with the pancreatic lesion, share the same pathological conditions, and show a favorable response to glucocorticosteroid therapy, characteristics indicative of a common pathophysiological background. Among the variety of extrapancreatic diseases, lachrymal and salivary gland lesions are some of the most frequent, found in 23%-39% of patients with AIP[16,17]. Extrapancreatic lesions may mimic or be misdiagnosed as primary lesions of the corresponding organs, e.g., lachrymal and salivary gland lesions for SS. It is therefore necessary to differentiate between IgG4-related diseases and inherent diseases of the corresponding organ. When the pancreatic lesion is obscured, it may be difficult to detect these presumably IgG4-related extrapancreatic lesions[4].

IgG4 is the rarest of the 4 IgG subclasses in humans, with an incidence of about 4%. IgG antibodies are predominantly involved in the secondary immune response; complement activation is possibly their most important biological function. The main role of IgG4 is presumably to protect against the biological effects of the complement-fixing IgG subclasses and to act in parasitic infestation or various forms of atopy[18-20]. Serum IgG4 levels are frequently and significantly elevated in AIP patients[6] and an elevated level of serum IgG4 has been included among the laboratory criteria for the diagnosis of AIP[4,15]. AIP patients with 3 extrapancreatic lesions have been reported to have significantly higher IgG4 levels than those lacking such lesions[16]. The optimal cutoff value for discriminating AIP patients with extrapancreatic lesions from those without was demonstrated on the basis of receiver operator characteristic curves to be 2200 mg/L[7].

The serum IgG4 level was measured in 61 SAID patients in our study, 28% of whom proved to have an elevated serum level of IgG4. However, none of them could be diagnosed with AIP according to the HISORt criteria. What could be the reason for this?

One explanation is the composition of our patient cohort. In Japan AIP predominantly affects men, with a male:female ratio of 2.85:1[16]. Moreover, there was a male preponderance in the United Kingdom, European and US studies (100%, 66% and 65% male, respectively), similar to in reports from Japan[21-24]. In contrast, there was only one male in our patient population.

Lachrymal and salivary lesions associated with AIP were previously considered to be complications of SS. However, in contrast to those accompanying SS, the lachrymal and salivary gland lesions associated with AIP yield negative results for anti-SS-A/SS-B autoantibodies and show numerous IgG4-positive plasma cell infiltrations in the affected tissues. These lesions are currently thought to correspond to Mikulicz's disease[25]. The explanation for our negative results may be that there was only one patient with negative SS-A/SS-B autoantibodies in our study group.

Another point is that autoantibodies against FcεRIα are detected in the sera of patients with different autoimmune diseases (such as SLE, dermatomyositis, pemphigus and pemphigoid); these antibodies are from subclasses IgG2 and IgG4, but they are functionally inactive[26]. In our study, elevated IgG4 levels were found in 7 patients treated for SLE.

Moreover, our 17 SAID patients with elevated IgG4 levels included 6 who suffered from different concomitant diseases which could cause the increase in the serum level of IgG4. In one patient, nodular sclerosis Hodgkin lymphoma (HL) was diagnosed histologically. HL cells frequently express interleukin 13 (IL-13) and its receptor. Besides exerting several effects on B cells (e.g., promoting their survival and proliferation), IL-13 switches the Ig class to IgG4 and IgE[27]. In another patient, bullous pemphigoid was identified, which is among the most common blistering autoimmune skin lesions. One of the features of the disease is the presence of autoantibodies against hemidesmosomal antigens (i.e., bullous pemphigoid antigen 1 and 2) in the serum and in affected areas of the skin. The major types of these autoantibodies are IgG4 and IgE[28]. In a third patient, cutaneous lymphocytic vasculitis was diagnosed, which could also explain the serum IgG4 elevation[29]. In 2 patients, the underlying disease was accompanied by Hashimoto's thyroiditis, which can elevate the IgG4 level since thyroglobulin autoantibodies

are from subclasses IgG2 and IgG4[30]. There was also one patient with bronchial asthma, in which disease elevated titers of IgG4 can be found[31].

Finally, SS was diagnosed in the remaining 4 patients, one of whom was seronegative, while the others were seropositive. The elevated serum IgG4 level in patients with seronegative SS may possibly be explained by the presence of Mikulicz's disease[32]. Furthermore, an elevated serum IgG4 level has also been reported in SS[33].

However, not all AIP patients display elevated serum IgG and IgG4 levels. IgG4-negative AIP patients seem to occur more frequently in Europe[34]. Furthermore, some AIP cases improve spontaneously[4]. Hence, it cannot be ruled out that our SAID cohort included AIP patients who were not diagnosed by the measurement of serum IgG4 or in whom the morphology of the pancreas had already normalized by the time of our examination.

Overall, it can be concluded that the serum IgG4 level may be elevated in SAIDs, but as a consequence of the concomitant SAID rather than of AIP. The determination of serum IgG4 does not seem to be suitable for the differentiation between IgG4-related diseases and SAIDs.

COMMENTS

Background

Autoimmune pancreatitis (AIP) is frequently associated with some other autoimmune disease, suggesting that it may be a systemic disorder. The determination of serum immunoglobulin G4 (IgG4) is a sensitive marker to diagnose AIP and IgG4-related diseases.

Research frontiers

IgG4 is a sensitive marker in the diagnosis of AIP. The association of AIP and systemic autoimmune diseases (SAIDs), and the usefulness of the determination of serum IgG4 in the diagnosis of AIP in patients with SAIDs are not defined.

Innovations and breakthroughs

The authors revealed that the serum IgG4 level may be elevated in SAIDs without the presence of AIP. The determination of serum IgG4 does not seem to be suitable for the differentiation between IgG4-related diseases and SAIDs.

Applications

This study provides important data about the serum level of IgG4 in SAIDs. The determination of serum IgG4 does not seem to be suitable for the differentiation between IgG4-related diseases and SAIDs. The diagnosis of AIP in SAIDs should be made on the results of morphological and histological examination.

Terminology

AIP is an increasingly recognized distinct type of chronic pancreatitis with a presumed autoimmune etiology. Immunoglobulin G (IgG) has four subclasses (IgG1 through IgG4) and the IgG4 subclass accounts for 3%-6% of total serum IgG.

Peer review

This paper discusses the relationship between autoimmune pancreatitis and an elevated serum IgG4 level in autoimmune diseases, and presents interesting and potentially important information. The patients used in this study are few and biased (1 male and 60 female cases), but it is highly significant as a clinical pilot study. This study is well-designed and written, but there are some points to be clarified.

REFERENCES

1 **Detlefsen S**, Drewes AM. Autoimmune pancreatitis. *Scand J Gastroenterol* 2009; **44**: 1391-1407

2 **Shimosegawa T**, Kanno A. Autoimmune pancreatitis in Japan: overview and perspective. *J Gastroenterol* 2009; **44**: 503-517

3 **Park DH**, Kim MH, Chari ST. Recent advances in autoimmune pancreatitis. *Gut* 2009; **58**: 1680-1689

4 **Okazaki K**, Kawa S, Kamisawa T, Ito T, Inui K, Irie H, Irisawa A, Kubo K, Notohara K, Hasebe O, Fujinaga Y, Ohara H, Tanaka S, Nishino T, Nishimori I, Nishiyama T, Suda K, Shiratori K, Shimosegawa T, Tanaka M. Japanese clinical guidelines for autoimmune pancreatitis. *Pancreas* 2009; **38**: 849-866

5 **Hamano H**, Kawa S, Horiuchi A, Unno H, Furuya N, Akamatsu T, Fukushima M, Nikaido T, Nakayama K, Usuda N, Kiyosawa K. High serum IgG4 concentrations in patients with sclerosing pancreatitis. *N Engl J Med* 2001; **344**: 732-738

6 **Choi EK**, Kim MH, Lee TY, Kwon S, Oh HC, Hwang CY, Seo DW, Lee SS, Lee SK. The sensitivity and specificity of serum immunoglobulin G and immunoglobulin G4 levels in the diagnosis of autoimmune chronic pancreatitis: Korean experience. *Pancreas* 2007; **35**: 156-161

7 **Kamisawa T**, Imai M, Egawa N, Tsuruta K, Okamoto A. Serum IgG4 levels and extrapancreatic lesions in autoimmune pancreatitis. *Eur J Gastroenterol Hepatol* 2008; **20**: 1167-1170

8 **Okazaki K**, Uchida K, Koyabu M, Miyoshi H, Takaoka M. Recent advances in the concept and diagnosis of autoimmune pancreatitis and IgG4-related disease. *J Gastroenterol* 2011; **46**: 277-288

9 **Kamisawa T**, Okamoto A. Autoimmune pancreatitis: proposal of IgG4-related sclerosing disease. *J Gastroenterol* 2006; **41**: 613-625

10 **Kamisawa T**, Okazaki K, Kawa S, Shimosegawa T, Tanaka M. Japanese consensus guidelines for management of autoimmune pancreatitis: III. Treatment and prognosis of AIP. *J Gastroenterol* 2010; **45**: 471-477

11 **Vitali C**, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, Daniels TE, Fox PC, Fox RI, Kassan SS, Pillemer SR, Talal N, Weisman MH. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* 2002; **61**: 554-558

12 **Gill JM**, Quisel AM, Rocca PV, Walters DT. Diagnosis of systemic lupus erythematosus. *Am Fam Physician* 2003; **68**: 2179-2186

13 **Aletaha D**, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD, Combe B, Costenbader KH, Dougados M, Emery P, Ferraccioli G, Hazes JM, Hobbs K, Huizinga TW, Kavanaugh A, Kay J, Kvien TK, Laing T, Mease P, Ménard HA, Moreland LW, Naden RL, Pincus T, Smolen JS, Stanislawska-Biernat E, Symmons D, Tak PP, Upchurch KS, Vencovský J, Wolfe F, Hawker G. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum* 2010; **62**: 2569-2581

14 **Walker JG**, Pope J, Baron M, Leclercq S, Hudson M, Taillefer S, Edworthy SM, Nadashkevich O, Fritzler MJ. The development of systemic sclerosis classification criteria. *Clin Rheumatol* 2007; **26**: 1401-1409

15 **Chari ST**, Smyrk TC, Levy MJ, Topazian MD, Takahashi N, Zhang L, Clain JE, Pearson RK, Petersen BT, Vege SS, Farnell MB. Diagnosis of autoimmune pancreatitis: the Mayo Clinic experience. *Clin Gastroenterol Hepatol* 2006; **4**: 1010-1016; quiz 934

16 **Hamano H**, Arakura N, Muraki T, Ozaki Y, Kiyosawa K, Kawa S. Prevalence and distribution of extrapancreatic lesions complicating autoimmune pancreatitis. *J Gastroenterol* 2006; **41**: 1197-1205

17 **Naitoh I**, Nakazawa T, Ohara H, Ando T, Hayashi K, Tanaka H, Okumura F, Miyabe K, Yoshida M, Sano H, Takada H, Joh T. Clinical significance of extrapancreatic lesions in autoimmune pancreatitis. *Pancreas* 2010; **39**: e1-e5

18 **Nirula A**, Glaser SM, Kalled SL, Taylor FR. What is IgG4? A review of the biology of a unique immunoglobulin subtype. *Curr Opin Rheumatol* 2011; **23**: 119-124

19 **van der Zee JS**, van Swieten P, Aalberse RC. Inhibition of complement activation by IgG4 antibodies. *Clin Exp Immunol* 1986; **64**: 415-422

20 **Aalberse RC**, Schuurman J. IgG4 breaking the rules. *Immunology* 2002; **105**: 9-19

21 **Church NI**, Pereira SP, Deheragoda MG, Sandanayake N, Amin Z, Lees WR, Gillams A, Rodriguez-Justo M, Novelli M, Seward EW, Hatfield AR, Webster GJ. Autoimmune pancreatitis: clinical and radiological features and objective response to steroid therapy in a UK series. *Am J Gastroenterol* 2007; **102**: 2417-2425

22 **Zamboni G**, Lüttges J, Capelli P, Frulloni L, Cavallini G, Pederzoli P, Leins A, Longnecker D, Klöppel G. Histopathological features of diagnostic and clinical relevance in autoimmune pancreatitis: a study on 53 resection specimens and 9 biopsy specimens. *Virchows Arch* 2004; **445**: 552-563

23 **Raina A**, Yadav D, Krasinskas AM, McGrath KM, Khalid A, Sanders M, Whitcomb DC, Slivka A. Evaluation and management of autoimmune pancreatitis: experience at a large US center. *Am J Gastroenterol* 2009; **104**: 2295-2306

24 **Czakó L**, Gyökeres T, Topa L, Sahin P, Takács T, Vincze A, Dubravcsik Z, Szepes A, Pap A, Földesi I, Terzin V, Tiszlavicz L, Wittmann T. Autoimmune pancreatitis in Hungary: a multicenter nationwide study. *Pancreatology* 2011; **11**: 261-267

25 **Yamamoto M**, Harada S, Ohara M, Suzuki C, Naishiro Y, Yamamoto H, Takahashi H, Imai K. Clinical and pathological differences between Mikulicz's disease and Sjögren's syndrome. *Rheumatology* (Oxford) 2005; **44**: 227-234

26 **Fiebiger E**, Hammerschmid F, Stingl G, Maurer D. Anti-FcepsilonRIalpha autoantibodies in autoimmune-mediated disorders. Identification of a structure-function relationship. *J Clin Invest* 1998; **101**: 243-251

27 **Skinnider BF**, Elia AJ, Gascoyne RD, Trümper LH, von Bonin F, Kapp U, Patterson B, Snow BE, Mak TW. Interleukin 13 and interleukin 13 receptor are frequently expressed by Hodgkin and Reed-Sternberg cells of Hodgkin lymphoma. *Blood* 2001; **97**: 250-255

28 **Döpp R**, Schmidt E, Chimanovitch I, Leverkus M, Bröcker EB, Zillikens D. IgG4 and IgE are the major immunoglobulins targeting the NC16A domain of BP180 in Bullous pemphigoid: serum levels of these immunoglobulins reflect disease activity. *J Am Acad Dermatol* 2000; **42**: 577-583

29 **Kawassaki AM**, Haga H, Dantas TC, Musolino RS, Baldi BG, Carvalho CR, Kairalla RA, Mauad T. Adenopathy and pulmonary infiltrates in a Japanese emigrant in Brazil. *Chest* 2011; **139**: 947-952

30 **Fukuma N**, McLachlan SM, Petersen VB, Kau P, Bradbury J, Devey M, Bleasdale K, Grabowski P, Smith BR. Human thyroglobulin autoantibodies of subclasses IgG2 and IgG4 bind to different epitopes on thyroglobulin. *Immunology* 1989; **67**: 129-131

31 **Sprangers B,** Claes K. IgG4-related disease should be considered in cases of hypocomplementemic immune-complex tubulointerstitial nephritis. *Letters and Replies NDT Plus* 2010; **3:** 326-334

32 **Masaki Y**, Sugai S, Umehara H. IgG4-related diseases including Mikulicz's disease and sclerosing pancreatitis: diagnostic insights. *J Rheumatol* 2010; **37**: 1380-1385

33 **Suzuki S**, Kida S, Ohira Y, Ohba T, Miyata M, Nishimaki T, Morito T, Kasukawa R, Hojyo H, Wakasa H. [A case of Sjögren's syndrome accompanied by lymphadenopathy and IgG4 hypergammaglobulinemia]. *Ryumachi* 1993; **33**: 249-254

34 **Kamisawa T**, Takuma K, Tabata T, Inaba Y, Egawa N, Tsuruta K, Hishima T, Sasaki T, Itoi T. Serum IgG4-negative autoimmune pancreatitis. *J Gastroenterol* 2011; **46**: 108-116

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BRIEF ARTICLE

Clinical course of sub-centimeter-sized nodules detected during surveillance for hepatocellular carcinoma

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Abstract

AIM: To evaluate the outcome of sub-centimeter-sized nodules (SCSNs) detected during surveillance for hepatocellular carcinoma (HCC) in patients at risk.

METHODS: We retrospectively analyzed a total of 142 patients with liver cirrhosis or chronic hepatitis B or C without a prior history of HCC in whom a SCSN was detected during HCC surveillance. We calculated the rate of HCC development from SCSNs in the study population and analyzed the differences in the baseline clinical characteristics and imaging features between the patients with SCSNs that eventually developed into HCC and patients with SCSNs that did not develop into HCC.

RESULTS: During 667 person-years of follow-up, HCC developed in 33 patients. The calculated HCC development rate was 4.9% per year. The cumulative one-, two-, three- and five-year HCC development rates were 5.6%, 10.6%, 14.1% and 20.4%, respectively. Upon baseline comparison, the HCC group was older (54.4 ± 8.3 years *vs* 48.9 ± 9.4 years; $P = 0.003$) and had lower albumin levels (3.56 ± 0.58 g/dL *vs* 3.84 ± 0.55 g/dL; $P = 0.012$) and higher baseline alpha-fetoprotein (AFP) levels (8.5 ng/mL *vs* 5.4 ng/mL; $P = 0.035$) compared to the non-HCC group. Nodule pattern and initial radiologic diagnosis also differed between the two groups. Multivariate analysis revealed that age [$P = 0.012$, odds ratio (OR) =1.075, 95% confidence interval (CI) =1.016-1.137], sex ($P = 0.009$, OR = 3.969, 95% CI: 1.403-11.226), and baseline AFP level ($P = 0.024$, OR = 1.039, 95% CI: 1.005-1.073) were independent risk factors for developing HCC.

CONCLUSION: The overall risk of HCC development in patients with SCSNs is similar to that in liver cirrhosis patients. Patients with these risk factors need to be closely monitored during follow-up.

Key words: Chronic liver disease; Hepatocellular carcinoma; Risk factor; Sub-centimeter-sized nodule

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death in the world, and the ninth leading cause of cancer deaths in the United States[1-7]. The number of deaths per year from HCC is virtually identical to the incidence throughout the world, underscoring the high fatality rate of this aggressive disease[8]. The sole approach to achieve long-term survival is to detect the tumor at an early stage, when effective therapy can be applied[9]. Accordingly, the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases recommend performing screening for HCC in patients at risk who would be treated if diagnosed with this condition[10-12]. Under these guidelines, imaging criteria for the diagnosis of HCC are established for lesions 1 cm or larger in patients at risk, but owing to a high false-positive rate, a wait-and-see policy is recommended for nodules smaller than 1 cm in diameter[11,12]. However, the possibility remains high that minute hepatic nodules detected during surveillance may become malignant over time[13,14]. In addition, a delay in the start of treatment of early-stage HCC may be associated with a poorer patient survival[15]. Nevertheless, clinicians have limited data on the clinical course of sub-centimeter-sized nodules (SCSNs) detected during surveillance.

A variety of important risk factors for the development of HCC have been identified. These include chronic hepatitis B and C virus infection and cirrhosis due to almost any cause[16-22]. Almost 80% of cases are due to underlying chronic hepatitis B and C virus infection[17]. Since patients with chronic hepatitis B who may not have fully developed cirrhosis or have regressed cirrhosis as well as patients with cirrhosis are at increased risk of developing HCC, an updated the American Association for the Study of Liver Diseases guidelines recommended surveillance in patients with chronic hepatitis B[12].

The purpose of our study was to evaluate the outcome of SCSNs detected during HCC surveillance in patients at risk and to determine the risk factors for development of those nodules into HCC.

MATERIALS AND METHODS

Patients

This retrospective study was conducted according to the principles of the Declaration of Helsinki. The study involved patients with liver cirrhosis of any etiology or chronic liver disease including chronic hepatitis B and C virus infection, without a prior history of HCC in whom a SCSN was detected during HCC surveillance with ultrasonography (US) or computed tomography (CT) of the liver at Samsung Medical Center, Seoul, South Korea between January 1, 2005 and April 30, 2005 (n = 198). At our institution, patients at risk for HCC were followed with alpha-fetoprotein (AFP) and US every 6 mo. In case of a difficult US, such as in obese individuals, CT and US were performed alternately for HCC surveillance. Even when an SCSN was detected, patients were usually followed with AFP and US every three or six mo as appropriate. However, if any SCSN enlarged or its appearance was typical of HCC, 3 mo surveillance was used for a certain period or other image modalities such as CT or magnetic resonance imaging (MRI) were performed additionally. The medical records of all patients were reviewed thoroughly. Patients who met any of the following criteria were excluded: (1) less than 12 mo of follow-up, except subjects who were diagnosed with HCC within 12 mo of follow-up; (2) subjects who were lost to follow-up and diagnosed with HCC at an outside hospital; and (3) any history of cancer. Thus, a total of 56 patients were excluded from the study. Forty patients had less than 12 mo of follow-up, seven patients were excluded because HCC was diagnosed at the time of inclusion in the study, and three patients were lost to follow-up. Additionally, three patients had hepatic nodules 1 cm or larger in size at inclusion, two patients had other types of cancer, and the etiology of liver disease in one patient was unclear.

Data collection

The following clinical and laboratory information was collected from each patient: age, sex, etiology of liver disease, presence of liver cirrhosis, the Child-Pugh classification, aspartate aminotransferase (AST), alanine aminotransferase (ALT), prothrombin time (PT), serum total bilirubin, platelet count, serum albumin, and baseline and follow-up AFP levels.

Image interpretation

The initial radiologic diagnosis of SCSNs was based on the results of US or CT during surveillance. In addition, all radiologic images were reviewed by one radiologist who had 11 years of experience in liver imaging interpretation. He did not participate in the initial patient selection and was blinded to the final diagnoses and clinical information such as AFP levels. Each detected lesion was evaluated for the number, location, and echogenicity/attenuation of nodules. Lesions were categorized as follows: (1) hypoechoic/low-attenuation; (2) hyperechoic/high-attenuation; and (3) mixed echoic/attenuation (Figure 1). All lesions were included in one of these three categories.

The diagnosis of HCC was based either on biopsy or the clinical criteria of the Korean Liver Cancer Study Group and the National Cancer Center, South Korea[3]. Briefly, the diagnosis of HCC was made when the AFP level was $\geqslant$ 400 ng/mL and at least one of the dynamic enhancement CT or MRI showed a vascular pattern typical of HCC in patients at risk including patients with HBV or HCV infection, or liver cirrhosis. If the AFP level was < 400 ng/mL, at least two of the dynamic enhancement CT, MRI or transarterial angiography must show vascular patterns typical of HCC in order to make a diagnosis of HCC.

Statistical analysis

Statistical analyses were conducted using PASW Statistics

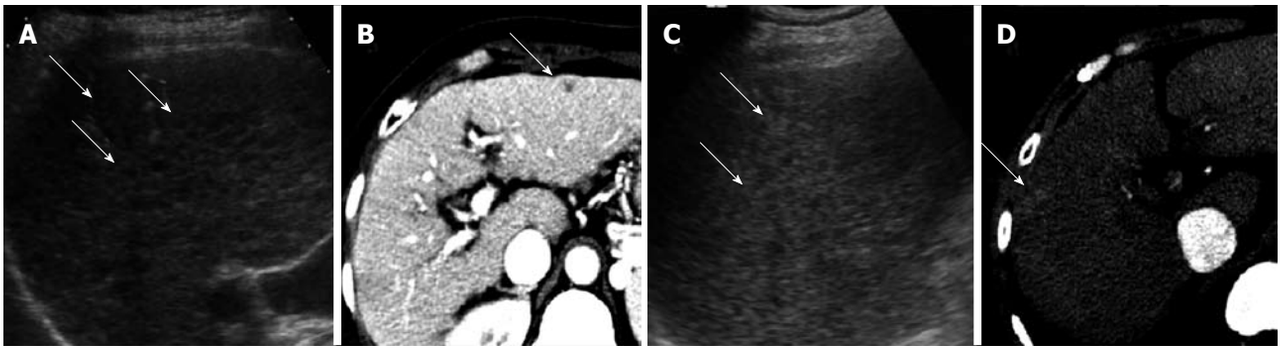

Figure 1 Representative cases according to ultrasonography and computed tomography findings. A: Ultrasonography shows scattered sub-centimeter-sized low echoic nodules; B: Arterial phase computed tomography (CT) scan shows a 5 mm sized low attenuated nodule in left lobe of liver; C: Ultrasonography shows a 7 mm sized hyperechoic nodule in right lobe of liver; D: Arterial phase CT scan shows a 5 mm sized high attenuated nodule in right lobe of liver.

18 for Windows (SPSS, Inc, Chicago, IL, United States). The statistical results are presented as the mean ± SD, median (range), or number (%) of patients. The differences in the baseline clinical characteristics and imaging features between the lesions that eventually developed into HCC and those that did not (non-HCC) were statistically analyzed to identify significant risk factors for the development of HCC from SCSNs detected during surveillance.

Continuous variables were compared parametrically using Student's *t*-test or non-parametrically using the Mann-Whitney *U*-test. Categorical variables were compared using the χ^2-test or Fisher's exact test as appropriate. Multiple logistic regression analysis was performed on variables that were different between the non-HCC and HCC groups in the univariate analysis ($P < 0.100$), in order to identify variables independently associated with the development of HCC. HCC development rates were calculated using the Kaplan-Meier method. A two-sided *P* value < 0.05 was considered statistically significant.

RESULTS

A total of 142 patients were included in this study. Their characteristics are summarized in Table 1. Eighty-four patients (59.2%) were male and the mean age was 50.2 ± 9.4 (SD) years. The etiology of liver disease was hepatitis B virus infection in 126 patients (88.7%), hepatitis C virus infection in 9 (6.3%), and alcoholic liver disease in 7 (5.0%). One hundred and eleven patients (78.2%) had cirrhotic liver. Ninety-eight patients (88.3%) were Child-Pugh class A, 10 (9.0%) were class B, and 3 (2.7%) were class C. A total of 33 patients had at least one SCSN: 23 patients were detected by US and 10 were detected by CT. There was one SCNS in 26 patients (18.3%), two SCSNs in 7 (5.0%), three in 3 (2.1%), four in 1 (0.7%), and more than four in 105 (73.9%). The SCSNs were hypoechoic/low-attenuation in 77 patients (54.3%), hyperechoic/high-attenuation in 31 (21.8%), and mixed echoic/attenuation in 34 (23.9%). Initial radiologic diagnosis of the hepatic nodules was regenerative nodule (RN)/dysplastic nodule (DN) in 119 patients (83.8%), hemangioma in 17 (12.0%), indeterminate nodule in 5 (3.5%), and arterioportal shunt in 1 (0.7%).

During 667 person-years of follow-up (mean, 28.5 ± 20.0 mo), HCC developed in 33 patients (23.2%). The mean durations of follow-up were 32.6 ± 19.5 and 64.3 ± 17.6 in the HCC and non-HCC groups, respectively. The mean time to diagnosis of HCC after detection of SCSNs was 33.1 ± 18.9 mo. Except for one biopsy-proven case, most of the HCC cases were diagnosed according to the clinical criteria of the Korean Liver Cancer Study Group and the National Cancer Center, South Korea[3], which were not same as the international guidelines[10,11] at that time. However, when retrospectively reevaluated, all diagnoses of HCC were satisfied with the updated American Association for the Study of Liver Diseases guidelines[12]. Following diagnosis, twelve patients (36.4%) underwent radiofrequency ablation, 13 (39.4%) underwent transarterial chemoembolization, 5 (15.2%) underwent surgical resection, 1 (3.0%) underwent liver transplantation, and 2 (6.0%) did not receive any treatment.

The calculated HCC development rate was 4.9% per year. The cumulative one-, two-, three- and five-year HCC development rates were 5.6%, 10.6%, 14.1% and 20.4%, respectively.

Clinical features and initial radiologic results of patients in the HCC and non-HCC groups

Patients diagnosed with HCC were older (54.4 ± 8.3 years *vs* 48.9 ± 9.4 years; $P = 0.003$) and had lower albumin levels (3.56 ± 0.58 g/dL *vs* 3.84 ± 0.55 g/dL; $P = 0.012$) and elevated baseline AFP levels [8.5 (range: 3.2-211.6) ng/mL *vs* 5.4 (range: 1.0-55.9) ng/mL; $P = 0.035$) compared to patients with non-HCC nodules. In terms of nodule pattern, patients diagnosed with HCC had more hypoechoic/low-attenuation nodules and less hyperechoic/high-attenuation nodules than patients with non-HCC nodules [23 (69.7%) *vs* 54 (49.5%) and 1 (3.0%) *vs* 30 (27.5%), respectively, $P = 0.011$]. In the initial radiologic diagnosis of hepatic nodules, RN/DN accounted for 31 (93.9%) in patients diagnosed with HCC, while RN/DN and hemangioma accounted for 88 (80.7%) and 17 (15.6%), respectively, in patients with non-HCC nodules ($P = 0.036$). There were no significant differences in

Table 1 Baseline characteristics of high-risk patients who had sub-centimeter-sized nodules (*n* = 142)

Baseline characteristics	Number of patients
Age (yr)	50.2 ± 9.4
Male	84 (59.2)
Etiology of liver disease	
Hepatitis B infection	126 (88.7)
Hepatitis C infection	9 (6.3)
Alcohol liver cirrhosis	7 (5.0)
Liver cirrhosis	111 (78.2)
Child-Pugh A	98 (88.3)
Child-Pugh B	10 (9.0)
Child-Pugh C	3 (2.7)
AST (UI/L)	47.9 ± 26.8
ALT (UI/L)	53.2 ± 42.5
PT (INR)	1.19 ± 0.17
Bilirubin (mg/dL)	1.24 ± 0.97
Platelets (10^9/L)	125.3 ± 60.3
Albumin (g/dL)	3.77 ± 0.56
Baseline AFP (ng/mL, range)	5.7 (1.0-211.6)
Number of nodules	
One	26 (18.3)
Two	7 (5.0)
Three	3 (2.1)
Four	1 (0.7)
Over four	105 (73.9)
Nodule pattern	
Hypoechoic/low-attenuation	77 (54.3)
Hyperechoic/high-attenuation	31 (21.8)
Mixed	34 (23.9)
Initial radiologic diagnosis	
RN/DN	119 (83.8)
Hemangioma	17 (12.0)
Indeterminate nodule	5 (3.5)
Arterioportal shunt	1 (0.7)

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PT: Prothrombin time; AFP: Alpha-fetoprotein; RN: Regenerative nodule; DN: Dysplastic nodule; INR: International normalized ratio. Data are shown as the mean ± SD, median (range) or *n* (%) of patients.

Table 2 Risk factors for the development of hepatocellular carcinoma from sub-centimeter-sized nodules

Variables	Diagnosis		*P* value
	HCC (*n* = 33)	Non-HCC (*n* = 109)	
Age (yr)	54.4 ± 8.3	48.9 ± 9.4	0.003
Male	24 (72.4)	60 (55.0)	0.070
Etiology of liver disease			0.364
Hepatitis B infection	27 (81.8)	99 (90.8)	
Hepatitis C infection	3 (9.1)	6 (5.5)	
Alcoholic liver cirrhosis	3 (9.1)	4 (3.7)	
Liver cirrhosis	29 (87.9)	82 (75.2)	0.123
AST (UI/L)	49.3 ± 20.4	47.5 ± 28.5	0.736
ALT (UI/L)	50.9 ± 36.5	53.9 ± 44.3	0.722
PT (INR)	1.23 ± 0.17	1.18 ± 0.17	0.088
Bilirubin (mg/dL)	1.27 ± 0.78	1.23 ± 1.02	0.831
Platelets (10^9/L)	110.1 ± 53.9	129.9 ± 61.6	0.099
Albumin (g/dL)	3.56 ± 0.58	3.84 ± 0.55	0.012
Number of nodules			0.390
One	4 (12.1)	22 (20.0)	
Two	1 (3.0)	6 (5.5)	
Three	0 (0.0)	3 (2.8)	
Four	0 (0.0)	1 (0.9)	
Over four	28 (84.8)	77 (70.6)	
Nodule pattern			0.011
Hypoechoic/low-attenuation	23 (69.7)	54 (49.5)	
Hyperechoic/high-attenuation	1 (3.0)	30 (27.5)	
Mixed	9 (27.3)	25 (22.9)	
Initial radiologic diagnosis			0.036
RN/DN	31 (93.9)	88 (80.7)	
Hemangioma	0 (0.0)	17 (15.6)	
Indeterminate nodule	2 (6.1)	3 (2.8)	
Arterioportal shunt	0 (0.0)	1 (0.9)	
Baseline AFP (ng/mL, range)	8.5 (3.2-211.6)	5.4 (1.0-55.9)	0.035

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PT: Prothrombin time; AFP: Alpha-fetoprotein; RN: Regenerative nodule; DN: Dysplastic nodule; INR: International normalized ratio; HCC: Hepatocellular carcinoma. Data are shown as the mean ± SD, median (range) or *n* (%) of patients.

sex, etiology of liver disease, presence of liver cirrhosis, Child-Pugh class, AST, ALT, PT, bilirubin, platelet count, or number of nodules between patients diagnosed with HCC and patients with non-HCC nodules (Table 2).

Multivariate analysis revealed that old age [*P* = 0.012, odds ratio (OR) = 1.075, 95% confidence interval (CI) = 1.016-1.137], male sex (*P* = 0.009, OR = 3.969, 95% CI: 1.403-11.226), and high baseline AFP level (*P* = 0.024, OR = 1.039, 95% CI: 1.005-1.073) were associated with an increased risk of developing HCC from SCSNs detected during surveillance (Table 3).

Table 3 Multivariate analysis of risk factors for the development of hepatocellular carcinoma from sub-centimeter-sized hepatic nodules

Variables	*P* value	OR	95% CI
Age (yr)	0.012	1.075	1.016-1.137
Male	0.009	3.969	1.403-11.226
PT (INR)	0.877	0.698	0.007-66.718
Platelets (10^9/L)	0.917	0.999	0.990-1.009
Albumin (g/dL)	0.478	0.624	0.169-2.298
Nodule pattern	0.081		
Nodule pattern (1)[1]	1.000	0.812	0.233-2.827
Nodule pattern (2)[2]	0.054	0.075	0.006-1.026
Baseline AFP (ng/mL)	0.024	1.039	1.005-1.073

[1]Between hypoechoic/low-attenuation and mixed echoic/attenuation; [2]Between hyperechoic/high-attenuation and mixed echoic/attenuation. OR: Odds ratio; CI: Confidence interval; PT: Prothrombin time; AFP: Alpha-fetoprotein; INR: International normalized ratio. Data are shown as the mean ± SD, median (range) or number (%) of patients.

DISCUSSION

The purpose of our study was to evaluate the outcome of SCSNs detected during surveillance in patients at risk and to determine risk factors for developing HCC from those nodules. The current practice guidelines recommend follow-up of SCSNs every few months in order to detect growth suggestive of malignant transformation[10-12]. However, early diagnosis of HCC has a significant impact on survival because it enables the timely implementation of effective treatment strategies, including hepatic resection, loco-regional ablative therapy, and liver transplantation[23,24]. In addition, even in cases of HCC that are

detected early and can be treated with radiofrequency ablation, a delay (more than five weeks) in treatment may be associated with poorer patient survival[15]. Therefore, in the present study, we focused on the SCSNs, which have not been investigated so far, even though occasionally encountered in practice, and identified clinical risk factors for the development of HCC from SCSNs.

Several studies have reported an HCC yearly incidence in HBV or HCV infection, which is between 2%-8% per year depending on the study population[12,18,21,25-33]. In the present study, the annual HCC incidence from SCSNs was 4.9% per year, which is similar to above-mentioned HCC incidences of 2%-8%/year in chronic HBV or HCV infection. Thus, although the detection of SCSNs during surveillance is not infrequent and their management could be a major clinical challenge, it seems that the HCC incidence does not increase significantly in patients with SCSNs compared to patients without SCSNs.

There have been a few studies of sub-centimeter-sized HCC[34,35]. Park *et al*[34] reported that small (5-10 mm) arterially enhancing nodules at the hepatic arterial phase of CT in surveillance for HCC have a 29.5% probability of developing into HCC over a mean 35.7 mo of follow-up on a per-person basis. They also identified the presence of HCC treatment history, a larger size of small (5-10 mm) arterially enhancing nodules, presence of coexistent HCC, and absence of coexistent typical arterioportal shunts as independent risk factors for future development of HCC. In our study, SCSNs had a 23.2% probability of developing into HCC over a mean of 28.5 mo of follow-up. The unique feature of the present study that differentiates it from that of Park *et al*[34] is that our study population had no prior HCC history and included 77 (54.3%) patients who had hypoechoic/low-attenuation SCSNs. In addition, patients diagnosed with HCC had more hypoechoic/low-attenuation SCSNs than patients with non-HCC nodules (69.7% *vs* 49.5%; P = 0.011; Table 2), although the difference was not significant in the multivariate analysis (P = 0.081). This could be due to hemangiomas, which are mainly hyperechoic/high-attenuation and benign, because 17 patients with a hemangioma were included only in the non-HCC group (Table 2). Therefore, we selected patients who had RN/DN and performed a subgroup analysis. The proportion of patients with hypoechoic/low-attenuation SCSNs did not differ between the two groups (70.0% *vs* 56.5%, P = 0.134). According to our results, non-enhancing minute hepatic nodules also might have considerable malignant potential and should receive as much attention as enhancing nodules.

A study by Forner *et al*[36] evaluated the accuracy of contrast-enhanced US and dynamic MRI for the diagnosis of nodules 20 mm or smaller detected during US surveillance. The study included 89 patients with cirrhosis, of whom 13 patients (14.6%) had a SCSN. Among those with SCSNs, 2 (15.4%) were ultimately diagnosed with HCC. Significant differences were found in age, nodule size, and the presence of a halo between patients diagnosed with HCC and patients with non-HCC nodule in all subjects, although multivariate analysis was not performed. In our study, old age, male sex, and high baseline AFP levels were associated with an increased risk of developing HCC from SCSNs detected during surveillance. Among these variables, male sex was the strongest risk factor (P = 0.009, OR = 3.969, 95% CI: 1.403-11.226). Elevated baseline AFP levels may be affected by undiscovered HCC. Therefore, we excluded subjects who were diagnosed with HCC at the time of inclusion in the study. Additionally, we investigated the change in AFP levels and calculated the AFP ratio as the last AFP level divided by the baseline AFP level. The AFP ratios were also significantly elevated in the HCC group compared to the non-HCC group [1.0 (range: 0.2-74.3) *vs* 0.7 (range: 0.0-4.7); P = 0.040], even though baseline AFP levels were elevated. Thus, elevated AFP level at baseline could be considered a risk factor for developing HCC, and an increased AFP ratio during follow-up should be considered a critical warning sign for HCC development.

The present study had some limitations. First, the retrospective design likely introduced selection bias. Second, there was a lack of histological confirmation for the benign lesions, which were defined on the basis of radiologic images. However, it is unlikely that HCCs were incorrectly categorized as benign because our follow-up period was sufficiently long. Furthermore, pathological confirmation of these lesions would not be practical in clinical settings. Last, our assessments regarding the number, location, nodule pattern, and size of SCSNs had an element of subjectivity due to the small nodule sizes and sometimes ill-defined margins. To overcome this limitation, all radiologic images were reviewed by an experienced radiologist who was blinded to the final diagnoses.

In conclusion, the overall risk of HCC development in patients with SCSNs is similar to that in liver cirrhosis patients. However, since old age, male sex, and high baseline AFP level are associated with an increased risk of developing HCC from SCSNs, patients with these risk factors need to be closely monitored during follow-up.

COMMENTS

Background

During hepatocellular carcinoma (HCC) surveillance, the detection of sub-centimeter-sized nodules (SCSNs) is not infrequent and their management is a major clinical challenge. Owing to a high false-positive rate, a wait-and-see policy is recommended for those nodules. However, the possibility remains high that small nodules detected during surveillance may become malignant over time and a delay in the start of treatment of even early-stage HCC may be associated with a poorer patient survival.

Research frontiers

Clinicians have limited data on the clinical course of SCSNs. In this study, the authors investigated outcomes of SCSNs detected during HCC surveillance in patients at risk.

Innovations and breakthroughs

This is the first report to evaluate the outcome of SCSNs detected during surveillance in patients with cirrhosis or chronic liver disease and to determine

risk factors for developing HCC from those nodules. Therefore, the study could provide valuable information to clinicians managing patients with chronic liver disease.

Applications

The study results suggest that patients with risk factors such as old age, male sex and high baseline alpha-fetoprotein need to be closely monitored during follow-up.

Peer review

This study is very informative for clinicians because the detection of SCSNs during surveillance is frequently encountered in practice setting. In addition, their results have scientific relevance for understanding the epidemiology of the disease.

REFERENCES

1 **Parkin DM**, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74-108

2 **Altekruse SF**, McGlynn KA, Reichman ME. Hepatocellular carcinoma incidence, mortality, and survival trends in the United States from 1975 to 2005. *J Clin Oncol* 2009; **27**: 1485-1491

3 **Bosch FX**, Ribes J, Díaz M, Cléries R. Primary liver cancer: worldwide incidence and trends. *Gastroenterology* 2004; **127**: S5-S16

4 **Stroffolini T**, Andreone P, Andriulli A, Ascione A, Craxi A, Chiaramonte M, Galante D, Manghisi OG, Mazzanti R, Medaglia C, Pilleri G, Rapaccini GL, Simonetti RG, Taliani G, Tosti ME, Villa E, Gasbarrini G. Characteristics of hepatocellular carcinoma in Italy. *J Hepatol* 1998; **29**: 944-952

5 **El-Serag HB**, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. *N Engl J Med* 1999; **340**: 745-750

6 **Deuffic S**, Poynard T, Buffat L, Valleron AJ. Trends in primary liver cancer. *Lancet* 1998; **351**: 214-215

7 **Taylor-Robinson SD**, Foster GR, Arora S, Hargreaves S, Thomas HC. Increase in primary liver cancer in the UK, 1979-94. *Lancet* 1997; **350**: 1142-1143

8 **Parkin DM**. Global cancer statistics in the year 2000. *Lancet Oncol* 2001; **2**: 533-543

9 **Forner A**, Reig ME, de Lope CR, Bruix J. Current strategy for staging and treatment: the BCLC update and future prospects. *Semin Liver Dis* 2010; **30**: 61-74

10 **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. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421-430

11 **Bruix J**, Sherman M. Management of hepatocellular carcinoma. *Hepatology* 2005; **42**: 1208-1236

12 **Bruix J**, Sherman M. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022

13 **Fracanzani AL**, Burdick L, Borzio M, Roncalli M, Bonelli N, Borzio F, Maraschi A, Fiorelli G, Fargion S. Contrast-enhanced Doppler ultrasonography in the diagnosis of hepatocellular carcinoma and premalignant lesions in patients with cirrhosis. *Hepatology* 2001; **34**: 1109-1112

14 **Takayama T**, Makuuchi M, Hirohashi S, Sakamoto M, Okazaki N, Takayasu K, Kosuge T, Motoo Y, Yamazaki S, Hasegawa H. Malignant transformation of adenomatous hyperplasia to hepatocellular carcinoma. *Lancet* 1990; **336**: 1150-1153

15 **Chen WT**, Fernandes ML, Lin CC, Lin SM. Delay in treatment of early-stage hepatocellular carcinoma using radiofrequency ablation may impact survival of cirrhotic patients in a surveillance program. *J Surg Oncol* 2011; **103**: 133-139

16 **Davila JA**, Morgan RO, Shaib Y, McGlynn KA, El-Serag HB. Hepatitis C infection and the increasing incidence of hepatocellular carcinoma: a population-based study. *Gastroenterology* 2004; **127**: 1372-1380

17 **Perz JF**, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol* 2006; **45**: 529-538

18 **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

19 **Tsukuma H**, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T, Nakanishi K, Fujimoto I, Inoue A, Yamazaki H. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N Engl J Med* 1993; **328**: 1797-1801

20 **Yu MW**, Chen CJ. Hepatitis B and C viruses in the development of hepatocellular carcinoma. *Crit Rev Oncol Hematol* 1994; **17**: 71-91

21 **Sherman M**, Peltekian KM, Lee C. Screening for hepatocellular carcinoma in chronic carriers of hepatitis B virus: incidence and prevalence of hepatocellular carcinoma in a North American urban population. *Hepatology* 1995; **22**: 432-438

22 **Chen JD**, Yang HI, Iloeje UH, You SL, Lu SN, Wang LY, Su J, Sun CA, Liaw YF, Chen CJ. Carriers of inactive hepatitis B virus are still at risk for hepatocellular carcinoma and liver-related death. *Gastroenterology* 2010; **138**: 1747-1754

23 **Gonzalez SA**, Keeffe EB. Diagnosis of hepatocellular carcinoma: role of tumor markers and liver biopsy. *Clin Liver Dis* 2011; **15**: 297-306, vii-x

24 **Ishikawa M**, Yogita S, Miyake H, Fukuda Y, Harada M, Wada D, Tashiro S. Differential diagnosis of small hepatocellular carcinoma and borderline lesions and therapeutic strategy. *Hepatogastroenterology* 2002; **49**: 1591-1596

25 **Sakuma K**, Saitoh N, Kasai M, Jitsukawa H, Yoshino I, Yamaguchi M, Nobutomo K, Yamumi M, Tsuda F, Komazawa T. Relative risks of death due to liver disease among Japanese male adults having various statuses for hepatitis B s and e antigen/antibody in serum: a prospective study. *Hepatology* 1988; **8**: 1642-1646

26 **McMahon BJ**, Alberts SR, Wainwright RB, Bulkow L, Lanier AP. Hepatitis B-related sequelae. Prospective study in 1400 hepatitis B surface antigen-positive Alaska native carriers. *Arch Intern Med* 1990; **150**: 1051-1054

27 **Fattovich G**, Giustina G, Degos F, Tremolada F, Diodati G, Almasio P, Nevens F, Solinas A, Mura D, Brouwer JT, Thomas H, Njapoum C, Casarin C, Bonetti P, Fuschi P, Basho J, Tocco A, Bhalla A, Galassini R, Noventa F, Schalm SW, Realdi G. Morbidity and mortality in compensated cirrhosis type C: a retrospective follow-up study of 384 patients. *Gastroenterology* 1997; **112**: 463-472

28 **Degos F**, Christidis C, Ganne-Carrie N, Farmachidi JP, Degott C, Guettier C, Trinchet JC, Beaugrand M, Chevret S. Hepatitis C virus related cirrhosis: time to occurrence of hepatocellular carcinoma and death. *Gut* 2000; **47**: 131-136

29 **Villeneuve JP**, Desrochers M, Infante-Rivard C, Willems B, Raymond G, Bourcier M, Côté J, Richer G. A long-term follow-up study of asymptomatic hepatitis B surface antigen-positive carriers in Montreal. *Gastroenterology* 1994; **106**: 1000-1005

30 **Manno M**, Cammà C, Schepis F, Bassi F, Gelmini R, Giannini F, Miselli F, Grottola A, Ferretti I, Vecchi C, De Palma M, Villa E. Natural history of chronic HBV carriers in northern Italy: morbidity and mortality after 30 years. *Gastroenterology* 2004; **127**: 756-763

31 **Hsu YS**, Chien RN, Yeh CT, Sheen IS, Chiou HY, Chu CM, Liaw YF. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. *Hepatology* 2002; **35**: 1522-1527

32 **de Franchis R**, Meucci G, Vecchi M, Tatarella M, Colombo M, Del Ninno E, Rumi MG, Donato MF, Ronchi G. The natural history of asymptomatic hepatitis B surface antigen carriers.

Ann Intern Med 1993; **118**: 191-194
33 **Sánchez-Tapias JM**, Costa J, Mas A, Bruguera M, Rodés J. Influence of hepatitis B virus genotype on the long-term outcome of chronic hepatitis B in western patients. *Gastroenterology* 2002; **123**: 1848-1856
34 **Park MJ**, Kim YS, Lee WJ, Lim HK, Rhim H, Lee J. Outcomes of follow-up CT for small (5-10-mm) arterially enhancing nodules in the liver and risk factors for developing hepatocellular carcinoma in a surveillance population. *Eur Radiol* 2010; **20**: 2397-2404
35 **Kim JE**, Kim SH, Lee SJ, Rhim H. Hypervascular hepatocellular carcinoma 1 cm or smaller in patients with chronic liver disease: characterization with gadoxetic acid-enhanced MRI that includes diffusion-weighted imaging. *AJR Am J Roentgenol* 2011; **196**: W758-W765
36 **Forner A**, Vilana R, Ayuso C, Bianchi L, Solé M, Ayuso JR, Boix L, Sala M, Varela M, Llovet JM, Brú C, Bruix J. Diagnosis of hepatic nodules 20 mm or smaller in cirrhosis: Prospective validation of the noninvasive diagnostic criteria for hepatocellular carcinoma. *Hepatology* 2008; **47**: 97-104

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BRIEF ARTICLE

Family history influences the early onset of hepatocellular carcinoma

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Abstract

AIM: To evaluate the relationship between a positive family history of primary liver cancer and hepatocellular carcinoma (HCC) development in Korean HCC patients.

METHODS: We studied a total of 2242 patients diagnosed with HCC between January 1990 and July 2008, whose family history of primary liver cancer was clearly described in the medical records.

RESULTS: Of the 2242 patients, 165 (7.4%) had a positive family history of HCC and 2077 (92.6%) did not. The male to female ratio was 3.6:1, and the major causes of HCC were chronic hepatitis B virus (HBV) infection in 75.1%, chronic hepatitis C virus infection in 13.2% and alcohol in 3.1%. The median ages at diagnosis in the positive- and negative-history groups were 52 years (range: 29-79 years) and 57 years (range: 18-89 years), respectively ($P < 0.0001$). Furthermore, among 1713 HCC patients with HBV infection, the number of patients under 45 years of age out of 136 patients with positive family history was 26 (19.1%), whereas those out of 1577 patients with negative family history was 197 (12.5%), suggesting that a positive family history may be associated with earlier development of HCC in the Korean population ($P = 0.0028$).

CONCLUSION: More intensive surveillance maybe recommended to those with a positive family history of HCC for earlier diagnosis and proper management especially when HBV infection is present.

Key words: Liver cancer; Hepatocellular carcinoma; Family history; Epidemiology

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INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for up to 90% of primary liver cancers. It is the fifth most common can-

cer and the third most common cause of cancer-related death worldwide[1-3]. The major risk factors for the development of HCC include liver cirrhosis of any etiology, chronic hepatitis B virus (HBV) infection, chronic hepatitis C virus (HCV) infection, heavy alcohol consumption and non-alcoholic steatohepatitis[2,4].

Familial clustering has been reported in many types of cancer including pancreas, colon, stomach, lung and breast cancers based on either meta-analyses or registry-based studies throughout the world[5-9]. However, no data on familial clustering for HCC is available in Korea to date although a few studies are reported in some other countries[10-15]. The development of HCC in Caucasian populations are reported to be less related to chronic HBV infection, but the clustering of HBV infection among family members was reported to be the major cause associated with family histories of HCC in Asia[3,12,14,16]. Still, the impact of family history of HCC in the development of HCC remains to be determined along with the possible confounding effects of important risk factors for HCC. Here, we report a large retrospective cohort study evaluating the effect of family history of HCC on its development among Korean patients with various risk factors.

MATERIALS AND METHODS

Study population

This study was a single-hospital-based study; cases were retrospectively evaluated. The data were recruited retrospectively from the medical records of 2242 patients who had first been diagnosed with and treated for HCC between January 1990 and July 2008. Before the analysis, the diagnosis of HCC was reconfirmed based on the American Association for the Study of Liver Diseases Practice Guidelines for Management of HCC[17], with either positive histopathology on liver biopsy and/or non-invasive criteria of hepatic imaging demonstrating one or more space-occupying lesions showing arterial hypervascularization on triphasic computed tomography and/or magnetic resonance imaging with or without an elevated alpha-fetoprotein (AFP) level.

All patients were screened for hepatitis virus infection when diagnosed with HCC, and if a patient had no evidence of chronic viral hepatitis, he or she underwent studies for autoimmune hepatitis and metabolic and/or genetic disorders such as Wilson's disease, hemochromatosis, primary biliary cirrhosis. HBV infection was diagnosed by testing for hepatitis B surface antigen, hepatitis core IgG, and HBV DNA, and HCV infection was diagnosed by testing for anti-HCV antibodies and HCV RNA; testing was performed at the central lab of Seoul St. Mary's Hospital. Alcohol-related cirrhosis was clinically diagnosed based on a compatible history of sustained alcohol consumption over 75 g/d in the absence of any other cause for liver disease.

A family history of HCC was determined based on medical records written during personal interviews on admission.

Tumor staging

Tumor staging for HCC was based on a classification system modified from the Union for International Cancer Control staging classification[18-20].

Statistical analysis

The results are presented as frequency (*n*) and percentage for categorical data and median (minimum to maximum) for continuous data.

To compare the general characteristics according to HCC family history, categorical variables were analyzed using either the χ^2 test or Fisher's exact test, as appropriate. Continuous variables were compared using the Mann-Whitney *U*-test. To identify differences in HCC family history according to HBV and HCV infections, statistical analyses were performed by the χ^2 test, Fisher's exact test, or the Mann-Whitney *U*-test, as appropriate.

All statistical analyses were performed using SAS software, Version 9.1 (SAS Institute Inc., Cary, NC). A 2-tailed *P*-value of < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

The demographic features of the HCC patients are summarized in Table 1. A total of 2242 cases with HCC were recruited by a retrospective chart review. The number of male patients ($n = 1765$) was approximately 3.6 times that of female patients (78.7% *vs* 21.3%, respectively). The median age at the time of diagnosis was 57 years (18-89 years), and the median age at the peak incidence of HCC was in the sixth decade ($n = 722$, 32.2%) followed by the seventh ($n = 640$, 28.6%) for all cases. When classified by gender, male patients were most commonly diagnosed with HCC in their fifties, whereas female patients were most commonly diagnosed in their sixties.

The most common single cause of HCC was HBV ($n = 1683$, 75.1%); the second was HCV, and the fourth was alcohol. The group classified as "unknown" was the third largest group and included the patients with non-B, non-C, and non-alcoholic liver cirrhosis and those for whom the cause of HCC could not be identified even after thorough evaluation (Table 1). In patients with chronic hepatitis B, the peak incidence of HCC was observed in the sixth decade of life; however, it was observed 10 years later in patients for whom HCC was caused by chronic hepatitis C or alcohol (data not shown). In 32 (1.4%) of 2242 patients, more than one etiology was identified. HBV co-infection with HCV was the most common combination and affected 17 patients; HBV with alcohol was the second most common and affected 13 patients; the remaining two cases were caused by HCV with alcohol. When the patients were classified based on whether a patient had HBV infection, the HBV-positive group consisted of 1713 patients (76.4%), whereas the HBV-negative group included only 529 (23.6%) patients. The patients were then reclassified into HCV-positive and

Table 1 General characteristics of the study population *n* (%)

		Total *n* = 2242	FHx (-) *n* = 2077 (92.6%)	FHx (+) *n* = 165 (7.4%)	*P* value
Gender	Male	1765 (78.7)	1639 (78.9)	126 (76.4)	0.441
	Female	477 (21.3)	438 (21.1)	39 (23.6)	
Age	Median (min-max)	57.0 (18.0-89.0)	57.0 (18.0-89.0)	52.0 (29.0-79.0)	< 0.0001
	< 20	1 (0.1)	1 (0.1)	0 (0.0)	< 0.001
	20-29	13 (0.5)	12 (0.6)	1 (0.6)	
	30-39	112 (5.0)	99 (4.8)	13 (7.9)	
	40-49	458 (20.4)	410 (19.7)	48 (29.1)	
	50-59	722 (32.2)	661 (31.8)	61 (37.0)	
	60-69	640 (28.6)	606 (29.2)	34 (20.6)	
	70-79	256 (11.4)	248 (11.9)	8 (4.8)	
	⩾ 80	40 (1.8)	40 (1.9)	0 (0.0)	
Etiology	HBV	1683 (75.1)	1548 (74.5)	135 (81.8)	0.236
	HCV	296 (13.2)	280 (13.5)	16 (9.6)	
	Alcohol	70 (3.1)	68 (3.3)	2 (1.2)	
	Combined	32 (1.4)	31 (1.5)	1 (0.6)	
	Others	50 (2.2)	45 (2.2)	5 (3.0)	
	Unknown	111 (5.0)	105 (5.0)	6 (3.6)	
HBV	HBV (+)	1713 (76.4)	1577 (75.9)	136 (82.4)	0.058
	HBV (-)	529 (23.6)	500 (24.1)	29 (17.6)	
HCV	HCV (+)	315 (14.1)	298 (14.3)	17 (10.3)	0.15
	HCV (-)	1927 (85.9)	1779 (86.7)	148 (89.6)	
Stage	Ⅰ	185 (8.3)	167 (8.0)	18 (10.9)	0.221
	Ⅱ	655 (29.2)	602 (29.0)	54 (32.7)	
	Ⅲ	648 (28.9)	610 (29.4)	38 (23.1)	
	Ⅳa and Ⅳb	753 (33.6)	698 (33.6)	55 (33.3)	
Lab,	ALT (U/L)	47.0 (3.0-3505.0)	47.0 (3.0-3505.0)	47.5 (3.0-840.0)	0.987
median (min-max)	TB (mg/mL)	1.1 (0.1-47.6)	1.1 (0.1-47.6)	1 (0.1-26.0)	0.868
	Alb (g/dL)	3.5 (1.2-9.6)	3.5 (1.2-9.6)	3.7 (1.6-5.0)	0.106
	Platelet (x 10^3/μL)	121.5 (8.0-1084.0)	121.0 (18.0-1084.0)	133.0 (8.0-635.0)	0.181
Tumor markers,	AFP (ng/ mL)	69.1 (0.0-529 470.0)	67.00 (0.0-529 470.0)	110.8 (1.1-53 606.0)	0.972
median (min-max)	PIVKA-Ⅱ (Mau/mL)	88.0 (1.0-16 636.8)	92.0 (1.0-16 636.8)	80.0 (6.0-2000.0)	0.990

AFP: Alpha-fetoprotein; Alb: Serum albumin; ALT: Alanine transaminase; HBV: Hepatitis B virus; HCV: Hepatitis C virus; FHx: Family history; PIVKA-Ⅱ: Protein induced by vitamin K deficiency-Ⅱ; TB: Serum total bilirubin.

-negative groups; the HCV-positive group included only 14.1% (*n* = 315) of all patients.

By the modified International Union Against Cancer (UICC) staging classification, only 185 patients (8.3%) were diagnosed at stage Ⅰ, followed by 655 (29.2%) patients at stage Ⅱ, 648 (28.9%) patients at stage Ⅲ, and 753 (33.6%) patients at stage Ⅳ. Thus, over half of the patients (62.5%) were diagnosed at advanced stages of HCC, including stages Ⅲ and Ⅳ.

Laboratory data at diagnosis did not indicate severe hepatic dysfunction. The medians of serum alanine transaminase, serum total bilirubin, serum albumin, and platelet count were 47 U/L (3.0-3505.0), 1.1 mg/mL (0.1-58), 3.5 g/dL (1.2-4.98), and 121 500/μL (8000-1 084 000), respectively. With regard to tumor markers, the median serum AFP level was 69.1 ng/mL (0.0-529 470.0), and the median level of serum protein induced by vitamin K deficiency (PIVKA-Ⅱ) was 88.0 Mau/mL (1.0-16 636.8). No statistical difference between the positive- and negative-family-history groups was found.

Family history of HCC

As shown in Table 1, 165 (7.4%) of 2242 patients had a positive family history of HCC in one or more family members, whereas 2077 (92.6%) patients had no family history of any HCC. Because the main concern was the influence of a positive family history in the development of HCC, positive histories in the offspring of the patients were not considered. Among those 165 patients with positive family histories, 159 had a positive family history in one or more first-degree relatives, including parents and siblings. Of the remaining six patients (all of these patients as well as their mothers had HBV infection), five had a positive family history of HCC in a brother or sister of the patient's mother, and one had a positive family history in the grandmother and in an uncle on the mother's side. We considered these patients to have positive family histories because vertical transmission of HBV and its oncogenic effects on the affected individual and on his or her family members cannot be omitted in a population with a high prevalence of HBV infection. The number of patients with a positive family history in a single family member was 143. A father, a mother, or a sibling was a single family member with a history of HCC in 25, 29 and 89 patients, respectively. The number of patients with a positive family history in two or more family members were as follows: both mother and father in two, a father and a sibling in two, a mother and a sibling in 10, a mother and two siblings in one, two siblings in one, a grandmother and an uncle in one, a sibling and

Table 2 Distribution of patients with positive family history by age and tumor staging *n* (%)

	n	Age (yr, median)	Range	*P* value
Family members				
Father	29	53	29.0-78.0	0.101
Mother	42	48.5	33.0-79.0	
Siblings	102	39	30.0-48.0	
HBV infection		HBV (+)	HBV (-)	
		(*n* = 136)	(*n* = 29)	
Age	Median	52.0	57.0	0.065
	(min-max)	(30.0-78.0)	(29.0-79.0)	
	< 20	0 (0.0)	0 (0.0)	0.001
	20-29	0 (0.0)	1 (3.4)	
	30-39	12 (8.8)	1 (3.4)	
	40-49	39 (28.7)	9 (31.0)	
	50-59	56 (41.2)	5 (17.5)	
	60-69	26 (19.1)	8 (27.6)	
	70-79	3 (2.2)	5 (17.2)	
	⩾ 80	0 (0.0)	0 (0.0)	
Stage	1	17 (12.5)	1 (3.4)	0.056
	2	46 (33.8)	8 (27.6)	
	3	26 (19.1)	12 (41.4)	
	4	47 (33.6)	8 (27.6)	
HCV infection		HCV (+)	HCV (-)	
		(*n* = 18)	(*n* = 147)	
Age	Median	54.0	52.0	0.629
	(min-max)	(29.0-75.0)	(30.0-79.0)	
	< 20	0 (0.0)	0 (0.0)	0.026
	20-29	1 (5.9)	0 (0.0)	
	30-39	1 (5.9)	12 (8.1)	
	40-49	5 (29.4)	43 (29.1)	
	50-59	3 (17.6)	58 (39.2)	
	60-69	6 (35.3)	28 (18.9)	
	70-79	1 (5.9)	7 (4.7)	
	⩾ 80	0 (0.0)	0 (0.0)	
Stage	1	18 (12.2)	0 (0.0)	0.335
	2	49 (33.1)	5 (29.4)	
	3	32 (21.6)	6 (35.3)	
	4	49 (33.1)	6 (35.3)	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; FHx: Family history.

an uncle in two, and a mother, grandmother, and an aunt in one. Considering 159 the patients positive family history only in the first degree relative, when grouped into HBV-positive and -negative, the median age at diagnosis of HCC was 51.5 and 57.0, respectively ($P = 0.049$).

Age at diagnosis*:* Regardless of the family history of HCC, both groups had a peak incidence of HCC diagnosis in their fifties. However, the median age at diagnosis was 52 years (range: 29-79 years) for those with positive family histories, which was significantly younger ($P < 0.0001$) than that for those with negative family histories (57; range: 18-89 years). In the positive-family-history group, 61 (37.0%) patients were diagnosed in their fifties, and 48 (29.1%) in their forties. In the negative-family-history group, 661 (31.8%) patients were diagnosed in their fifties, and 606 (29.2%) in their sixties ($P < 0.001$) (Table 1).

Among the patients in the positive-family-history group, the median age at diagnosis in the HBV-positive patients ($n = 136$) *vs* the HBV-negative patients ($n = 29$) was 52 *vs* 57, respectively; this difference was not significant ($P = 0.065$). HBV-positive patients were most frequently diagnosed with HCC in their fifties ($n = 56$, 41.2%), followed by their forties ($n = 39$, 28.7%). The median age at diagnosis in the HCV-positive patients ($n = 18$) *vs* the HCV-negative patients ($n = 147$) was 54 *vs* 52 years, respectively; this difference also failed to reach statistical significance ($P = 0.629$) (Table 2).

Risk factors*:* HBV and HCV were two important causes of HCC development with or without a family history of HCC. Among patients with a positive family history, 135 cases were caused by HBV infection only, and 16 cases were caused by HCV only; one patient had both HBV and HCV infections (Table 1). In the negative-family-history group, 1548 (74.5%) cases were caused by HBV infection, followed by 280 (13.5%) cases of HCV infection, and these percentages were not significantly different from those found in the positive-family-history group ($P = 0.236$) (Table 1).

Among those with a positive family history of HCC, the median age at diagnosis of 136 patients with HBV infection was 52 years (range: 30-78 years), whereas that of 29 patients without HBV infection was 57 years (range: 29-79 years) ($P = 0.132$). With regard to HCV infection among those with a positive family history, the median age at diagnosis was 54 years (range: 29-75 years) in the HCV-infected group and 52 years (range: 30-79 years) in the HCV-noninfected group ($P = 0.562$) (Table 2).

Staging at diagnosis: HCC staging was based on a modified UICC staging system, with UICC stages ⅣA and ⅣB defined here as stage IV. Stages Ⅰ, Ⅱ, Ⅲ and Ⅳ included 18 (10.9%), 54 (32.7%), 38 (23.0%) and 55 (33.4%) patients, respectively, in the positive-family-history group, and 167 (8.0%), 602 (29.0%), 610 (29.4%) and 698 (33.6%) patients, respectively, in the negative-family-history group ($P = 0.221$). When the stages were reclassified into earlier (stages Ⅰ and Ⅱ) *vs* advanced stages (stages Ⅲ and Ⅳ), the early and advanced stages included 72 (43.6%) and 93 (56.4%) patients in the positive-family-history group and 768 (37.0%) and 1308 (63.0%) patients in the negative family history group ($P = 0.090$), respectively.

HBV infection and family history of HCC

As shown in Table 3, 1711 patients had HBV infections, and 134 (7.8%) of these had a positive family history of HCC. The median age of these 134 patients was 52 years (30-78 years), and among those without a family history ($n = 1577$, 92.1%), the median age was 57 years (range: 18.0-89.0 years), showing a significant statistical difference between the two groups ($P < 0.0001$). Among 529 patients without HBV infection, 29 (5.5%) had a positive family history of HCC, and their median age was 57 years (range: 29-79 years), whereas the remaining 500 patients without a family history had a median age of 61 years (range: 22-88 years) ($P = 0.164$). The patients were then divided into two groups based on age: a younger group (ages under 45 years) and an older group (ages 45 years or more). Among the HBV-positive patients, the numbers

Table 3 Characteristics of study subjects based on hepatitis B virus infection and family history *n* (%)

		HBV (+) (*n* = 1713)		*P* value	HBV (-) (*n* = 529)		*P* value
		FHx (+) (*n* = 136)	FHx (-) (*n* = 1577)		FHx (+) (*n* = 29)	FHx (-) (*n* = 500)	
Age, median (range)		52 (30.0-78.0)	57 (18.0-89.0)	< 0.0001	57 (29.0-79.0)	61 (22.0-88.0)	0.164
Age group	< 45	26 (19.1)	197 (12.5)	0.028	5	57	0.244
	⩾ 45	110 (80.9)	1380 (87.5)		24	443	
Stage	1	17 (12.5)	122 (7.7)	0.018	1 (2.2)	45 (9.0)	0.364
	2	46 (33.8)	443 (28.1)		8 (27.6)	159 (31.8)	
	3	26 (19.1)	473 (30.0)		12 (41.4)	137 (27.4)	
	4	47 (34.6)	539 (34.2)		8 (27.6)	159 (31.8)	
non-HBV	HCV				16 (55.2)	280 (56.0)	0.481
	Alcohol				2 (6.9)	68 (13.6)	
	Combined				0 (0.0)	2 (0.4)	
	Others				5 (17.2)	45 (9.0)	
	Unknown				6 (20.7)	105 (21.0)	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; FHx: Family history.

of patients in the younger group with positive and negative family histories were 26 and 197, respectively, *vs* 110 and 1380, respectively, in the older group ($P = 0.028$). In the HBV-negative group with 529 patients, no significant difference was observed between the younger and older groups either in positive- (5 *vs* 24) or negative-family-history group (57 *vs* 443).

Considering the tumor staging at diagnosis, the numbers of patients with HBV infection and a positive family history were 17 (12.5%), 46 (33.8%), 26 (19.1%) and 47 (34.6%) in stages Ⅰ, Ⅱ, Ⅲ and Ⅳ, respectively. These frequencies were significantly different ($P = 0.018$) from those among patients with HBV infection and a negative family history, for whom the corresponding numbers were 122 (7.7%), 443 (28.1%), 473 (30.0%) and 539 (34.2%). The patients without HBV infection and with a positive family history at each stage numbered 1 (3.2%), 8 (25.8%), 12 (38.7%) and 8 (32.3%), and those without HBV infection and with a negative family history numbered 45 (9.0%), 159 (31.8%), 137 (27.4%) and 159 (31.8%) in stages Ⅰ, Ⅱ, Ⅲ and Ⅳ, respectively ($P = 0.425$).

DISCUSSION

This is the first extensive investigation of the relationship between a family history of HCC and the risk of HCC development in Korea, with further considerations regarding major risk factors for HCC development. We observed that 7.4% ($n = 165$) of 2242 patients with HCC reported having a positive family history of HCC.

The most significant finding in this study was that the median age at diagnosis was 5 years younger among patients with a positive family history than among those with a negative family history ($P < 0.0001$). Also, the age distribution was significantly different between the groups (Table 1, $P < 0.001$). The age at diagnosis of HCC had been analyzed in a previous study evaluating the association of family history of liver cancer with HCC development in the United States, but in that study, the mean age at diagnosis in patients with a positive family history (mean age: 64.1 years, $n = 21$) and in patients without a family history (mean age: 59.9 years, $n = 156$) did not differ significantly ($P = 0.1$)[12,21]. It was said that the lack of significant association between HCC and affected parents or offspring in the study can be related to the small numbers[12]. On the other hand, the age difference of 5 years was significant in our study, and was younger in patients with a positive family history, we conclude that the significance was partly influenced by the large number of cases recruited in our study. Considering only the HBV-positive patients, we observed that the age at diagnosis was also significantly younger by 5 years in patients with a positive family history of liver cancer compared with those with a negative family history ($P < 0.0001$). This may be a natural corollary, as several reports have reported an association between the development of HCCs in infants and children and vertical transmission of HBV[22,23]. From these observations, we also concluded that the age of diagnosis with HCC may be influenced by the family history of HCC regardless of whether it is related to HBV infection and that infection with HBV earlier in life is not the only factor affecting the earlier development of HCC in HBV endemic regions[24-26]. This may also imply that a person with a history of HCC in any family member should pay special attention to screening for the development of HCC. Still, the effects of genetic backgrounds in these patients remain to be evaluated in the future.

The age recommendation for HCC surveillance in Asian males with HBV is over 40 years, and the recommendation for Asian females is over 50 years[3,4,16,27]. In our study, when the HBV-positive patients were grouped into younger (ages under 45 years) *vs* older (ages 45 years or more) age groups, the patients with positive family histories were diagnosed with HCC at earlier ages compared with those with negative family histories ($P = 0.042$). These results are not surprising and support the common assumption that prolonged exposure to HBV seems to be a possible explanation for a relatively earlier occurrence of HCC[21,25,26,28-32].

Another important finding in this study was the significant association between tumor staging at diagnosis and positive family history among HBV-infected patients

(P = 0.018). Because HBV is the most common risk factor for HCC, and the vertical transmission of HBV is a medical concern in Korea (although well controlled), these findings may suggest that earlier surveillance for HCC, perhaps earlier than typically recommended, in HBV-positive patients with a positive family history of HCC may allow these patients to be diagnosed at earlier stages; this would facilitate better control of HCC and better treatment outcomes in Korean HCC patients[33-35].

Despite the current increased efforts in HBV vaccination and in prevention of vertical transmission in Korea, HBV was the most prevalent hepatitis virus in our study population, as is expected in many Asian countries. This may reflect the effect on HCC development of vertical transmission several decades ago, before vaccination and prevention were available in Korea, and future study results may differ from ours.

This single-hospital-based study has limitations. The first is the limitation on HCC patients studied, as a single hospital cannot represent the whole country; fortunately, the general characteristics of our study subjects were not much skewed compared with other reports on HCC epidemiology in Korean patients[30]. Second, the medical records describing family histories of primary liver cancer were solely dependent on each patient's memory. Also, the data collection was not performed prospectively, but by reviewing the medical records retrospectively, which may have led to some misclassification. Because this was, in part, a cohort study, we restricted the cases to those with clearly detailed medical records to minimize any misleading information. Third, it is possible that the findings may have been influenced by the surveillance program for HBV infection in Korea, which may have assisted in diagnosis at early ages and at early stages of HCC among patients with HBV infection and positive family histories. It is possible that chronic HBV carriers were diagnosed with HCC earlier than are patients without HBV infection because many of them had regular follow-ups at a liver clinic. However, at this point, because a patient would not be able to be diagnosed with HCC if he or she did not visit a doctor, this bias may be unavoidable unless the whole population was examined. Finally, the genetic characteristics of those who develop HCC must be evaluated in the future because not all patients with HBV infection are prone to developing HCC.

In conclusion, a positive family history of liver cancer may influence the age at diagnosis of HCC, and this difference was also in patients with HBV infection. Furthermore, we cautiously recommend more intensive check-ups earlier in life and at shorter intervals for patients who have positive family histories of liver cancer, as this may foster detection of HCC at more treatable stages.

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COMMENTS

Background

Hepatocellular carcinoma (HCC), which is the fifth most common cancer and the third most common cause of cancer-related death worldwide, has some well known risk factors for its development. The three well-known causes for this devastating disease are hepatitis B virus (HBV), hepatitis C virus (HCV), and alcoholic liver disease. Recent reports showed familial clustering of this disease based on the perinantal transmission of the HBV.

Research frontiers

Although the clustering of HBV infection among family members, which is related to vertical transmission, was reported to be the major cause associated with family histories of liver cancer and HCC, the family history of liver cancer affecting HCC development and its familial aggregation along with the possible confounding effects of important risk factors remains to be determined.

Innovations and breakthroughs

This extensive investigation revealed a significant finding that the median age at diagnosis of HCC was 5 years younger among patients with a positive family history than among those without a family history of HCC. Another important finding in this study was the significant association between tumor staging at diagnosis and positive family history in HBV-infected patients.

Applications

The study suggests that a positive family history of liver cancer may influence the age at diagnosis of HCC, and cautiously proposed that earlier surveillance for HCC, perhaps earlier than typically recommended, especially in HBV-positive patients with a positive family history of liver cancer may allow these patients to be diagnosed at earlier stages, as this may foster detection of HCC at more treatable stages.

Terminology

Familial clustering of cancers: Whether based on genetic background, environmental factors, or vertical transmission of a particular infection, familial clustering of cancers has been reported in many types of cancer, including pancreas, colon, stomach, lung and breast cancers.

Peer review

In this retrospective study, the authors have evaluated the impact of a positive family history on the development of hepatocellular carcinoma in a group of Korean patients. 7.4% of 2242 patients had a positive family history of HCC. The median age at diagnosis was significantly lower in those that had a positive family history. This result was maintained even after analyzing separately the population with non-HBV etiology. The authors conclude that a positive family history may be associated with earlier appearance of HCC, warranting a stricter surveillance.

REFERENCES

1 **Sherman M**. Epidemiology of hepatocellular carcinoma. *Oncology* 2010; **78** Suppl 1: 7-10
2 **Parkin DM**, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74-108
3 **Yang JD**, Roberts LR. Epidemiology and management of hepatocellular carcinoma. *Infect Dis Clin North Am* 2010; **24**: 899-919, viii
4 **Sherman M**. Hepatocellular carcinoma: epidemiology, surveillance, and diagnosis. *Semin Liver Dis* 2010; **30**: 3-16
5 **Maisonneuve P**, Lowenfels AB. Epidemiology of pancreatic cancer: an update. *Dig Dis* 2010; **28**: 645-656
6 **Butterworth AS**, Higgins JP, Pharoah P. Relative and absolute risk of colorectal cancer for individuals with a family history: a meta-analysis. *Eur J Cancer* 2006; **42**: 216-227
7 **Yaghoobi M**, Bijarchi R, Narod SA. Family history and the risk of gastric cancer. *Br J Cancer* 2010; **102**: 237-242
8 **Brenner DR**, Hung RJ, Tsao MS, Shepherd FA, Johnston MR, Narod S, Rubenstein W, McLaughlin JR. Lung cancer risk in never-smokers: a population-based case-control study of epidemiologic risk factors. *BMC Cancer* 2010; **10**: 285
9 **Gramling R,** Lash TL, Rothman KJ, Cabral HJ, Silliman R, Roberts M, Stefanick ML, Harrigan R, Bertoia ML, Eaton CB.

Family history of later-onset breast cancer, breast healthy behavior and invasive breast cancer among postmenopausal women: a cohort study. *Breast Cancer Res* 2010; **12**: R82
10 **Hemminki K**, Vaittinen P, Kyyrönen P. Age-specific familial risks in common cancers of the offspring. *Int J Cancer* 1998; **78**: 172-175
11 **Zhang JY**, Wang X, Han SG, Zhuang H. A case-control study of risk factors for hepatocellular carcinoma in Henan, China. *Am J Trop Med Hyg* 1998; **59**: 947-951
12 **Hassan MM**, Spitz MR, Thomas MB, Curley SA, Patt YZ, Vauthey JN, Glover KY, Kaseb A, Lozano RD, El-Deeb AS, Nguyen NT, Wei SH, Chan W, Abbruzzese JL, Li D. The association of family history of liver cancer with hepatocellular carcinoma: a case-control study in the United States. *J Hepatol* 2009; **50**: 334-341
13 **Cai RL**, Meng W, Lu HY, Lin WY, Jiang F, Shen FM. Segregation analysis of hepatocellular carcinoma in a moderately high-incidence area of East China. *World J Gastroenterol* 2003; **9**: 2428-2432
14 **Donato F**, Gelatti U, Chiesa R, Albertini A, Bucella E, Boffetta P, Tagger A, Ribero ML, Portera G, Fasola M, Nardi G. A case-control study on family history of liver cancer as a risk factor for hepatocellular carcinoma in North Italy. Brescia HCC Study. *Cancer Causes Control* 1999; **10**: 417-421
15 **Roberts SK**, Kemp W. Hepatocellular carcinoma in an Australian tertiary referral hospital 1975-2002: change in epidemiology and clinical presentation. *J Gastroenterol Hepatol* 2007; **22**: 191-196
16 **McClune AC**, Tong MJ. Chronic hepatitis B and hepatocellular carcinoma. *Clin Liver Dis* 2010; **14**: 461-476
17 **Bruix J**, Sherman M. Management of hepatocellular carcinoma. *Hepatology* 2005; **42**: 1208-1236
18 **Ueno S**, Tanabe G, Nuruki K, Hamanoue M, Komorizono Y, Oketani M, Hokotate H, Inoue H, Baba Y, Imamura Y, Aikou T. Prognostic performance of the new classification of primary liver cancer of Japan (4th edition) for patients with hepatocellular carcinoma: a validation analysis. *Hepatol Res* 2002; **24**: 395-403
19 **Llovet JM**, Brú C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. *Semin Liver Dis* 1999; **19**: 329-338
20 **Izumi R**, Shimizu K, Ii T, Yagi M, Matsui O, Nonomura A, Miyazaki I. Prognostic factors of hepatocellular carcinoma in patients undergoing hepatic resection. *Gastroenterology* 1994; **106**: 720-727
21 **Yun EH**, Lim MK, Oh JK, Park JH, Shin A, Sung J, Park EC. Combined effect of socioeconomic status, viral hepatitis, and lifestyles on hepatocelluar carcinoma risk in Korea. *Br J Cancer* 2010; **103**: 741-746
22 **Chen WJ**, Lee JC, Hung WT. Primary malignant tumor of liver in infants and children in Taiwan. *J Pediatr Surg* 1988; **23**: 457-461
23 **Chang MH**, Chen DS, Hsu HC, Hsu HY, Lee CY. Maternal transmission of hepatitis B virus in childhood hepatocellular carcinoma. *Cancer* 1989; **64**: 2377-2380
24 **Fan JG**, Farrell GC. Prevention of hepatocellular carcinoma in nonviral-related liver diseases. *J Gastroenterol Hepatol* 2009; **24**: 712-719
25 **Yang HI**, Sherman M, Su J, Chen PJ, Liaw YF, Iloeje UH, Chen CJ. Nomograms for risk of hepatocellular carcinoma in patients with chronic hepatitis B virus infection. *J Clin Oncol* 2010; **28**: 2437-2444
26 **Chang PE**, Ong WC, Lui HF, Tan CK. Is the prognosis of young patients with hepatocellular carcinoma poorer than the prognosis of older patients? A comparative analysis of clinical characteristics, prognostic features, and survival outcome. *J Gastroenterol* 2008; **43**: 881-888
27 Prevention of hepatocellular carcinoma in the Asia-Pacific region: consensus statements. *J Gastroenterol Hepatol* 2010; **25**: 657-663
28 **Song IH**, Kim KS. Current status of liver diseases in Korea: hepatocellular carcinoma. *Korean J Hepatol* 2009; **15** Suppl 6: S50-S59
29 **Hann HW**, Kim CY, London WT, Whitford P, Blumberg BS. Hepatitis B virus and primary hepatocellular carcinoma: family studies in Korea. *Int J Cancer* 1982; **30**: 47-51
30 **Lee HS**, Han CJ, Kim CY. Predominant etiologic association of hepatitis C virus with hepatocellular carcinoma compared with hepatitis B virus in elderly patients in a hepatitis B-endemic area. *Cancer* 1993; **72**: 2564-2567
31 **Carr BI**, Pancoska P, Branch RA. HCC in young adults. *Hepatogastroenterology* 2010; **57**: 436-440
32 **Kim SR**, Kudo M, Hino O, Han KH, Chung YH, Lee HS. Epidemiology of hepatocellular carcinoma in Japan and Korea. A review. *Oncology* 2008; **75** Suppl 1: 13-16
33 **Thomas MB**, Jaffe D, Choti MM, Belghiti J, Curley S, Fong Y, Gores G, Kerlan R, Merle P, O'Neil B, Poon R, Schwartz L, Tepper J, Yao F, Haller D, Mooney M, Venook A. Hepatocellular carcinoma: consensus recommendations of the National Cancer Institute Clinical Trials Planning Meeting. *J Clin Oncol* 2010; **28**: 3994-4005
34 **Cho SJ**, Yoon JH, Hwang SS, Lee HS. Do young hepatocellular carcinoma patients with relatively good liver function have poorer outcomes than elderly patients? *J Gastroenterol Hepatol* 2007; **22**: 1226-1231
35 **Cabibbo G**, Craxì A. Epidemiology, risk factors and surveillance of hepatocellular carcinoma. *Eur Rev Med Pharmacol Sci* 2010; **14**: 352-355

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BRIEF ARTICLE

Risk modification of colorectal cancer susceptibility by interleukin-8 -251T>A polymorphism in Malaysians

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Abstract

AIM: To investigate the allele and genotype frequencies and associated risk of interleukin (*IL*)-8 -251T>A polymorphism on colorectal cancer (CRC) susceptibility risk.

METHODS: Peripheral blood samples of 255 normal controls and 255 clinically and histopathologically confirmed CRC patients were genotyped for *IL-8* -251T>A polymorphism employing allele-specific polymerase chain reaction. The relative association of variant allele and genotypes with CRC susceptibility risk was determined by calculating the odds ratios (ORs). Corresponding χ^2 tests on the CRC patients and controls were carried out and 95% confidence intervals (CIs) were determined using Fisher's exact test. The allele frequencies and its risk association were calculated using FAMHAP, haplotype association analysis software.

RESULTS: On comparing the frequencies of genotypes of patients and controls, the homozygous variant AA was significantly higher in CRC patients (P = 0.002) compared to controls. Investigation on the association of the polymorphic genotypes with CRC susceptibility risk, showed that the homozygous variant *IL-8* -251AA had a significantly increased risk with OR 3.600 (95% CI: 1.550-8.481, P = 0.001). In the case of allele frequencies, variant allele A of *IL-8* -251 showed a significantly increased risk of CRC predisposition with OR 1.32 (95% CI: 1.03-1.69, P = 0.003).

CONCLUSION: Variant allele and genotype of *IL-8* (-251 T>A) was significantly associated with CRC susceptibility risk and could be considered as a high-risk variant for CRC predisposition.

Key words: Interleukin-8 -251T>A; Polymorphism; Colorectal cancer; Malaysians

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INTRODUCTION

Colorectal cancer (CRC), the incidence of which has been increasing worldwide for the past few years, represents a significant cause of morbidity and mortality. CRC develops as a result of progressive accumulation of genetic and epigenetic alterations that lead to a series of histo-

pathological changes, initiated by transition from normal mucosa to adenoma to carcinoma. Excluding inherited types of CRC, the susceptibility of a certain individual to development of sporadic CRC remains largely undetermined. Sporadic CRC is a multifactorial disease, therefore, environmental factors, host genotype and immunological factors all could significantly contribute to initiation and even progression of this malignancy.

Recently, chronic inflammation has been linked to increased risk of various types of cancer[1,2]. Epidemiological observations, animal models and clinical studies have established an association between continuous inflammatory conditions and CRC[1,3,4]. Patients with inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, are at increased risk of developing CRC[5]. The associations between inflammatory response genes and IBD make them attractive candidate susceptibility genes for CRC because approximately 1:6 individuals with IBD are estimated to develop colorectal malignancy[6]. Despite this evidence strongly implicating chronic inflammation as a culprit in colorectal carcinogenesis, surprisingly little research has directly addressed the genetic predisposing factors that mediate inflammatory response and favor CRC development.

Genetic polymorphisms have emerged in recent years as important determinants of disease susceptibility and severity. Polymorphic variants of several genes are thought to play a key role in determining how individuals respond at the cellular level to various environment conditions including inflammation. If inflammation constitutes one of the molecular networks underlying susceptibility to CRC, genes that mediate inflammatory responses might be a group of candidate genes for CRC predisposition. Few genes are known to be important for inflammation of the colorectum, and their allelic variants have been shown to have biological effects[7,8]. Interleukin (*IL*)-8 is a chemokine and one of the major mediators of inflammatory responses, and is believed to play a role in the pathogenesis of cancer. Several polymorphisms have been detected in the *IL-8* gene, and a common polymorphism at the -251 position (251T>A) of the promoter region has been associated with transcriptional activity of the gene. A case-control study was designed to investigate the *IL-8* -251T>A polymorphic allele and genotype frequencies in healthy controls and sporadic CRC patients in the Malaysian population, and to determine the influence of the polymorphic genotype of *IL-8* -251T>A on sporadic CRC susceptibility risk.

MATERIALS AND METHODS

Recruitment of subjects

The study was approved by the Research Review Board and Ethics Committee of Universiti Sains Malaysia and Ministry of Health (MOH) Malaysia. In this case-control study, cases comprised 255 CRC patients (139 male and 116 female), recruited from Hospital Universiti Sains Malaysia, and from a few hospitals under the MOH, Malaysia like Hospital Sultanah Bahiyah, Alor Setar, Kedah and Hospital Raja Perempuan Zainab Ⅱ, Kota Bharu, Kelantan, Malaysia. An equal number of sex- and age-matched (± 5-10 years) normal healthy individuals (115 male and 140 female) were also recruited as controls. The ages of the patients ranged from 27 to 77 years with a mean age of 57.26 years. For the controls, the age ranged from 33 to 78 years with a mean age of 48.91 years. The sex and age distribution of the study subjects are shown in Table 1.

Table 1 Distribution of sex and age in cases and controls

	Patients	Controls
Sex, *n* (%)		
Female	116 (45.5)	140 (54.9)
Male	139 (54.5)	115 (45.1)
Age (mean ± SD)	57.26 ± 7.074	48.91 ± 12.020

DNA extraction

Peripheral blood samples of 255 normal controls and 255 clinically diagnosed and histopathologically confirmed CRC patients were collected in EDTA tubes, after obtaining written informed consent. The collected samples were stored at -20 ℃ till use. Genomic DNA was extracted using commercial DNA extraction kit (QIAGEN, Hilden, Germany) and the gene of interest was amplified using appropriate primers. Single nucleotide polymorphism -251 T>A in the *IL-8* gene was determined using allele-specific polymerase chain reaction (PCR).

Genotyping

For genotyping, the allele-specific primers used were 5' -CCACAATTTGGTGAATTATCAAT-3' and (251A) or 5'-TGCCCCTTCACTCTGTTAAC-3' (251T). The consensus primer used was 5'-TGCCCCTTCACTCTGTTAAC-3' (giving a PCR product of 366 bp). The *IL-8* -251T>A polymorphic sequence was amplified using PCR with composition master mix using 100 ng DNA template, primer (0.2 μmol/L), 2.0 mmol/L $MgCl_2$, 10 × buffer, 10 mmol dNTP (0.2 μL) and 5 U *Taq* DNA polymerase (Applied Biosystems, Foster City, CA, United States) with a total volume of 25 μL PCR mixture. The annealing temperature was 56.7 ℃, and 35 PCR cycles were carried out. The PCR products were isolated on 2% agarose gels and visualized with SYBR Green. The *IL-8* -251T>A polymorphic genotypes were categorized into homozygous wild, heterozygous and homozygous variant.

Statistical analysis

The difference in various genotype frequencies of *IL-8* among the cases and controls was calculated. The relative associations of various genotypes with CRC susceptibility risk was determined by calculating the odds ratios (ORs). Corresponding χ^2 tests on the CRC patients and controls were carried out and 95% confidence intervals (CIs) were determined using Fisher's exact test. Statistical analysis was carried out using SPSS version 18. The allele frequen-

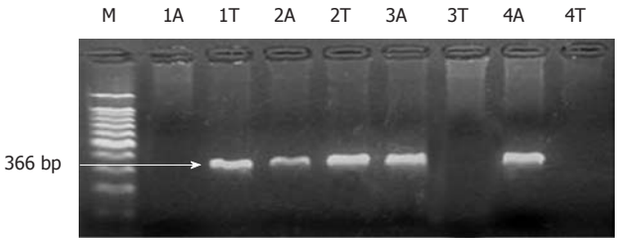

Figure 1 Various interleukin-8 -251T>A genotype patterns in sporadic colorectal cancer patients observed after genotyping using allele-specific polymerase chain reaction. The genotype was considered as homozygous wild type [interleukin (*IL*)-*8* -251TT] when only one band appeared at the T allele. When two bands appeared, each at the A allele and T allele, it was considered as heterozygous variant (*IL-8* -251TA). The homozygous variant genotype was characterized by the appearance of only one band at the A allele. M: DNA ladder; 1: Homozygous wild type (*IL-8* -251TT); 2: Heterozygous (*IL-8* -251TA); 3 and 4: Homozygous variant (*IL-8* -251AA).

cies and risk associations were calculated using FAMHAP, haplotype association analysis software to derive ORs.

RESULTS

Visualization of PCR product of IL-8 -251T>A

Various *IL-8* -251T>A genotype patterns in CRC patients in the Malaysian population are showed in Figure 1. A sample was considered as homozygous wild type (*IL-8* -251TT) when only one band appeared at the T allele. When two bands appeared at both the T allele and A allele, it was considered as heterozygous variant (*IL-8* -251TA). For the homozygous variant genotype (*IL-8* -251AA), only one band appeared at the A allele.

Frequencies of IL-8 -251T>A genotypes in CRC cases and controls

The frequencies of *IL-8* -251T>A genotypes in cases and controls are shown in Table 2. Among the 255 controls, the homozygous wild TT genotype was observed in 54 (21.18%), the heterozygous TA genotype was observed in 189 (74.12%) and the homozygous variant genotype AA was detected in 12 (4.7%) individuals. In the case of 255 CRC patients, 40 (15.69%) showed homozygous wild-type TT genotype (15.69% *vs* 21.18%, $P = 0.11$), 183 (71.76%) showed heterozygous variant TA genotype (71.76% *vs* 74.12%, $P = 0.55$), and 32 (12.55%) showed homozygous variant AA genotype. On comparing the frequencies of the polymorphic genotypes among the cases and controls, the homozygous variant genotype frequency was significantly higher among CRC cases (12.55% *vs* 4.7%, $P = 0.002$).

Association risk of IL-8 -251T>A genotypes with CRC susceptibility

Table 2 shows the associated risk of *IL-8* -251T>A genotypes with CRC susceptibility in this population. When the association of the polymorphic genotypes with CRC susceptibility risk was investigated, the *IL-8* homozygous variant genotype (AA) showed significantly increased risk with OR 3.600 (95% CI: 1.550-8.481, $P = 0.001$).

Table 2 Association risk and frequencies of T and A alleles of interleukin-8 -251T>A genotypes with colorectal cancer susceptibility

	Patients	Controls	OR (95% CI)	*P* value
IL-8 genotype				
Wild type -251TT	40 (15.69)	54 (21.18)	[1] (Ref.)	-
Hetero -251TA	183 (71.76)	189 (74.12)	1.307 (0.808-2.117)	0.250
Variant -251AA	32 (12.55)	12 (4.7)	3.600 (1.550-8.481)	0.001
Allele				
Wild type	263 (51.6)	297 (58.2)	0.76 (0.59-0.97)	-
Variant	247 (48.4)	213 (41.8)	1.32 (1.03-1.69)	0.003

IL-8: Interleukin-8; OR: Odd ratio; CI: Confidence intervals.

Allele frequencies and risk association of inflammation response genes with CRC susceptibility

The allele frequencies and risk association of T and A alleles in cases and controls in the Malaysian population are showed in Table 2. The frequency of wild-type allele T was 51.6% and the frequency of variant allele A was 48.4% among the CRC cases. In the case of controls, the frequency of the wild-type allele T was 58.2% and 41.8% showed variant allele A. When the risk association of variant allele was examined, variant allele A of *IL-8* showed a significantly higher risk with OR 1.32 (95% CI: 1.03-1.69, $P = 0.003$).

The patient group included patients from different parts of Malaysia and so the clinicopathological features of many of these patients could not be collected. Therefore, these details could not be specified. For the same reason, the association of genotype frequencies with patient prognostic subgroups could not be evaluated.

DISCUSSION

Inflammation, which is part of the immune response, may also induce or exaggerate some diseases through production of proinflammatory cytokines. Inflammatory cytokines are major inducers of chemokines that play a central role in leukocyte recruitment to sites of inflammation. Chemokines have pleiotropic biological effects that can play several roles in cancer progression, including angiogenesis, inflammation, cell recruitment and migration. *IL-8* or chemokine CXC ligand 8 is the prototype member of the CXC chemokine family. Evidence has shown that the individual level of cytokine production is affected by single nucleotide polymorphisms in cytokine genes, and the observed differences in cytokine production among individuals can be at least partially explained by gene polymorphisms. Genetic polymorphisms might directly influence inter-individually in the magnitude of inflammatory response, and this might contribute to an individual's ultimate clinical outcome. Genetic polymorphisms of cytokine genes have been identified to play a role in susceptibility to various diseases including cancer[9]. A common polymorphism in the -251 position (251T>A) of the promoter region of *IL-8* has been identified.

We investigated the frequencies and potential risk mo-

dification of *IL-8* -251T>A polymorphic genotype and allele on CRC susceptibility in the Malaysian population. Compared to controls, the prevalence of homozygous variant AA genotype was significantly higher in CRC patients (4.70% *vs* 12.55%, $P = 0.002$), whereas for the homozygous wild-type genotypes (TT) and heterozygous variant genotypes (TA), there was no significant difference in frequencies between the two groups. In a study by Yang *et al*[10], on the association of *IL-8* -251T>A polymorphism with prostate cancer, there was no significant difference in the distribution of *IL-8* polymorphic genotypes between prostate cancer cases and controls.

It has been suggested that *IL-8* and its receptors are crucial to the development and progression of many malignancies[11]. Genetic polymorphisms of the *IL-8* gene have been implicated in the susceptibility to a range of cancers including oral cancer[12], breast carcinoma[13] and gastric cancer[14]. Our interest was to investigate whether the genetic variants are related to the CRC risk in the Malaysian population. Our results showed that the polymorphism in the *IL-8* gene was significantly associated with the risk of CRC. The -251AA genotype was associated with a significantly increased risk of CRC as compared with the -251TT genotype (OR: 3.600, 95% CI: 1.550-8.481, $P = 0.001$). Similarly, for allele frequencies, such an association was observed. When compared with wild-type allele T of SNP *IL-8* -251, the variant allele A *IL-8* -251 showed significantly increased risk for CRC predisposition with OR 1.32 (95% CI: 1.03-1.69, $P = 0.003$). The strong association that we observed in CRC patients prompts us to suggest that *IL-8* gene -251AA polymorphism could contribute significantly to CRC susceptibility.

A few other molecular genetic epidemiological studies in diverse ethnic populations have found consistent as well as inconsistent results with ours. The *IL-8* -251T>A polymorphism has been associated with the risk of gastric cancer and gastric ulcer in Japanese patients with *Helicobacter pylori* infection[15]. Taguchi *et al*[14] also have reported that *IL-8* -251T>A polymorphism is associated with higher expression of *IL-8* protein, more severe neutrophil infiltration, and increased risk of atrophic gastritis and gastric cancer in the Japanese population. In the study of Gunter *et al*[16], homozygous variant genotype of the *IL-8* -251T>A had a 2.7-fold increased risk of colorectal adenoma compared to the homozygous wild type in the American population. In the French population, Küry *et al*[17] have reported that the heterozygous and homozygous variants of *IL-8* c -352 T>A are associated with an elevated risk of CRC compared to the homozygous major genotype. Li *et al*[18] have found that the risk of gastric cancer in the Chinese population is significantly elevated in patients with the *IL-8* -251AA genotype with OR 2.02 (95% CI: 1.27-3.21). In contrast with our study, Landi *et al*[19] have reported that the *IL-8* -251T>A genotypes have a protective role against CRC predisposition in the Spanish population, and Theoropoulus has reported that this SNP has no effect on CRC susceptibility risk in the Greek population[8]. A few other studies have suggested that the *IL-8* -251AA genotype is associated with an increased risk of prostate cancer in the Caucasian population[20] and Kaposi's sarcoma in the Dutch population[21], but a decreased risk of CRC[4].

The 251T>A polymorphism at the promoter region of *IL-8* gene is associated with the transcriptional activity of the gene[22], and to influence production and expression of *IL-8*[14,15]. Disrupted gene expression or altered protein formation of the *IL-8* gene may contribute positively or negatively to the establishment or progression of CRC. According to Wei *et al*[22], higher promoter activity of *IL-8* -251AA polymorphism might increase production and expression of *IL-8*, inducing a Th1-predominant immune response and leading to more susceptibility to CRC.

Free radicals generated as a result of oxidative stress produced by inflamed tissues may cause alteration in many metabolic reactions such as regulation of DNA, RNA and lipids, and thus can lead to cancer development[23,24]. Meira *et al*[25] have demonstrated that in inflamed colon cancer tissues, reactive oxygen species (ROS) and reactive nitrogen species are produced from activated inflammatory cells, and that these two species enhance DNA damage and this can cause mutation in areas of inflammation. Moreover, in CRC-associated colitis, chronic inflammation has been reported to cause oxidative damage to DNA, influence mutations in the *p53* gene in the inflamed tissues, and drive the cells to malignant transformation[26]. Oxidative stress produced by inflammatory cells in inflamed tissues in the intestinal tract has been reported to influence the development of CRC in patients with IBD[27]. These studies have clearly highlighted the importance of *IL-8* in modulating inflammation of the colorectum. According to Okada *et al*[28], ROS produced by inflammatory cells cause not only direct DNA damage but also indirect effects such as dysregulation of cell proliferation and apoptosis, stimulation of angiogenesis, and modification of gene/protein expression and protein activities that will cause cancer. Therefore, the relevance of *IL-8* -251T>A polymorphism in CRC susceptibility could be explained by the enhanced transcriptional activity of the gene, resulting in functional alteration of the gene product.

To the best of our knowledge, this is the first study on the association of the *IL-8* gene -251 T>A polymorphism and CRC risk in the Malaysian population. Our results show that the genetic variation of *IL-8* gene influences susceptibility to CRC in the Malaysian population, and suggest inflammation-mediated pathways in the process of colorectal carcinogenesis.

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COMMENTS

Background

Colorectal cancer (CRC), the incidence of which has been increasing worldwide for the past few years, represents a significant cause of morbidity and mortality. Genetics has a key role in predisposition to CRC and in its initiation and progression. Identifying predisposing genetic variations is important for our understanding of the carcinogenic process. Recently, chronic inflammation has been linked to increased risk to various types of cancer including CRC. Despite evidence strongly implicating chronic inflammation as a culprit in colorectal carcinogenesis, surprisingly little research has directly addressed the genetic predisposing factors that mediate inflammatory response and favor CRC development. Thus, it was of interest to explore the contribution of single nucleotide polymorphisms (SNPs) in inflammation genes as predisposing factors for CRC susceptibility.

Research frontiers

If inflammation constitutes one of the molecular networks underlying susceptibility to CRC, genes that mediate inflammatory response might be a group of candidate genes for CRC predisposition. Interleukin (IL)-8 is a chemokine and one of the major mediators of inflammatory responses, and is believed to play a role in the pathogenesis of cancer. IL-8 is a proangiogenic cytokine that is overexpressed in many human cancers and its expression promotes tumor growth, angiogenesis and metastasis. A polymorphism at -251 position (251T>A) of the promoter region of IL-8 has been associated with transcriptional activity of the gene. This study was designed to determine the frequencies and influence of the *IL-8* -251T>A polymorphic genotype and alleles on sporadic CRC susceptibility risk in the Malaysian population.

Innovations and breakthroughs

There are not many data available on the contribution of SNPs in inflammation response genes in mediating CRC predisposition risk, especially from the Asian population, and none from the Malaysian population. This is believed to be the first report of an association of genetic variation of *IL-8* with CRC susceptibility risk in the Malaysian population. We observed that the genetic diversity of the *IL-8* gene influences patient susceptibility to CRC and implies the importance of inflammation-mediated pathways in the process of colorectal carcinogenesis. From our results, it is reasonable to pronounce that *IL-8* gene may be an important candidate in the modulation of colorectal inflammation and the *IL-8* 251AA (homozygous variant genotype) could be considered as an important high-risk variant for CRC predisposition in the Malaysian population.

Applications

Early diagnosis is important for successful management of CRC patients and is facilitated by both invasive and noninvasive means of surveillance. Identification of genetic predisposition factors of CRC will help with better understanding of the colorectal carcinogenic process and in the design of diagnostic, therapeutic and preventive strategies. Understanding the genes and pathways that control the earliest steps of the disease and individual susceptibility can contribute to clinical management in the near future. In the future, study can be extended to a population level and individuals with high-risk predisposition genotypes can be identified. Once identified, they can be enrolled in cancer surveillance programs that will help in CRC prevention strategies.

Peer review

In this case-control study to determine the influence of the polymorphic genotype on sporadic CRC susceptibility risk in the Malaysian population, the *IL-8* -251T>A polymorphic allele and genotype frequencies were evaluated in 255 patients with sporadic CRC and 255 normal healthy controls. The frequency of the homozygous variant AA was significantly higher in CRC patients compared to controls. Furthermore, the homozygous variant *IL-8* -251AA was significantly associated with increased risk of CRC predisposition. This study is of interest because previous studies on the relationships between *IL-8* -251T>A polymorphism and CRC have yielded contradictory results.

REFERENCES

1 **Balkwill F**, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001; **357**: 539-545
2 **Coussens LM**, Werb Z. Inflammation and cancer. *Nature* 2002; **420**: 860-867
3 **Fitzpatrick FA**. Inflammation, carcinogenesis and cancer. *Int Immunopharmacol* 2001; **1**: 1651-1667
4 **Landi S**, Moreno V, Gioia-Patricola L, Guino E, Navarro M, de Oca J, Capella G, Canzian F. Association of common polymorphisms in inflammatory genes interleukin (IL)6, IL8, tumor necrosis factor alpha, NFKB1, and peroxisome proliferator-activated receptor gamma with colorectal cancer. *Cancer Res* 2003; **63**: 3560-3566
5 **Munkholm P**. Review article: the incidence and prevalence of colorectal cancer in inflammatory bowel disease. *Aliment Pharmacol Ther* 2003; **18** Suppl 2: 1-5
6 **Lakatos PL**, Hitre E, Szalay F, Zinober K, Fuszek P, Lakatos L, Fischer S, Osztovits J, Gemela O, Veres G, Papp J, Ferenci P. Common NOD2/CARD15 variants are not associated with susceptibility or the clinicopathologic characteristics of sporadic colorectal cancer in Hungarian patients. *BMC Cancer* 2007; **7**: 54
7 **Macarthur M**, Hold GL, El-Omar EM. Inflammation and Cancer II. Role of chronic inflammation and cytokine gene polymorphisms in the pathogenesis of gastrointestinal malignancy. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G515-G520
8 **Theodoropoulos G**, Papaconstantinou I, Felekouras E, Nikiteas N, Karakitsos P, Panoussopoulos D, Lazaris ACh, Patsouris E, Bramis J, Gazouli M. Relation between common polymorphisms in genes related to inflammatory response and colorectal cancer. *World J Gastroenterol* 2006; **12**: 5037-5043
9 **Platz EA**, De Marzo AM. Epidemiology of inflammation and prostate cancer. *J Urol* 2004; **171**: S36-S40
10 **Yang HP**, Woodson K, Taylor PR, Pietinen P, Albanes D, Virtamo J, Tangrea JA. Genetic variation in interleukin 8 and its receptor genes and its influence on the risk and prognosis of prostate cancer among Finnish men in a large cancer prevention trial. *Eur J Cancer Prev* 2006; **15**: 249-253
11 **Xie K**. Interleukin-8 and human cancer biology. *Cytokine Growth Factor Rev* 2001; **12**: 375-391
12 **Vairaktaris E**, Yapijakis C, Serefoglou Z, Derka S, Vassiliou S, Nkenke E, Vylliotis A, Wiltfang J, Avgoustidis D, Critselis E, Neukam FW, Patsouris E. The interleukin-8 (-251A/T) polymorphism is associated with increased risk for oral squamous cell carcinoma. *Eur J Surg Oncol* 2007; **33**: 504-507
13 **Snoussi K**, Mahfoudh W, Bouaouina N, Ahmed SB, Helal AN, Chouchane L. Genetic variation in IL-8 associated with increased risk and poor prognosis of breast carcinoma. *Hum Immunol* 2006; **67**: 13-21
14 **Taguchi A**, Ohmiya N, Shirai K, Mabuchi N, Itoh A, Hirooka Y, Niwa Y, Goto H. Interleukin-8 promoter polymorphism increases the risk of atrophic gastritis and gastric cancer in Japan. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 2487-2493
15 **Ohyauchi M**, Imatani A, Yonechi M, Asano N, Miura A, Iijima K, Koike T, Sekine H, Ohara S, Shimosegawa T. The polymorphism interleukin 8 -251 A/T influences the susceptibility of Helicobacter pylori related gastric diseases in the Japanese population. *Gut* 2005; **54**: 330-335
16 **Gunter MJ**, Canzian F, Landi S, Chanock SJ, Sinha R, Rothman N. Inflammation-related gene polymorphisms and colorectal adenoma. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 1126-1131
17 **Küry S**, Buecher B, Robiou-du-Pont S, Scoul C, Colman H, Le Neel T, Le Houérou C, Faroux R, Ollivry J, Lafraise B, Chupin LD, Sébille V, Bézieau S. Low-penetrance alleles predisposing to sporadic colorectal cancers: a French case-controlled

genetic association study. *BMC Cancer* 2008; **8**: 326
18 **Li A**, Varney ML, Valasek J, Godfrey M, Dave BJ, Singh RK. Autocrine role of interleukin-8 in induction of endothelial cell proliferation, survival, migration and MMP-2 production and angiogenesis. *Angiogenesis* 2005; **8**: 63-71
19 **Landi S**, Moreno V, Gioia-Patricola L, Guino E, Navarro M, de Oca J, Capella G, Canzian F. Association of common polymorphisms in inflammatory genes interleukin (IL)6, IL8, tumor necrosis factor alpha, NFKB1, and peroxisome proliferator-activated receptor gamma with colorectal cancer. *Cancer Res* 2003; **63**: 3560-3566
20 **McCarron SL**, Edwards S, Evans PR, Gibbs R, Dearnaley DP, Dowe A, Southgate C, Easton DF, Eeles RA, Howell WM. Influence of cytokine gene polymorphisms on the development of prostate cancer. *Cancer Res* 2002; **62**: 3369-3372
21 **van der Kuyl AC**, Polstra AM, Weverling GJ, Zorgdrager F, van den Burg R, Cornelissen M. An IL-8 gene promoter polymorphism is associated with the risk of the development of AIDS-related Kaposi's sarcoma: a case-control study. *AIDS* 2004; **18**: 1206-1208
22 **Wei YS**, Lan Y, Tang RG, Xu QQ, Huang Y, Nong HB, Huang WT. Single nucleotide polymorphism and haplotype association of the interleukin-8 gene with nasopharyngeal carcinoma. *Clin Immunol* 2007; **125**: 309-317
23 **Hussain SP**, Hofseth LJ, Harris CC. Radical causes of cancer. *Nat Rev Cancer* 2003; **3**: 276-285
24 **Marnett LJ**. Oxyradicals and DNA damage. *Carcinogenesis* 2000; **21**: 361-370
25 **Meira LB**, Bugni JM, Green SL, Lee CW, Pang B, Borenshtein D, Rickman BH, Rogers AB, Moroski-Erkul CA, McFaline JL, Schauer DB, Dedon PC, Fox JG, Samson LD. DNA damage induced by chronic inflammation contributes to colon carcinogenesis in mice. *J Clin Invest* 2008; **118**: 2516-2525
26 **Kraus S**, Arber N. Inflammation and colorectal cancer. *Curr Opin Pharmacol* 2009; **9**: 405-410
27 **Roessner A**, Kuester D, Malfertheiner P, Schneider-Stock R. Oxidative stress in ulcerative colitis-associated carcinogenesis. *Pathol Res Pract* 2008; **204**: 511-524
28 **Okada F**, Shionoya H, Kobayashi M, Kobayashi T, Tazawa H, Onuma K, Iuchi Y, Matsubara N, Ijichi T, Dugas B, Hosokawa M. Prevention of inflammation-mediated acquisition of metastatic properties of benign mouse fibrosarcoma cells by administration of an orally available superoxide dismutase. *Br J Cancer* 2006; **94**: 854-862

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BRIEF ARTICLE

Eradication of *Helicobacter pylori* increases childhood growth and serum acylated ghrelin levels

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Abstract

AIM: To determine whether *Helicobacter pylori* (*H. pylori*)-infected children have reduced body weight (BW) and height (BH) growth, and if *H. pylori* eradication may restore growth while improving serum acylated ghrelin.

METHODS: This longitudinal cohort study with one-year follow-up enrolled 1222 children aged 4 to 12 years old into an observation cohort (18 with and 318 without *H. pylori*) and intervention cohort (75 with and 811 without). The 7-d triple therapy was used for eradication in the intervention cohort. The net increases of BW and BH as well serum acylated ghrelin after one-year follow-up were compared between successful eradicated *H. pylori*-infected children and controls.

RESULTS: In the observation cohort, the *H. pylori*-infected children had lower z score of BW (-1.11 ± 0.47 *vs* 0.35 ± 0.69, $P = 0.01$) and body mass index (BMI) (0.06 ± 0.45 *vs* 0.44 ± 0.73, $P = 0.02$) at enrollment and lower net BW gain after one-year follow-up (3.3 ± 2.1 kg *vs* 4.5 ± 2.4 kg, $P = 0.04$) than the non-infected controls. In the intervention cohort, the *H. pylori*-infected children had lower z score of BMI (0.25 ± 1.09 *vs* 0.68 ± 0.87, $P = 0.009$) and serum acylated ghrelin levels (41.8 ± 35.6 pg/mL *vs* 83.6 ± 24.2 pg/mL, $P < 0.001$) than the non-infected controls. In addition to restoring decreased serum ghrelin levels (87.7 ± 38.0 pg/mL *vs* 44.2 ± 39.0 pg/mL, $P < 0.001$), the *H. pylori*-infected children with successful eradication had higher net gains ($P < 0.05$) and increase of z scores ($P < 0.05$) of both BW and BH as compared with non-infected controls after one-year follow-up.

CONCLUSION: *H. pylori*-infected children are associated with low serum acylated ghrelin and growth retardation. Successful eradication of *H. pylori* restores ghrelin levels and increases growth in children.

Key words: Child; Clinical trial; Ghrelin; Growth retardation; *Helicobacter pylori*

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INTRODUCTION

Primary infection with *Helicobacter pylori* (*H. pylori*) usually occurs during childhood[1]. This organism has been proven to cause chronic gastritis, peptic ulcer diseases, and had a high correlation with gastric cancer in humans[2,3]. In children, the *H. pylori* prevalence rate was relatively lower than adults[4,5]. Besides the link with gastric diseases, the association between *H. pylori* infection and growth retardation in children has raised clinical attention to this issue and caused some debate recently. Some cross-sectional analyses have indicated that *H. pylori*-infected children had subnormal growth retardation as compared with non-infected children[6-8], but some others did not support such findings[9,10]. Long-term observational studies have reported that children with persistent *H. pylori* infection have reduced body weight (BW) and height (BH) growth than the non-infected peers[11-13]. Therefore, to further support the causal relationship between *H. pylori* infection and growth retardation in children, interventional trials involving *H. pylori* eradication may provide new insights using a rigorous study design.

Ghrelin, a growth-hormone-releasing peptide biosynthesized mainly in the fundic mucosa, regulates appetite and body composition and is affected by inflammatory and atrophic events associated with *H. pylori* infection[14,15]. Previous studies showed conflicting results regarding the correlation between plasma ghrelin levels and *H. pylori* infection after eradication of bacteria[16-18]. This controversy may be caused by the measurement of total plasma ghrelin, which contains both acylated and desacylated forms. Acylated ghrelin is a more potent agonist on the growth-hormone-stimulating receptor than the desacylated form and undergoes a compensatory elevation in patients with chronic atrophic gastritis[19-21]. This study seeks to examine active ghrelin levels and its relationship with growth in patients before and after *H. pylori* eradication.

Although eradication of *H. pylori* can restore body mass index (BMI) and serum albumin in adult patients with infection[22,23], such improvement has not yet been documented in *H. pylori*-infected children. Moreover, it is unclear whether the improving growth parameters after *H. pylori* eradication are subsequently linked to increase serum acylated ghrelin levels. Therefore, this study sought to examine whether *H. pylori* eradication improves BW and BH growth in children in parallel with increases in serum acylated ghrelin levels.

MATERIALS AND METHODS

Subject enrollments in the two cohorts

This study enrolled 1292 students, aged 4 to 12 years old from three elementary schools and their associated pre-school kindergartens in Tainan City, Taiwan. The participants were consecutively enrolled into two study cohorts. Each participant provided informed consent documentation that was signed by her/his parents.

The first cohort (observation cohort) enrolled 400 children in 2005 to screen for the *H. pylori* infection, and they were then scheduled to return for follow-up growth status by a half-year interval of up to one year. The second group was an interventional cohort which enrolled 892 children in 2006 to screen for the *H. pylori* infection. Moreover, the *H. pylori*-infected subjects were invited to receive one-week of triple therapy for *H. pylori* eradication. As well, the children in the 2nd cohort were scheduled to return for follow-up growth status by a half-year interval of up to one year.

In each cohort, both the enrolled children and their parents were reviewed with a questionnaire to record data on underling medical diseases, *H. pylori* infection status, and a range of demographic variables, including socio-economic status, such as number of family members[8], and annual household income (low income indicated less than $15 000 US/year). The same nursing assistant provided the introduction of questionnaire to the enrolled subjects. Children with pre-established and severe medical/organic conditions predisposing to the failure of thrive, such as genetic/metabolic disorders and cyanotic congenital heart diseases, were not included. The study also excluded children with a known past history to receive anti-*H. pylori* therapy and children underwent eradication therapy or acid suppressors, during the follow-up period in the observation group. In both groups, the control cases were randomly selected (1:4 in the observation and 1:3 in the interventional cohorts) and were matched by age and gender to children with ^{13}C-labeled urea breath test (^{13}C-UBT)-confirmed *H. pylori* infection. Moreover, for the *H. pylori*-infected (confirmed by a positive ^{13}C-UBT) children at entry, the *H. pylori* status was assessed with a ^{13}C-UBT after 6 mo (intervention cohort) and one year follow-up (both cohort).

BMI and z scores of weight, height and BMI

For each participant, the overnight fasting BW and BH were serially measured at enrollment and at the follow-up period on the 6th mo and the 12th mo, respectively. The BMI was defined as BW in kilograms/squared of body length in meter (kg/m^2). The z scores (SD scores) of BW, BH and BMI were calculated using the reference population of 2003 Taiwanese boys and girls based on health-related physical fitness and based on 2006 World Health Organization standards[24]. The net changes of BW, BH and BMI were calculated by the value of each parameter at follow-up minus the corresponding value at enrollment. We also defined the increase of z score means that z scores of BW, BH and BMI were upgrade at the one-year follow-up than at the enrollment (the net change > 0).

Serological screening of H. pylori infection and confirmation by urea breath test

In each enrolled child, the serum was tested for anti-*H. pylori*

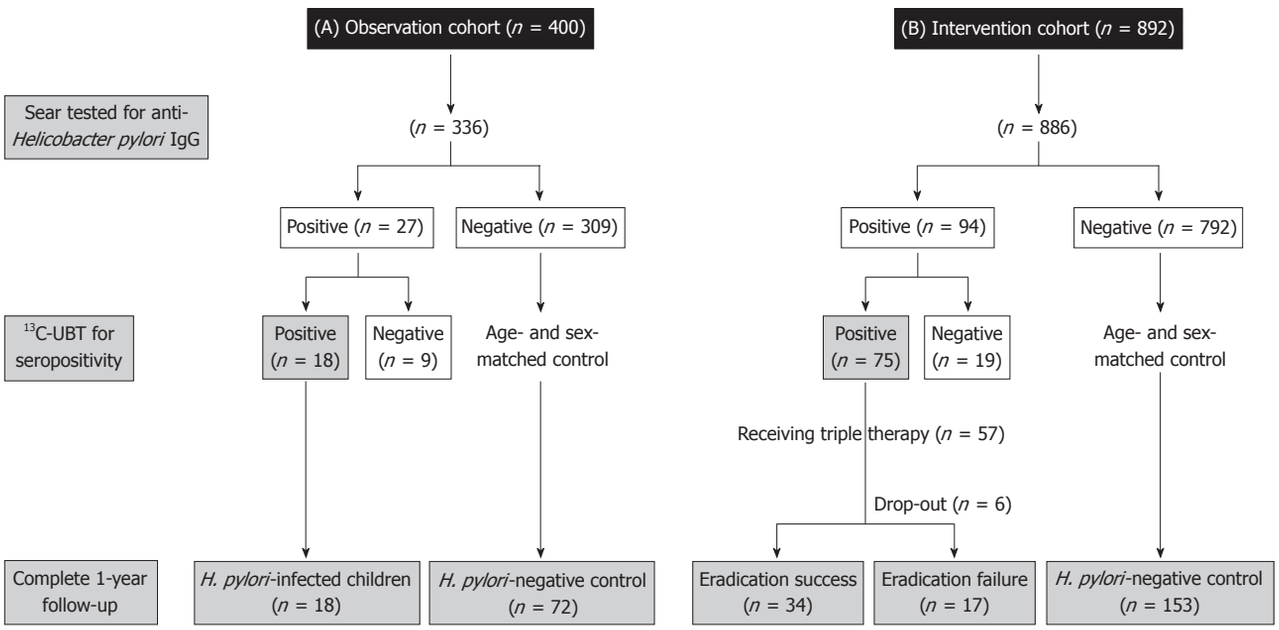

Figure 1 The study flow diagram and the case numbers were listed for the different study follow-up periods. *H. pylori*: *Helicobacter pylori*; ^{13}C-UBT: [13C]-labeled urea breath test.

IgG antibodies (HEL-p TEST™ Ⅱ, AMRAD Biotech, Australia) by enzyme-linked immunosorbent assay (ELISA) methods. The serologic kit has been validated with a favorable sensitivity and specificity (> 90%) in detecting *H. pylori* infection in our previous studies[25]. The seropositive children further confirmed by ^{13}C-UBT to diagnose ongoing *H. pylori* infection[8]. The cut-off value of positive ^{13}C-UBT was defined as excess $^{13}CO_2/^{12}CO_2$ ratio more than 3.5‰[8,26].

Eradication therapy for H. pylori-infected children

For the *H. pylori*-infected children in the intervention cohort, lansoprazole (1 mg/kg per day, max. 30 mg bid), amoxicillin (50 mg/kg per day, max. 1 g bid), and clarithromycin (15 mg/kg per day, max. 500 mg bid) were prescribed for one week[26]. We have educated the participants and their parents for the compliance and report of complications. Successful eradication therapy was defined by a negative result of ^{13}C-UBT on both the 6th and the 12th mo follow-up, respectively[27].

Serial serum acylated ghrelin levels before and after H. pylori eradications

The serum acylated ghrelin levels of the interventional cohorts at enrollment were compared between children with and without *H. pylori* infection. In addition, the serial serum acylated ghrelin levels of the children with *H. pylori* eradication collected at enrollment, the 6th mo, and the 12th mo follow-up were compared. Each blood sample of child was collected in the morning before breakfast and was incubated in the ice-bath container immediately. The sera were separated by centrifugation within 2-3 h and were stored in a -80 ℃ refrigerator until use. These samples' serum acylated ghrelin levels were analyzed in duplicate by a commercial kit (LINCO Research, St. Charles, Missouri, United States), using ELISA methods.

Statistical analysis

The χ^2 test with the odds ratio (OR) and 95% conference interval (CI) and logistic regression test were applied as an estimate of the possibly related factors between *H. pylori*-infected and non-infected children. The Student's *t* test and one-way analysis of variance with least significant difference test correction were used as appropriate to compare the differences of ghrelin, BW, BH, BMI and their net changes during one-year follow-up periods among different study groups. The paired *t* test was used to analyze the difference of the serial serum acylated ghrelin levels before and after eradication therapy within the same study group. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Participants and H. pylori infection

There were 84% (336/400) children in the observation and 99% (886/892) children in the intervention cohorts who completed the questionnaires and provided their sera for the anti-*H. pylori* IgG antibodies tested, respectively. In Figure 1, the case numbers of each cohort were serially summarized during the one-year follow-up. One hundred and twenty-one (27 in the observation cohort, 94 in the intervention cohort) were defined with seropositivity of *H. pylori* infection. Among them, 113 children received ^{13}C-UBT, of which only 93 (82%) children were positive (18 in the observation cohort and 75 in the intervention cohort). Accordingly, the overall *H. pylori* prevalence was 7.6% in these two cohorts.

For the 18 *H. pylori*-positive children in the observation cohort, the infection was persisted with a positive ^{13}C-UBT until the end of follow-up on the 1st year. Among the 75 *H. pylori*-infected children in the intervention cohort, 57 children were enrolled to receive the 7-d eradica-

Table 1 Comparison of demographic background, baseline body parameters, and the changes of growth after one-year follow-up between the *Helicobacter pylori*-infected and non-infected matched controls of the observation cohort (mean ± SD)

Groups	*H. pylori*-positive subjects	*H. pylori*-negative controls	*P* value
n	18	18	
Age (yr)	9.1 ± 1.6	9.4 ± 1.7	0.57
Sex (female:male)	8:10	27:45:00	0.59
Body weight (kg)			
At enrollment	29.9 ± 7.0	35.0 ± 10.1	0.02
z score at enrollment	-1.11 ± 0.47	0.35 ± 0.69	0.01
The 1st year	33.2 ± 7.5	39.5 ± 11.3	0.007
z score at the 1st year	-0.17 ± 0.45	0.38 ± 0.67	0.002
Net change	3.3 ± 2.1	4.5 ± 2.4	0.04
Body height (cm)			
At enrollment	132.3 ± 11.4	136.8 ± 12.3	0.15
z score at enrollment	-0.17 ± 0.49	0.15 ± 0.73	0.07
The 1st year	138.0 ± 11.4	142.5 ± 12.4	0.15
z score at the 1st year	-0.14 ± 0.48	0.13 ± 0.71	0.1
Net change	5.7 ± 0.9	5.8 ± 1.9	0.81
BMI (kg/m^2)			
At enrollment	16.8 ± 1.6	18.4 ± 2.8	0.03
z score at enrollment	0.06 ± 0.45	0.44 ± 0.73	0.02
The 1st year	17.2 ± 1.7	19.0 ± 3.0	0.01
z score at the 1st year	0 ± 0.38	0.45 ± 0.75	0.01
Net change	0.35 ± 0.8	0.69 ± 1.0	0.14

BMI: Body mass index; *H. Pylori*: *Helicobacter pylori*; Net change: The growth parameters at the 1st year minus that at enrollment.

tion therapy. A total of 6 subjects withdrew or were lost to follow-up. All of the finally eligible subjects had good drugs compliance (taking drugs at least 6 d) and none had major adverse complications. The intention-to-treat and per-protocol eradication rate of *H. pylori*-infected children were 60% (34/57) and 67% (34/51), respectively.

A lower BW, HW and BMI in the H. pylori infected subject

In the observation cohort, there were 72 age- and gender-matched subjects selected to serve as *H. pylori*-negative controls. In Table 1, the *H. pylori*-infected children had lower BW (29.9 ± 7.0 kg *vs* 35 ± 10.1 kg, $P = 0.02$), z score of BW (-1.11 ± 0.47 *vs* 0.35 ± 0.69, $P = 0.01$), BMI (16.8 ± 1.6 kg/m^2 *vs* 18.4 ± 2.8 kg/m^2, $P = 0.03$) and z score of BMI (0.06 ± 0.45 *vs* 0.44 ± 0.73, $P = 0.02$) than those non-infected controls. Moreover, after one-year follow-up, the *H. pylori*-infected children had a significantly lower BW (33.2 ± 7.5 kg *vs* 39.5 ± 11.3 kg, $P = 0.007$), z score of BW (-0.17 ± 0.45 *vs* 0.38 ± 0.67, $P = 0.002$), BMI (17.2 ± 1.7 kg/m^2 *vs* 19 ± 3.0 kg/m^2, $P = 0.01$), and z score of BMI (0 ± 0.38 *vs* 0.45 ± 0.75, $P = 0.01$) than the non-infected ones. Also in Table 1, there was a significantly lower net BW gain in the *H. pylori*-infected children than that in the non-infected controls (3.3 ± 2.1 kg *vs* 4.5 ± 2.4 kg, $P = 0.04$) after one-year follow-up. However, the net changes of BH, and BMI were not different between the children with and without *H. pylori* infections ($P > 0.05$).

In the intervention cohort (57 *H. pylori*-infected and 153 controls), children with *H. pylori* infection had significantly lower BMI (17.7 ± 3.8 kg/m^2 *vs* 19.0 ± 3.7 kg/m^2, $P = 0.02$) and z score of BMI (0.25 ± 1.09 *vs* 0.68 ± 0.87, $P = 0.009$) than controls at the enrollment. In Table 2, there was no difference with regards to patients' demographic background among the eradication failure, eradication success, and control groups at enrollment. In comparison to the observation cohort, the z score of BMI at enrollment was significantly lower in successful eradication group (0.21 ± 1.14 *vs* 0.68 ± 0.87, $P = 0.007$) than in the non-infected controls. The baseline BW and BH were still lower in the *H. pylori*-infected children (either with eradication success or failure) than in controls, although it is not statistically significant.

Table 2 Comparison of the demographic background, baseline body parameters, and the changes of growth among the different groups of the intervention cohort completed the one-year follow-up (mean ± SD)

Groups	*H. pylori* eradication failure	*H. pylori* eradication success	*H. pylori*-negative controls
n	17	34	153
Age (yr)	8.4 ± 1.9	8.7 ± 2.0	9.0 ± 1.7
Sex (female:male)	10:07	19:15	81 : 72
Family peptic ulcer history (%)	44.4	29.4	21
Intra-familial members ⩾ 5 (%)	33.3	29.4	32.1
Low income (%)	28.6	53.3	44.1
Baseline serum acylated ghrelin (pg/mL)[a,c]	37.2 ± 31.4	44.2 ± 37.9	83.6 ± 24.2
Body weight (kg)			
At enrollment	32.2 ± 10.6	32.9 ± 11.4	36.1 ± 11.4
z score at enrollment	0.44 ± 1.00	0.35 ± 1.04	0.60 ± 0.87
The 1st year	37.2 ± 11.7	38.7 ± 13.8	41.0 ± 12.6
z score at the 1st year	0.56 ± 0.88	0.49 ± 1.03	0.61 ± 0.88
Net changec	5.03 ± 2.77	5.84 ± 3.37	4.84 ± 2.35
Increase of z score (%)[c]	23.5	38.2	17.6
Body height (cm)			
At enrollment	134.6 ± 12.7	134.3 ± 11.7	135.7 ± 12.5
z score at enrollment	0.62 ± 0.99	0.44 ± 0.88	0.33 ± 0.77
The 1st year	141.8 ± 11.8	142.3 ± 12.8	141.5 ± 13.0
z score at the 1st year[a,c]	0.77 ± 0.94	0.63 ± 0.75	0.32 ± 0.79
Net change[c,e]	7.20 ± 2.85	8.00 ± 2.78	5.85 ± 1.81
Increase of z score (%)[c]	35.3	35.3	15.7
BMI (kg/m^2)			
At enrollment[a]	17.1 ± 2.6	17.7 ± 4.1	19.0 ± 3.7
z score at enrollment[c]	0.24 ± 0.75	0.21 ± 1.14	0.68 ± 0.87
The 1st year	18.1 ± 3.1	18.8 ± 4.7	20.0 ± 3.8
z score at the 1st year[c]	0.35 ± 0.82	0.27 ± 1.12	0.67 ± 0.87
Net change	0.98 ± 1.57	1.10 ± 1.56	0.99 ± 1.03
Increase of z score (%)	35.3	32.4	17

Low income: Indicated < $15000 US/year. Increase of z score means that z scores of body weight, height and body mass index (BMI) were upgrade at the one-year follow-up than at the enrollment (the net change > 0). The difference of the body weight, height, BMI and ghrelin level among the three groups were analyzed by oneway analysis of variance model with least significant difference correction. The difference of the up-shift of the z scores of body weight, height and BMI were analyzed by χ^2 test. [a]$P < 0.05$ between *H. pylori*-positive subjects with eradication failure and controls, [c]$P < 0.05$ between *H. pylori*-positive subjects with eradication success and controls.

Successful H. pylori eradication improves body growth of children within one year

There were 34 children with successful *H. pylori* eradica-

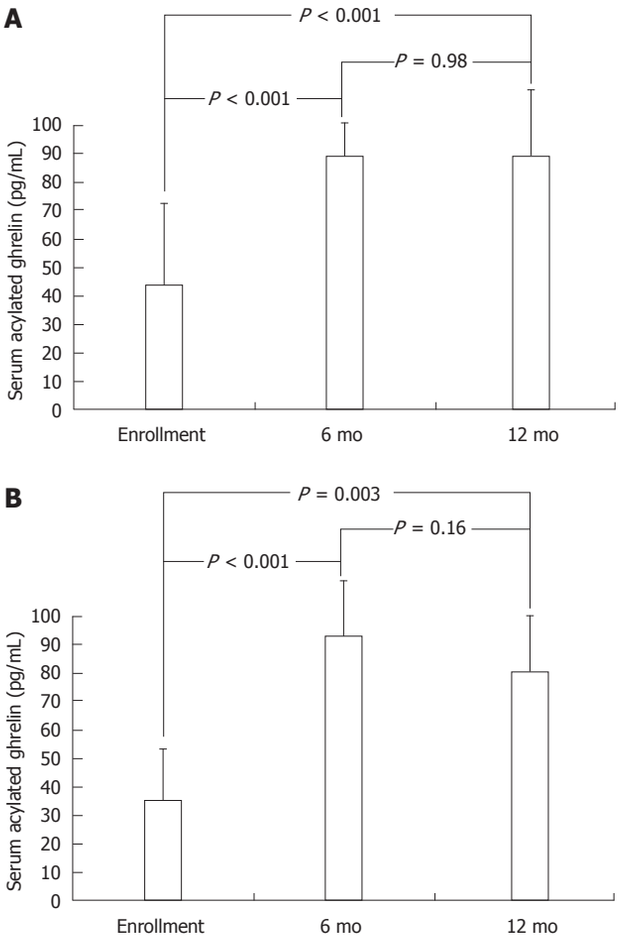

Figure 2 Comparison of the serum acylated ghrelin levels (mean) at enrollment, at 6th mo and at 12th mo follow-ups between the two groups of *Helicobacter pylori*-infected children with success (A) and with failure (B) of eradication therapy. The significance was analyzed by paired *t* test.

tion and 17 children with failure of who completed the one-year follow-up study. Moreover, we completed the one-year follow-up to the 153 age- and sex-matched non-infected controls. One-year after eradication therapy, the *H. pylori*-infected children with successful eradication had significantly higher net increases of BW (5.84 ± 3.37 kg *vs* 4.84 ± 2.85 kg, $P = 0.04$) and BH (8.00 ± 2.78 cm *vs* 5.85 ± 1.81 cm, $P < 0.001$) than the *H. pylori*-negative controls. Moreover, the rates of increase of z scores of BW (38.2% *vs* 17.6%, $P = 0.02$) and BH (35.3% *vs* 15.7%, $P = 0.02$) were significantly higher in children with successful eradication than controls. We further analyzed the enrolled age as a confounder for the increase of z scores of BW and BH by multiple logistic regression analysis. The results confirmed the successful eradication of *H. pylori* was an independent factor to predict the increase of BW ($P = 0.01$) and BH ($P = 0.01$) in the intervention cohort. Although triple therapy failed to achieve successful eradication, these *H. pylori*-infected children still had a higher net increase of BH (7.20 ± 2.85 cm *vs* 5.85 ± 1.81 cm, $P = 0.01$) than the non-infected controls. However, these *H. pylori*-infected children and non-infected controls had no difference in the net increase of BW (5.03 ± 2.77 kg *vs* 4.84 ± 2.35 kg, $P = 0.78$) and increase of z scores of BW (23.5% *vs* 17.6%, $P = 0.79$) during one-year follow-up.

Lower serum acylated ghrelin correlated to H. pylori infection and lower BW

In the intervention cohort, the *H. pylori*-infected children had a significantly lower serum acylated ghrelin level (41.8 ± 35.6 pg/mL *vs* 83.6 ± 24.2 pg/mL, $P < 0.001$) than the controls. In Table 2, at enrollment, both the *H. pylori*-infected children with and without eradication success during follow-up had lower serum acylated ghrelin levels than the non-infected controls (both $P < 0.001$). As the *H. pylori*-infected children exhibited lower BW and lower serum acylated ghrelin, the study further tested whether the children with lower BW, within the age ranges, could have a lower serum acylated ghrelin. We compared the serum acylated ghrelin levels of the children between those with BW and z score of BW above and below the selected cut-off point in the different age ranges (Table 3). Only in the *H. pylori*-infected children with age ranges as 8-12 years, the serum acylated ghrelin level was lower in the children with BW below the cut-off point than that with BW above (23.8 ± 22.1 pg/mL *vs* 51.8 ± 40.9 pg/mL, $P = 0.02$).

Improvement of the serum acylated ghrelin level after eradication therapy

In addition to the baseline serum acylated ghrelin levels, the study subjects of the intervention cohort underwent testing for serial serum acylated ghrelin levels after triple therapy. In Figure 2A, for the children with successful *H. pylori* eradication, the serum acylated ghrelin levels were significantly increased after eradication therapy as early as the 6th mo (88.2 ± 17.3 pg/mL *vs* 44.2 ± 38.1 pg/mL, $P < 0.001$), until the 12th mo (87.7 ± 38.0 pg/mL *vs* 44.2 ± 38.1 pg/mL, $P < 0.001$). In Figure 2B, for the children with failure of *H. pylori* eradication, the serum acylated ghrelin levels could be also significantly increased by eradication therapy at the 6th mo (93.2 ± 31.6 pg/mL *vs* 37.2 ± 30.9 pg/mL, $P < 0.001$) and at the 12th mo (80.6 ± 28.8 pg/mL *vs* 37.2 ± 30.9 pg/mL, $P = 0.003$).

DISCUSSION

Extra-gastric diseases related to *H. pylori* infection are emerging in importance, such as iron deficiency anemia and growth retardation in children[28]. Other studies have argued that lower socioeconomic status is conjunction with the presence of *H. pylori* accounts for poor growth in children[29]. For overcoming the influencing bias of poor socioeconomic status, indicated by low income, to child growth, multiple logistic regression confirmed that *H. pylori* infection was closely related to both z scores of BW and BMI independent to socioeconomic status. Accordingly, the current study should have not encountered significant bias of social backgrounds on growth limitation in children.

Based on the data of the observation cohort, the *H. pylori*-

Table 3 The differences of the baseline serum acylated ghrelin levels between the children with body weight above and below the cut-off point selected based on the different age ranges of children (mean ± SD)

	H. pylori infection		Non-*H. pylori* infection	
Age ranges (yr)	4-7	8-12	4-7	8-12
BW cut-off point, kg (*n*)	26 (21)	36 (32)	26 (47)	36 (87)
Baseline serum acylated ghrelin (pg/mL)				
Above or equal to the BW cut-off point	51.3 ± 38.6	51.8 ± 40.9	78.2 ± 12.0	85.9 ± 26.9
Below the BW cut-off point	47.8 ± 36.5	23.8 ± 22.1	82.5 ± 17.2	83.8 ± 31.2
[1]*P* value	0.93	0.02	0.31	0.78
z score of BW cut-off point (*n*)	0.5 (18)	0.5 (35)	0.5 (28)	0.5 (106)
Baseline serum acylated ghrelin (pg/mL)				
Above or equal to the z score of BW cut-off point	53.4 ± 40.6	46.8 ± 38.9	81.3 ± 14.1	81.7 ± 19.7
Below the z score of BW cut-off point	45.9 ± 33.6	27.3 ± 26.5	81.9 ± 16.6	89.6 ± 36.4
[1]*P* value	0.68	0.09	0.91	0.15

[1]The *P* value indicated the difference of serum acylated ghrelin levels between the children with body weight (BW) and z score of BW above or equal to the cut-off point and those with below the cut-off point within the same age ranges, analyzed by the Student's *t* test. The BW (z score of BW) cut-off point was determined by the mean (median) of non-*Helicobacter pylori* (*H. pylori*) infected children within the same age ranges.

infected children had significantly lower BW and BMI than gender- and age-matched controls at enrollment. In addition, the BH of *H. pylori*-infected children was 4.5 cm less than that of the non-infected children. After one-year follow-up, the *H. pylori*-infected children had profoundly lower BW, BMI, and net BW gain than the non-infected children. These findings comparing infected and matched disease-free subjects suggest that *H. pylori* infection exerted a negative effect on childhood growth. One possibility is that the presence of *H. pylori* leads to chronic gastric inflammation and thereby decreases food intake[30]. Alternatively, *H. pylori* infection may modify hormones related with the appetite, such as ghrelin[19-21,31,32].

Besides having a lower BW, the *H. pylori*-infected children had a significantly lower baseline serum acylated ghrelin level than that of the gender- and age-matched controls. These data not only suggest a relationship between *H. pylori* infection and lower BW, but that infection is linked to reduced ghrelin levels. Accordingly, it is important to determine whether children with a lower BW had lower serum acylated ghrelin. Supported by the findings in Table 3, only in the *H. pylori*-infected children with age ranging from 8-12 years, the serum acylated ghrelin levels were lower in children with BW below the cut-off point than that with BW above. This finding indicates a lower serum acylated ghrelin, which is induced by the *H. pylori* infection, could be related to induce a lower BW in children aged 8-12 years old.

Eradication of *H. pylori* in symptomatic adult patients has been reported to increase BMI[22,23], and to restore decreased ghrelin levels induced by *H. pylori* infection[16,17]. However, there is still limited evidence to prove such effects in children. Recently, a trial demonstrated *H. pylori* eradication may result in a significant increase in BMI, but with a decrease in the circulating ghrelin levels in a small group of children[33]. Consistent with these findings, our study supported *H. pylori* eradication to address positive impact on the improvement of childhood growth concomitant with an increase of serum acylated ghrelin level during the one-year long-term follow-up. Therefore, this study is the first to support that *H. pylori* eradication can restore decreased serum acylated ghrelin in children with lower BW. In the future, a longer follow-up study may help to determine whether ongoing elevations in BMI will ultimately lower serum ghrelin via a feedback loop. Our study was nonetheless sufficient to show that within one-year of *H. pylori* eradication, the children could achieve normal growth stature with concomitant restoration of the decreased serum acylated ghrelin induced by the *H. pylori* infection.

Even though some *H. pylori*-infected children had a failure of triple therapy, there was still existed an increase of BW, BH, and serum acylated ghrelin levels at the 6th and the 12th mo. Triple therapy can decrease bacterial loads or gastric inflammation[15,33]. We have analyzed the 51 pairs of ^{13}C-UBT and ghrelin levels (at enrollment, the 6th and 12th mo follow-up) in 17 children with a failure of triple therapy. The result shows the bacterial loads, indicated by the values of ^{13}C-UBT are not correlated well to the ghrelin levels ($r^2 = 0.03$, $P = 0.25$). Therefore, it is possibly due to transient improvement of gastric inflammation to restore serum acylated ghrelin levels. Lack of endoscopic evidence in children with failure of therapy is the limitation in this study. A longer follow-up period is thus needed to clarify this transient improving effect in children with failure of therapy.

In summary, *H. pylori* infection can be associated with decreased serum acylated ghrelin levels, BW and BH in children. Successful *H. pylori* eradication can restore ghrelin levels and the growth of BW and BH in the infected children with growth retardation.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection in children causes not only gastric inflammation and peptic ulcer diseases but also extragastric disorder. Longitudinal observational have found that children with persistent *H. pylori* infection have reduced body weight (BW) and height (BH) growth than the non-infected

ones. In addition, previous studies showed conflicting results regarding the correlation between plasma ghrelin levels and *H. pylori* infection after eradication of bacteria. Therefore, long-term follow up the childhood growth as well ghrelin levels in *H. pylori*-infected children after eradication therapy can illustrate the causal relationship between *H. pylori* infection and growth retardation in children.

Research frontiers

Growth retardation in *H. pylori*-infected children without any organic diseases remains controversial for eradication therapy. The authors aimed to establish a new indication for treating *H. pylori* infection in children with growth retardation and to explore the serum acylated ghrelin levels correlated to eradication therapy.

Innovations and breakthroughs

This study demonstrated that *H. pylori* infection can be associated with decreased serum acylated ghrelin levels, BW and BH in children. In the interventional study, successful *H. pylori* eradication can restore serum acylated ghrelin levels and the growth of BW and BH in the infected children with growth retardation at the 1-year follow-up.

Applications

This study confirmed the causal relationship of *H. pylori* infection and childhood growth retardation. Therefore, we supposed that eradication therapy should be considered as a treatment strategy in *H. pylori*-infected children with growth retardation, which was not related to other organic diseases.

Terminology

Growth retardation is indicated by poor BW and BH growth as compared to the age- and gender-matched normal population. Eradication therapy means that a treatment strategy to eradicate *H. pylori* from stomach. The first-line regiment consists of one proton pump inhibitor and two antibiotics.

Peer review

This is an interesting study aimed at determining whether *H. pylori*-infected children have reduced growth rates and lower levels of ghrelin compared to uninfected and if *H. pylori* eradication may reverse those changes. The study is well written and well designed.

REFERENCES

1 **Malaty HM**, Kumagai T, Tanaka E, Ota H, Kiyosawa K, Graham DY, Katsuyama T. Evidence from a nine-year birth cohort study in Japan of transmission pathways of Helicobacter pylori infection. *J Clin Microbiol* 2000; **38**: 1971-1973

2 **Marshall BJ**, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **1**: 1311-1315

3 **Suerbaum S**, Michetti P. Helicobacter pylori infection. *N Engl J Med* 2002; **347**: 1175-1186

4 **Yang YJ**, Wang SM, Chen CT, Huang MC, Chang CJ, Liu CC. Lack of evidence for fecal-oral transmission of Helicobacter pylori infection in Taiwanese. *J Formos Med Assoc* 2003; **102**: 375-378

5 **Lin DB**, Lin JB, Chen CY, Chen SC, Chen WK. Seroprevalence of Helicobacter pylori infection among schoolchildren and teachers in Taiwan. *Helicobacter* 2007; **12**: 258-264

6 **Büyükgebiz A**, Dündar B, Böber E, Büyükgebiz B. Helicobacter pylori infection in children with constitutional delay of growth and puberty. *J Pediatr Endocrinol Metab* 2001; **14**: 549-551

7 **Choe YH**, Kim SK, Hong YC. Helicobacter pylori infection with iron deficiency anaemia and subnormal growth at puberty. *Arch Dis Child* 2000; **82**: 136-140

8 **Yang YJ**, Sheu BS, Lee SC, Yang HB, Wu JJ. Children of Helicobacter pylori-infected dyspeptic mothers are predisposed to H. pylori acquisition with subsequent iron deficiency and growth retardation. *Helicobacter* 2005; **10**: 249-255

9 **Chimonas MA**, Baggett HC, Parkinson AJ, Muth PT, Dunaway E, Gessner BD. Asymptomatic Helicobacter pylori infection and iron deficiency are not associated with decreased growth among Alaska Native children aged 7-11 years. *Helicobacter* 2006; **11**: 159-167

10 **Sauvé-Martin H**, Kalach N, Raymond J, Senouci L, Benhamou PH, Martin JC, Briet F, Maurel M, Flourié B, Dupont C. The rate of Helicobacter pylori infection in children with growth retardation. *J Pediatr Gastroenterol Nutr* 1999; **28**: 354-355

11 **Patel P**, Mendall MA, Khulusi S, Northfield TC, Strachan DP. Helicobacter pylori infection in childhood: risk factors and effect on growth. *BMJ* 1994; **309**: 1119-1123

12 **Passaro DJ**, Taylor DN, Gilman RH, Cabrera L, Parsonnet J. Growth slowing after acute Helicobacter pylori infection is age-dependent. *J Pediatr Gastroenterol Nutr* 2002; **35**: 522-526

13 **Bravo LE**, Mera R, Reina JC, Pradilla A, Alzate A, Fontham E, Correa P. Impact of Helicobacter pylori infection on growth of children: a prospective cohort study. *J Pediatr Gastroenterol Nutr* 2003; **37**: 614-619

14 **Neary NM**, Small CJ, Wren AM, Lee JL, Druce MR, Palmieri C, Frost GS, Ghatei MA, Coombes RC, Bloom SR. Ghrelin increases energy intake in cancer patients with impaired appetite: acute, randomized, placebo-controlled trial. *J Clin Endocrinol Metab* 2004; **89**: 2832-2836

15 **Isomoto H**, Nakazato M, Ueno H, Date Y, Nishi Y, Mukae H, Mizuta Y, Ohtsuru A, Yamashita S, Kohno S. Low plasma ghrelin levels in patients with Helicobacter pylori-associated gastritis. *Am J Med* 2004; **117**: 429-432

16 **Isomoto H**, Ueno H, Saenko VA, Mondal MS, Nishi Y, Kawano N, Ohnita K, Mizuta Y, Ohtsuru A, Yamashita S, Nakazato M, Kohno S. Impact of Helicobacter pylori infection on gastric and plasma ghrelin dynamics in humans. *Am J Gastroenterol* 2005; **100**: 1711-1720

17 **Nwokolo CU**, Freshwater DA, O'Hare P, Randeva HS. Plasma ghrelin following cure of Helicobacter pylori. *Gut* 2003; **52**: 637-640

18 **Gokcel A**, Gumurdulu Y, Kayaselcuk F, Serin E, Ozer B, Ozsahin AK, Guvener N. Helicobacter pylori has no effect on plasma ghrelin levels. *Eur J Endocrinol* 2003; **148**: 423-426

19 **Kojima M**, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999; **402**: 656-660

20 **Campana D**, Nori F, Pagotto U, De Iasio R, Morselli-Labate AM, Pasquali R, Corinaldesi R, Tomassetti P. Plasma acylated ghrelin levels are higher in patients with chronic atrophic gastritis. *Clin Endocrinol* (Oxf) 2007; **67**: 761-766

21 **Osawa H**. Ghrelin and Helicobacter pylori infection. *World J Gastroenterol* 2008; **14**: 6327-6333

22 **Furuta T**, Shirai N, Xiao F, Takashima M, Hanai H. Effect of Helicobacter pylori infection and its eradication on nutrition. *Aliment Pharmacol Ther* 2002; **16**: 799-806

23 **Yang YJ**, Sheu BS, Chang WL, Cheng HC, Yang HB. Increased body mass index after H. pylori eradication for duodenal ulcer predisposes to erosive reflux esophagitis. *J Clin Gastroenterol* 2009; **43**: 705-710

24 **Chen W**, Chang MH. New growth charts for Taiwanese children and adolescents based on World Health Organization standards and health-related physical fitness. *Pediatr Neonatol* 2010; **51**: 69-79

25 **Sheu BS**, Lin CY, Lin XZ, Shiesh SC, Yang HB, Chen CY. Long-term outcome of triple therapy in Helicobacter pylori-related nonulcer dyspepsia: a prospective controlled assessment. *Am J Gastroenterol* 1996; **91**: 441-447

26 **Gold BD**, Colletti RB, Abbott M, Czinn SJ, Elitsur Y, Hassall E, Macarthur C, Snyder J, Sherman PM. Helicobacter pylori infection in children: recommendations for diagnosis and treatment. *J Pediatr Gastroenterol Nutr* 2000; **31**: 490-497

27 **Sheu BS**, Lee SC, Yang HB, Wu HW, Wu CS, Lin XZ, Wu JJ. Lower-dose (13)C-urea breath test to detect Helicobacter pylori infection-comparison between infrared spectrometer and mass spectrometry analysis. *Aliment Pharmacol Ther* 2000; **14**: 1359-1363

28 **Malfertheiner P**, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, Hunt R, Rokkas T, Vakil N, Kuipers EJ.

Current concepts in the management of Helicobacter pylori infection: the Maastricht III Consensus Report. *Gut* 2007; **56**: 772-781

29 **Sood MR**, Joshi S, Akobeng AK, Mitchell J, Thomas AG. Growth in children with Helicobacter pylori infection and dyspepsia. *Arch Dis Child* 2005; **90**: 1025-1028

30 **Weigt J**, Malfertheiner P. Influence of Helicobacter pylori on gastric regulation of food intake. *Curr Opin Clin Nutr Metab Care* 2009; **12**: 522-525

31 **Ozçay F**, Demir H, Ozen H, Gürakan F, Saltik IN, Yüce A, Koçak N. Normal growth in young children with Helicobacter pylori infection. *J Pediatr Gastroenterol Nutr* 2002; **35**: 102

32 **Perri F**, Pastore M, Leandro G, Clemente R, Ghoos Y, Peeters M, Annese V, Quitadamo M, Latiano A, Rutgeerts P, Andriulli A. Helicobacter pylori infection and growth delay in older children. *Arch Dis Child* 1997; **77**: 46-49

33 **Pacifico L**, Anania C, Osborn JF, Ferrara E, Schiavo E, Bonamico M, Chiesa C. Long-term effects of Helicobacter pylori eradication on circulating ghrelin and leptin concentrations and body composition in prepubertal children. *Eur J Endocrinol* 2008; **158**: 323-332

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BRIEF ARTICLE

Colorectal cancer screening: Comparison of transferrin and immuno fecal occult blood test

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Abstract

AIM: To evaluate the sensitivity and specificity of transfesrrin dipstick test (Tf) in colorectal cancer (CRC) screening and precancerous lesions screening.

METHODS: Eight hundreds and sixty-one individuals at high-risk for CRC were recruited. Six hundreds and eleven subsequently received the three fecal occult blood tests and colonoscopy with biopsy performed as needed. Fecal samples were obtained on the day before colonoscopy. Tf, immuno fecal occult blood test (IFOBT) and guaiac fecal occult blood test (g-FOBT) were performed simultaneously on the same stool. To minimize false-negative cases, all subjects with negative samples were asked to provide an additional stool specimen for a second test even a third test. If the results were all negative after testing three repeated samples, the subject was considered a true negative. The performance characteristics of Tf for detecting CRC and precancerous lesions were examined and compared to those of IFOBT and the combination of Tf, IFOBT and g-FOBT.

RESULTS: A total of six hundreds and eleven subjects met the study criteria including 25 with CRC and 60 with precancerous lesions. Sensitivity for detecting CRC was 92% for Tf and 96% for IFOBT, specificities of Tf and IFOBT were both 72.0% (95% CI: 68.2%-75.5%; χ^2 = 0.4, P > 0.05); positive likelihood ratios of those were 3.3 (95% CI: 2.8-3.9) and 3.4 (95% CI: 2.9-4.0), respectively. In precancerous lesions, sensitivities for Tf and IFOBT were 50% and 58%, respectively (χ^2 = 0.8, P > 0.05); specificities of Tf and IFOBT were 71.5% (95% CI: 67.6%-75.1%) and 72.2% (95% CI: 68.4%-75.8%); positive likelihood ratios of those were 1.8 (95% CI: 1.3-2.3) and 2.1 (95% CI: 1.6-2.7), respectively; compared to IFOBT, g-FOBT+ Tf+ IFOBT had a significantly higher positive rate for precancerous lesions (83% *vs* 58%, respectively; χ^2 = 9.1, P < 0.05). In patients with CRC and precancerous lesions, the sensitivities of Tf and IFOBT were 62% and 69% (χ^2 = 0.9, P > 0.05); specificities of those were 74.5% (95% CI: 70.6%-78.1%) and 75.5% (95% CI: 71.6%-79.0%); positive likelihood ratios of those were 2.5 (95% CI: 2.0-3.1) and 2.8 (95% CI: 2.3-3.5). Compared to IFOBT alone, combining g-FOBT, IFOBT and Tf led to significantly increased sensitivity for detecting CRC and cancerous lesions (69% *vs* 88%, respectively; χ^2 = 9.0, P < 0.05).

CONCLUSION: Tf dipstick test might be used as an additional tool for CRC and precancerous lesions screening in a high-risk cohort.

Key words: Transferrin; Immuno fecal occult blood test; Colorectal cancer; Precancerous lesions; Transferrin dipstick test

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INTRODUCTION

Colorectal cancer (CRC) is one of the major diseases threatening human health. In the United States, CRC is the third most frequently diagnosed cancer among men and women, and the third leading cause of cancer death[1]. In China, the prevalence of CRC has risen in recent years, possibly attributable to changes in the population's lifestyle and dietary habits[2,3]. In most cases, CRC is believed to arise within precancerous lesions that develop slowly over many years[4,5].

Currently, many tools are be used for CRC screening. CRC screening tests recommended by the American Cancer Society (ACS) can be grouped into 2 categories: (1) tests that primarily detect CRC, which include tests that look for blood, such as guaiac fecal occult blood test and fecal immunochemical test, or exfoliated DNA [single-strand DNA (sDNA)] in stools; and (2) tests that can detect cancer and advanced lesions, which include endoscopic and radiological exams, i.e., colonoscopy, double-contrast barium enema (DCBE), and computed tomography colonography (CTC) (or virtual colonoscopy)[6]. However, these tests all have certain limitations.

Several published randomized trials have showed that the most widely accepted test method, fecal occult blood test (FOBT), can reduce CRC incidence[7] and mortality rate[8]. However, guaiac fecal occult blood test (g-FOBT) has been criticized for its high false positive because it detects non-human haem in food[9,10]. Compared with that of g-FOBT, the sensitivity of immuno fecal occult blood test (IFOBT) is significantly higher[11-13]. IFOBT specifically detects human hemoglobin (Hb) in stool by antibody-antigen reaction, which has no restrictions on diet or drug intake. However, Hb is unstable in feces because it can be degraded by bacteria. Furthermore, Hb can not be used to detect lesions that are not accompanied by bleeding[14-17]. Fecal DNA test was developed based on the molecular genetics of CRC. It is suggested that the occurrence of most CRCs has close relationship with chromosomal instability, with mutations progressively accumulating in the *adenomatous polyposis coli* gene, the p53 tumor-suppressor gene, and the K-ras oncogene[18]. Despite relatively high specificity[19], Fecal DNA test has many problems[20], including the lack of adequate fecal DNA makers, complex extraction steps, and so on. Furthermore, population-based studies showing the capability of the method to decrease mortality of CRC have been lacking[21]. Other non-invasive methods include testing for faecal calprotectin, which has high sensitivity but low specificity[22].

DCBE is a preferred method for screening in children, old people and those who can not undergo colonoscopy. However, its false positive and false negative ratios are both higher than those of colonoscopy[15,23]. Colonoscopy can detect CRC in the entire colonic lumen and is the most sensitive and specific test. A report showed that the incidence and mortality of CRC rate were reduced to 67% and 65%, respectively, after colonoscopy screening in an average-risk cohort[24]. However, colonoscopy is invasive and has risks to certain extent[25]. High costs and painful procedure has prevented colonoscopy from being used as a method for large-scale screening of CRC. Practically it is only used for final diagnostic test of positive patients. In 2008, two additional tests have been added to CRC screening guidelines of the ACS[26]: sDNA and CTC. CTC is a minimally invasive method for examination of the whole colon. It is safe and the entire colon can be examined thoroughly. A recently study shows that for $\geqslant$ 10 mm colorectal lesions, the sensitivity of CTC is similar to that of colonoscopy. However, for < 10 mm and flat neoplasms, the sensitivity of CTC is lower than colonoscopy[27]. Additionally, CTC can not perform biopsy and is an expensive procedure. For these reasons, our study sought to develop a method to improve the sensitivities and specificities of CRC and precancerous lesions screening.

Transferrin (Tf), which is present in plasma by the release of neutrophil-specific granules, is undetectable in normal human gastrointestinal tract. Detection of Tf in feces or contents in the stomach indicates bleeding in gastrointestinal tract. Unlike hemoglobin, Tf is resistant to degradation by digestive enzymes and bacteria. Thus, compared to hemoglobin, Tf is more stable in feces[28]. It has been reported that fecal Tf is elevated in patients with colorectal tumor, compared to healthy individuals[29]. Recently, a number of proteomic studies showed that Tf could be used as a marker expressing in a number of cancers[30,31]. Saitoh *et al*[32] and Hirata *et al*[33] compared fecal Tf with IFOBT in clinical studies and found that Tf was as useful as IFOBT in diagnosing colorectal diseases. However, these two studies did not analyze patients with precancerous lesions. Sheng *et al*[34] compared fecal Tf with IFOBT for their sensitivities in detecting CRC and precancerous lesions in CRC patients. However, the subjects of this study were CRC patients, and specificity was not analyzed.

So far, Tf has not been recommended as a method for CRC screening by the ACS. Based on the above studies, we assumed that the sensitivity and specificity of Tf in detecting CRC and precancerous lesions were equal or superior to IFOBT. Using a combination of the three measurements (g-FOBT, Tf and IFOBT) appears to increase the sensitivity of diagnosis in high-risk population. In order to investigate whether Tf can be applied in the screening of CRC and precancerous lesions, we conducted this study to compare the effectiveness of Tf and IFOBT in the detection of colorectal cancer and precancerous lesions.

MATERIALS AND METHODS

Study materials

The stool specimen collection, colonoscopy and pathologic examination were performed in the Eighth Hospital of Wuhan City which is a hospital specializing in anorectal diseases. G-FOBT, IFOBT and Tf kits were purchased from Baso Diagnostics Inc and WHPM Inc.

Study group

From January 2010 to September 2010, 861 subjects at high-risk (a personal history of curative-intent resection of CRC or intestinal polyps; family history of colorectal cancer; having the following two or more: chronic diarrhea, chronic constipation, abdominal pain, dark stool, blood or mucus on stool) were recruited. The inclusion criteria were as following: age over 14 years, male or female. Subjects with age < 14 years were excluded. All participants provided written informed consent and were instructed on diet and drug restrictions three days before and during the period of stool collection.

Fecal samples collection and IFOBT and Tf analysis

All fecal samples were collected the day before colonoscopy and processed in accordance with manufacturer' s instructions. We applied the fecal sample on the strip and the result was read out within 5 min (the result was invalid after 5 min). A red bar in control area (C) only was considered as negative. A red bar in both the testing area (T) and the control area (C), was considered as positive. If there was no red bar in the control area(C), the test was considered invalid. Tf, IFOBT and g-FOBT were performed simultaneously on the same stool. To minimize false-negative cases, all subjects with negative samples were asked to provide an additional stool specimen for a second test; if the second test still gave negative result, a third test would be conducted. As long as one of the three tests showed positive results, the subject was considered to have a positive sample. If the results were all negative after testing three repeated samples, the subject was considered a true negative. Approximately 10% of the samples were repeated and the concordance was 100%.

Statistical analysis

The positive rate of Tf alone, IFOBT alone, Tf combined with IFOBT (Tf + IFOBT), Tf and IFOBT combined with g-FOBT (Tf + IFOBT + g-FOBT), as well as their respective specificity, likelihood ratio, odd ratio and 95% confidence interval were calculated to compared the sensitivity of Tf, IFOBT, Tf+ IFOBT and Tf + IFOBT + g-FOBT in detecting CRC and precancerous lesions. χ^2 and McNemar' s test were conducted to determine the significance of difference. $P < 0.05$ in a two-tailed test was considered statistically significant. Analyses were performed using SPSS version 17.0.

RESULTS

Subject enrollment flow is described in Figure 1. Of the 861 participants in this study, 250 subjects who have taken neither FOBTs nor colonoscopy, or have taken only one of the tests were excluded in this survey. Six hundred and eleven subsequently received both FOBTs and colonoscopy with biopsy performed as needed. Among them, 286 were found to have abnormalities by colonoscopy, while 447 were classified as low risk population including no abnormalities (325 cases) and benign lesions (122 cases). Benign lesions included chronic enteritis, chronic schistosomiasis bowel disease, intestinal diverticula, colorectal erosive inflammation (a total of 112 cases) and inflammatory intestinal mucosa by biopsy (10 cases). One hundred and seventy-four subjects were found to have polyps or neoplasm. Pathological examination showed CRC (25 cases), precancerous lesions (60 cases), inflammatory intestinal mucosa (10 cases); Polyps (79 cases) that were less than 3 mm in diameter, broad-based, sessile and flat were not subjected to biopsy. Precancerous lesions included tubular adenoma, villous adenoma, tubular villous adenoma and hyperplastic polyp with moderate-severe dysplasia (with histological confirmation).

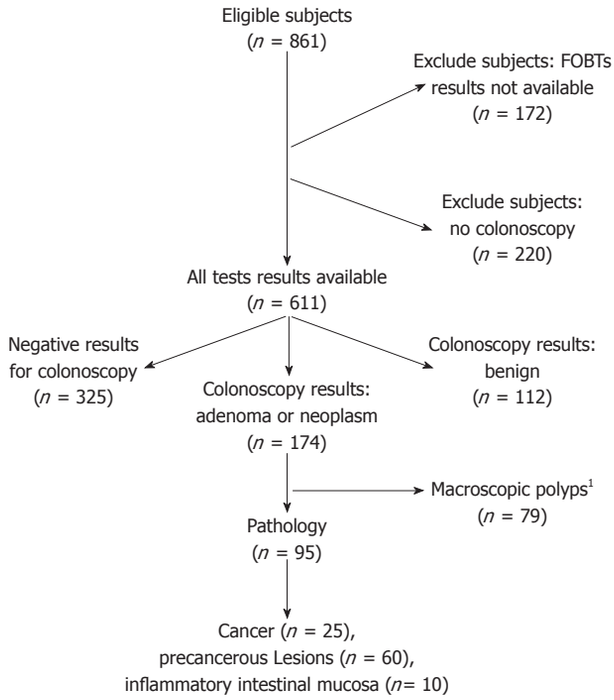

Figure 1 Flow diagram of the study. [1]Includes polyps that were less than 3 mm in diameter, broadbased, sessile and fat.

The overall demographic information of 611 subjects (Table 1). There were 310 men and 301 women among the participants, with a median age of 50 years (range 14-85 years). Among them, 10 men and 15 women had CRC, with a median age of 62 years; 35 men and 25 women had precancerous lesions, with a median age of 56 years.

The positive rate of g-FOBT, Tf, Tf+ IFOBT, and g-FOBT+ Tf+ IFOBT in fecal samples from five groups of participants is shown in Table 2. In CRC, the positive rates of Tf and IFOBT were 92% and 96%, respectively ($\chi^2 = 0.4$, $P > 0.05$). In precancerous lesions, the positive

Table 1 Patient demographics

Characteristic	No. of participants	Colorectal cancer	Precancerous lesions	Polyp	Abnormality	Low risk
Total	611	25	60	79	286	447
Sex						
Male	310	10	35	49	152	216
Female	301	15	25	30	134	231
Age, yr						
Median	50	62	56	53	53	48
Range	14-85	39-85	24-84	25-84	17-85	14-81

rates for Tf and IFOBT were 50% and 58%, respectively ($\chi^2 = 0.8$, $P > 0.05$); compared to IFOBT, g-FOBT+ Tf+ IFOBT had a significantly higher positive rate for precancerous lesions (83% *vs* 58%, respectively; $\chi^2 = 9.1$, $P < 0.05$). In CRC and precancerous lesions, the positive rates for Tf and IFOBT were 62% and 69% ($\chi^2 = 0.9$, $P > 0.05$), whereas g-FOBT+ Tf+ IFOBT also provided significantly higher positive rate compared to IFOBT alone (88% *vs* 69%, respectively; $\chi^2 = 9.0$, $P < 0.05$). For Tf alone, a difference in positive rate was observed for detecting CRC and precancerous lesions (92% *vs* 50%, respectively; $\chi^2 = 13.3$, $P < 0.05$).

The performance characteristics of various tests examined by our study (Table 3). For detecting CRC, The specificities of Tf and IFOBT were both 72.0% (95% CI: 68.2%-75.5%); positive likelihood ratios of those were 3.3 (95% CI: 2.8 - 3.9) and 3.4 (95% CI: 2.9-4.0), respectively. For detecting precancerous lesions, specificities of Tf and IFOBT were 71.5% (95% CI: 67.6%-75.1%) and 72.2% (95% CI: 68.4%- 75.8%); positive likelihood ratios of those were 1.8 (95% CI: 1.3-2.3) and 2.1 (95% CI: 1.6-2.7), respectively. For detecting both CRC and precancerous lesions, specificities of Tf and IFOBT were 74.5% (95% CI: 70.6%-78.1%) and 75.5% (95% CI: 71.6%-79.0%); positive likelihood ratios of those were 2.5 (95% CI: 2.0-3.1) and 2.8 (95% CI: 2.3-3.5), respectively. In these tests, the specificity of Tf and IFOBT for detecting CRC was the same. Likelihood ratio can accurately reflect how likely it is that patients with CRC will test positive. The likelihood ratio showed that Tf and IFOBT detected CRC (3.3 and 3.4, respectively) more effectively than they detected precancerous lesions (1.8 and 2.1, respectively).

DISCUSSION

The data from our study demonstrated that the sensitivities and specificities of Tf and IFOBT were similar in the detection of colorectal cancer and precancerous lesions in high-risk cohort. These results suggest that when using Tf alone, the sensitivity and specificity have no visible difference compared to using IFOBT alone; when combining these three methods, the sensitivity can be enhanced.

There had been several comparative studies of Tf and IFOBT previously. Saitoh *et al*[32] found that the sensitivities of Tf and IFOBT for detecting CRC were similar (53.8% and 61.5%, respectively). The study used enzyme-linked immunosorbent assay (ELISA) kit for fecal Tf and Latex agglutination for IFOBT. Hirata *et al*[33] found that the sensitivities of Tf and IFOBT were both 50%, whereas combining both methods gave a slightly higher sensitivity of 61.1%. The study measured the Tf and Hb quantitatively by sandwich ELISA. Both studies analyzed the sensitivity for detecting colorectal diseases (colon cancer, colorectal polyps, ulcerative colitis, Crohn's disease, *etc*.) but not precancerous lesions. In addition, in both previous studies, each patient was tested only once with one stool specimen. In contrast, our study strove to minimize false negative results by testing up to three stool specimens from a single patient, hence achieving a more accurate estimation of sensitivity. Sheng *et al*[34] found that the positive ratio of Tf and IFOBT for detecting colorectal cancer were 80% and 75%, respectively. For detecting precancerous lesions, the positive ratios were 72% (Tf) and 44% (IFOBT). The difference is statistically significant. Combining the two methods gave a positive ratio of 78% in detecting precancerous lesions. Three possible reasons might explain the differences between Sheng *et al*[34] and our study. First, the tested subjects were different. The previous study tested CRC patients. Our study tested those who are at high-risk. Second, the sample size was different. Our study had 611 samples, compared to 110 in the previous study. Third, the design of the studies was different. The previous study took only one stool specimen from an individual patient and retested the sample if the result was negative. We took at least one specimen from every participant and up to three specimens from those showing negative results. None of the three previous studies analyzed the specificities of colorectal cancer and precancerous lesions detection. The difference in specificity may be caused by variation in other factors, such as degradation of hemoglobin, samples, experiment and the quality of reagents, *etc*.

The study shows that Tf and IFOBT both have false positive and false negative results in colorectal cancer and precancerous lesions screening. IFOBT specifically detects the Hb in stool by antibody-antigen reaction. Anti-Hb antibody do not react with animal blood, fruits and vegetables in the testing material, and do not confer peroxidase activity, which obviously reduce the false positive rate. However, the test has several problems, including (1) some participants' hemoglobin may not be recognized by the anti-Hb antibody used in the test; (2) hemoglobin can be degraded by bacteria, resulting in the loss of antigen; (3) the symptom of bleeding in early colorectal lesions is intermittent; and (4) the massive bleeding causes an excessive amount of antigen to be present in the reaction system and hence the "pre-band phenomenon". These are all possible causes of false negative results in the detection using IFOTB. Tf, a type of β1 globulin with a molecular weight of 77 KD, transports extracellular iron into cells through membrane receptor-mediated endocytosis[35]. Tf can resist degradation caused by digestive enzymes and bacteria, and is more stable than hemoglobin in stool. But Tf can only be detected at a concentration

Table 2 Positive rate of three fecal occult blood tests in fecal samples from colorectal cancer patients, precancerous lesions subjects, polyp subjects, abnormality subjects and low risk subjects *n* (%)

Disease (N)	g-FOBT		Tf		IFOBT		Tf+ IFOBT		g-FOBT+ Tf+ IFOBT	
	+[1]	-[1]	+[1]	-[1]	+[1]	-[1]	+[1]	-[1]	+[1]	-[1]
CRC (25)	25 (100)	0 (0)	23 (92)	2 (8)	24 (96)	1 (4)	25 (100)	0 (0)	25 (100)	0 (0)
Precancerous lesions (60)	36 (60)	24 (40)	30 (50)	30 (50)	35 (58)	25 (42)	40 (67)	20 (33)	50 (83)	10 (17)
Polyp (79)	35 (44)	44 (56)	29 (37)	50 (63)	20 (25)	59 (75)	34 (43)	45 (57)	49 (62)	30 (38)
Abnormality (286)	153 (53)	133 (47)	126 (44)	160 (56)	128 (45)	158 (55)	162 (57)	124 (43)	203 (71)	83 (29)
Low risk (447)	148 (33)	299 (67)	105 (23)	342 (77)	109 (24)	338 (76)	154 (34)	293 (66)	221 (49)	226 (51)

[1]*n* (*n*/N × 100%). Tf: Transferrin; IFOBT: Immuno fecal occult blood test; g-FOBT: Guaiac-fecal occult blood test; CRC: Colorectal cancer.

Table 3 Sensitivity, specificity, positive likelihood ratio and odd ratio of three fecal occult blood tests for detection of colorectal cancer, precancerous lesions and colorectal cancer + precancerous lesions

Test	No. of neoplasms detected	Sensitivity%		Specificity%		Likely ratio(+) (95% CI)	Odd ratio (95% CI)
		n/total	% (95% CI)	*n*/total	% (95% CI)		
CRC							
g-FOBT	25	25/25	100 (86.7-100)	367/586	62.6 (58.6-66.5)	2.7 (2.4-3.0)	-
Tf	25	23/25	92 (75.0-97.8)	422/586	72 (68.2-75.5)	3.3 (2.8-3.9)	29.6 (6.8-126.9)
IFOBT	25	24/25	96 (80.5-99.3)	422/586	72 (68.2-75.5)	3.4 (2.9-4.0)	61.8 (8.3-460.2)
Tf+ IFOBT	25	25/25	100 (86.7-100)	358/586	61.1 (57.1-65.0)	2.6 (2.3-2.8)	-
g-FOBT+ Tf+ IFOBT	25	25/25	100 (86.7-100)	266/586	45.4 (41.4-49.4)	1.8 (1.7-2.0)	-
Precancerous lesions							
g-FOBT	60	36/60	60 (47.4-71.4)	343/551	62.3 (58.1-66.2)	1.6 (1.3-2.0)	2.5 (1.4-4.3)
Tf	60	30/60	50 (37.7-62.3)	394/551	71.5 (67.6-75.1)	1.8 (1.3-2.3)	2.5 (1.5-4.3)
IFOBT	60	35/60	58.3 (45.7-70.0)	398/551	72.2 (68.4-75.8)	2.1 (1.6-2.7)	3.6 (2.1-6.3)
Tf+ IFOBT	60	40/60	66.7 (54.1-65.3)	338/551	61.3 (57.2-65.3)	1.7 (1.4-2.1)	3.2 (1.8-5.6)
g-FOBT+ Tf+ IFOBT	60	50/60	83.3 (72.0-90.7)	256/551	46.5 (42.3-50.6)	1.6 (1.4-1.8)	4.3 (2.2-8.7)
CRC+ precancerous lesions							
g-FOBT	85	61/85	71.8 (61.4-80.2)	343/526	65.2 (61.0-69.2)	2.1 (1.7-2.5)	4.8 (2.9-7.9)
Tf	85	53/85	62.4 (51.7-72.0)	392/526	74.5 (70.6-78.1)	2.5 (2.0 -3.1)	4.8 (3.0-7.8)
IFOBT	85	59/85	69.4 (59.0-78.2)	397/526	75.5 (71.6-79.0)	2.8 (2.3-3.5)	7.0 (4.2-11.5)
Tf+ IFOBT	85	65/85	76.5 (66.4-84.2)	338/526	64.3 (60.1-68.2)	2.1 (1.8-2.5)	5.8 (3.4-9.9)
g-FOBT+ Tf+ IFOBT	85	75/85	88.2 (79.7-93.5)	256/526	48.7 (44.4-53.0)	1.7 (1.5-1.9)	7.1 (3.6-14.1)

Tf: Transferrin; IFOBT: Immuno fecal occult blood test; g-FOBT: Guaiac-fecal occult blood test; CRC: Colorectal cancer; CI: Confidence interval.

greater than 10 ng/mL. The ratio of hemoglobin and Tf is 5.4:1 in specimens containing blood. Thus, if the subject has low level of Tf, or the bleeding is very trivial, the testing threshold can not be reached and false negative results will be the outcome. Our study tested the stool specimen repeatedly, therefore reduced the error rate. All subjects underwent standard colonoscopic examination with biopsy performed as needed. In this way, an accurate test was performed to examine the sensitivities and the specificities of the three methods.

The results of this study have a significant implication for CRC screening. A number of studies showed that early detection based on fecal occult blood test helped decrease CRC mortality by 15%-25%[4,7,36]. Mandel *et al*[7,37] found that screening once every year or once every two years with g-FOBT or IFOTB can decrease the mortality of CRC and CRC related diseases, compared to no screening. In our test, for 65 subjects, IFOBT showed negative result while Tf were positive. Hence, Tf is appropriate for the screening of CRC and precancerous lesions. Positive likelihood ratio, which involves both sensitivity and specificity of screening, can fully evaluate the diagnosing value of screening. It is very stable and not subject to morbidity. Results of our study demonstrated that the positive likelihood ratio of Tf detecting CRC was similar with that of IFOBT in various populations, which indicates that Tf has a similar value with IFOBT and is fit for the CRC screening in an average- risk population. Further, the findings of the analysis suggest that a combination of Tf, IFOBT and g-FOBT enables compensation of the inadequacy of single tests, which will reduce false negative rate and improve the positive ratio. So, in order to enhance the sensitivities of detecting CRC and precancerous lesions, all three methods should be used simultaneously.

Our study does have some limitations, and the first is its study subject. The sensitivity and specificity of Tf had been calculated in this study, those of that in an average-risk group are yet to be further determined. Prospective studies in an average-risk group are needed to validate these results. Nevertheless, hardly everyone at average-risk group can undergo colonoscopy, leading that the specificities of fecal occult blood tests can not be evaluated. We prepare to apply computed tomographic virtual

colonoscopy to screen patients who are at average-risk for CRC. The second is the range of age in this study is very wide. The third major limitation of our study is that the three stool occult blood tests were all qualitative and certain amount of deviation was existed compared to quantitative test.

In conclusion, Tf dipstick test can be applied to screen for CRC and precancerous lesions and the efficacy is approximately the same as that of IFOBT in high risk cohort. By combining g-FOBT, Tf and IFOBT, the sensitivity can be improved significantly while the specificity is sacrificed. Large-scale and prospective clinical studies will be needed to determine whether Tf dipstick test can be used as a screening method for CRC and precancerous lesions in different screening population.

COMMENTS

Background

Fecal occult blood test (FOBT) is a simple and convenient tool for colorectal cancer (CRC) screening. Immuno fecal occult blood test (IFOBT) has limited sensitivities and specificities for detecting CRC and precancerous lesions.

Research frontiers

FOBT, a non-invasive method, can reduce CRC incidence and mortality rate. However, hemoglobin (Hb) is unstable in feces because it can be degraded by bacteria. Furthermore, Hb can not be used to detect lesions that are not accompanied by bleeding. Transferrin (Tf), which is present in plasma by the release of neutrophil-specific granules, is undetectable in normal human gastrointestinal tract. Tf can resist degradation caused by digestive enzymes and bacteria, and is more stable than hemoglobin in stool.

Innovations and breakthroughs

Tf dipstick test was found to be as sensitive and specific as IFOBT in the detection of CRC and precancerous lesions in high-risk cohort. Combining guaiac fecal occult blood test, IFOBT and Tf enhanced the sensitivity.

Applications

Tf dipstick test can be applied to screen for CRC and precancerous lesions and the efficacy is approximately the same as that of IFOBT in high risk cohort.

Terminology

Transferin (Tf), a type of β1 globulin with a molecular weight of 77 KD, transports extracellular iron into cells through membrane receptor-mediated endocytosis. Detection of Tf in feces or contents in the stomach indicates bleeding in gastrointestinal tract.

Peer review

The study seeks to evaluate biomarkers for colorectal cancer screening. To develop non-invasive method such as fecal test for cancer screening is clinically relevant. The study has tested a reasonable size of cohorts and found combined test of several markers let to significantly increased sensitivity of detecting cancerous lesions compared to the commonly used method. The results suggest that the transferrin dipstick test might be used as an additional tool for colorectal cancer and precancerous lesions screening in a high-risk cohort.

REFERENCES

1 **Jemal A**, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin* 2009; **59**: 225-249

2 **Wei YS**, Lu JC, Wang L, Lan P, Zhao HJ, Pan ZZ, Huang J, Wang JP. Risk factors for sporadic colorectal cancer in southern Chinese. *World J Gastroenterol* 2009; **15**: 2526-2530

3 **Lei T**, Mao WM, Yang HJ, Chen XZ, Lei TH, Wang X, Ying Q, Chen WQ, Zhang SW. [Study on cancer incidence through the Cancer Registry Program in 11 Cities and Counties, China.]. *Zhonghua Liuxingbingxue Zazhi* 2009; **30**: 1165-1170

4 **Hewitson P**, Glasziou P, Watson E, Towler B, Irwig L. Cochrane systematic review of colorectal cancer screening using the fecal occult blood test (hemoccult): an update. *Am J Gastroenterol* 2008; **103**: 1541-1549

5 **Logan RF**. Review: faecal occult blood test screening reduces risk of colorectal cancer mortality. *Evid Based Med* 2009; **14**: 15

6 **Smith RA**, Cokkinides V, Brooks D, Saslow D, Brawley OW. Cancer screening in the United States, 2010: a review of current American Cancer Society guidelines and issues in cancer screening. *CA Cancer J Clin* 2010; **60**: 99-119

7 **Mandel JS**, Church TR, Bond JH, Ederer F, Geisser MS, Mongin SJ, Snover DC, Schuman LM. The effect of fecal occult-blood screening on the incidence of colorectal cancer. *N Engl J Med* 2000; **343**: 1603-1607

8 **Labianca R**, Beretta GD, Kildani B, Milesi L, Merlin F, Mosconi S, Pessi MA, Prochilo T, Quadri A, Gatta G, de Braud F, Wils J. Colon cancer. *Crit Rev Oncol Hematol* 2010; **74**: 106-133

9 **Levin B**, Brooks D, Smith RA, Stone A. Emerging technologies in screening for colorectal cancer: CT colonography, immunochemical fecal occult blood tests, and stool screening using molecular markers. *CA Cancer J Clin* 2003; **53**: 44-55

10 **Collins JF**, Lieberman DA, Durbin TE, Weiss DG. Accuracy of screening for fecal occult blood on a single stool sample obtained by digital rectal examination: a comparison with recommended sampling practice. *Ann Intern Med* 2005; **142**: 81-85

11 **Allison JE**, Sakoda LC, Levin TR, Tucker JP, Tekawa IS, Cuff T, Pauly MP, Shlager L, Palitz AM, Zhao WK, Schwartz JS, Ransohoff DF, Selby JV. Screening for colorectal neoplasms with new fecal occult blood tests: update on performance characteristics. *J Natl Cancer Inst* 2007; **99**: 1462-1470

12 **Oort FA**, Terhaar Sive Droste JS, Van Der Hulst RW, Van Heukelem HA, Loffeld RJ, Wesdorp IC, Van Wanrooij RL, De Baaij L, Mutsaers ER, van der Reijt S, Coupe VM, Berkhof J, Bouman AA, Meijer GA, Mulder CJ. Colonoscopy-controlled intra-individual comparisons to screen relevant neoplasia: faecal immunochemical test vs. guaiac-based faecal occult blood test. *Aliment Pharmacol Ther* 2010; **31**: 432-439

13 **Parra-Blanco A**, Gimeno-García AZ, Quintero E, Nicolás D, Moreno SG, Jiménez A, Hernández-Guerra M, Carrillo-Palau M, Eishi Y, López-Bastida J. Diagnostic accuracy of immunochemical versus guaiac faecal occult blood tests for colorectal cancer screening. *J Gastroenterol* 2010; **45**: 703-712

14 **Young GP**, Cole S. New stool screening tests for colorectal cancer. *Digestion* 2007; **76**: 26-33

15 **Kronborg O**, Regula J. Population screening for colorectal cancer: advantages and drawbacks. *Dig Dis* 2007; **25**: 270-273

16 **Uchida K**, Matsuse R, Miyachi N, Okuda S, Tomita S, Miyoshi H, Hirata I, Tsumoto S, Ohshiba S. Immunochemical detection of human blood in feces. *Clin Chim Acta* 1990; **189**: 267-274

17 **Burton RM**, Landreth KS, Barrows GH, Jarrett DD, Songster CL. Appearance, properties, and origin of altered human hemoglobin in feces. *Lab Invest* 1976; **35**: 111-115

18 **Lengauer C**, Kinzler KW, Vogelstein B. Genetic instability in colorectal cancers. *Nature* 1997; **386**: 623-627

19 **Tagore KS**, Lawson MJ, Yucaitis JA, Gage R, Orr T, Shuber AP, Ross ME. Sensitivity and specificity of a stool DNA multitarget assay panel for the detection of advanced colorectal neoplasia. *Clin Colorectal Cancer* 2003; **3**: 47-53

20 **Woolf SH**. A smarter strategy? Reflections on fecal DNA screening for colorectal cancer. *N Engl J Med* 2004; **351**: 2755-2758

21 **Hakama M**, Coleman MP, Alexe DM, Auvinen A. Cancer screening: evidence and practice in Europe 2008. *Eur J Cancer* 2008; **44**: 1404-1413

22 **Hoff G**, Grotmol T, Thiis-Evensen E, Bretthauer M, Gondal G, Vatn MH. Testing for faecal calprotectin (PhiCal) in the Norwegian Colorectal Cancer Prevention trial on flexible sigmoidoscopy screening: comparison with an immunochemical test for occult blood (FlexSure OBT). *Gut* 2004; **53**: 1329-1333

23 **McDonald S**, Lyall P, Israel L, Coates R, Frizelle F. Why

barium enemas fail to identify colorectal cancers. *ANZ J Surg* 2001; **71**: 631-633
24 **Kahi CJ**, Imperiale TF, Juliar BE, Rex DK. Effect of screening colonoscopy on colorectal cancer incidence and mortality. *Clin Gastroenterol Hepatol* 2009; **7**: 770-775; quiz 711
25 **Rabeneck L**, Paszat LF, Hilsden RJ, Saskin R, Leddin D, Grunfeld E, Wai E, Goldwasser M, Sutradhar R, Stukel TA. Bleeding and perforation after outpatient colonoscopy and their risk factors in usual clinical practice. *Gastroenterology* 2008; **135**: 1899-1906, 1906.e1
26 **Smith RA**, Cokkinides V, Brawley OW. Cancer screening in the United States, 2008: a review of current American Cancer Society guidelines and cancer screening issues. *CA Cancer J Clin* 2008; **58:** 161-179
27 **Pox CP**, Schmiegel W. Role of CT colonography in colorectal cancer screening: risks and benefits. *Gut* 2010; **59**: 692-700
28 **Chiang CH**, Jeng JE, Wang WM, Jheng BH, Hsu WT, Chen BH. A comparative study of three fecal occult blood tests in upper gastrointestinal bleeding. *Kaohsiung J Med Sci* 2006; **22**: 223-228
29 **Sugi K**, Saitoh O, Hirata I, Katsu K. Fecal lactoferrin as a marker for disease activity in inflammatory bowel disease: comparison with other neutrophil-derived proteins. *Am J Gastroenterol* 1996; **91**: 927-934
30 **Ward DG**, Suggett N, Cheng Y, Wei W, Johnson H, Billingham LJ, Ismail T, Wakelam MJ, Johnson PJ, Martin A. Identification of serum biomarkers for colon cancer by proteomic analysis. *Br J Cancer* 2006; **94**: 1898-1905
31 **Ahmed N**, Oliva KT, Barker G, Hoffmann P, Reeve S, Smith IA, Quinn MA, Rice GE. Proteomic tracking of serum protein isoforms as screening biomarkers of ovarian cancer. *Proteomics* 2005; **5**: 4625-4636
32 **Saitoh O**, Kojima K, Kayazawa M, Sugi K, Tanaka S, Nakagawa K, Teranishi T, Matsuse R, Uchida K, Morikawa H, Hirata I, Katsu K. Comparison of tests for fecal lactoferrin and fecal occult blood for colorectal diseases: a prospective pilot study. *Intern Med* 2000; **39**: 778-782
33 **Hirata I**, Hoshimoto M, Saito O, Kayazawa M, Nishikawa T, Murano M, Toshina K, Wang FY, Matsuse R. Usefulness of fecal lactoferrin and hemoglobin in diagnosis of colorectal diseases. *World J Gastroenterol* 2007; **13**: 1569-1574
34 **Sheng JQ**, Li SR, Wu ZT, Xia CH, Wu X, Chen J, Rao J. Transferrin dipstick as a potential novel test for colon cancer screening: a comparative study with immuno fecal occult blood test. *Cancer Epidemiol Biomarkers Prev* 2009; **18**: 2182-2185
35 **Lönnerdal B**, Iyer S. Lactoferrin: molecular structure and biological function. *Annu Rev Nutr* 1995; **15**: 93-110
36 **Chew MH**, Suzanah N, Ho KS, Lim JF, Ooi BS, Tang CL, Eu KW. Colorectal cancer mass screening event utilising quantitative faecal occult blood test. *Singapore Med J* 2009; **50**: 348-353
37 **Mandel JS**, Church TR, Ederer F, Bond JH. Colorectal cancer mortality: effectiveness of biennial screening for fecal occult blood. *J Natl Cancer Inst* 1999; **91**: 434-437

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BRIEF ARTICLE

Health-related quality of life evaluated by tumor node metastasis staging system in patients with hepatocellular carcinoma

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Abstract

AIM: To investigate and evaluate the change in health-related quality of life (HRQoL) by tumor node metastasis (TNM) staging system in patients with hepatocellular carcinoma (HCC).

METHODS: A total of 140 patients diagnosed with HCC between June 2008 and April 2009 in our department were enrolled to this study. One hundred and thirty-five (96.5%) patients had liver cirrhosis secondary to hepatitis B virus (HBV) infection, 73 (54.07%) of them being HBV DNA positive; the other etiologies of liver cirrhosis were alcoholic liver disease (1.4%), hepatitis C (1.4%) or cryptogenic (0.7%). All subjects were fully aware of their diagnosis and provided informed consent. HRQoL was assessed before treatment using the functional assessment of cancer therapy-hepatobiliary (FACT-Hep) questionnaire. Descriptive statistics were used to evaluate demographics and disease-specific characteristics of the patients. One-way analysis of variance and independent samples *t* tests were used to compare the overall FACT-Hep scores and clinically distinct TNM stages. Scores for all FACT-Hep items were analyzed by frequency analyses. The mean scores obtained from the FACT-Hep in different Child-Pugh classes were also evaluated.

RESULTS: The mean FACT-Hep scores were reduced significantly from TNM Stage Ⅰ to Stage Ⅱ, Stage ⅢA, Stage ⅢB group (687 ± 39.69 *vs* 547 ± 42.57 *vs* 387 ± 51.24 *vs* 177 ± 71.44, $P = 0.001$). Regarding the physical and emotional well-being subscales, scores decreased gradually from Stage Ⅰ to Stage ⅢB ($P = 0.002$ *vs* Stage Ⅰ; $P = 0.032$ *vs* Stage Ⅱ; $P = 0.033$ *vs* Stage ⅢA). Mean FACT-Hep scores varied by Child-Pugh class, especially in the subscales of physical well-being, functional well-being and the hepatobiliary cancer ($P = 0.001$ *vs* Stage I; $P = 0.036$ *vs* Stage Ⅱ; $P = 0.032$ *vs* Stage ⅢA). For the social and family well-being subscale, only Stage ⅢB scores were significantly lower as compared with Stage Ⅰ scores ($P = 0.035$). For the subscales of functional well-being and hepatobiliary cancer, there were significant differences for Stages ⅡI, ⅢA and ⅢB ($P = 0.002$ *vs* Stage Ⅰ).

CONCLUSION: HRQoL of patients with HCC worsens gradually with progression of TNM stages. The most impaired subscales of HRQoL, as measured by FACT-Hep, were physical and emotional well-being.

Key words: Hepatocellular carcinoma; Tumor node metastasis staging; Functional assessment of cancer therapy-hepatobiliary; Health-related quality of life; Cross-sectional study

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INTRODUCTION

In recent years, there has been increased interest in quality of life as it pertains to patients' health status. Many different questionnaires, such as the functional assessment of cancer therapy-hepatobiliary (FACT-Hep), the Health-Related Quality of Life (HRQoL), and the Short Form (36) Health Survey are becoming key instruments in the evaluation of patients' health status. HRQoL results may be more relevant than length of life, as patients are frequently more concerned about life quality than longevity.

The incidence of hepatocellular carcinoma (HCC) in China is increasing. HCC is now the second leading cause of death of cancers[1]; about 15%-40% of the hepatitis B virus carriers may develop cirrhosis and HCC[2]. Approximately 80% of the patients diagnosed with HCC are unable to undergo surgical resection or transplantation[3]. Non-surgical treatment, such as transcatheter chemo-embolization or chemotherapy may improve the patients' prognosis to varying degrees[4-8]. Symptoms can be extremely variable in advanced HCC; the compensated patients may be symptomatic for months or decades. The impact is significant on patients' functioning and well-being. Patients may experience anxiety because of emotional concerns associated with the disease and treatment. Complications and extra-hepatic manifestations of advanced disease may reduce the quality of life, as therapeutic interventions may restrain outdoor activities. These challenges may negatively affect the quality of life, including physical, emotional, and functional well-beings[9].

Assessment of HRQoL with cancer has become an important outcome indicator during the last two decades[10]. The FACT-Hep is the most widely used evaluation tool focusing on hepatobiliary cancer such as HCC, pancreatic cancer and cancers of the gallbladder[11]. In clinical studies, the FACT-Hep performs well in assessing the quality of life of patients with HCC, and is considered to be of utmost importance for improving survival rates and quality of life[11].

The FACT-Hep[12] is a new and important index for evaluation of prognosis of the patients and the results of clinical trials, complementing the traditional end-points assessment, such as tumor response rate and survival time[13-15].

As HCC worsens, quality of life generally decreases, but two unanswered questions remain. First, FACT-Hep has not been used to assess prognosis with a large series of HCC patients at different tumor node metastasis (TNM) stages. Second, quality of life is a broad concept, and no data are available to examine the impact of disease on patients' self-perception and the factors which are associated with poor HRQoL on the FACT-Hep.

The aim of this study was to determine the HRQoL of patients at different TNM tumor stages of HCC by FACT-Hep, and to determine the factors associated with impaired HRQoL. The TNM Classification of Malignant Tumors was based on physical exam findings, imaging studies (ultrasound, computed tomography or magnetic resonance imaging) and other tests[16]. For solid tumors, such as HCC, TNM is the most commonly used staging system.

MATERIALS AND METHODS

Patients

Most patients admitted to our department had primary liver cancers. On admission, patients' quality of life was evaluated using the Chinese version of the FACT-Hep. During an 11-mo period from June 2008 to April 2009, we studied 140 patients with HCC (133 males, 7 females; aged 28-75 years). Patients completed the study questionnaire following the diagnosis of HCC, and prior to therapeutic intervention. All subjects were fully aware of their diagnosis and provided informed consent to participate. The ethical committees of the participating centers approved the study. The TNM staging system[17] (edition 6 published in 2002; and instituted in 2003) was used in this study. Patients were selected based on the demographic characteristics or clinical status.

Inclusion criteria

(1) Patients aged ⩾ 18 years; (2) diagnosis of HCC was established by imaging examinations (ultrasound or computed tomography) and confirmed by α1-variable fetoprotein levels exceeding 10 times the normal values, or liver biopsy; (3) patients who had no prior history of malignancy with encephalopathy and no cognitive impairment (as judged by the attending clinician); (4) patients who should speak, read, understand and write Chinese; (5) Karnofsky ⩾ 60. The expected survival was at least six months; (6) patients who voluntarily agreed and were able to make the decision to participate in the study; and (7) patients who provided written informed consent.

Exclusion criteria

(1) Illiteracy; (2) current psychosis or homicidal ideation; (3) serious visual or auditory disease; (4) evidence of cognitive impairment or psychiatric disturbance that would prevent informed consent; (5) physical condition too poor to complete the required the 20-25 min of questionnaires; and (6) patient's family requests that the patient's condition should be kept a secret from the patients.

FACT-Hep

The FACT-Hep is a 45-item, self-report instrument designed to measure HRQoL in patients with HCC. The FACT-Hep consists of 27-item FACT-General (FACT-G), which assesses generic HRQoL concerns using five subscales, and the newly validated 18-item hepatobiliary subscale, which assesses specific symptoms of hepatobiliary

Table 1 Patient characteristics *n* (%)

Factors		*P* value
Age (yr), median (range)	52 (28–75)	0.3509
Sex		< 0.001
Male	133 (95.0)	
Female	7 (5.0)	
Level of education		< 0.0001
Primary school	20 (14.0)	
Secondary school	56 (40.0)	
Commercial or vocational school	64 (46.0)	
Tumor, node, metastasis stage		
Ⅰ	49 (35.0)	0.0023
Ⅱ	35 (25.0)	
ⅢA	29 (20.7)	
ⅢB	27(19.3)	
ⅢC	0	
Ⅳ	0	
Etiology		
Hepatitis B	135 (96.5)	
Hepatitis C	2 (1.4)	
Alcoholic	2 (1.4)	
Cryptogenic	1 (0.7)	
Child-Pugh class		
A	84 (60.0)	
B	29 (20.7)	
C	27 (19.3)	

cancer and side effects of treatment. The five FACT-Hep subscales are: (1) physical well-being (PWB, 7 questions); (2) social and family well-being (SFWB, 7 questions); (3) emotional well-being (EWB, 6 questions); and (4) functional well-being (FWB, 7 questions); and (5) hepatobiliary cancer subscale (HepCS, 20 questions).

The FACT-Hep shows a high internal consistency at initial assessment (Cronbach's alpha range: 0.72-0.94) and retesting (Cronbach's alpha range: 0.81-0.94)[11]. Measurement stability is also high for all aggregated scales (test-retest correlation range: 0.84-0.91; interclass correlation coefficient range: 0.82-0.90)[11]. The FACT-Hep can be used independently as a brief measure of disease-related symptoms and functioning in assessing HRQoL of patients with HCC[11]. In this study, the FACT-Hep was translated into Chinese and adjusted appropriately based on the local cultural background. Early research using the Chinese version of the FACT-Hep showed a better reliability and validity (a reliability > 0.5 and a validity > 0.73)[11]. The present study aimed to assess the relationship between the HRQoL in HCC patients as measured by the Chinese version of the FACT-Hep and TNM tumor stage.

All FACT items were rated on 5-point scales ranging from 1 to 5. Converse items should be unified before analysis. The PWB, FWB, SFWB and EWB were summed to get the FACT-G total score. The FACT-G and HepCS scores were summed to obtain the FACT-Hep total score. Higher scores on all subscales of the FACT-Hep reflect higher functioning and fewer symptoms.

Patients completed the FACT-Hep after receiving uniform written instructions from a medical doctor (Li MD) or nurse (Dong HJ). Following FACT-Hep, another doctor (Lang QB) reviewed the forms for missing items. If there were more than three missing items, we requested that the patient complete the FACT-Hep again.

Table 2 Functional assessment of cancer therapy-hepatobiliary mean scores (*n* = 140)

FACT-Hep subscales	mean ± SD	Sum of scores	*F*	*P* value
PWB	16.47 ± 4.27	276.86	16.99	0.000
SFWB	14.52 ± 3.35	37.08	1.71	0.184
EWB	12.74 ± 3.39	131.33	7.11	0.001
FWB	17.57 ± 3.93	165.32	5.84	0.004
HepCS	41.46 ± 9.52	754.79	5.72	0.004

PWB: Physical well-being; SFWB: Social and family well-being; EWB: Emotional well-being; FWB: Functional well-being; HepCS: Hepatobiliary cancer subscale; FACT-Hep: Functional assessment of cancer therapy-hepatobiliary.

Statistical analysis

SPSS 13.0 software was used to process and analyze the data. Descriptive statistics were used to evaluate demographic and disease-specific characteristics. One-way analysis of variance and independent samples *t* tests ($P < 0.05$) were used to compare FACT-Hep scores between clinically distinct groups.

RESULTS

Sample size

FACT-Hep questionnaires were issued to 145 patients. Two patients dropped out of the study due to disease exacerbation and three questionnaires were omitted from analysis because of missing data (> 5 questions). In total, 140 questionnaires were completed (with a completion rate of 98.19%). The average time for finishing a FACT-Hep was 13.50 ± 2 min.

Patient characteristics

Of the 140 patients, 133 were male (95.0%) and the mean age at diagnosis was 52.34 ± 9.73 years (range: 28-75). The TNM tumor stages were as follows: Stage Ⅰ: 49 cases; Stage Ⅱ: 35 cases; Stage ⅢA: 29 cases; and Stage Ⅲ B: 27 cases. All patients had cirrhosis. One hundred and thirty-five (96.5%) subjects had liver cirrhosis secondary to hepatitis B virus (HBV) infection, with 73 (54.07%) of them being HBV DNA positive; the other etiologies of liver cirrhosis included alcoholic liver disease (2), hepatitis C (2) or cryptogenic (1). Demographic and clinical characteristics are shown in Table 1.

FACT-Hep scores and TNM stage

Median FACT-Hep scores decreased as TNM stage advanced, and FACT-Hep scores were strongly associated with TNM stage (Figure 1).

Differences in FACT-Hep items

Mean FACT-Hep subscale scores were: PWB 16.47 ± 4.272; SFWB 14.52 ± 3.351; EWB 12.74 ± 3.394; FWB 17.90 ± 4.06; and HepCS 41.46 ± 9.52. The overall mean differences are presented in Table 2.

Table 3 Functional assessment of cancer therapy-hepatobiliary subscale results by tumor node metastasis stage of hepatocellular carcinoma and by Child-Pugh class (mean ± SD)

	n	PWB	SFWB	EWB	FWB	HepCS
TNM stage						
Ⅰ	49	21 ± 2.15[d,f]	15.81 ± 3.68	15.11 ± 2.79[d,f]	19.96 ± 3.20[d,f]	48.52 ± 8.36[d,f]
Ⅱ	35	19.07 ± 3.41[b,f]	14.69 ± 2.82	13.38 ± 3.01[b]	19.24 ± 2.21[b]	45.45 ± 10.01[b]
ⅢA	29	16.29 ± 2.24[b,d]	14.66 ± 3.3	13.06 ± 3.08[b]	18.37 ± 3.98[b]	41.29 ± 7.05[b]
ⅢB	27	12.41 ± 2.88[b,d,f]	13.61 ± 3.32[a]	10.84 ± 3.15[b,d,f]	14.69 ± 3.39[b,d]	35.35 ± 7.44[b,d]
Child-Pugh class						
A	73	19.56 ± 2.79	15.29 ± 3.34	14.12 ± 2.89	19.58 ± 3.26[j]	46.12 ± 8.79[j]
B	40	16.65 ± 2.79[h]	14.25 ± 3.08	11.8 ± 3.05	16.48 ± 2.98[h]	38.88 ± 6.61[h]
C	27	10.52 ± 2.41[h]	12.85 ± 3.21[h]	10.41 ± 3.49[h,j]	13.78 ± 3.48[h,j]	32.7 ± 7.38[h,j]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* Stage Ⅰ; [d]$P < 0.01$ *vs* Stage Ⅱ; [f]$P < 0.01$ *vs* Stage ⅢA; [h]$P < 0.01$ *vs* Child A; [j]$P < 0.01$ *vs* Child B. PWB: Physical well-being; SFWB: Social and family well-being; EWB: Emotional well-being; FWB: Functional well-being; HepCS: Hepatobiliary cancer subscale.

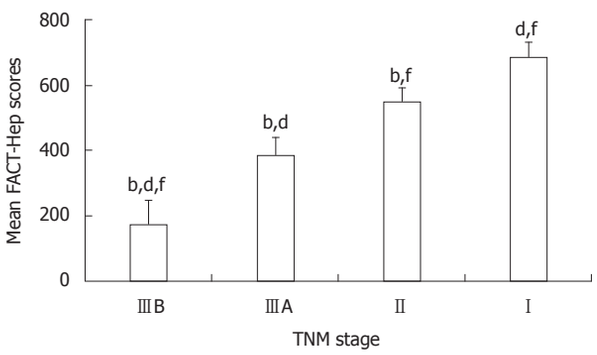

Figure 1 Mean functional assessment of cancer therapy-hepatobiliary scores by tumor node metastasis stage for hepatocellular carcinoma patients (*n* = 140, mean ± SD). [b]$P < 0.01$ *vs* Stage Ⅰ; [d]$P < 0.01$ *vs* Stage Ⅱ; [f]$P < 0.01$ *vs* Stage ⅢA. FACT-Hep: Functional assessment of cancer therapy-hepatobiliary.

Child-Pugh classification and FACT-Hep

Scores for each FACT-Hep item worsened with increasing severity of hepatic cirrhosis, based on the Child-Pugh classification (Table 3). Further analyses of factors impacting specific HRQoL were performed using percent analysis.

Physical well-being: Lack of physical strength was reported by 86% of patients; nausea by 51%; illness affecting the role at home by 83%; pain by 66%; recent uncomfortable feelings by 92%; and frequent bed rest by 55%. Patients had variable decreases in daily exercise capacity.

Social and family well-being: Patients kept close contact with friends (92%); received emotional support at home (59%); had support from friends (69%); had family who understood the patient's condition (49%); communicated about their condition with family (69%); and had close ties with a lover (43%). Only five patients answered questions regarding their sex lives (3.5%).

Emotional well-being: Sorrow and sad emotions were reported by 83% of patients; fear of death by 79%; and concern about disease progression by 74%. Only 53% of patients completed the item regarding current treatment and losing confidence in overcoming the current disease.

Functional well-being: Working ability was reported by 41%; sense of satisfaction with work by 51%; enjoyment of life by 55%; acceptance of the disease by 59%; insomnia by 95%; enjoyment from hobbies by 63%; and quality of life satisfaction by 52%.

Hepatobiliary cancer subscale: Patients reported stomach distension (65%); weight loss (57%); abnormal intestinal function (87%); digestive dysfunction (87%); diarrhea (64%); good appetite (36%); concern regarding appearance (61%); shoulder and back pain (71%); constipation (54%); fatigue (84%); ability to independently accomplish daily affairs (47%); jaundice 60 cases (43%); fever (36%); itching (27%); food taste changes (48%); cold sensitivity (61%); dry mouth (76%); stomach pain (63%); and swollen ankles (20%). No patients had bile drainage tubes.

DISCUSSION

Our study showed that patients with HCC had a perceived health status which varies by TNM stage for most FACT-Hep items. This conclusion was based on questionnaires widely used in Chinese clinical studies which indicate that the quality of life is an important prognostic factor and predicts survival time in patients with HCC[18].

HCC, an end-stage complication of liver disease, is expected to affect quality of life, but limited results have been reported previously[19]. Both disease-related and treatment-related symptomatic relief has been the primary goal in advanced HCC management because of the low survival rate of the patients. Alleviating clinical symptoms and improving quality of life have become targets for HCC treatment[20]. Quality of life and related factors in patients with liver cancer have been reported in some studies as indicators of treatment efficacy[21-23]. The FACT-Hep has been widely used in clinical studies to assess HRQoL[11]. Although traditional clinical diagnostic indicators (survival time and tumor response rates) and patients' subjective feelings are the primary components of quality of life for patients with HCC, the FACT-Hep can provide more comprehensive clinical evaluations[24,25].

There are several staging systems for HCC, such as the Japan Integrated Staging score, the new barcelona

clinic liver cancer (BCLC) staging classification, and the Tokyo score. These proposed staging systems consider both tumor size and liver function for HCC evaluation[26].

We classified patients' disease status using the BCLC and found that advancing BCLC stage was associated with a decreasing trend for FACT-Hep scores, because BCLC staging is related to liver function. Patients with cirrhosis had lower FACT-Hep scores (lower scores represent lower quality of life, Table 3). Previous studies have assessed the relationship between liver function and HRQoL deterioration and fatigue in patients with quantified inflammatory activity and degree of fibrosis[27]. Additionally, quality of life in patients with cirrhosis secondary to primary biliary cirrhosis or chronic hepatitis C has been reported previously[28-30].

The FACT-HepG mean scores showed that HRQoL in HCC patients significantly declined from TNM Stage Ⅰ to Stage Ⅱ to Stage ⅢA to Stage ⅢB. Thus, FACT-Hep scores could reflect varying levels of HCC disease severity.

Our results demonstrate that HCC has a significant and potentially adverse impact on physical health and psychological well-being, causing disruptions to patients' normal lives. Because the SFWB status of patients with HCC was impaired, family and friends' emotional support becomes particularly important. Although these symptoms may appear minor in the clinical setting, these factors may significantly predict the poor quality of life. Unfortunately, relevant information about sex life on the FACT-Hep questionnaire was limited and controversial; only five patients completed these questions. This limited response may be due to Chinese cultural norms regarding discussion of sexual activity. Throughout the five FACT-Hep items, we found that EWB was variably impaired. Thus, it appears that patients who have an established diagnosis of HCC experience a wide range of negative emotional symptoms such as sorrow and fear of death. Patients reported various levels of FWB based on the disease stage. On the HepCS, patients most commonly reported abnormal intestinal function, digestive dysfunction and fatigue.

Some limitations must be considered in evaluating these results. To avoid the variability of different therapeutic effects, we chose a simple analysis of untreated patients on admission. However, we believe that this approach may more accurately reflect the relationship between FACT-Hep results and TNM stage. Additionally, it may be more significant, in both clinical work and clinical perspective studies, to use the FACT-Hep as a tool to evaluate the patients' quality of life prior to treatment. Careful consideration of digestive dysfunction and emotional support is needed, as symptoms greatly impact HRQoL. Only 3.5% of patients responded to questions regarding sexual activity on the FACT-Hep scale. Cultural influences may preclude the use of these questions and it may be more instructive to focus on HCC patients' psychological states in tumor remission and long-term quality of life, which are other important elements of FACT-Hep. Regarding sleep, the sleep disorders in patients with advanced HCC may be due to the increased burden of physical and psychological factors.

Frequency percents of depression, anxiety and other psychiatric symptoms were significantly higher than other factors. This may be related to the disease itself, which is often associated with a range of negative emotional responses at the time of diagnosis. Furthermore, the poor prognosis, short survival time, need for repeated treatment, and high treatment cost may directly affect patients' mental health status, as demonstrated by the FACT-Hep results for stress, anxiety, fear of death and disease progression.

In conclusion, this study demonstrated that advancing TNM tumor stages were associated with the declining patient quality of life. These results may be useful for physicians to adjust HCC management according to patients' physical condition, extent of disease, and quality of life. Results of this study could be used to provide improved health services to meet the needs of HCC patients at different TNM tumor stages prior to initiating the treatment. In addition, more attention should be paid to the sad emotions, digestive dysfunction, fatigue and insomnia. Despite these findings, more definite evidence of the benefits of FACT-Hep questionnaire is required to justify its use. We plan to perform a long-term follow-up of these HCC patients to closely monitor the quality of life changes and to explore and predict their trends.

COMMENTS

Background

The incidence of hepatocellular carcinoma (HCC) in China is increasing, and HCC is now the second leading cause of death of cancers. Although many therapies have improved survival rates, symptoms become extremely variable in advanced HCC disease; compensated patients may be symptomatic for months or decades. In recent years, there has been increased interest in quality of life as it pertains to patients' health status. Many different questionnaires, such as the functional assessment of cancer therapy-hepatobiliary (FACT-Hep), the health-related quality of life (HRQoL), and the short form (36) health survey are becoming key components in the evaluation of patients' health status. HRQoL results may be more relevant than length of life, as patients are frequently more concerned about life quality than longevity.

Research frontiers

The Chinese version of the FACT-Hep is a new and important tool for the evaluation of prognosis and clinical trials, complementing the traditional end-point methods such as tumor response rate and survival time. This study demonstrated that advancing tumor node metastasis (TNM) stages were associated with the declining patient quality of life. These results may be useful for physicians to adjust HCC management according to patients' physical condition, extent of disease, and quality of life.

Innovations and breakthroughs

As HCC disease worsens, quality of life generally decreases, but two unanswered questions remain. First, FACT-Hep has not been used to assess prognosis with a large series of HCC patients at different TNM tumor stages. Second, quality of life is a broad concept, and no data are available to examine the impact of disease on patients' self-perception and the factors associated with poor HRQoL on the FACT-Hep.

Applications

This study demonstrated that advancing TNM tumor stages were associated with the declining patient quality of life. These results may be useful for physicians to adjust HCC management according to patients' physical condition, extent of disease, and quality of life. Results of this study could be used to provide improved health services to meet the needs of HCC patients at different TNM tumor stages prior to initiating the treatment

Terminology

The FACT-Hep is a 45-item, self-report instrument designed to measure HRQoL in patients with HCC. The FACT-Hep consists of 27-item FACT-G, which assesses generic HRQoL concerns using five subscales, and the newly validated 18-item hepatobiliary subscale, which assesses specific symptoms of hepatobiliary cancer and side effects of treatment. The five FACT- Hep subscales are: (1) physical well-being (7 questions); (2) social and family well-being (7 questions); (3) emotional well-being (6 questions); and (4) functional well-being (7 questions); and (5) hepatobiliary cancer subscale (20 questions).

Peer review

The paper quantifies the quality of life of 140 consecutive and unselected patients with HCC. They have used the FACT-Hep questionnaire and classified their patients according to TNM staging system.

REFERENCES

1 **Parkin DM,** Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA. *Cancer J Clin* 2005; **55:** 74-108
2 **Liu J**, Fan D. Hepatitis B in China. *Lancet* 2007; **369**: 1582-1583
3 **Edwards BK**, Brown ML, Wingo PA, Howe HL, Ward E, Ries LA, Schrag D, Jamison PM, Jemal A, Wu XC, Friedman C, Harlan L, Warren J, Anderson RN, Pickle LW. Annual report to the nation on the status of cancer, 1975-2002, featuring population-based trends in cancer treatment. *J Natl Cancer Inst* 2005; **97**: 1407-1427
4 **Geschwind JF**, Ramsey DE, Choti MA, Thuluvath PJ, Huncharek MS. Chemoembolization of hepatocellular carcinoma: results of a metaanalysis. *Am J Clin Oncol* 2003; **26**: 344-349
5 **Lau WY**, Lai EC. Hepatocellular carcinoma: current management and recent advances. *Hepatobiliary Pancreat Dis Int* 2008; **7**: 237-257
6 **Llovet JM**, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: Chemoembolization improves survival. *Hepatology* 2003; **37**: 429-442
7 **El-Serag HB**, Marrero JA, Rudolph L, Reddy KR. Diagnosis and treatment of hepatocellular carcinoma. *Gastroenterology* 2008; **134**: 1752-1763
8 **Cahill BA**, Braccia D. Current treatment for hepatocellular carcinoma. *Clin J Oncol Nurs* 2004; **8**: 393-399
9 **Tuinman MA**, Hoekstra HJ, Sleijfer DT, Fleer J, Vidrine DJ, Gritz ER, Hoekstra-Weebers JE. Testicular cancer: a longitudinal pilot study on stress response symptoms and quality of life in couples before and after chemotherapy. *Support Care Cancer* 2007; **15**: 279-286
10 **Ohara-Hirano Y**, Kaku T, Hirakawa T, Noguchi Y, Hirata N, Shinkoda H, Kitahara E, Saito T, Amada S, Ohki M. Uterine cervical cancer: a holistic approach to mental health and it's socio-psychological implications. *Fukuoka Igaku Zasshi* 2004; **95**: 183-194
11 **Heffernan N**, Cella D, Webster K, Odom L, Martone M, Passik S, Bookbinder M, Fong Y, Jarnagin W, Blumgart L. Measuring health-related quality of life in patients with hepatobiliary cancers: the functional assessment of cancer therapy-hepatobiliary questionnaire. *J Clin Oncol* 2002; **20**: 2229-2239
12 **Zhu ZC**, Lang QB, Chen Z, Li DT, Ling CQ. [Evaluation of Chinese version of the Functional Assessment of Cancer Therapy-Hepatobiliary questionnaire]. *Zhongxiyi Jiehe Xuebao* 2008; **6**: 341-345
13 **Que HF**, Chen HF, Xu JN, Liu S, Lu DM, Tang HJ. [Discussion of relationship between quality of life and clinical effect assessment of malignant tumor treated with traditional Chinese medicine]. *Zhongxiyi Jiehe Xuebao* 2005; **3**: 253-256
14 **Steel J**, Baum A, Carr B. Quality of life in patients diagnosed with primary hepatocellular carcinoma: hepatic arterial infusion of Cisplatin versus 90-Yttrium microspheres (Therasphere). *Psychooncology* 2004; **13**: 73-79
15 **You J**.[Significance and necessity of developing quality of life questionnaire for cancer patients adapting to traditional Chinese medicine]. *Zhongxiyi Jiehe Xuebao* 2006; **4**: 473-477
16 **Huang YH**, Chen CH, Chang TT, Chen SC, Wang SY, Lee HS, Lin PW, Huang GT, Sheu JC, Tsai HM, Lee PC, Chau GY, Lui WY, Lee SD, Wu JC. Evaluation of predictive value of CLIP, Okuda, TNM and JIS staging systems for hepatocellular carcinoma patients undergoing surgery. *J Gastroenterol Hepatol* 2005; **20**: 765-771
17 Sobin LH, Gospodarowicz MK, Wittekind C. Editors. TNM Classification of Malignant Tumors. 7th ed. Oxford: John Wiley and Sons, 2009
18 **Poon RT**, Fan ST, Yu WC, Lam BK, Chan FY, Wong J. A prospective longitudinal study of quality of life after resection of hepatocellular carcinoma. *Arch Surg* 2001; **136**: 693-699
19 **Steel JL,** Chopra K, Olek MC, Carr BI. Health-related quality of life: Hepatocellular carcinoma, chronic liver disease, and the general population. *Qual Life Res* 2007; **16:** 203-215
20 **Sun V**, Ferrell B, Juarez G, Wagman LD, Yen Y, Chung V. Symptom concerns and quality of life in hepatobiliary cancers. *Oncol Nurs Forum* 2008; **35**: E45-E52
21 **Fielding R**, Wong WS. Quality of life as a predictor of cancer survival among Chinese liver and lung cancer patients. *Eur J Cancer* 2007; **43**: 1723-1730
22 **Steel JL**, Geller DA, Carr BI. Proxy ratings of health related quality of life in patients with hepatocellular carcinoma. *Qual Life Res* 2005; **14**: 1025-1033
23 **Lai HL**, Lin SY, Yeh SH. [Exploring uncertainty, quality of life and related factors in patients with liver cancer]. *Huli Zazhi* 2007; **54**: 41-52
24 **Wang YB**, Chen MH, Yan K, Yang W, Dai Y, Yin SS. Quality of life after radiofrequency ablation combined with transcatheter arterial chemoembolization for hepatocellular carcinoma: comparison with transcatheter arterial chemoembolization alone. *Qual Life Res* 2007; **16**: 389-397
25 **Steel JL**, Eton DT, Cella D, Olek MC, Carr BI. Clinically meaningful changes in health-related quality of life in patients diagnosed with hepatobiliary carcinoma. *Ann Oncol* 2006; **17**: 304-312
26 **Chung H**, Kudo M, Takahashi S, Hagiwara S, Sakaguchi Y, Inoue T, Minami Y, Ueshima K, Fukunaga T, Matsunaga T. Comparison of three current staging systems for hepatocellular carcinoma: Japan integrated staging score, new Barcelona Clinic Liver Cancer staging classification, and Tokyo score. *J Gastroenterol Hepatol* 2008; **23**: 445-452
27 **Teuber G**, Schäfer A, Rimpel J, Paul K, Keicher C, Scheurlen M, Zeuzem S, Kraus MR. Deterioration of health-related quality of life and fatigue in patients with chronic hepatitis C: Association with demographic factors, inflammatory activity, and degree of fibrosis. *J Hepatol* 2008; **49**: 923-929
28 **Bonkovsky HL,** Snow KK, Malet PF, Back-Madruga C, Fontana RJ, Sterling RK, Kulig CC, Di Bisceglie AM, Morgan TR, Dienstag JL, Ghany MG, Gretch DR. Health-related quality of life in patients with chronic hepatitis C and advanced fibrosis. *J Hepatol* 2007; **46:** 420-431
29 **Poupon RE**, Chrétien Y, Chazouillères O, Poupon R, Chwalow J. Quality of life in patients with primary biliary cirrhosis. *Hepatology* 2004; **40**: 489-494
30 **Montali L**, Tanaka A, Riva P, Takahashi H, Cocchi C, Ueno Y, Miglioretti M, Takikawa H, Vecchio L, Frigerio A, Bianchi I, Jorgensen R, Lindor KD, Podda M, Invernizzi P. A short version of a HRQoL questionnaire for Italian and Japanese patients with Primary Biliary Cirrhosis. *Dig Liver Dis* 2010; **42**: 718-723

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BRIEF ARTICLE

Effect of intensive *vs* conventional insulin therapy on perioperative nutritional substrates metabolism in patients undergoing gastrectomy

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Abstract

AIM: To investigate the effect of intensive *vs* conventional insulin therapy on perioperative nutritional substrates metabolism in patients undergoing radical distal gastrectomy.

METHODS: Within 24 h of intensive care unit management, patients with gastric cancer were enrolled after written informed consent and randomized to the intensive insulin therapy (IIT) group to keep glucose levels from 4.4 to 6.1 mmol/L or the conventional insulin therapy (CIT) group to keep levels less than 10 mmol/L. Resting energy expenditure (REE), respiratory quotient (RQ), resting energy expenditure per kilogram (REE/kg), and the lipid oxidation rate were monitored by the indirect calorimeter of calcium citrate malate nutrition metabolism investigation system. The changes in body composition were analyzed by multi-frequency bioimpedance analysis. Blood fasting glucose and insulin concentration were measured for assessment of Homeostasis model assessment of insulin resistance.

RESULTS: Sixty patients were enrolled. Compared with preoperative baseline, postoperative REE increased by over 22.15% and 11.07%; REE/kg rose up to 27.22 ± 1.33 kcal/kg and 24.72 ± 1.43 kcal/kg; RQ decreased to 0.759 ± 0.034 and 0.791 ± 0.037; the lipid oxidation ratio was up to 78.25% ± 17.74% and 67.13% ± 12.76% supported by parenteral nutrition solutions from 37.56% ± 11.64% at the baseline; the level of Ln-HOMA-IR went up dramatically ($P < 0.05$, respectively) on postoperative days 1 and 3 in the IIT group. Meanwhile the concentration of total protein, albumin and triglyceride declined significantly on postoperative days 1 and 3 compared with pre-operative levels ($P < 0.05$, respectively). Compared with the CIT group, IIT reduced the REE/kg level (27.22 ± 1.33 kcal/kg *vs* 29.97 ± 1.47 kcal/kg, $P = 0.008$; 24.72 ± 1.43 kcal/kg *vs* 25.66 ± 1.63 kcal/kg, $P = 0.013$); and decreased the Ln-HOMA-IR score ($P = 0.019$, 0.028) on postoperative days 1 and 3; IIT decreased the level of CRP on postoperative days 1 and 3 ($P = 0.017$, 0.006); the total protein and albumin concentrations in the IIT group were greater than those in the CIT group ($P = 0.023$, 0.009). Postoperative values of internal cell fluid (ICF), fat mass, protein mass (PM), muscle mass, free fat mass and body weight decreased obviously on postoperative 7th day compared with the preoperative baseline in the CIT group ($P < 0.05$, respectively). IIT reduced markedly consumption of fat mass, PM and ICF compared with CIT ($P = 0.009$ to 0.026).

CONCLUSION: There were some benefits of IIT in decreasing the perioperative insulin resistance state, reducing energy expenditure and consumption of proteins and lipids tissue in patients undergoing gastrectomy.

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BRIEF ARTICLE

GABA stimulates human hepatocellular carcinoma growth through overexpressed GABAA receptor theta subunit

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Abstract

AIM: To investigate the function of gamma-aminobutyric acid (GABA) and gamma-aminobutyric acid A receptor θ subunit (GABRQ) in hepatocellular carcinoma (HCC).

METHODS: Semiquantitative polymerase chain reaction was used for detecting the expression of GABRQ receptor among HCC cell line HepG2, normal liver cell line L-02, non-malignant Chang's liver cells, 8 samples of HCC tissues and paired non-cancerous tissues. HepG2 cells were treated with GABA at serial concentrations (0, 1, 10, 20, 40 and 60 μmol/L), and their proliferating abilities were analyzed with the methyl thiazolyl tetrazolium assay, cell cycle analysis and tumor implanted in nude mice. Small interfering RNA was used for knocking down the endogenous GABRQ in HepG2. Proliferating abilities of these cells treated with or without GABA were analyzed.

RESULTS: We identified the overexpression of GABRQ in HCC cell lines and half of the tested HCC tissues. Knockdown of endogenous GABRQ expression in HepG2 attenuated HCC cell growth, suggesting its role in HCC cell viability. We studied the effect of GABA in the proliferation of GABRQ-positive cell lines *in vitro* and *in vivo*, and found that GABA increased HCC growth in a dose-dependent manner. Notably, the addition of GABA into the cell culture medium promoted the proliferation of GABRQ-expressing HepG2 cells, but not GABRQ-knockdown HepG2 cells, which means that GABA stimulates HepG2 cell growth through GABRQ.

CONCLUSION: GABRQ play important roles in HCC development and progression and could be a promising molecular target for the development of new diagnostic and therapeutic strategies of HCC.

Key words: Hepatocellular carcinoma; Proliferation; Gamma-aminobutyric acid; Gamma-aminobutyric receptor θ; small interfering RNA

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common

primary liver cancer and one of the most common malignancies in the world, accounting for approximately one million deaths per year[1,2]. Although liver resection and local ablation are regarded as potentially curative treatment[3], its prognosis is poor. Most of the patients are diagnosed with advanced disease at presentation for which palliative therapy forms the mainstay of treatment[4].

To improve this situation, the development of novel molecular therapies against effective targets is an urgent issue. Toward this direction, we previously used a method combining an in silico screen and experimental verification to identify genes that are differentially expressed in cancers compared with their corresponding normal tissues[5]. Among genes that are overexpressed in HCC cells, we focused on the gamma-aminobutyric acid (*GABA*) gene. Gamma-aminobutyric acid A receptor θ subunit (GABRQ) is a subunit of gamma-aminobutyric acid A (GABAA) receptors that may associate with other GABAA receptor subunits to form a functional chloride channel which mediates inhibitory synaptic transmission in the mature central nervous system (CNS). GABA primarily functions as an inhibitory neurotransmitter in the mature CNS by activating the GABA receptor, but it can also modulate the proliferation, migration and differentiation of neuronal cells during CNS development[6-9] and the proliferation of peripheral non-neuronal cells[10,11]. GABA and GABAA receptors are also present in peripheral tissues, including cancerous cells, but their precise functions are poorly defined.

This study demonstrates that GABRQ is overexpressed in HCC and that GABA promotes the proliferation of cancer cells through GABRQ.

MATERIALS AND METHODS

Cell lines

HCC cell line HepG2 and normal liver cell lines Chang's liver and L-02 were maintained by our lab and cultured in Dulbecco-modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). Cells were maintained at 37 ℃ atmosphere of humidified air with 5% CO_2.

Collection of tissues

All samples of HCC tissues and paired non-cancerous tissues (5 cm away from tumor) were obtained during surgical resection from the Xiangya Hospital of Central South University. Written consent was obtained from the patients, who agreed to the collection of tissue samples. The resected tissue samples were immediately cut into small pieces and snapfrozen in liquid nitrogen until use. All tumor tissue and paired non-cancerous tissue samples were pathologically confirmed.

Semiquantitative polymerase chain reaction

RNA isolated from cells was reverse-transcribed and amplified using the One-Step reverse transcription polymerase chain reaction (RT-PCR) System (Fermentas, Vilnius, Lithuania). The sets of primers for GABRQ receptor subunit are Sense 5'-TCGAGTTCTCCTCTGCTGTG-3', Antisense 5'-TATGCAGATCCAGGGACAA-3' (465 bp); Sense 5'-AATCCCATCACCATCTTCCA-3' and antisense 5'-CCTGCTTCACCACCTTCTTG-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 580 bp). After heating at 95 ℃ for 1 min, samples were exposed to 30 cycles (GAPDH, 25 cycles) of 95 ℃ for 30 s, 60 ℃ for 30 s and 68 ℃ for 1 min 30 s with a final extension at 68 ℃ for 10 min. Reaction products were separated on 1.5% agarose gels containing ethidium bromide and the level of amplification was analyzed using a Phosphor Imager.

RNA interference

To knockdown GABRQ expression, we used pGCsi-U6/Neo/GFP vector encoding a small hairpin RNA directed against the target gene in HepG2.The target sequences for GABRQ were: 5'-taGCAAGGAGGTGTATTTCTA-3' (Si-1), 5'-caGCTATGGTGTTCGCTTTAA-3'(Si-2), 5'-caGGCTGATGACAGTA TTATT-3'(Si-3), 5'-aaGGATGCTTTCGTGCATGAT-3'(Si-3). As a negative control, we used shRNA vector without hairpin oligonucleotides (Si-Mock).

Cell transfection

Human HCC cell line HepG2 was plated onto 6-well plates, and transfected with these small interfering RNA (siRNA) expression vectors using FuGENE6 (Roche) according to the instructions of the manufacturer, followed by 800 μg/mL of neomycin selection. The cells were harvested 10 d later to analyze the knockdown effect on GABRQ by RT-PCR using the primers shown above and by flow cytometry using rabbit anti-human polyclonal antibody against GABRQ (Chemicon).

Impact of GABRQ-siRNA on the growth of hepatocellular carcinoma cells

HepG2/Si-1, HepG2/Si-Mock cells were seeded with serum-free medium at a density of 10^3 cells/well in 96-well plates (n = 6), grown overnight, washed in phosphate-buffered saline (PBS), and incubated with 10% FBS with or without 40 μmol/L GABA DMEM at 37 ℃, 5% CO_2, for varying periods and exposed to fresh media every other day. During the last 4 h of each day's culture, the cells were treated with methyl thiazolyl tetrazolium (MTT, 50 μg per well, Sigma, United States). The generated formazan was dissolved in dimethyl sulfoxide (DMSO) and the ODs at 490 nm were measured for detecting the cell viability.

The effect of GABRQ silencing on the colony formation of HepG2 cells was analyzed by colony formation assay. HepG2/Si-1, HepG2/Si-Mock cells at 100 cells per well in 6-cm plates were incubated with serum-fee medium for 24 h, and then cultured in 10% FBS with or without 40 μmol/L GABA DMEM at 37 ℃, 5% CO_2, for 3 wk. The cell colonies were washed twice with PBS, fixed by 4% paraformaldehyde for 15 min and stained with Giemsa for 30 min. Individual clones with more than 50

Table 1 Cell cycle of HepG-2/Si-Mock and HepG-2/Si-1 (mean ± SD, *n* = 3)

	Si-1	Si-Mock
G0/G1 (%)	53.95 ± 3.22	49.95 ± 3.56
G2/M (%)	22.38 ± 2.79	21.61 ± 3.83
S (%)	24.29 ± 3.32	28.74 ± 3.85[a]

[a]$P < 0.05$ *vs* Si-1.

cells were counted. Clone forming efficiency for individual type of cells was calculated, according to the number of colonies/number of inoculated cells × 100%.

To evaluate the impact of GABRQ silencing on the HepG2 cells and the effect of GABA stimulation on the HepG2 cells, cell cycle was examined by flow cytometry analysis. HepG2/Si-1, HepG2/Si-Mock cells were incubated with serum-fee medium for 24 h, and then cultured in DMEM with 10% FBS with or without 40 μmol/L GABA, then harvested at 70%-80% confluence and resuspended in fixation fluid at a density of 10^6/mL; 1500 μL propidium iodide (PI) solution was added, and the cell cycle was detected by FACS Caliber (Becton-Dickinson).

Effect of gamma-aminobutyric acid on the growth of hepatocellular carcinoma cells

To study the effect of GABA on the proliferation of GABRQ-expressing HCC cells, cell proliferation was tested *in vitro*. In the MTT assay, HepG2 cells were seeded with serum-free medium at a density of 10^3 cells/well in 96-well plates ($n = 6$), grown overnight, washed in PBS, and incubated with GABA (Sigma-Aldrich) at serial concentrations (0, 1, 10, 20, 40 and 60 μmol/L) in appropriate medium supplemented with 1% FBS. The samples were tested every 24 h for 6 d. MTT was added (50 μg/well) for 4 h. Formazan products were solubilized with DMSO, and the optical density was measured at 490 nm.

In the flow cytometry assay, HepG2 cells were incubated with serum-fee medium for 24 h, and then cultured in DMEM with 10% FBS and serial concentrations (0, 1, 10, 20, 40 and 60 μmol/L) GABA for 48 h. Cells were harvested and resuspended in fixation fluid at a density of 10^6/mL, 1500 μL PI solution was added, and the cell cycle was detected by FACS Caliber (Becton Dickinson).

Tumor formation in nude mice

The influence of GABRQ silencing and GABA stimulation on the tumor development of HCC *in vivo* was examined. Briefly, HepG2, HepG2/Si-Mock and HepG2/Si-1 cells were treated with or without GABA (40 μmol/L) for 24 h first, and then the cells (3×10^6) were suspended in 0.2 mL of extracellular matrix gel and injected subcutaneously in the left back flank of the animals. The 8-wk-old BALB/c nude (nu/nu) mice (Slac Laboratory Animal Center, Shanghai, China) were divided into six groups: (1) the mice were injected with HepG2 and treated with 0.9% NaCl injection (150 μL) into the implanted tumor (HepG2, $n = 4$); (2) the mice were injected with HepG2 and treated with GABA injections (40 μmol/L in 150 μL of 0.9% NaCl) into the implanted tumor (HepG2 + GABA, $n = 4$); (3) the mice were injected with HepG2/ Si-Mock and treated with 0.9% NaCl injection (150 μL) into the implanted tumor (HepG2/Si-Mock, $n = 4$); (4) the mice were injected with HepG2/Si-Mock and treated with GABA injections (40 μmol/L in 150 μL of 0.9% NaCl) into the implanted tumor (HepG2/Si-Mock + GABA, $n = 4$); (5) the mice were injected with HepG2/Si-1 and treated with 0.9% NaCl injection (150 μL) into the implanted tumor (HepG2/Si-1, $n = 4$); and (6) the mice were injected with HepG2/Si-1 and treated with GABA injections (40 μmol/L in 150 μL of 0.9% NaCl) into the implanted tumor (HepG2/Si-1 + GABA, $n = 4$). The same operator carried out the injections every other day starting from "day 0" when the tumors were implanted. Tumor variables were measured every 3 d by an electronic caliper, and tumor volume was calculated using a standard formula[12,13]: tumor volume = width^2 × length × 0.5. At the end of the experiment, all mice were sacrificed and individual tumor weights were measured.

Statistical analysis

All data were expressed as mean ± SD. Differences among groups were determined by analysis of variance analysis and comparison between two groups was analyzed by the Student's *t* test using the GraphPad Prism soft-ware version 4.0 (GraphPad Software, Inc, San Diego, CA). A value of $P < 0.05$ was used to indicate statistical significance.

RESULTS

Expression of GABRQ receptors

We documented GABRQ mRNA expression in HepG2, Chang's liver and L-02 cell lines as well as in 8 pairs of HCC and adjacent non-tumor tissues. The results of semi-quantitative RT-PCR show GABRQ receptor subunit was detected in HepG2 and in Chang's liver cells, but not in normal cell line L-02 (Figure 1A). GABRQ receptor subunit was also detected in HCC tissues (6/8), but not in adjacent non-tumor tissues (Figure 1B).

Impact of GABRQ-siRNA on the growth of hepatocellular carcinoma cells

To investigate the biological significance of GABRQ overexpression in HCC cells, we constructed four siRNA expression vectors (Si-1, Si-2, Si-3 and Si-4) specific to GABRQ transcripts and transfected them into HepG2 cells that endogenously expressed high levels of GABRQ, as shown in Figure 1. A knockdown effect was observed by RT-PCR when we transfected Si-1, but not Si-2, Si-3, Si-4 or a negative control Si-Mock (Figure 2A). MTT assay (Figure 2B) revealed a drastic reduction in the number of cells transfected with Si-1 compared with Si-Mock for which no knockdown effect was observed. Cell proliferation was detected by flow cytometry; results showed HepG2 cells with GABRQ siRNA blocked the cell cycle in G1 phase, which may inhibit the growth of HepG2 cells (Table 1). This result was consistent with the MTT analysis.

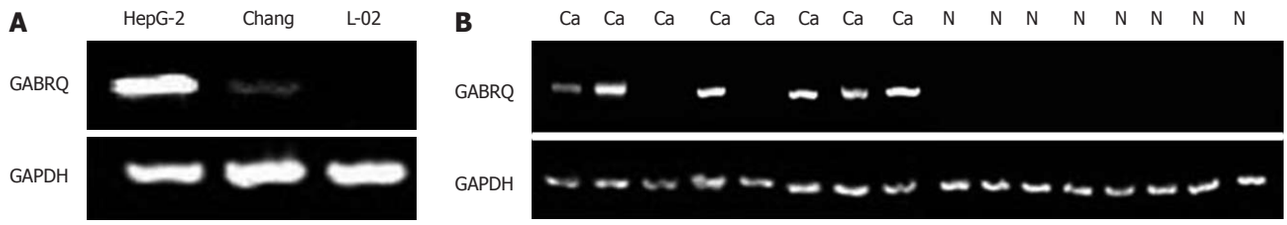

Figure 1 Expression of gamma-aminobutyric acid A receptor θ subunit in different cell lines, in liver cancerous tissues (Ca) and adjacent tissues of liver cancers (N) by reverse transcription polymerase chain reaction. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

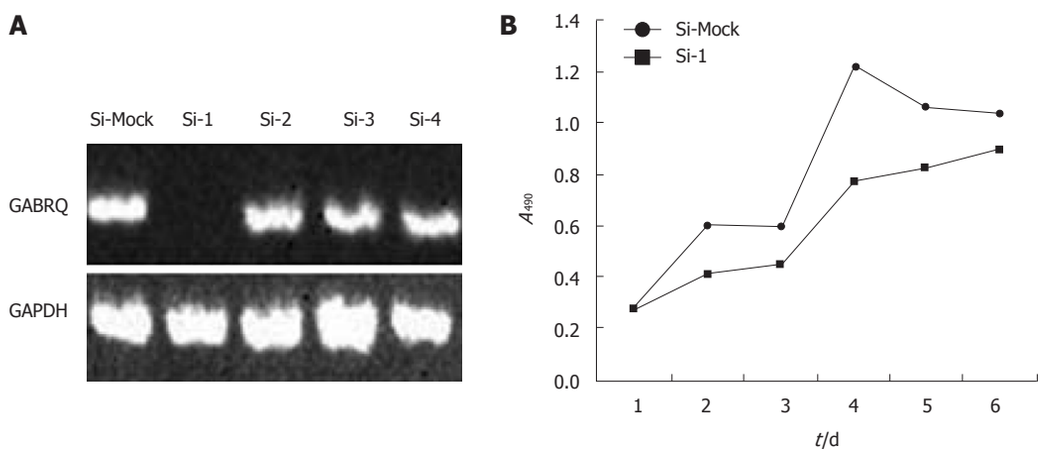

Figure 2 The impact of gamma-aminobutyric acid A receptor θ subunit-siRNA on the growth of hepatocellular carcinoma cells. A: Reverse transcription polymerase chain reaction (RT-PCR) verified the RNAi effect on gamma-aminobutyric acid A receptor θ subunit (GABRQ); B: Methyl thiazolyl tetrazolium assay HepG2 cells transfected with Si-1 and negative vectors to GABRQ. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Table 2 Cell cycle of HepG-2 treated with gamma-aminobutyric acid at serial concentrations (mean ± SD, *n* = 3, μmol/L)

	0	1	10	20	40	60
G0/G1 (%)	40.9 ± 2.92	39.6 ± 2.73	33.8 ± 2.79	31.7 ± 2.56	30.2 ± 2.17	47.2 ± 2.93
G2/M (%)	18.1 ± 0.84	19.8 ± 0.97	20.7 ± 0.85	21.3 ± 0.79	20.3 ± 1.18	18.5 ± 1.23
S (%)[a]	31.0 ± 1.89	30.6 ± 1.94	35.5 ± 2.28	37.0 ± 2.17	39.5 ± 2.34	34.3 ± 2.02

[a]$P < 0.05$ *vs* HepG-2 treated with gamma-aminobutyric acid.

A RT-PCR verified the knockdown effect on GABRQ expression by Si-1, but not by Si-2, Si-3, Si-4 and a negative control Si-Mock in HepG2 cells. GPDH was used to quantify RNAs; Figure 2B illustrates MTT assay of HepG2 cells transfected with Si-1 vectors to GABRQ and a negative control vector (Si-Mock). Y-axis: Average value of absorbance at 490 nm, measured with a microplate reader ($n = 6$, $P < 0.05$).

Effect of gamma-aminobutyric acid on the growth of hepatocellular carcinoma cells

Results displayed in Figure 3A show the addition of GABA in the culture media enhanced the proliferation of HepG2 cells in a dose-dependent manner. The promoting effect on HCC cell proliferation was more evident with the GABA concentration ranging from 1 μmol/L to 40 μmol/L. When the GABA concentration was increased to 60 μmol/L, the promoting effect became insignificant.

The promoting effect on HCC cell proliferation was also detected by flow cytometry analysis. After treating with GABA at serial concentrations, the G0/G1-phase fraction of HepG2 cells significantly decreased; on the contrary, S-phase cells significantly increased, especially at the concentration of 20 μmol/L and 40 μmol/L (Figure 3B, Table 2); this result was consistent with the results above.

In the nude mice implanted with tumors (injected with HepG2 cells), the development of solid HCC tumors was monitored for 40 d. As a result, a significant difference in tumor weight was found in GABA-treated (at the concentration of 40 μmol/L) mice compared with mice injected with 0.9% NaCl only (Figure 3C and D).

Effect of GABA on the growth of hepatocellular carcinoma cells after down-regulated expression of GABRQ

To examine the function of GABRQ as a GABA receptor on the growth of GABRQ-expressing HCC cells, we treat-

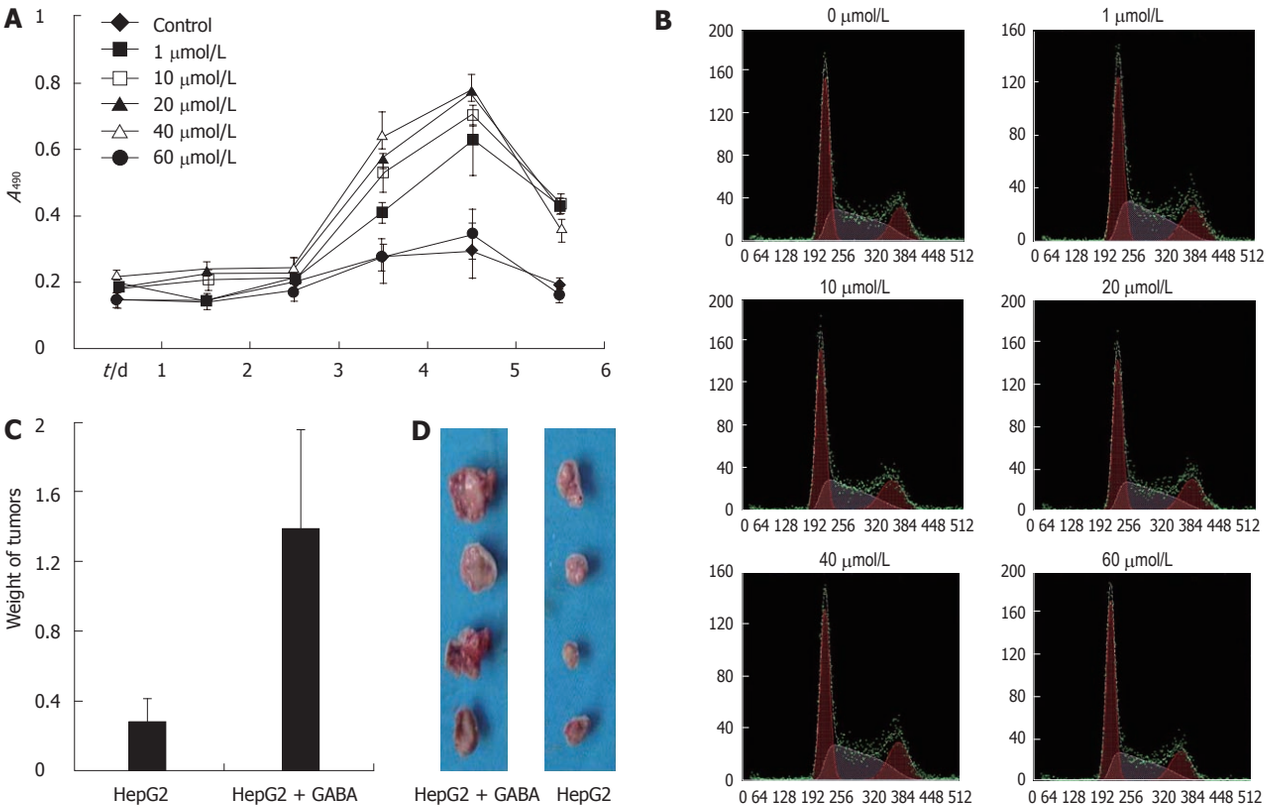

Figure 3 The effect of gamma-aminobutyric acid on the growth of hepatocellular carcinoma cells. A: The effects of serial concentration gamma-aminobutyric acid (GABA) on proliferation of HepG-2 cells; B: Cell cycle measured by flow cytometry; C: Forty days after tumor cell injection, mice were sacrificed and tumor weight was recorded (n = 4, P < 0.01); D: Comparison of tumor weight (the left was tumor of mice injected with 0.9% NaCl only, the right was the tumor of mice treated with 40 μmoL/L GABA).

Table 3 Cell cycle of HepG-2/Si-Mock and HepG-2/Si-1 treated with or without gamma-aminobutyric acid at concentration of 40 μmol/L (mean ± SD, n = 3)

	Si-Mock	Si-Mock + GABA	Si-1	Si-1 + GABA
G0/G1 (%)	49.95 ± 3.16	46.21 ± 2.98	53.95 ± 2.62	48.68 ± 2.49
G2/M (%)	21.61 ± 3.43	20.82 ± 2.43	22.38 ± 2.19	21.45 ± 2.26
S (%)	28.74 ± 3.35	33.64 ± 3.76[a]	24.29 ± 2.72[b]	27.43 ± 1.95

[a]P < 0.05, [b]P < 0.01 *vs* Si-Mock.

ed HepG2/Si-1 and HepG2/Si-Mock cells with or without GABA (40 μmol/L). The results are shown in Figure 4A: GABA enhanced the growth of HepG2/Si-Mock compared with the HepG2/Si-Mock without GABA. On the other hand, the proliferating ability of HepG2/Si-1, which did not express GABRQ, was not enhanced by GABA. In the nude mice injected with HepG2/Si-Mock, the tumor weight of the mice treated with GABA was much larger than that of the mice treated without GABA, while the mice injected with HepG2/Si-1 did not present such differences (Figure 4C and D).

To further explore GABA stimulation of HepG2 cell growth through GABRQ, we examined the effects of Si-1 and Si-Mock on cell cycle. After treatment with 40 μmol/L GABA, the G0/G1-phase fraction of HepG2/Si-Mock cells significantly decreased; in contrast, S-phase cells significantly increased, but this event did not occur in HepG2/Si-1 cells (Table 3, Figure 4B).

The other illustration of growth effect of reduced GABRQ expression in HepG2 cells was achieved in a colony formation assay (Figure 4E and F). As a result, the average colony number of Si-1 cells was decreased compared with Si-Mock cells. After treatment with 40 μmol/L GABA, the numbers of cell colonies of HepG2/Si-Mock cells significantly increased, but this did not occur in HepG2/Si-1 cells. These data indicate that GABA stimulates HepG2 cell growth through GABRQ.

DISCUSSION

In this study, we validated the overexpression of GABRQ in more than half of the tested HCC tissues compared with the adjacent non-tumor liver tissues; GABRQ was expressed in malignant liver cell lines HepG2 and moderately expressed in normal cell line Chang's liver, but not in normal cell line L-02, implicating that GABRQ may be a good molecular target for the diagnosis of HCC. Functional analysis using siRNA of GABRQ strongly supported its involvement in the development and progression of HCC. In our study, the proliferation rate of HepG2 cells after GABRQ knockdown was significantly reduced, whereas proliferation of Si-Mock cells was not inhibited. This result indicated that GABRQ may increase the pro-

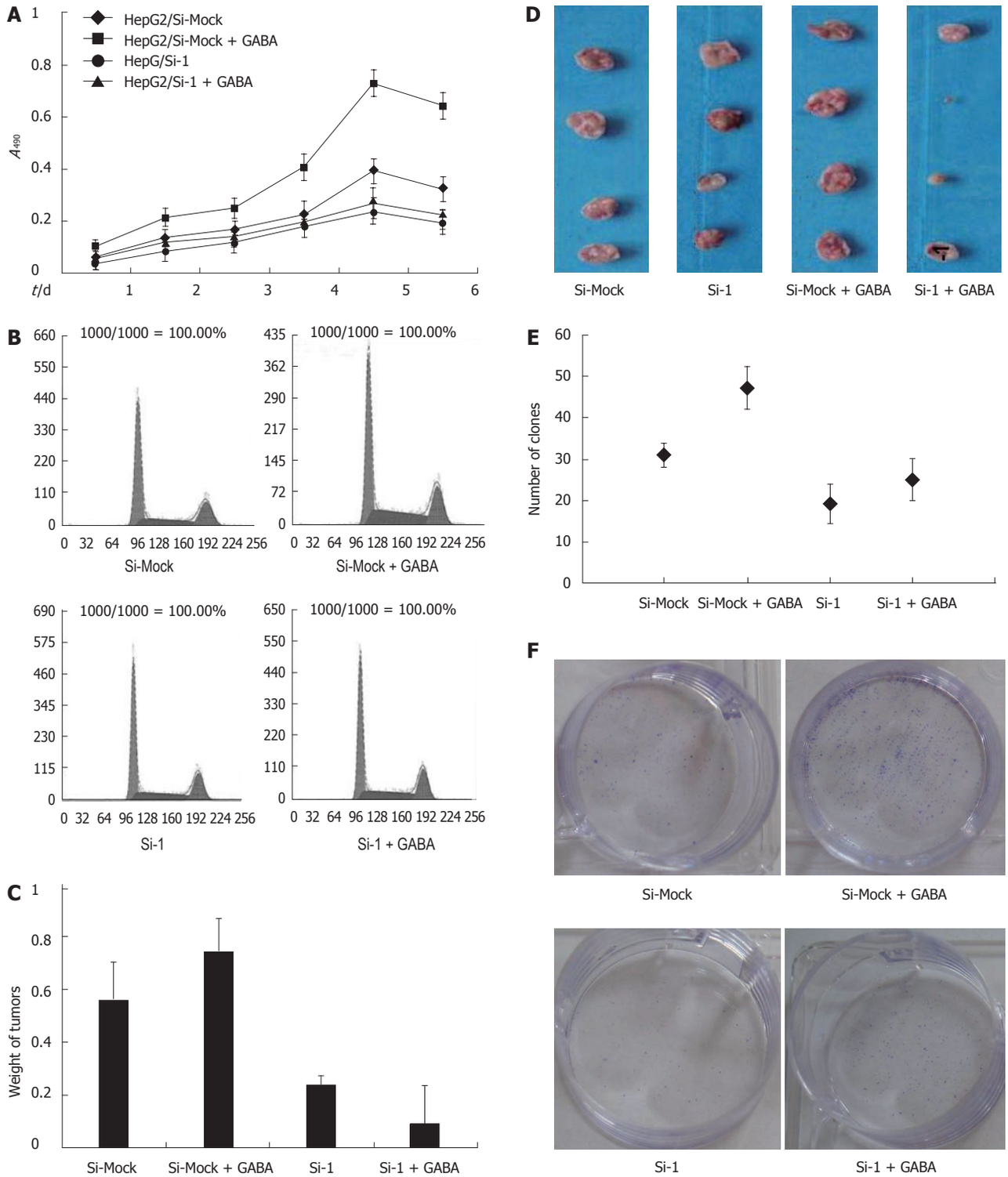

Figure 4 The effect of gamma-aminobutyric acid on the growth of hepatocellular carcinoma cells after down-regulated expression of gamma-aminobutyric acid A receptor θ subunit. A: The effects of gamma-aminobutyric acid (GABA) on proliferation of HepG2/Si-Mock and HepG2/Si-1 cells; B: Analysis of cell cycles of HepG-2 cells by flow cytometry; C: Forty days after tumor cell injection, mice were sacrificed and tumor weight was recorded (n = 4, P < 0.01 for Si-Mock *vs* Si-Mock + GABA; n = 4, P < 0.01 for Si-1 *vs* Si-1 + GABA); D: Comparison of tumor weight (the left two ones were tumor of mice injected HepG2/Si-Mock and HepG2/Si-1 with 0.9% NaCl, the right two ones were the tumor of mice HepG2/Si-Mock and HepG2/Si-1 treated with 40 μmoL/L GABA); E and F: Colony formation assay (n = 3, P < 0.05 for HepG-2/Si-Mock *vs* HepG-2/Si-Mock + GABA).

liferation ability of hepatocytes. Primarily, GABA and GABA receptors function as an inhibitory neurotransmitter in the mature CNS, but their precise functions in non-neuronal cells or tumor cells are unknown. Joseph *et al*[14] reported that GABA could inhibit colon cancer migration associated with the norepinephrine-induced pathway. On the other hand, another report showed that GABA and GABAB receptor pathways could be involved in

prostate cancer metastasis or invasion through the regulation of metalloproteinase production[15]. Therefore, it is controversial whether GABA-associated pathways could act positively or negatively in the regulation of cancer cell behavior. However, our findings in this study can clearly indicate evidence supporting the theory that GABA and GABAA receptor with GABRQ promote HCC cell proliferation.

By comparing the proliferative activity of the GABRQ-knockdown HepG2 cells treated with GABA, we found that GABA stimulated HepG2 cell growth through GABRQ. The proliferating ability of the cells treated with GABA was not enhanced compared with the cells without GABA treatment. Previous studies suggest that GABA stimulates collagen synthesis and proliferation of human fibroblasts[16]. Biju *et al*[17] reported that, in N-nitrosodiethylamine-induced neoplasia in the rat liver, GABAB receptors were increased and that the GABAB receptor agonist baclofen increased epidermal growth factor-mediated DNA synthesis in hepatocytes. Thus, GABA-associated pathways also could act positively in the regulation of cancer cell behavior. Our findings in this study also support the theory that GABA and GABRQ promote HepG2 cell proliferation *in vivo* and *in vitro*. Interestingly, GABAA receptor antagonist bicuculline methiodide could also promote the proliferation of HepG2 cells (data not shown), indicating that it might activate some other signal pathways[18].

Although GABA usually induces hyperpolarization in adult neurons, GABA has been shown to exert depolarizing responses in the immature CNS structures and CNS tumors[19,20]. In particular, GABA increased the proliferation of immature cerebellar granule cells through the activation of GABAA receptors and voltage-dependent calcium channels[21,22]. Takehara *et al*[23] reported that GABA stimulated pancreatic cancer growth through GABRP by increasing intracellular Ca^{2+} levels and activating the mitogen-activated protein kinase/extracellular signal-regulated kinase cascade. Also, Minuk *et al*[24] reported that human HCC tissues were depolarized compared with adjacent non-tumor tissues. From the results above, we deduce that GABA may promote the HepG2 cell proliferation through GABRQ by voltage-dependent calcium channels. Interestingly, GABA inhibited the growth of the GABRQ-knockdown HepG2 cells. This indicates that GABA activates some other receptors to inhibit the proliferation without GABRQ, which is identical to some previous reports[25-27].

In conclusion, compared with adjacent non-tumor tissues, HCC tissues have increased GABRQ receptor expression. Knockdown of GABRQ expression in receptor-expressing malignant hepatocytes results in attenuated *in vitro* and *in vivo* tumor growth. Moreover, GABA promotes hepatocyte proliferation through GABRQ. These findings highlight the importance of elucidating the role of GABAergic activity in the pathogenesis of HCC. They also raise the potential for new therapeutic and diagnostic approaches to human HCC.

COMMENTS

Background

Gamma-aminobutyric acid A receptor θ subunit (GABRQ) is a subunit of the gamma-aminobutyric acid A (GABAA) receptors that may associate with other GABAA receptor subunits to form a functional chloride channel which mediates inhibitory synaptic transmission in the mature central nervous system (CNS). gamma-aminobutyric acid (GABA) functions as an inhibitory neurotransmitter for activating GABA receptors.

Research frontiers

Recently, abnormal levels of gene and protein expression of some GABA receptor subunits have been detected in many malignant tumors. This research indicates that GABAergic system may play an important role in the pathogenesis and development of malignant tumors.

Innovations and breakthroughs

This study demonstrated the overexpression of GABRQ in hepatocellular carcinoma (HCC), which has not been previously described, and illustrated that GABA stimulates HCC cell proliferation through GABRQ.

Applications

Further characterization of GABRQ will provide new insights into the role of GABRQ in the molecular pathogenesis and therapy of HCC.

Terminology

GABA stands for gamma-aminobutyric acid, which is an inhibitory neurotransmitter. GABRQ stands for gamma-aminobutyric acid A receptor θ subunit.

Peer review

The authors have analyzed the expression and the role of GABRQ in hepatocellular carcinoma. The manuscript is well-written and the study is conducted appropriately in order to understand the molecular mechanisms that control hepatocarcinogenesis, and also raise the potential for new therapeutic and diagnostic approaches to human HCC.

REFERENCES

1 **Hao K**, Luk JM, Lee NP, Mao M, Zhang C, Ferguson MD, Lamb J, Dai H, Ng IO, Sham PC, Poon RT. Predicting prognosis in hepatocellular carcinoma after curative surgery with common clinicopathologic parameters. *BMC Cancer* 2009; **9**: 389

2 **Huang J**, Li Y, Guo F, Tong Y, Wang J, Hu J, Li G. Expression of scFv SA3 against hepatoma fused with enhanced green fluorescent protein and its targeted ability in vivo. *Zhongnan Daxue Xuebao Yixueban* 2011; **36**: 979-986

3 **Song TJ**, Ip EW, Fong Y. Hepatocellular carcinoma: current surgical management. *Gastroenterology* 2004; **127**: S248-S260

4 **Paul SB**, Gamanagatti SR, Mukund A, Abbas SZ, Acharya SK. Transarterial chemoembolization for hepatocellular carcinoma: significance of extrahepatic collateral supply. *Indian J Cancer* 2011; **48**: 339-344

5 **Liu Y**, Li YH, Guo FJ, Wang JJ, Sun RL, Hu JY, Li GC. Gamma-aminobutyric acid promotes human hepatocellular carcinoma growth through overexpressed gamma-aminobutyric acid A receptor alpha 3 subunit. *World J Gastroenterol* 2008; **14**: 7175-7182

6 **Haydar TF**, Wang F, Schwartz ML, Rakic P. Differential modulation of proliferation in the neocortical ventricular and subventricular zones. *J Neurosci* 2000; **20**: 5764-5774

7 **Behar TN**, Schaffner AE, Scott CA, Greene CL, Barker JL. GABA receptor antagonists modulate postmitotic cell migration in slice cultures of embryonic rat cortex. *Cereb Cortex* 2000; **10**: 899-909

8 **Neelands TR**, Zhang J, Macdonald RL. GABA(A) receptors expressed in undifferentiated human teratocarcinoma NT2 cells differ from those expressed by differentiated NT2-N cells. *J Neurosci* 1999; **19**: 7057-7065

9 **Meier J**, Akyeli J, Kirischuk S, Grantyn R. GABA(A) receptor activity and PKC control inhibitory synaptogenesis in CNS tissue slices. *Mol Cell Neurosci* 2003; **23**: 600-613

10 **Tamayama T**, Maemura K, Kanbara K, Hayasaki H, Yabumoto Y, Yuasa M, Watanabe M. Expression of GABA(A) and GABA(B) receptors in rat growth plate chondrocytes: activation of the GABA receptors promotes proliferation of mouse chondrogenic ATDC5 cells. *Mol Cell Biochem* 2005; **273**: 117-126

11 **Erlander MG**, Tobin AJ. The structural and functional heterogeneity of glutamic acid decarboxylase: a review. *Neurochem Res* 1991; **16**: 215-226

12 **Fava G**, Marucci L, Glaser S, Francis H, De Morrow S, Benedetti A, Alvaro D, Venter J, Meininger C, Patel T, Taffetani S, Marzioni M, Summers R, Reichenbach R, Alpini G. gamma-Aminobutyric acid inhibits cholangiocarcinoma growth by cyclic AMP-dependent regulation of the protein kinase A/extracellular signal-regulated kinase 1/2 pathway. *Cancer Res* 2005; **65**: 11437-11446

13 **Guo F**, Li Y, Liu Y, Wang J, Li Y, Li G. Inhibition of metastasis-associated lung adenocarcinoma transcript 1 in CaSki human cervical cancer cells suppresses cell proliferation and invasion. *Acta Biochim Biophys Sin* (Shanghai) 2010; **42**: 224-229

14 **Joseph J**, Niggemann B, Zaenker KS, Entschladen F. The neurotransmitter gamma-aminobutyric acid is an inhibitory regulator for the migration of SW 480 colon carcinoma cells. *Cancer Res* 2002; **62**: 6467-6469

15 **Azuma H**, Inamoto T, Sakamoto T, Kiyama S, Ubai T, Shinohara Y, Maemura K, Tsuji M, Segawa N, Masuda H, Takahara K, Katsuoka Y, Watanabe M. Gamma-aminobutyric acid as a promoting factor of cancer metastasis; induction of matrix metalloproteinase production is potentially its underlying mechanism. *Cancer Res* 2003; **63**: 8090-8096

16 **Scutt A**, Meghji S, Harvey W. Stimulation of human fibroblast collagen synthesis in vitro by gamma-aminobutyric acid. *Biochem Pharmacol* 1987; **36**: 1333-1335

17 **Biju MP**, Pyroja S, Rajeshkumar NV, Paulose CS. Enhanced GABA(B) receptor in neoplastic rat liver: induction of DNA synthesis by baclofen in hepatocyte cultures. *J Biochem Mol Biol Biophys* 2002; **6**: 209-214

18 **Mares P**, Chino M, Kubová H, Mathern P, Veliký M. Convulsant action of systemically administered glutamate and bicuculline methiodide in immature rats. *Epilepsy Res* 2000; **42**: 183-189

19 **Ganguly K**, Schinder AF, Wong ST, Poo M. GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. *Cell* 2001; **105**: 521-532

20 **Labrakakis C**, Patt S, Hartmann J, Kettenmann H. Functional GABA(A) receptors on human glioma cells. *Eur J Neurosci* 1998; **10**: 231-238

21 **Fiszman ML**, Borodinsky LN, Neale JH. GABA induces proliferation of immature cerebellar granule cells grown in vitro. *Brain Res Dev Brain Res* 1999; **115**: 1-8

22 **Fiszman ML**, Schousboe A. Role of calcium and kinases on the neurotrophic effect induced by gamma-aminobutyric acid. *J Neurosci Res* 2004; **76**: 435-441

23 **Takehara A**, Hosokawa M, Eguchi H, Ohigashi H, Ishikawa O, Nakamura Y, Nakagawa H. Gamma-aminobutyric acid (GABA) stimulates pancreatic cancer growth through overexpressing GABAA receptor pi subunit. *Cancer Res* 2007; **67**: 9704-9712

24 **Minuk GY**, Zhang M, Gong Y, Minuk L, Dienes H, Pettigrew N, Kew M, Lipschitz J, Sun D. Decreased hepatocyte membrane potential differences and GABAA-beta3 expression in human hepatocellular carcinoma. *Hepatology* 2007; **45**: 735-745

25 **Tatsuta M**, Iishi H, Baba M, Nakaizumi A, Ichii M, Taniguchi H. Inhibition by gamma-amino-n-butyric acid and baclofen of gastric carcinogenesis induced by N-methyl-N'-nitro-N-nitrosoguanidine in Wistar rats. *Cancer Res* 1990; **50**: 4931-4934

26 **Zhang M**, Gong Y, Assy N, Minuk GY. Increased GABAergic activity inhibits alpha-fetoprotein mRNA expression and the proliferative activity of the HepG2 human hepatocellular carcinoma cell line. *J Hepatol* 2000; **32**: 85-91

27 **Tatsuta M**, Iishi H, Baba M, Yano H, Uehara H, Nakaizumi A. Effect of selective and non-selective muscarinic blockade on baclofen inhibition of gastric carcinogenesis induced by N-methyl-N'-nitro-N-nitrosoguanidine in Wistar rats. *Carcinogenesis* 1996; **17**: 293-296

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BRIEF ARTICLE

Phosphatase and tensin homolog expression related to cetuximab effects in colorectal cancer patients: A meta-analysis

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Abstract

AIM: To investigate the correlation between expression of phosphatase and tensin homolog (PTEN) and cetuximab effects in colorectal cancer.

METHODS: We searched PubMed, EMBASE and ASCO to identify eligible studies. Finally, 8 randomized control studies were included in the meta-analysis. STATA 10.0 Software was used to investigate heterogeneity among individual studies and to summarize all the studies. Risk ratios (RRs) and hazard ratios (HRs) with 95% confidence intervals (CIs) were used to assess the strength of the association.

RESULTS: Compared with 20 of 266 patients with loss of PTEN, 206 of 496 patients with intact PTEN protein expression had a better objective response rate to cetuximab-based therapy (RR, 4.75; 95% CI, 2.59-8.72; $P < 0.001$). PTEN positivity was associated with better progression-free survival (PFS) (HR, 0.675; 95% CI, 0.473-0.964; $P = 0.031$) but not with better overall survival (OS) (HR, 0.608; 95% CI, 0.411-0.899; $P = 0.013$). In patients with KRAS wild-type status, PTEN positivity did not predict a longer PFS or OS (PFS: HR, 0.707; 95% CI, 0.440-1.138; $P = 0.154$; OS: HR, 0.943; 95% CI, 0.646-1.377; $P = 0.761$).

CONCLUSION: Expression of PTEN is related to the effect of cetuximab in colorectal cancer patients and should be considered in treatment with cetuximab.

Key words: Phosphatase and tensin homolog; Cetuximab; Colorectal cancer; Prognosis; Meta-analysis

Peer reviewer: Julio M Mayol, MD, PhD, Department of Digestive Surgery, Hospital Clinico San Carlos, Martin-lagos S/n, 28040 Madrid, Spain

Shen Y, Yang J, Xu Z, Gu DY, Chen JF. Phosphatase and tensin homolog expression related to cetuximab effects in colorectal cancer patients: A meta-analysis. *World J Gastroenterol* 2012; 18(21): 2712-2718 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i21/2712.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i21.2712

INTRODUCTION

Colorectal cancer (CRC) is the fourth common malignancy and the second leading cause of cancer death in Western countries[1]. More than half of CRC patients will develop metastatic lesions (mCRC), which are often found in the liver[2]. Although novel pharmaceutical and surgical interventions have been introduced to treat mCRC, the 5-year survival rate for mCRC remains below 10%[3,4]. Recently cetuximab, a monoclnonal antibody that targets the epidermal growth factor receptor (EGFR) has

been proven to be efficacious in mCRC patients[3]. Cetuximab binds to EGFR *via* its ligand-binding domain to inhibit the activation of EGRF signaling. In clinical trials, cetuximab has been reported to achieve a response rate of 10% as a single agent and of 23%-25% in combination with chemotherapy[5,6]. The addition of cetuximab to chemotherapies enhances their antitumor activity[7]. The proposed mechanisms include: reducing tumor cell proliferation, angiogenesis, and DNA repair capacity; increasing apoptosis; and inducing cell cycle arrest at treatment-sensitive points[5]. These effects may enhance and restore tumor sensitivity to cytotoxic agents[8].

In CRC patients, EGFR is overexpressed in 75% of the tumors and its overexpression is associated with worse outcome[3,9]. EGFR was accordingly an obvious candidate for targeted therapy in this malignancy[5]. The tumor suppressor phosphatase and tensin homolog (PTEN) is an important negative regulator of cell-survival signaling[1]. To date, there is evidence to suggest that loss of expression of PTEN has negative association with the prognosis of CRC, especially mCRC. Loss of PTEN expression results in increased phosphatidylinositol phosphate-3 concentration, which induces subsequent protein kinase B hyperphosphorylation, thus protecting cancer cells from apoptotic stimuli[10-12]. In Addition, underexpression of PTEN confers resistance to cetuximab-induced apoptosis[10].

It is important to reveal the relation between the expression of PTEN and the prognosis of mCRC patients treated with cetuximab, as this will be helpful for adopting appropriate targeted therapy for patients[13]. At present, there are many studies which have reported the clinical outcomes of cetuximab in mCRC patients with loss of expression of PTEN. Hence, we carried out a meta-analysis to analyze the relation between the expression of PTEN and prognosis of CRC patients treated with cetuximab.

MATERIALS AND METHODS

Eligibility criteria

The purpose of this research was to systematically review the published articles of cetuximab-based chemotherapy in CRC (both primary and metastatic). Studies which reported the patients' PTEN status and compared the prognosis, were included in the analysis. The primary outcomes of interest were overall survival (OS) and progression-free survival (PFS). Care was taken to include only primary data or data that superseded earlier work.

Identification of studies

The search for studies was performed using the electronic database PubMed with the keywords "colorectal cancer", "cetuximab" and "PTEN". We also referred to the electronic database ASCO and EMBASE. All studies matching the eligibility criteria were retrieved and their bibliographies were checked for other relevant publications. Review articles and bibliographies of other relevant studies were identified through hand-searching to identify the additional studies. Data from review articles, case reports, abstracts, and letters were not included. Pharmaceutical industries and authors were not contacted. Characteristics of the studies were extracted from published articles and summarized in a consistent manner to aid comparison[14].

Statistical analysis

The meta-analysis was conducted by using Stata software (version 10.0; StataCorp Lakeway, College Station, TX, United States). Before performing the analyses, data of each published study were carefully checked and verified for coherence with the original publications. The strength of the association between status of PTEN and response of cetuximab-based therapy was measured by the risk ratio (RR) with 95% confidence intervals (CIs). Individual trial level time-to-event data was summarized by the hazard ratio (HR) with 95% CIs. Pooled estimations of RR and HR were obtained by calculating a weighted average of RR and HR from each study.

Statistical heterogeneity between studies was evaluated with the χ^2 test with significance set at a P value of 0.05. The percentage of total variation across the studies, with higher values indicating a greater degree of heterogeneity, was measured by the I^2 statistic. If the P value was $\leqslant 0.05$, the assumption of homogeneity was deemed invalid, and the DerSimonian-Laird method[15] (random-effects model) was used after exploring the causes of the heterogeneity; otherwise, the Mantel-Haenszel method[16] (fixed-effects model) was used. In the absence of heterogeneity, the fixed-effects and random-effects models provided similar results. I^2 lay between 0% and 100%, and a value of 0% indicated no observed heterogeneity, while larger values indicated increasing heterogeneity[17].

Findings of the meta-analysis are depicted in classical Forest plots, with point estimates and 95% CIs for each trial and overall size of the squares proportional to the effect size[18]. It was statistically significant when the two-tailed P value < 0.05. Publication bias was adjusted using the trim-and-fill method, and assessed by visual inspection of funnel plots (Figure 1)[19].

RESULTS

Description of studies

After exclusion of duplicate and irrelevant studies (Figure 2), our search yielded 8 eligible published studies that were retrieved for more detailed evaluation and meta-analysis[3,5,9,10,20-23]. The main characteristics of these selected studies are summarized in Table 1, and the description of PTEN status listed in Table 2. Most of the patients received a cetuximab-based therapy as second-line or later therapy after chemotherapy failure. All 8 studies including a total of 698 patients, of whom 513 were allocated to cetuximab plus irinotecan and others to cetuximab only or with various regimens as shown in detail in Table 1. The outcome measures of the above studies were evaluated based on the Response Evaluation Criteria in Solid Tu-

Table 1 The main characteristics of the 8 selected studies

First Author	Year	Type of study	*n*	Chemotherapy regimen						
				Ctx only	Ctx plus iri	Ctx plus folfiri	Ctx plus folfox	Pan	Ctx plus oxa	Ctx plus oxa and cap
Sartore-Bianchi *et al*[3]	2009	Cohort study	110	14	74	0	0	22	0	0
Negri *et al*[5]	2009	Retrospective study	50	0	36	0	0	0	14	0
Laurent-Puig *et al*[9]	2009	Retrospective study	173	3	141	28	0	0	0	0
Loupakis *et al*[10]	2009	Retrospective cohort study	102	2	100	0	0	0	0	0
Perrone *et al*[21]	2009	Cohort study	32	0	32	0	0	0	0	0
Frattini *et al*[20]	2007	Cohort study	27	0	23	0	0	0	0	4
Razis *et al*[22]	2008	Retrospective study	72	1	13	27	18	-	-	-
Sartore-Bianchi *et al*[23]	2009	Cohort study	132	15	94	0	0	23	0	0

CTX: Cetuximab; Pan: Panitumumab; oxa: Oxaliplaten; cap: Capecitabine.

Table 2 Description of phosphatase and tensin homolog status

No.	Title of the study	Method
1	Analysis of PTEN, BRAF and EGFR status in determining benefit from cetuximab therapy in wild-type KRAS metastatic colon cancer	IHC
2	PI3KCA/PTEN deregulation contributes to impaired responses to cetuximab in metastatic colorectal cancer patients	FISH
3	PTEN expression and KRAS mutations on primary tumors and metastases in the prediction of benefit from cetuximab plus irinotecan for patients with metastatic colorectal cancer	IHC
4	PTEN status in advanced colorectal cancer treated with cetuximab	FISH
5	PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies	IHC
6	PTEN loss of expression predicts cetuximab efficacy in metastatic colorectal cancer patients	IHC
7	Potential value of PTEN in predicting cetuximab response in colorectal cancer: An exploratory study	FISH
8	Multi-determinants analysis of molecular alterations for predicting clinical benefit to EGFR-targeted monoclonal antibodies in colorectal cancer	IHC

PTEN: Phosphatase and tensin homolog; BRAF: V-raf murine sarcoma viral oncogene homolog; EGFR: Epidermal growth factor receptor; IHC: Immunohistochemistry; FISH: Fluorescence *in situ* hybridization.

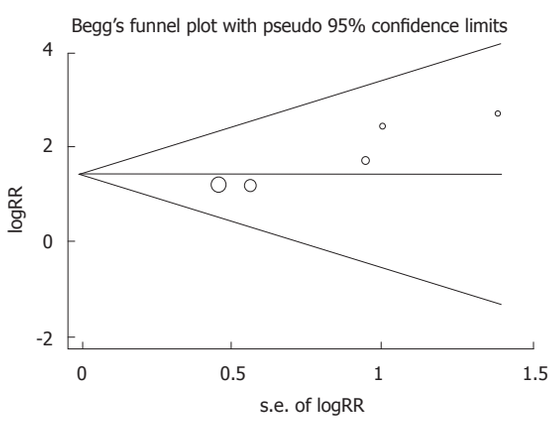


Figure 1 Begg's funnel plot of publication bias.

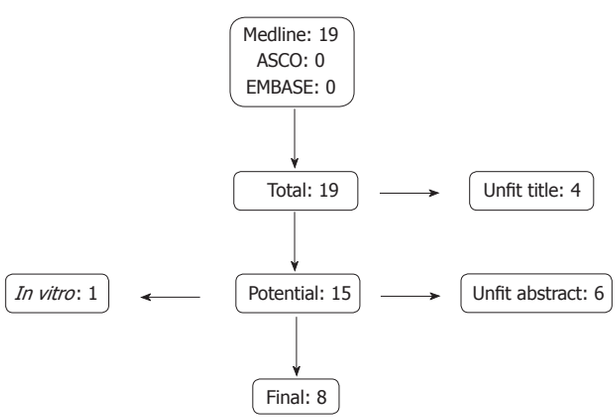
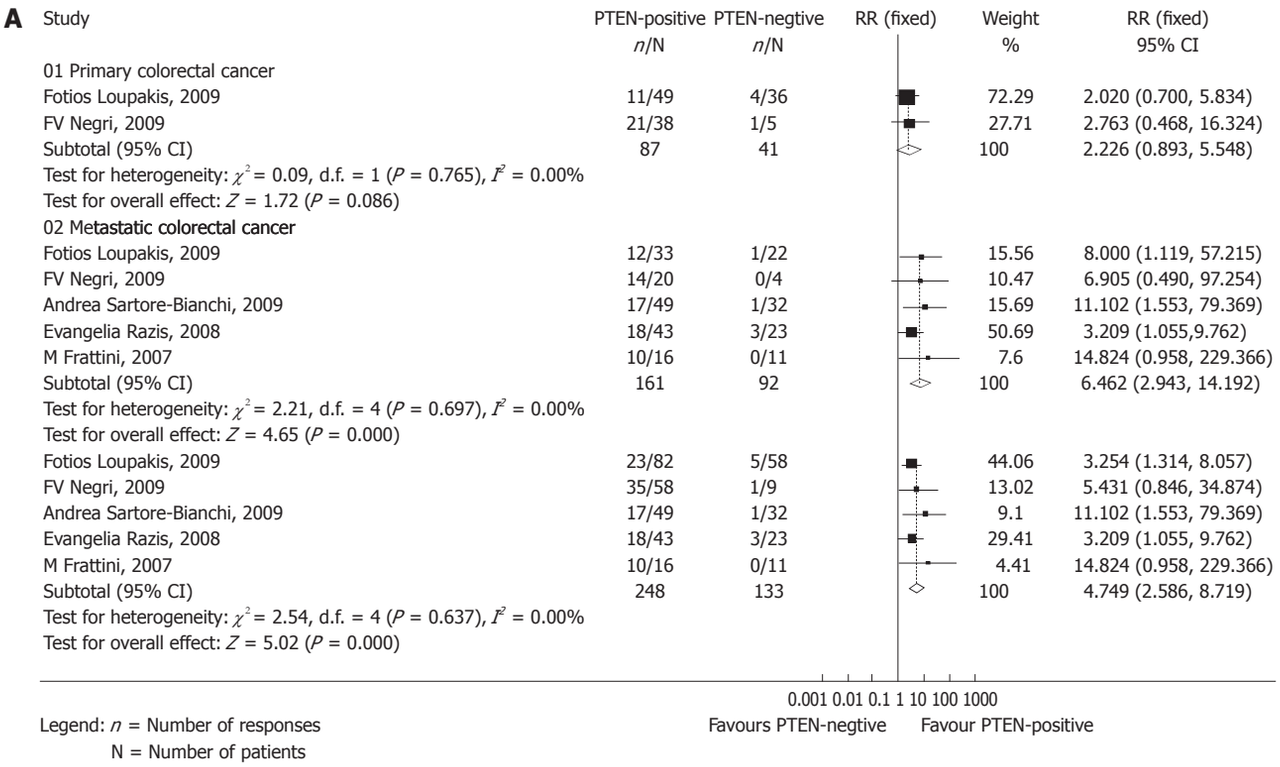


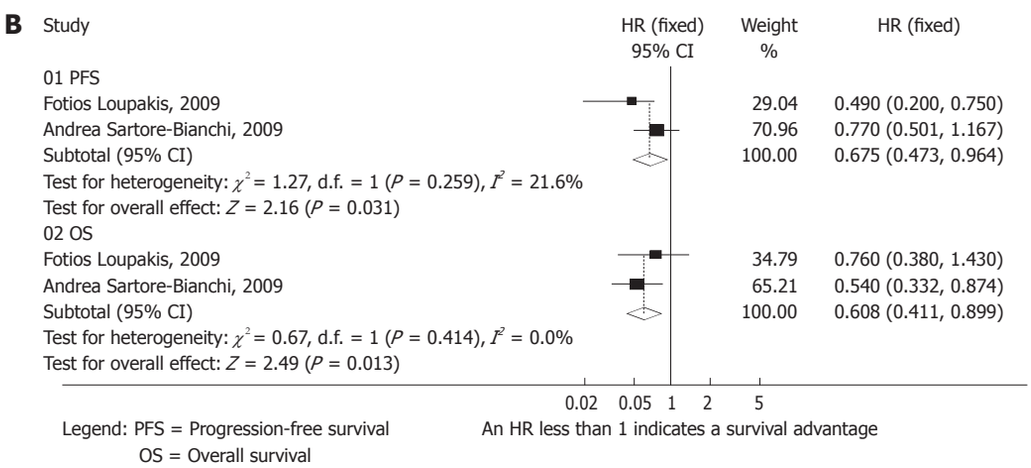
Figure 2 Selection of the studies.

mor criteria, PFS and OS. Patients with stable disease or progression of disease were defined as non-responders. Results are presented for the comparisons with the available data.

Analysis of status of the PTEN homolog and objective response

Five articles documented the response rate of cetuximab-based therapy (Figure 3A). There were 266 patients with loss of PTEN and 496 patients with normal expression of PTEN. In total, compared with 20 of 266 patients with loss of PTEN, 206 of 496 patients with intact protein expression had an objective response rate to cetuximab-based therapy (RR, 4.75; 95% CI, 2.59-8.72; $P < 0.001$). There was no heterogeneity between trials ($P = 0.637$, $I^2 = 0.0\%$). We also analyzed the response to cetuximab-based therapy in metastatic and primary colorectal tumors. Cetuximab-based therapy achieved significantly higher RR among patients with PTEN expression for metastatic tumors (RR, 6.46; 95% CI, 2.94-14.19; $P < 0.001$). In contrast, among 128 assessable primary tumors, 32 of 87 PTEN-positive and 5 of 41 PTEN-negative patients were





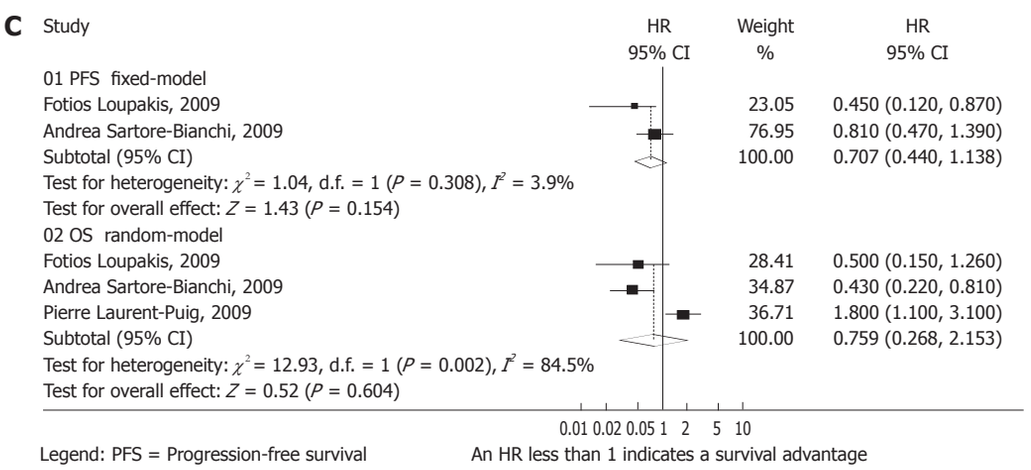


Figure 3 Analysis of status of the phosphatase and tensin homolog homolog. A: Analysis of status of the phosphatase and tensin homolog (PTEN) homolog and objective response; B: Analysis of status of the PTEN homolog and survival; C: Combined analysis of the PTEN homolog and Kirsten rat sarcoma 2 viral oncogene homolog (KRAS) status and survival. RR: Risk ratio; CI: Confidence interval; HR: Hazard ratio; OS: Overall survival; PFS: Progression-free survival.

responders, and there was no significant difference observed (RR, 2.226; 95% CI, 0.893-5.548; $P = 0.086$). There was no evidence for heterogeneity between the studies ($P = 0.697$, $I^2 = 0.0\%$; $P = 0.765$, $I^2 = 0.0\%$; respectively).

Analysis of status of the phosphatase and tensin homolog and survival

Only two trials involving 170 patients were included in this comparison, because none of the other eligibility criteria had sufficient follow-up data listed (Figure 3B). The HR summarizes survival for PTEN-positive compared with PTEN-negative patients after cetuximab-based therapy, with an HR of less than 1 indicating a survival advantage for expression of PTEN in colorectal tumors. As for PFS, PTEN positivity was associated with better survival (HR, 0.675; 95% CI, 0.473-0.964; $P = 0.031$). The analysis for OS confirmed that loss of PTEN was significantly associated with poor clinical outcome (HR, 0.608; 95% CI, 0.411-0.899; $P = 0.013$). There was no significant inter-trial heterogeneity for the end points of PFS ($P = 0.259$, $I^2 = 21.6\%$) or OS ($P = 0.414$, $I^2 = 0.0\%$).

Combined analysis of the PTEN homolog and KRAS status and survival

The studies selected for this analysis are listed in Figure 3C. The HR summarizes survival for PTEN-positive/wtKRAS *vs* PTEN-negative/wtKRAS, with an HR of less than 1 indicating a survival advantage for PTEN-positive/wtKRAS. Overall, among patients with KRAS wild-type status, PTEN positivity did not predict a longer PFS or OS (PFS: HR, 0.707; 95% CI, 0.440-1.138; $P = 0.154$; OS: HR, 0.943; 95% CI, 0.646-1.377; $P = 0.761$). Heterogeneity was not found among trials for the analysis of PFS ($P = 0.308$, $I^2 = 3.9\%$). However, there was marked inter-group heterogeneity for the combined analysis of OS making it difficult to obtain a clear conclusion ($P = 0.002$, $I^2 = 84.5\%$). To adjust for this bias, the trim-and-fill method was implemented. The adjusted estimates for OS were obtained by using the random-effects model (HR, 0.759; 95% CI, 0.268-2.153; $P = 0.604$). In the results from the data, there was no difference between the fixed-effects and the random-effects model, indicating the reliability of this meta-analysis, so we can reach a real conclusion.

DISCUSSION

Nowadays, there is a trend towards individualized treatment in tumor therapy. The optimized application of cetuximab has paved a way for individualized treatment of CRC[24]. In recent years, cetuximab has been widely used in the patients with mCRC, and most of the patients have better prognosis than those treated with combined chemotherapy alone. However, personalized cancer medication is based on the genetics of individual colorectal tumors[24]. Hence, the effects of molecular alterations, especially the activating mutations in the KRAS protein, and the corresponding therapeutic effect of cetuximab have been widely discussed[25-27]. KRAS mutation testing is used in the setting of EGFR-targeted therapy for metastatic disease worldwide[28]. Nevertheless, an intact KRAS is necessary but not sufficient to obtain benefit from EGFR inhibition[29-32]. Alterations in other downstream effectors of EGFR, such as BRAF and PIK3CA/PTEN have been found to give rise to cetuximab resistance[1,33]. Therefore, there is a deep need to reveal possible interactions between targeted agents, so that we can better select patients likely to respond to cetuximab-based treatment[28,29,34].

In this study, we focused on the association between the alteration of PTEN protein expression and the therapeutic effects of cetuximab in CRC patients. In addition, patients treated with panitumumab were also listed in the study search, because the two EGFR inhibitors, cetuximab (the chimeric IgG1 monoclonal antibody) and panitumumab (the humanized IgG2 monoclonal antibody), are currently approved in medication for CRC[34,35]. Both of the molecules bind to the EGFR, leading to inhibition of its downstream signaling and providing some clinical benefit.

PTEN is a tumor suppressor protein, which works as a negative regulator of PI3K/PTEN/Akt, which is a cell-survival signaling pathway[36]. Loss of PTEN expression was associated with the aggressive capacity of CRC, and that understanding the biologic mechanisms responsible for regulation of PTEN expression may allow better translational treatment of CRC patients. Furthermore, CRC patients with loss of PTEN expression show resistance to cetuximab[1].

In our selected studies, patients with normal PTEN expression had higher RR in all CRC with cetuximab-based therapy (especially in mCRC). Also we revealed that patients with PTEN normal expression with cetuximab treatment have better prognosis than those without cetuximab treatment and statistical analysis (OS and PFS) also presents significant differences ($P < 0.05$). In these studies we concluded that PTEN be proposed as an independent predictive factor[1] of cetuximab efficacy. We suggested that PTEN could help to predict prognosis and efficacy of cetuximab. Diagnostic evaluation of PTEN expression might provide additional guidelines for the treatment strategies for CRC patients and valuable prognostic information.

On the other hand, we did a combined analysis of PTEN and KRAS status on OS and PFS. Unfortunately, among patients with wild-type KRAS, PTEN positivity did not predict longer PFS and OS. Only one report showed the interaction between KRAS mutations with or without expression of PTEN in CRC. Thus, we could not perform a meta-analysis. The conclusion obtained in the report was that the PFS and OS of PTEN-positive patients with KRAS mutations were not significantly longer than in all other patients who presented with KRAS mutations and were PTEN-negative[10]. Survival analyses by Loupakis *et al*[10] demonstrated that BRAF mutations (HR, 3.75; $P = 0.015$) but not PIK3CA mutations (HR, 1.20; $P = 0.672$), were significantly associated with decreased OS, whereas neither of these alterations was significantly

associated with PFS. Further clinical data are necessary to identify a certain genes-alteration signature to predict the therapeutic effects of cetuximab-based therapy.

There are some limitations in this meta-analysis. First, the numbers of published studies were not adequate for a comprehensive analysis. Second, only 3 trials reported data of PFS and OS, and a lack of the original data in some studies limited our evaluation of survival, which may cause serious confounding bias. Third, although significant heterogeneity in some end-point variables were at least partly overcome by random-effects analysis, there was still heterogeneity between the relevant studies for inclusion, which may have affected the final results.

In conclusion, our meta-analysis showed an important role of PTEN status in determining the application of cetuximab-based targeted therapy. More clinical trials are warranted in this field to obtain more accurate results. Further improvement in the tailoring of EGFR targeted therapies needs more studies on molecular dissection of the EGFR-initiated oncogenic signaling cascade.

COMMENTS

Background

Cetuximab as a monoclnonal antibody (mAb) that has been used in colorectal cancer (CRC) patients. However, the responses vary in different individuals. Phosphatase and tensin homolog (PTEN) is an important negative regulator and its downregulation has been found in many CRC patients. The relationship between PTEN expression and the effects of cetuximab in CRC patients is still uncertain. The aim of this meta-analysis was to obtain a correlation.

Research frontiers

Cetuximab is a mAb that targets the epidermal growth factor receptor (EGFR). It binds to EGFR *via* its ligand-binding domain to inhibit the activation of EGRF signaling. Cetuximab has been reported to achieve a response rate of 10% as a single agent and of 23%-25% in combination chemotherapy. PTEN is an important negative regulator of cell-survival signaling and underexpression of PTEN confers resistance to cetuximab-induced apoptosis.

Innovations and breakthroughs

Many studies have reported the clinical outcomes of cetuximab in CRC patients with loss expression of PTEN. An exact conclusion has not been achieved mainly because of the limitation of sample size. This is the first study to report the relation between the expression of PTEN and prognosis of CRC patients treated with cetuximab.

Applications

This study may be helpful for adopting appropriate target therapy of cetuximab in patients with CRC.

Terminology

PTEN is the tumor suppressor phosphatase and tensin homolog that plays as an important negative regulator of cell-survival signaling.

Peer review

This is an interesting manuscript presenting a systematic analysis of the impact of PTEN expression on CRC response to cetuximab. It has clear limitations due to the quality of published papers. However, its findings are interesting.

REFERENCES

1 **Sawai H**, Yasuda A, Ochi N, Ma J, Matsuo Y, Wakasugi T, Takahashi H, Funahashi H, Sato M, Takeyama H. Loss of PTEN expression is associated with colorectal cancer liver metastasis and poor patient survival. *BMC Gastroenterol* 2008; **8**: 56

2 **Folprecht G**, Gruenberger T, Bechstein WO, Raab HR, Lordick F, Hartmann JT, Lang H, Frilling A, Stoehlmacher J, Weitz J, Konopke R, Stroszczynski C, Liersch T, Ockert D, Herrmann T, Goekkurt E, Parisi F, Köhne CH. Tumour response and secondary resectability of colorectal liver metastases following neoadjuvant chemotherapy with cetuximab: the CELIM randomised phase 2 trial. *Lancet Oncol* 2010; **11**: 38-47

3 **Sartore-Bianchi A**, Martini M, Molinari F, Veronese S, Nichelatti M, Artale S, Di Nicolantonio F, Saletti P, De Dosso S, Mazzucchelli L, Frattini M, Siena S, Bardelli A. PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies. *Cancer Res* 2009; **69**: 1851-1857

4 **Amado RG**, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD, Chang DD. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 2008; **26**: 1626-1634

5 **Negri FV**, Bozzetti C, Lagrasta CA, Crafa P, Bonasoni MP, Camisa R, Pedrazzi G, Ardizzoni A. PTEN status in advanced colorectal cancer treated with cetuximab. *Br J Cancer* 2010; **102**: 162-164

6 **Hebbar M**, Wacrenier A, Desauw C, Romano O, Cattan S, Triboulet JP, Pruvot FR. Lack of usefulness of epidermal growth factor receptor expression determination for cetuximab therapy in patients with colorectal cancer. *Anticancer Drugs* 2006; **17**: 855-857

7 **Assenat E**, Desseigne F, Thezenas S, Viret F, Mineur L, Kramar A, Samalin E, Portales F, Bibeau F, Crapez-Lopez E, Bleuse JP, Ychou M. Cetuximab plus FOLFIRINOX (ERBIRINOX) as first-line treatment for unresectable metastatic colorectal cancer: a phase II trial. *Oncologist* 2011; **16**: 1557-1564

8 **Gerber DE**, Choy H. Cetuximab in combination therapy: from bench to clinic. *Cancer Metastasis Rev* 2010; **29**: 171-180

9 **Laurent-Puig P,** Cayre A, Manceau G, Buc E, Bachet JB, Lecomte T, Rougier P, Lievre A, Landi B, Boige V, Ducreux M, Ychou M, Bibeau F, Bouché O, Reid J, Stone S, Penault-Llorca F. Analysis of PTEN, BRAF, and EGFR status in determining benefit from cetuximab therapy in wild-type KRAS metastatic colon cancer. *J Clin Oncol* 2009; **27:** 5924-5930

10 **Loupakis F**, Pollina L, Stasi I, Ruzzo A, Scartozzi M, Santini D, Masi G, Graziano F, Cremolini C, Rulli E, Canestrari E, Funel N, Schiavon G, Petrini I, Magnani M, Tonini G, Campani D, Floriani I, Cascinu S, Falcone A. PTEN expression and KRAS mutations on primary tumors and metastases in the prediction of benefit from cetuximab plus irinotecan for patients with metastatic colorectal cancer. *J Clin Oncol* 2009; **27**: 2622-2629

11 **Van Cutsem E**, Köhne CH, Hitre E, Zaluski J, Chang Chien CR, Makhson A, D'Haens G, Pintér T, Lim R, Bodoky G, Roh JK, Folprecht G, Ruff P, Stroh C, Tejpar S, Schlichting M, Nippgen J, Rougier P. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med* 2009; **360**: 1408-1417

12 **Di Cristofano A**, Pandolfi PP. The multiple roles of PTEN in tumor suppression. *Cell* 2000; **100**: 387-390

13 **Moroni M**, Veronese S, Benvenuti S, Marrapese G, Sartore-Bianchi A, Di Nicolantonio F, Gambacorta M, Siena S, Bardelli A. Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study. *Lancet Oncol* 2005; **6**: 279-286

14 **Popat S**, Matakidou A, Houlston RS. Thymidylate synthase expression and prognosis in colorectal cancer: a systematic review and meta-analysis. *J Clin Oncol* 2004; **22**: 529-536

15 **DerSimonian R**, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177-188

16 **Demets DL**. Methods for combining randomized clinical trials: strengths and limitations. *Stat Med* 1987; **6**: 341-350

17 **Higgins JP**, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. *BMJ* 2003; **327**: 557-560

18 **Ibrahim EM**, Zekri JM, Bin Sadiq BM. Cetuximab-based

therapy for metastatic colorectal cancer: a meta-analysis of the effect of K-ras mutations. *Int J Colorectal Dis* 2010; **25**: 713-721
19 **Song F**, Gilbody S. Bias in meta-analysis detected by a simple, graphical test. Increase in studies of publication bias coincided with increasing use of meta-analysis. *BMJ* 1998; **316**: 471
20 **Frattini M**, Saletti P, Romagnani E, Martin V, Molinari F, Ghisletta M, Camponovo A, Etienne LL, Cavalli F, Mazzucchelli L. PTEN loss of expression predicts cetuximab efficacy in metastatic colorectal cancer patients. *Br J Cancer* 2007; **97**: 1139-1145
21 **Perrone F**, Lampis A, Orsenigo M, Di Bartolomeo M, Gevorgyan A, Losa M, Frattini M, Riva C, Andreola S, Bajetta E, Bertario L, Leo E, Pierotti MA, Pilotti S. PI3KCA/PTEN deregulation contributes to impaired responses to cetuximab in metastatic colorectal cancer patients. *Ann Oncol* 2009; **20**: 84-90
22 **Razis E,** Briasoulis E, Vrettou E, Skarlos DV, Papamichael D, Kostopoulos I, Samantas E, Xanthakis I, Bobos M, Galanidi E, Bai M, Gikonti I, Koukouma A, Kafiri G, Papakostas P, Kalogeras KT, Kosmidis P, Fountzilas G. Potential value of PTEN in predicting cetuximab response in colorectal cancer: an exploratory study. *BMC Cancer* 2008; **8:** 234
23 **Sartore-Bianchi A**, Di Nicolantonio F, Nichelatti M, Molinari F, De Dosso S, Saletti P, Martini M, Cipani T, Marrapese G, Mazzucchelli L, Lamba S, Veronese S, Frattini M, Bardelli A, Siena S. Multi-determinants analysis of molecular alterations for predicting clinical benefit to EGFR-targeted monoclonal antibodies in colorectal cancer. *PLoS One* 2009; **4**: e7287
24 **Bardelli A**, Siena S. Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. *J Clin Oncol* 2010; **28**: 1254-1261
25 **Parsons DW**, Wang TL, Samuels Y, Bardelli A, Cummins JM, DeLong L, Silliman N, Ptak J, Szabo S, Willson JK, Markowitz S, Kinzler KW, Vogelstein B, Lengauer C, Velculescu VE. Colorectal cancer: mutations in a signalling pathway. *Nature* 2005; **436**: 792
26 **Li FH**, Shen L, Li ZH, Luo HY, Qiu MZ, Zhang HZ, Li YH, Xu RH. Impact of KRAS mutation and PTEN expression on cetuximab-treated colorectal cancer. *World J Gastroenterol* 2010; **16**: 5881-5888
27 **Petrelli F**, Borgonovo K, Cabiddu M, Ghilardi M, Barni S. Cetuximab and panitumumab in KRAS wild-type colorectal cancer: a meta-analysis. *Int J Colorectal Dis* 2011; **26**: 823-833
28 **De Roock W**, Biesmans B, De Schutter J, Tejpar S. Clinical biomarkers in oncology: focus on colorectal cancer. *Mol Diagn Ther* 2009; **13**: 103-114
29 **Silvestris N**, Tommasi S, Petriella D, Santini D, Fistola E, Russo A, Numico G, Tonini G, Maiello E, Colucci G. The dark side of the moon: the PI3K/PTEN/AKT pathway in colorectal carcinoma. *Oncology* 2009; **77** Suppl 1: 69-74
30 **Meriggi F**, Di Biasi B, Abeni C, Zaniboni A. Anti-EGFR therapy in colorectal cancer: how to choose the right patient. *Curr Drug Targets* 2009; **10**: 1033-1040
31 **Bouchahda M**, Karaboué A, Saffroy R, Innominato P, Gorden L, Guettier C, Adam R, Lévi F. Acquired KRAS mutations during progression of colorectal cancer metastases: possible implications for therapy and prognosis. *Cancer Chemother Pharmacol* 2010; **66**: 605-609
32 **Moosmann N**, von Weikersthal LF, Vehling-Kaiser U, Stauch M, Hass HG, Dietzfelbinger H, Oruzio D, Klein S, Zellmann K, Decker T, Schulze M, Abenhardt W, Puchtler G, Kappauf H, Mittermüller J, Haberl C, Schalhorn A, Jung A, Stintzing S, Heinemann V. Cetuximab plus capecitabine and irinotecan compared with cetuximab plus capecitabine and oxaliplatin as first-line treatment for patients with metastatic colorectal cancer: AIO KRK-0104--a randomized trial of the German AIO CRC study group. *J Clin Oncol* 2011; **29**: 1050-1058
33 **Souglakos J**, Philips J, Wang R, Marwah S, Silver M, Tzardi M, Silver J, Ogino S, Hooshmand S, Kwak E, Freed E, Meyerhardt JA, Saridaki Z, Georgoulias V, Finkelstein D, Fuchs CS, Kulke MH, Shivdasani RA. Prognostic and predictive value of common mutations for treatment response and survival in patients with metastatic colorectal cancer. *Br J Cancer* 2009; **101**: 465-472
34 **Ortega J**, Vigil CE, Chodkiewicz C. Current progress in targeted therapy for colorectal cancer. *Cancer Control* 2010; **17**: 7-15
35 **Saadeh CE**, Lee HS. Panitumumab: a fully human monoclonal antibody with activity in metastatic colorectal cancer. *Ann Pharmacother* 2007; **41**: 606-613
36 **Kim JG**, Chae YS, Sohn SK, Kang BW, Moon JH, Lee SJ, Jeon SW, Park JS, Park JY, Choi GS. Clinical significance of genetic variations in the PI3K/PTEN/AKT/mTOR pathway in Korean patients with colorectal cancer. *Oncology* 2010; **79**: 278-282

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BRIEF ARTICLE

Aberrant methylation and downregulation of *sall3* in human hepatocellular carcinoma

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Abstract

AIM: To investigated whether *sall3* transcription was regulated by promoter CpG island hypermethylation in hepatocellular carcinoma (HCC).

METHODS: The cell lines Huh7, HepG2, SK-HEP1, SMMC7721, Bel7402, QGY7703 and a cohort of 38 HCC tissue specimens and corresponding nontumorous tissues were subjected to analysis for *sall3* promoter CpG island methylation and mRNA transcription. *sall3* promoter CpG island methylation levels were determined using the MassARRAY platform and mRNA transcription levels of the gene were detected by quantitative real-time polymerase chain reaction.

RESULTS: The levels of *sall3* mRNA were decreased by more than twofold in 33 of 38 tumor tissues compared to adjacent noncancerous tissues. Among these 33 tumor tissues with lower levels of *sall3* mRNA, 24 showed higher levels of methylation. Based on these results, we hypothesized that the decrease in *sall3* mRNA transcription level was likely due to promoter CpG island hypermethylation. Changes in *sall3* mRNA transcription and promoter CpG island methylation were determined in the above six cell lines after treatment with 0, 0.1, 0.5 and 2.5 μmol 5-aza-2-deoxycytidine, a demethylating agent. Promoter CpG island methylation levels decreased in a dose-dependent manner in all six cell lines, while the mRNA transcription level increased dose-dependently in Huh7, HepG2, SK-HEP1 and SMMC7721 cells and irregularly in Bel7402 and QGY7703 cells.

CONCLUSION: These results indicated that promoter CpG island hypermethylation contributes to the down-regulation of *sall3* mRNA transcription in HCC.

Key words: Hepatocellular carcinoma; *sall3*; Aberrant methylation; Down regulation mRNA transcription

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, representing a major public health issue, especially in Asia[1]. The pathogenesis of HCC involves chronic hepatitis virus infection and activation of oncogenes and/or inactivation of tumor suppressor genes by mutations and epigenetic modification[2]. Epigenetic inactivation of tumor suppressor genes by DNA hypermethylation plays an important role in carcinogenesis[3]. Many groups have reported that promoter hypermethylation of CpG islands is associated with development, stage, recurrence, progression and survival in HCC[4,5]. In many cases, aberrant methylation of promoter regions within genes is correlated with a loss of gene expression[6]. Furthermore, in contrast to mutations, epigenetic changes may be reversible, raising the possibility of developing therapeutics based on restoring a normal epigenetic state in cancer-associated genes[7,8].

sal was originally identified as a region-specific homeotic gene in *Drosophila*[9]. *sall3* is one of four mammalian members of the sal-like (sall) gene family (*sall1*, *sall2*, *sall3* and *sall4*), which are involved in embryonic development[10]. *sall3* is one of several genes deleted in 18q deletion syndrome, characterized by hearing loss, mental retardation, midfacial hypoplasia, delayed growth, and limb abnormalities[11]. Loss of the *sall3* gene leads to palate deficiency, abnormalities in cranial nerves, and perinatal lethality[12]. Recently, it was reported that sall3 can interact with DNMT3A and shows the ability to inhibit CpG island methylation in HCC[13]. However, when scanning the nucleotide sequences of *sall3*, we found a CpG island in the promoter. It has been reported that *sall3* gene methylation levels are significantly increased in bladder cancer compared to nontumorous controls, and may be a new biomarker for the sensitive and specific detection of bladder cancer[14]. Furthermore, it has been reported that the *sall3* gene CpG island has a higher frequency of hypermethylation in HCC tumors compared with adjacent noncancerous tissues as determined by a qualitative methylation method[15]. However, the decreased *sall3* mRNA transcription levels in human HCC tissues and whether this is caused by promoter CpG island hypermethylation have not been fully examined.

Here, we show that sall3 mRNA transcription was downregulated in most (33/38) tumor tissues examined compared with adjacent noncancerous tissues. Most (24/33) downregulation of mRNA transcription was strongly associated with hypermethylation of the promoter CpG island. This association was further confirmed by subsequent cell line experiments; treatment of the cell lines with the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine reversed promoter CpG island hypermethylation and restored *sall3* mRNA transcription. These results indicated that promoter CpG island hypermethylation is the main reason for the downregulation of *sall3* mRNA transcription in HCC.

MATERIALS AND METHODS

Tissue specimens and cell lines

Thirty-eight paired clinical samples of HCC, including tumor tissues and adjacent noncancerous tissues, were collected from surgical specimens at the Department of Hepatobiliary Surgery, Nanfang Hospital (16 cases), and the Cancer Institute of Sun Yat-sen University (22 cases), both in Guangzhou, China. All specimens were obtained immediately after surgical resection and were stored at -70 ℃ until DNA/RNA extraction.

Written informed consent was obtained from all patients prior to inclusion in the study. The study protocol was approved by the Nanfang Hospital Ethics Committee at Southern Medical University and the Sun Yat-sen Cancer Center Ethics Committee at Sun Yat-sen University.

Cell culture and 5-aza-CdR treatment

Six HCC cell lines (Huh7, HepG2, SMMC-7721, Bel-7402, SK-HEP1, QGY7703) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin and incubated in a 5% CO_2 atmosphere at 37 ℃. The demethylating agent, 5-aza-2-deoxycytidine (5-aza-CdR; Sigma, St. Louis, MO), was freshly prepared in ddH_2O. HepG2 cells (3×10^5 cells/well) and other hepatoma cells (1×10^5 cells/well) in exponential growth phase were seeded in 6-well plates. After 24 h of culture, cells were treated with 5-aza-CdR at 0, 0.1, 0.5 and 2.5 mol for 3 d. The culture medium was replaced every 24 h with fresh media containing 5-aza-CdR. Total RNA and genomic DNA were extracted for real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and DNA methylation level analysis.

Detection of sall3 CpG Island DNA hypermethylation

Genomic DNA was extracted from cells and HCC samples using a QIAamp DNA Minikit (Qiagen, Valencia, CA). Genomic DNA (1 μg) was modified with sodium bisulfite using the EZ DNA methylation kit (Zymo Research, Orange, CA). DNA methylation levels of clinical samples and cell lines were determined using the MassARRAY platform (Sequenom, San Diego, CA) as described previously[16]. Briefly, two fragments covering 38 CpG sites from *sall3* were amplified from bisulfite-modified DNA. A 10-mer tag sequence was added to the forward primer, and a T7-promoter tag was added to the reverse primer to balance the PCR primer length. The primers used were 5'-AGGAAGAGAGGGATTGTTTGGATTTGATTTTAATTT-3' (sense) and 5'-CAGTAATACGACTCACTATAGGGAGAAGGCTCACAAATAACCTCCTAAAACTTCCC-3' (antisense); 5'-AGGAAGAGAGTTTTAAGGTTGGTTTTATTTTGTTT-3' (sense) and 5'-CAGTAATACGACTCACTATAGGGAGAAGGCTTCTCAAAAATAATCTCAAACCCCTA-3' (antisense). Methylation data for individual units (1-3 CpG sites per unit) were analyzed using the EpiTyper software (Sequenom).

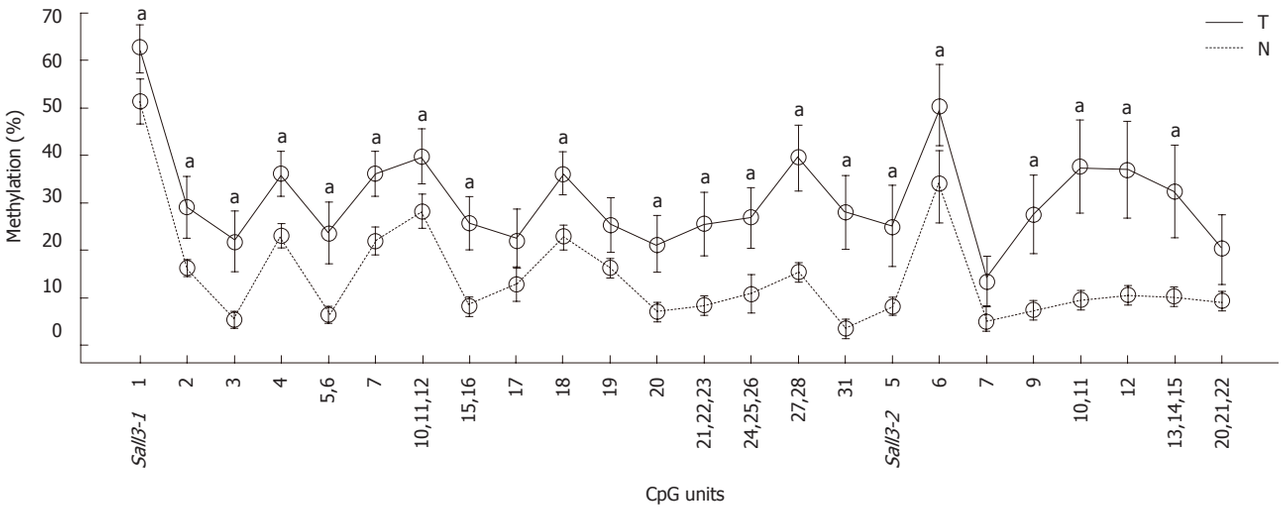


Figure 1 Average methylation levels were calculated from 38 tumors (T) and adjacent non-cancerous tissues (N) on 24 CpG units from *sall3* CpG island respectively. The data was analyzed by Wilcox rank sum test ($^aP < 0.05$ *vs* non-cancerous tissues). Error bar, 95% confidence interval.

RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from cell lines and tissue samples using the Trizol reagent (Invitrogen), according to the manufacturer's protocol. First-strand cDNA was generated using a SYBR PrimeScript RT-PCR Kit (TaKaRa, Kyoto, Japan). *sall3* mRNA expression was detected by qRT-PCR using a SYBR Premix Ex *Taq* Kit (TaKaRa) on an ABI 7500 Real-Time PCR System (Applied Biosystems). β-actin was used as an internal control. The primers used were as follows: *sall3* forward primer: 5'-GCTGCCTTCTCAGTTATTTGACC-3', reverse primer: 5'-TGACCGTTCACTTCCATTTTGA-3'; β-actin forward primer: 5'-TTGTTACAGGAAGTCGCTTGCC-3', reverse primer: 5'-ATGCTATCACCTCCCCTGTGTGT-3'. Relative levels of *sall3* mRNA were calculated and expressed as $2^{-\Delta\Delta Ct}$[17].

Statistical analysis

qRT-PCR results in different groups were analyzed by Student's *t* test. The methylation levels of Oct-6 in HCC tumors and adjacent noncancerous tissues were compared using the Wilcox rank sum test. All tests were two-sided. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

RESULTS

sall3 promoter CpG island aberrant methylation and sall3 mRNA transcription in human HCC tissues

To determine whether *sall3* promoter CpG island hypermethylation changes leads to decreased *sall3* mRNA transcription in human HCC tissues, the methylation levels of the *sall3* promoter CpG island in 38 HCC tumors and adjacent noncancerous tissues were examined using the MassARRAY platform (Sequenom). Of the 38 tumors, 27 (71%) showed higher methylation levels at the *sall3* CpG island compared with adjacent noncancerous tissue, while methylation levels were similar at the *sall3* CpG island in tumor tissue and adjacent noncancerous tissue in 11 cases (29%). None of the tumors showed lower methylation levels at the *sall3* CpG island compared with adjacent noncancerous tissues. Average methylation levels for each CpG unit in the 38 tumor tissues and adjacent noncancerous tissues are listed in Figure 1. Among the total of 24 CpG units (1-3 CpG sites per unit), the average methylation levels of 20 CpG units in 38 tumors were significantly higher than those in adjacent noncancerous tissues.

To determine whether aberrant CpG island DNA methylation in HCC tissues may be correlated with the decreased *sall3* mRNA transcription, *sall3* mRNA levels in 38 paired samples were determined by qRT-PCR (Figure 2). The relative ratio between methylation and mRNA expression levels of *sall3* are negatively correlated in 26 of 38 tumor tissues *vs* corresponding adjacent noncancerous tissues (Figure 3). Of the 38 tumor tissues, 33 (86.8%) showed a decreased *sall3* mRNA level compared to adjacent noncancerous tissues (Figure 2). Among these 33 tumor tissues with lower mRNA levels, 24 showed higher methylation levels and a negative association was found between CpG island methylation and mRNA expression (Figure 3); nine tumor tissues showed methylation levels that were not significantly different from adjacent noncancerous tissues. Of the 38 tumor tissues examined, three (7.9%) showed higher *sall3* mRNA expression than adjacent noncancerous tissues (Figure 2). Methylation level was similar to the adjacent noncancerous tissue in one of these three tumor tissues, while the methylation levels in the remaining two tumor tissues were higher than those in the adjacent noncancerous tissue. Of the 38 tumor tissues, two (5.3%) showed similar *sall3* mRNA expression to adjacent noncancerous tissues (Figure 2). Of these two tumor tissues, one showed no significant difference in methylation level compared with adjacent noncancerous tissues, while the methylation level in the other was higher than that the in the adjacent noncancer-

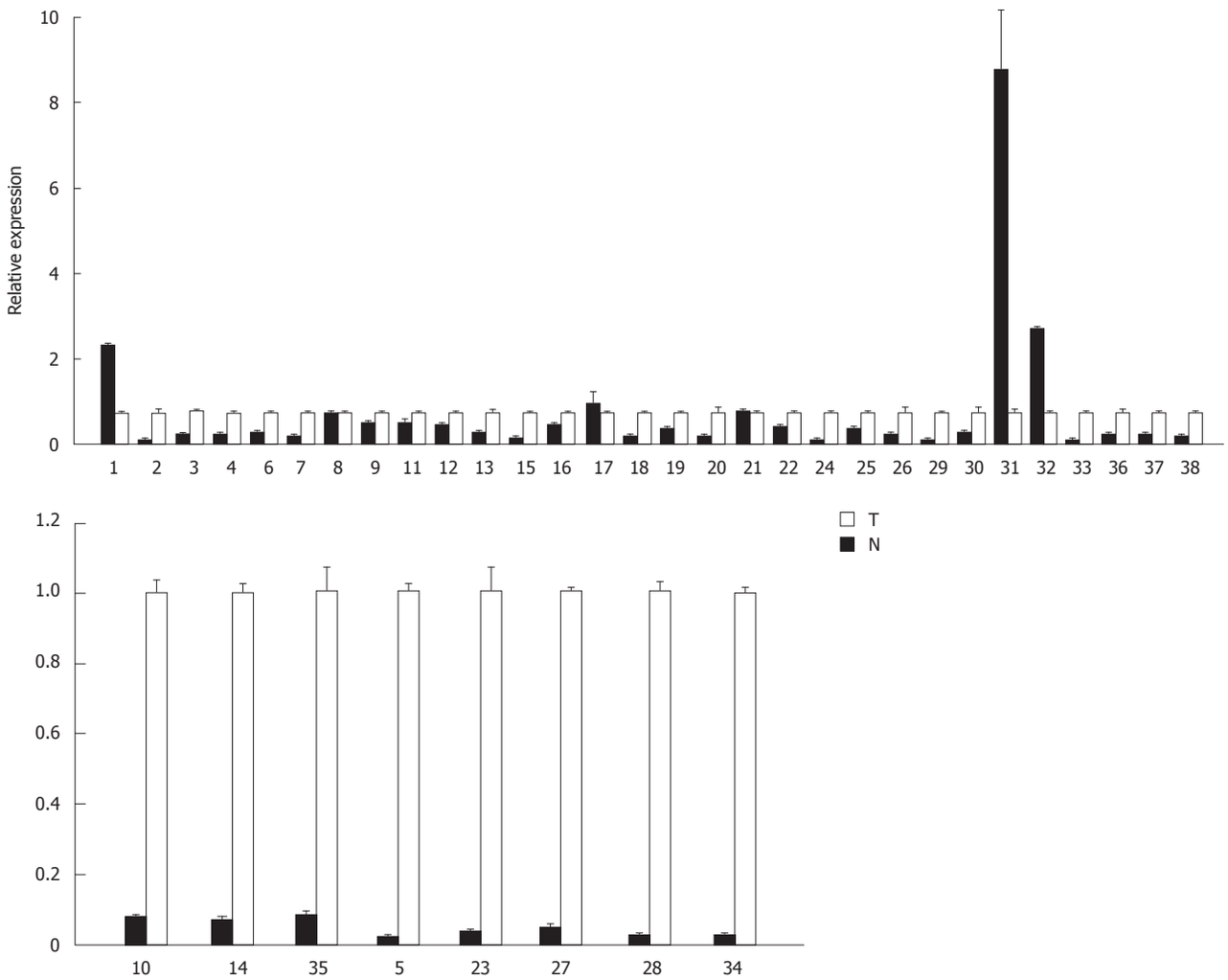


Figure 2 *sall3* mRNA is analyzed in 38 tumor tissues (T) and adjacent non-cancerous tissues (N). Error bars, SD from triplicates.

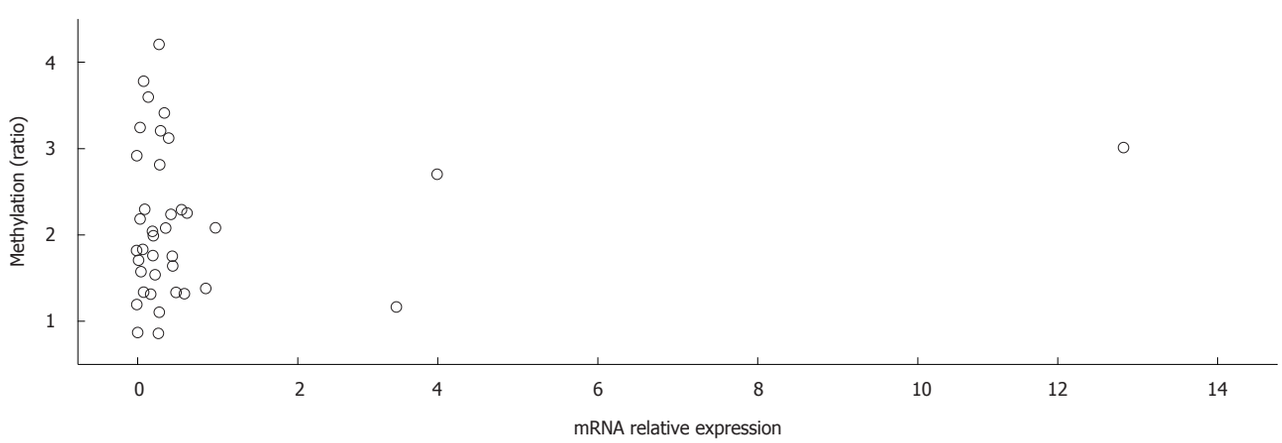
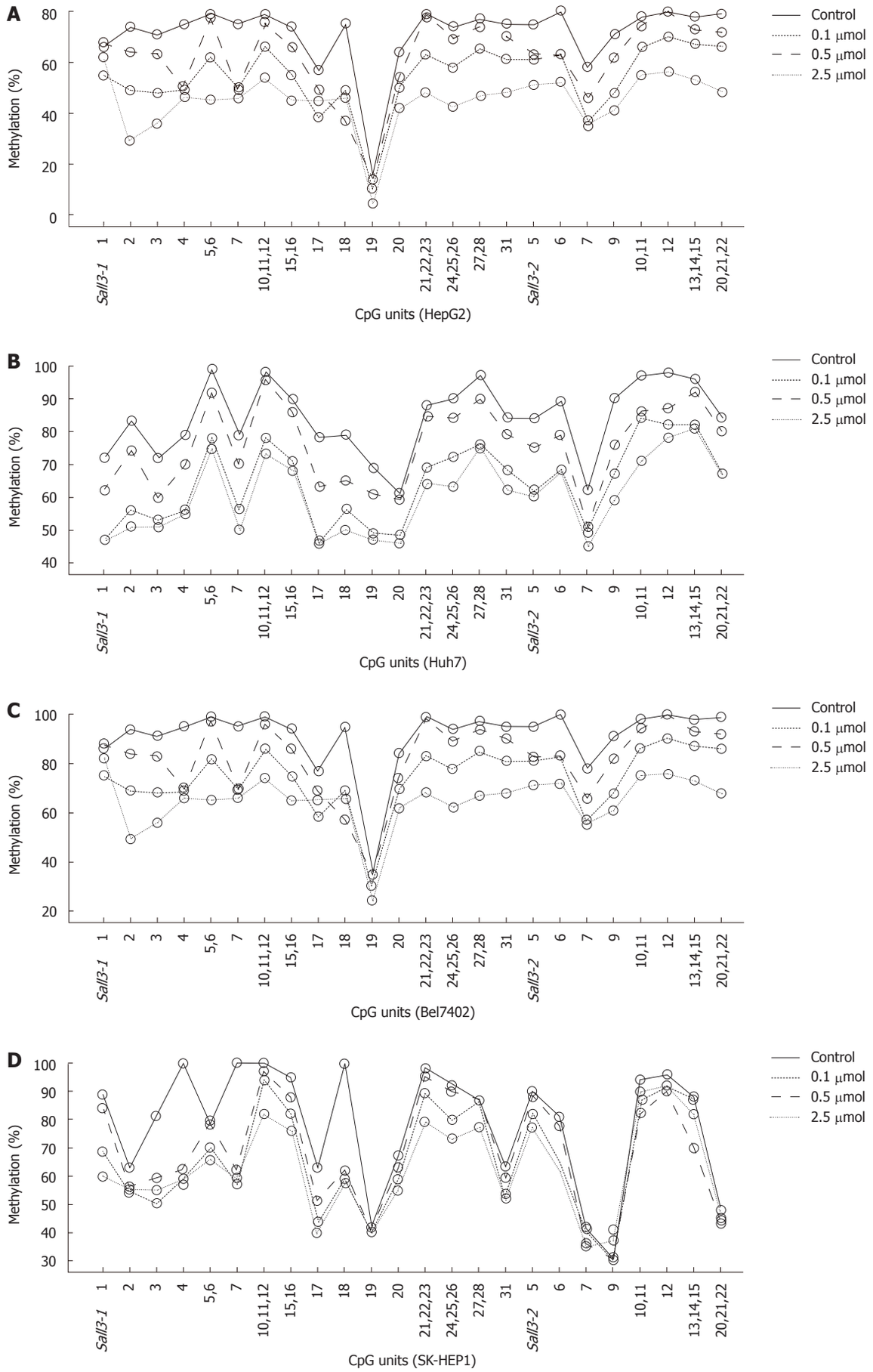


Figure 3 Correlation between methylation levels and mRNA expression of *sall3* in 24 tumor tissues and corresponding adjacent noncancerous tissues.

ous tissue. Together, these results indicated a negative correlation between *sall3* promoter CpG island hypermethylation and *sall3* mRNA transcription in 24 tumor tissues and their adjacent noncancerous tissues (Figure 3). *sall3* mRNA transcription in other tissues showed no association with *sall3* promoter CpG island hypermethylation, suggesting regulatory mechanisms other than those involved in HCC.

sall3 promoter CpG island hypermethylation correlates its mRNA transcription in human HCC cell lines

To determine whether decreased *sall3* mRNA transcrip-


A
Methylation (%)
Control
0.1 μmol
0.5 μmol
2.5 μmol
Sall3-1
Sall3-2
CpG units (HepG2)
B
CpG units (Huh7)
C
CpG units (Bel7402)
D
CpG units (SK-HEP1)

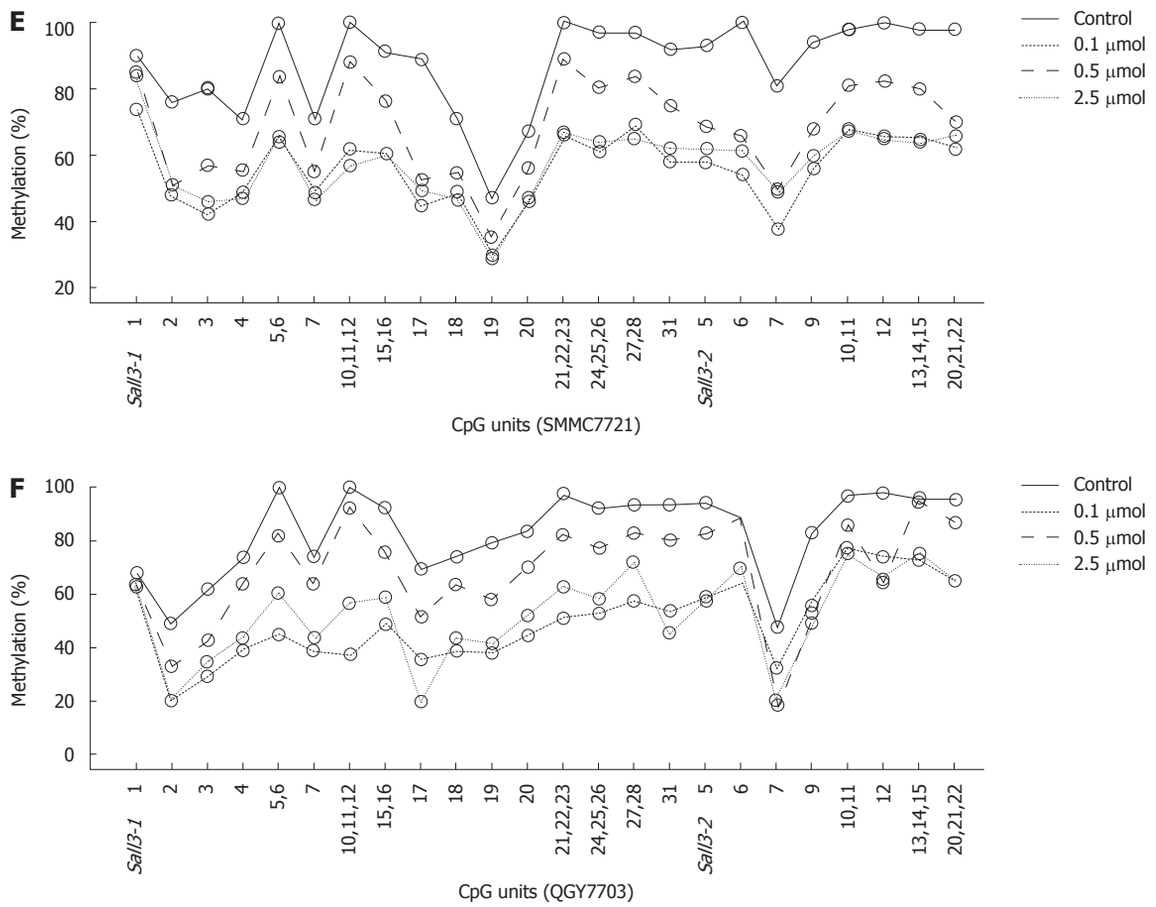


Figure 4 Quantitative methylation analysis on each CpG unit of sall3 CpG island in HCC cells after 5-Aza-CdR treatment or control (A-F).

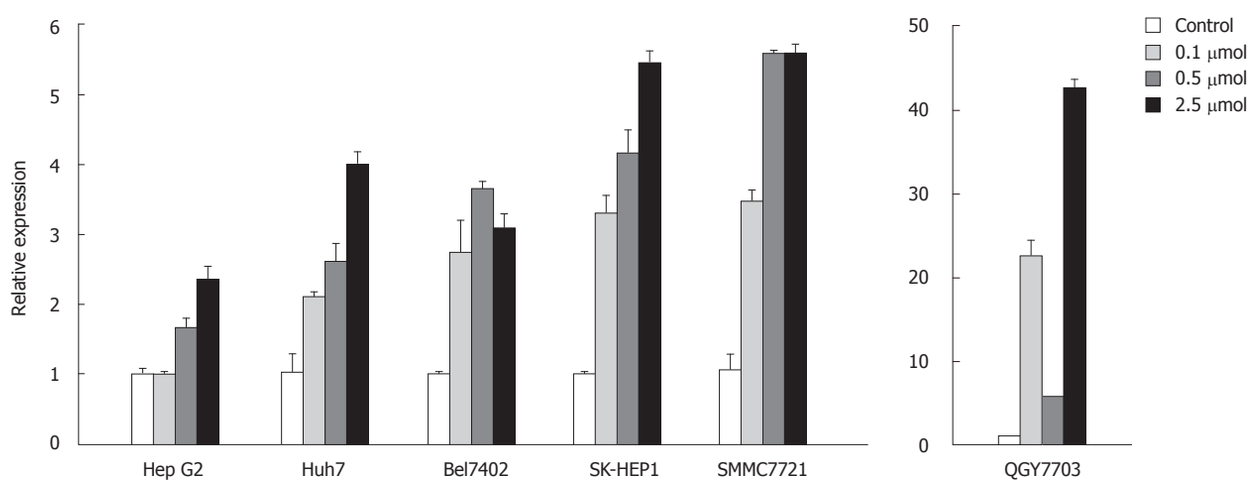


Figure 5 Relative sall3 expression in six hepatocellular carcinoma cells after 5-AZA-CdR treatment. Error bars, SD from triplicates.

tion levels in human HCC tissues are due to promoter CpG island hypermethylation, six human HCC cell lines (Huh7, HepG2, SK-HEP1, SMMC7721, Bel7402 and QGY7703) were exposed to 0, 0.1, 0.5 or 2.5 μmol 5-aza-CdR, an inhibitor of DNMTs, for 72 h. As expected, after treatment with 0.1, 0.5 or 2.5 μmol 5-aza-CdR, promoter CpG island methylation levels showed dose-dependent downregulation in all six cell lines (Figure 4). *sall3* mRNA levels also showed dose-dependent upregulation in Huh7, HepG2, SK-HEP1 and SMMC7721 cells, while it was irregularly upregulated in Bel7402 and QGY7703 cells (Figure 5). These data indicated that *sall3* promoter CpG island hypermethylation is likely responsible for the decrease in *sall3* mRNA transcription level.

DISCUSSION

HCC is one of the most common gastrointestinal ma-

lignancies, ranking fifth in the occurrence of common cancers, and third in common causes of cancer-related death[18]. Early-stage HCC is potentially curable with surgical resection or hepatic transplantation[19,20]. However, most patients present with advanced disease and are not amenable to surgical resection or transplantation. Therefore, they have a poor prognosis[21], and improved methods for early diagnosis are urgently required. DNA methylation is a gene expression regulatory mechanism and plays a fundamental role in carcinogenic processes[22,23]. Aberrant CpG island methylation of tumor-related genes is an early and frequent event in the process of carcinogenesis, and DNA methylation status of tumor-related genes is a potential diagnostic marker[24-26]. As it is possible to restore the function of methylated tumor suppressor genes, combinations of epigenetic modifiers and other therapeutic agents may also become a promising alternative to conventional treatments[27-29].

Although an increasing number of genes undergoing aberrant CpG island methylation have been reported in HCC[4,5,30,31], the methylated genes have not yet been fully characterized. Yu *et al*[14] reported that *sall3* was a novel target of aberrant methylation in bladder cancer. Xia *et al*[15] reported that the hypermethylation frequency in tumor tissues was significantly higher than those in adjacent noncancerous tissues, but whether the decreased transcription of *sall3* was caused by hypermethylation of the promoter CpG island in HCC remained unknown.

In the present study, we found that the levels of *sall3* mRNA were decreased in 33 of 38 tumor tissues compared with those in adjacent noncancerous tissues. Twenty-four of these 33 tumor tissues with reduced *sall3* mRNA levels showed elevated methylation levels, suggesting that the decreased *sall3* mRNA transcription levels were likely caused by promoter CpG island hypermethylation. We also confirmed that six HCC cell lines (Huh7, HepG2, SK-HEP1, SMMC7721, Bel7402 and QGY7703) were hypermethylated at the *sall3* promoter. Using the demethylating agent 5-aza-CdR, the transcription of *sall3* could be restored in these cells when the *sall3* promoter region was partially demethylated. Taken together with the results in HCC tissue samples, it is clear that promoter hypermethylation in *sall3* was strongly associated with the reduced transcription of *sall3* mRNA. However, in more than 25% of the HCC cases (9/33), decreased mRNA transcription was observed with similar CpG island methylation levels. In addition, there were three tumor tissues with promoter hypermethylation without decreased mRNA transcription compared with corresponding nontumorous tissues; the levels of mRNA transcription were increased in two of these cases and similar in the remaining one case. These results suggested that other regulatory mechanisms unrelated to promoter hypermethylation may also be involved. Further studies are needed to clarify this issue. However, the overall strong association between promoter hypermethylation and decreased mRNA transcription suggests a causative role of aberrant *sall3* promoter methylation and decreased mRNA transcription in most cases of HCC.

In conclusion, we showed that *sall3* mRNA transcription was downregulated in most (33/38) tumor tissues compared with adjacent noncancerous tissues in HCC. Most cases (24/33) with downregulated mRNA transcription were strongly associated with promoter CpG island hypermethylation. This association was further confirmed by subsequent experiments in cell lines; treatment of the cell lines Huh7, HepG2, SK-HEP1, SMMC7721, Bel7402 and QGY7703 with the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine reversed promoter CpG island hypermethylation and restored *sall3* mRNA transcription. These results indicated that promoter CpG island hypermethylation is the main reason for downregulation of *sall3* mRNA transcription in HCC. These finding regarding *sall3* mRNA transcription and DNA methylation associated with human HCC provide new insights into the pathogenesis of HCC and may serve as a powerful molecular marker for detecting HCC in biopsy tissues of HCC patients.

COMMENTS

Background

Inactivation of tumor suppressor genes by promoter CpG island hypermethylation plays a key role in cancer pathogenesis.

Research frontiers

Aberrant CpG island methylation of tumor-related genes is a early and frequent event in carcinogenetic process and DNA methylation status of tumor-related genes is a potential diagnostic marker. Using demethylating agents to restore the function of methylated tumor suppressor genes also becomes a promising alternative to conventional treatments.

Innovations and breakthroughs

sall3 mRNA transcription was downregulated in most tumor tissues compared with adjacent noncancerous tissues. Downregulation of mRNA transcription was strongly associated with hypermethylation of the promoter CpG island. This association was further confirmed by subsequent cell line experiments; treatment of the cell lines with the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine reversed promoter CpG island hypermethylation and restored sall3 mRNA transcription.

Applications

The finding of sall3 mRNA transcription and DNA methylation associated with human hepatocellular carcinoma (HCC) provide new insights into pathogenesis of HCC and may serve as a powerful molecular marker for detecting HCC in biopsies tissues of HCC patients.

Terminology

To concisely and accurately describe, define or explain the specific, unique terms that are not familiar to majority of the readers, but are essential for the readers to understand the article. DNA methylation typically occurs at CpG sites (cytosine-phosphate-guanine sites, that is, where a cytosine is directly followed by a guanine in the DNA sequence). This methylation results in the conversion of the cytosine to 5-methylcytosine. Hypermethylation typically occurs at CpG islands in the promoter region and is associated with gene inactivation. Demethylation is the chemical process resulting in the removal a of methyl group (CH_3) from a molecule.

Peer review

This is a nice human study conducted by a group of competent researchers. The study is nicely done. Also, the paper is well written.

REFERENCES

1 **Lai EC**, Lau WY. The continuing challenge of hepatic cancer in Asia. *Surgeon* 2005; **3**: 210-215
2 **Farazi PA**, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat Rev Cancer* 2006; **6**: 674-687
3 **Jones PA**, Baylin SB. The fundamental role of epigenetic

events in cancer. *Nat Rev Genet* 2002; **3**: 415-428
4 **Zhu J**. DNA methylation and hepatocellular carcinoma. *J Hepatobiliary Pancreat Surg* 2006; **13**: 265-273
5 **Tischoff I**, Tannapfe A. DNA methylation in hepatocellular carcinoma. *World J Gastroenterol* 2008; **14**: 1741-1748
6 **Garinis GA**, Patrinos GP, Spanakis NE, Menounos PG. DNA hypermethylation: when tumour suppressor genes go silent. *Hum Genet* 2002; **111**: 115-127
7 **Strathdee G**, Brown R. Aberrant DNA methylation in cancer: potential clinical interventions. *Expert Rev Mol Med* 2002; **4**: 1-17
8 **Strathdee G**, Brown R. Epigenetic cancer therapies: DNA methyltransferase inhibitors. *Expert Opin Investig Drugs* 2002; **11**: 747-754
9 **Jürgens G**. Head and tail development of the Drosophila embryo involves spalt, a novel homeotic gene. *EMBO J* 1988; **7**: 189-196
10 **Mollereau B**, Dominguez M, Webel R, Colley NJ, Keung B, de Celis JF, Desplan C. Two-step process for photoreceptor formation in Drosophila. *Nature* 2001; **412**: 911-913
11 **Dostal A**, Nemeckova J, Gaillyova R. The 18q deletion syndrome and analysis of the critical region for orofacial cleft at 18q22.3. *J Craniomaxillofac Surg* 2009; **37**: 272-275
12 **Parrish M**, Ott T, Lance-Jones C, Schuetz G, Schwaeger-Nickolenko A, Monaghan AP. Loss of the Sall3 gene leads to palate deficiency, abnormalities in cranial nerves, and perinatal lethality. *Mol Cell Biol* 2004; **24**: 7102-7112
13 **Shikauchi Y**, Saiura A, Kubo T, Niwa Y, Yamamoto J, Murase Y, Yoshikawa H. SALL3 interacts with DNMT3A and shows the ability to inhibit CpG island methylation in hepatocellular carcinoma. *Mol Cell Biol* 2009; **29**: 1944-1958
14 **Yu J**, Zhu T, Wang Z, Zhang H, Qian Z, Xu H, Gao B, Wang W, Gu L, Meng J, Wang J, Feng X, Li Y, Yao X, Zhu J. A novel set of DNA methylation markers in urine sediments for sensitive/specific detection of bladder cancer. *Clin Cancer Res* 2007; **13**: 7296-7304
15 **Xia W,** Ni W, Fei Q, Sun J, Zhao Y, Zhang H, Gu J, He Y, Yu J. Methylation of Sall3 gene in hepatocellular carcinoma. *Zhenduanxue Lilun Yu Shijian* 2010; **9:** 491-494
16 **Ehrich M**, Zoll S, Sur S, van den Boom D. A new method for accurate assessment of DNA quality after bisulfite treatment. *Nucleic Acids Res* 2007; **35**: e29
17 **Livak KJ**, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402-408
18 **Villanueva A**, Minguez B, Forner A, Reig M, Llovet JM. Hepatocellular carcinoma: novel molecular approaches for diagnosis, prognosis, and therapy. *Annu Rev Med* 2010; **61**: 317-328
19 **Bergsland EK**, Venook AP. Hepatocellular carcinoma. *Curr Opin Oncol* 2000; **12**: 357-361
20 **Llovet JM**. Updated treatment approach to hepatocellular carcinoma. *J Gastroenterol* 2005; **40**: 225-235
21 **Thomas MB**, Abbruzzese JL. Opportunities for targeted therapies in hepatocellular carcinoma. *J Clin Oncol* 2005; **23**: 8093-8108
22 **Esteller M**, Hamilton SR, Burger PC, Baylin SB, Herman JG. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res* 1999; **59**: 793-797
23 **Tessema M**, Länger F, Dingemann J, Ganser A, Kreipe H, Lehmann U. Aberrant methylation and impaired expression of the p15(INK4b) cell cycle regulatory gene in chronic myelomonocytic leukemia (CMML). *Leukemia* 2003; **17**: 910-918
24 **Hua D**, Hu Y, Wu YY, Cheng ZH, Yu J, Du X, Huang ZH. Quantitative methylation analysis of multiple genes using methylation-sensitive restriction enzyme-based quantitative PCR for the detection of hepatocellular carcinoma. *Exp Mol Pathol* 2011; **91**: 455-460
25 **Lambert MP**, Paliwal A, Vaissière T, Chemin I, Zoulim F, Tommasino M, Hainaut P, Sylla B, Scoazec JY, Tost J, Herceg Z. Aberrant DNA methylation distinguishes hepatocellular carcinoma associated with HBV and HCV infection and alcohol intake. *J Hepatol* 2011; **54**: 705-715
26 **Jacinto FV**, Esteller M. MGMT hypermethylation: a prognostic foe, a predictive friend. *DNA Repair* (Amst) 2007; **6**: 1155-1160
27 **Ellis L**, Atadja PW, Johnstone RW. Epigenetics in cancer: targeting chromatin modifications. *Mol Cancer Ther* 2009; **8**: 1409-1420
28 **Ganesan A**, Nolan L, Crabb SJ, Packham G. Epigenetic therapy: histone acetylation, DNA methylation and anti-cancer drug discovery. *Curr Cancer Drug Targets* 2009; **9**: 963-981
29 **Cortez CC**, Jones PA. Chromatin, cancer and drug therapies. *Mutat Res* 2008; **647**: 44-51
30 **Lee S**, Lee HJ, Kim JH, Lee HS, Jang JJ, Kang GH. Aberrant CpG island hypermethylation along multistep hepatocarcinogenesis. *Am J Pathol* 2003; **163**: 1371-1378
31 **Moribe T**, Iizuka N, Miura T, Kimura N, Tamatsukuri S, Ishitsuka H, Hamamoto Y, Sakamoto K, Tamesa T, Oka M. Methylation of multiple genes as molecular markers for diagnosis of a small, well-differentiated hepatocellular carcinoma. *Int J Cancer* 2009; **125**: 388-397

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CASE REPORT

A pediatric non-protein losing Menetrier's disease successfully treated with octreotide long acting release

Giovanni Di Nardo, Salvatore Oliva, Marina Aloi, Federica Ferrari, Simone Frediani, Adriana Marcheggiano, Salvatore Cucchiara

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Adriana Marcheggiano, Department of Medical Science, Sapienza University of Rome, 00161 Rome, Italy
Author contributions: Di Nardo G performed the second endoscopy with mucosectomy and wrote the manuscript; Oliva S performed the first endoscopy and wrote the manuscript; Aloi M and Ferarri F performed all clinical investigations and followed-up the patient; Frediani S suggested the use of the octreotide as a possible therapeutic tool; Marcheggiano A performed all the histological examinations; Cucchiara S approved the use of octreotide and strongly revised a draft of the paper.
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Abstract

Pediatric Menetrier's disease (MD) is an uncommon, acute, self-limited hypertrophic gastropathy characterized by enlarged gastric folds associated with epithelial hyperplasia and usually accompanied by protein losing gastropathy. Gastric cytomegalovirus infection is found in one third of MD children and its treatment is often associated with remission. Diagnosis often requires full-thickness biopsy due to inability to detect typical histological findings with conventional endoscopic biopsy. We report an uncommon case of non self-limited pediatric MD needing endoscopic mucosal resection for diagnosis which was then successfully treated with octreotide long-acting release (LAR). To the best of our knowledge, this is the first pediatric MD case successfully treated with octreotide LAR. Our experience suggests octreotide LAR as treatment for refractory MD before gastrectomy.

Key words: Menetrier's disease; Octreotide; Endoscopic mucosal resection; Children

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INTRODUCTION

Menetrier's disease (MD) is a rare hypertrophic gastropathy characterized by enlarged gastric rugal folds and epithelial hyperplasia, accompanied by protein losing gastropathy[1]. Primarily seen in adults, MD can have a long clinical course and is associated with considerable morbidity and even mortality, which are related to surgical resection and potential risk for malignant transformation[2-4]. Pediatric MD often presents as oedema and hypoalbuminemia due to protein loss through the abnormal gastric mucosa and usually has a benign self-limited course with symptoms resolving within 5 wk[4,5]. Diagnosis often requires full-thickness biopsy in conjunction with endoscopic view of enlarged gastric folds[6] while histology includes foveolar hyperplasia with cystic

dilation of pits, accompanied by glandular atrophy of the gastric body[1,2]. Gastric cytomegalovirus (CMV) infection is found in one third of MD children and its treatment is often associated with remission[7,8]. We describe the first case of non self-limited and non protein losing MD, diagnosed with endoscopic mucosal resection (EMR) and successfully treated with octreotide long-acting release (LAR).

CASE REPORT

A 4-year-old boy was referred to our Unit for severe iron deficiency anemia (haemoglobin level: 4.7 g/dL and iron studies consistent with iron-deficiency anemia) already treated with blood transfusion in a territorial hospital. The child had a normal physical development with regular growth curve. Liver and spleen diseases were excluded on a clinical and biochemical basis. A grandfather underwent total gastrectomy due to MD. At upper gastrointestinal endoscopy there were marked thickened gastric folds with overlying erosions and exudate involving the corpus and fundus, whereas the antrum and pylorus were normal (Figure 1). Mucosal biopsies showed mild foveolar hyperplasia, dilation of some glands and were negative for *Helicobacter pylori* and CMV. The latter was also negative in the gastric juice, serum and urine. There was no clinical and laboratory evidence of protein-losing enteropathy and serum gastrin level was normal.

After 2 mo of unsuccessful treatment with omeprazole at 2 mg/kg per day, an EMR was done in the area of greatest gastric folds thickening to obtain a wider sample for a histological diagnosis. Thus, a diagnosis of MD was made. based on the presence of marked epithelial hyperplasia with tortuous and cystically dilated foveolar glands, discontinuous atrophy of stomach glands and significant reduction of parietal cells; a strong small vessel congestion with a diffuse edema was also observed within the lamina propria (Figure 2).

Following a lack of response to conventional therapies and due to preliminary reports on successful use of octreotide in adult MD[9-13], the patient was given ten doses of octreotide (50 μg subcutaneously two times a day), which was well tolerated. He then began octreotide LAR (5 mg intramuscularly every 28 d). Two months later haemoglobin and iron levels were normal. Six months later therapy was stopped and a rapid reduction of the haemoglobin level ensued. Thus, we continued octreotide with long-term normalization of the haemoglobin levels. Fifteen months later gastric folds were less prominent and no erosions at endoscopic follow-up were seen; however the histology was unchanged.

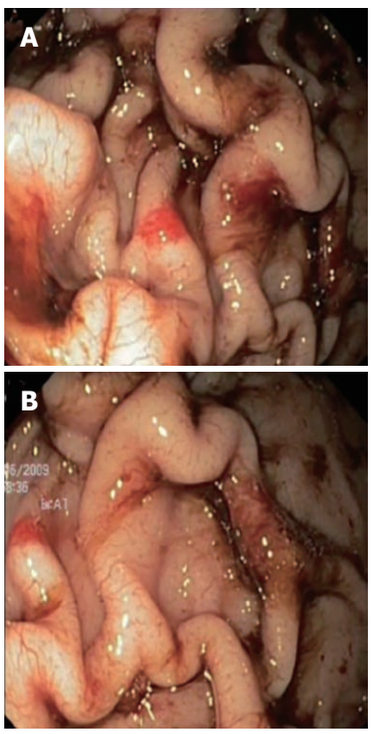

Figure 1 Endoscopic view of markedly thickened gastric folds, with overlying erosions and exudates involving fundus (A) and corpus (B).

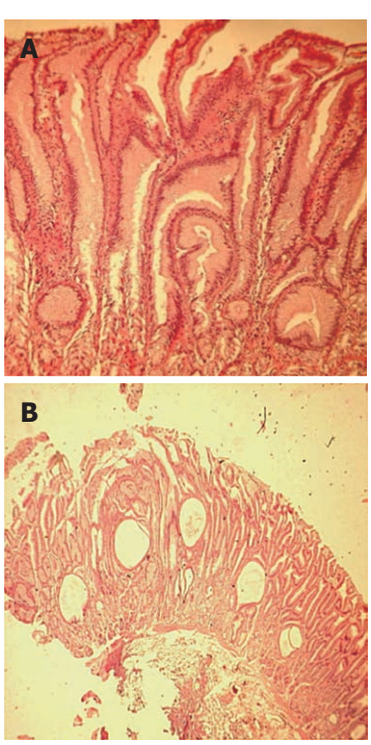

Figure 2 Histopathology from endoscopic mucosal resection (HE staining; A: 40×, B: 20×) shows elongated, tortuous and cystically dilated foveolar glands, discontinuous atrophy of gastric glands and significant reduction of parietal cells.

DISCUSSION

MD is characterized by hypertrophic folds in the body of the stomach, foveolar hyperplasia and hypoproteinemia[1]. While spontaneous remission is rare in adults and gastrectomy is not uncommonly required in refractory disease[2,3], pediatric MD is commonly an acute, self-limited, protein-losing gastropathy[5]. However, a hyperplastic hypersecretory variant of MD with normal or increased acid secretion and no protein loss is reported: it often

requires full-thickness biopsy due to an inability to detect typical histological findings with conventional endoscopic biopsy[6].

Therapy in children is supportive and includes adequate hydration, antisecretory agents (histamine 2 receptor antagonists and proton pump inhibitors) and albumin replacement[3-5]. Treatment of CMV, when detected, is usually associated with remission[7,8].

Interestingly, transforming growth factor α, one of the ligands for the epidermal growth factor receptor (EGF-R), has been implicated in the mechanisms underlying MD[14]: it causes dose-dependent *in vitro* proliferation of gastric epithelial cells and reduction of acid secretion, which are hallmarks of the disease[15,16].

Treatment with an experimental monoclonal antibody against EGF-R has been successful in 3 severe MD cases[17,18]. There is also evidence that somatostatin decreases the number of EGF binding sites at the cell surface[19]; thus, octreotide may modulate EGF-R signalling at several levels[20,21]. These mechanisms and preliminary reports on successful use of octreotide in adults with MD[9-13] prompted us to use this agent in our patient.

In conclusion, we describe an uncommon case of pediatric MD with atypical presentation (only anemia) and chronic unremitting course. MD should be considered in all children with thickened gastric folds at endoscopy, even in the absence of typical clinical and laboratory manifestations. EMR is a good alternative to full thickness biopsy for diagnosing disorders associated with gastric wall thickening such as MD.

To the best of our knowledge, this is the first pediatric MD case successfully treated with octreotide LAR. Our experience suggests octreotide LAR as treatment for refractory MD before gastrectomy.

REFERENCES

1 **Lee EL,** Feldman M. Gastritis and Gastropathies. **In: Feldman M, Friedman LS, Brandt LJ. Editors. Feldman: Sleisenger and Fordtran's Gastrointestinal and Liver Disease. 8th** ed. Philadelphia: **Saunders; 2006: 1068-1083**

2 **Sundt TM**, Compton CC, Malt RA. Ménétrier's disease. A trivalent gastropathy. *Ann Surg* 1988; **208**: 694-701

3 **Scharschmidt BF. The natural history of hypertrophic gastr**ophy (Menetrier's disease). Report of a case with 16 year follow-up and review of 120 cases from the literature. *Am J Med* 1977; **63**: 644-652

4 **Wolfsen HC**, Carpenter HA, Talley NJ. **Menetrier's disease:** a form of hypertrophic gastropathy or gastritis? *Gastroenterology* 1993; **104**: 1310-1319

5 **Blackstone MM**, Mittal MK. The edematous toddler: a case of pediatric Ménétrier disease. *Pediatr Emerg Care* 2008; **24**: 682-684

6 **Gleeson FC**, Mangan TF, Levy MJ. **Endoscopic ultrasound** and endoscopic mucosal resection features of a non-protein losing form of Ménétrier's disease. *Clin Gastroenterol Hepatol* 2008; **6**: e24-e25

7 **Eisenstat DD**, Griffiths AM, Cutz E, Petric M, Drumm B. Acute cytomegalovirus infection in a child with Ménétrier's disease. *Gastroenterology* 1995; **109**: 592-595

8 **Xiao SY**, Hart J. Marked gastric foveolar hyperplasia associated with active cytomegalovirus infection. *Am J Gastroenterol* 2001; **96**: 223-226

9 **Yeaton P**, Frierson HF. Octreotide reduces enteral protein losses in Ménétrier's disease. *Am J Gastroenterol* 1993; **88**: 95-98

10 **Ojeda E**, Ruiz J, Cosme A, Lobo C. [Menetrier disease associated with ulcerative colitis. Response to the treatment with octreotide. Review of the diagnostic criteria and etiopathogenesis]. *Gastroenterol Hepatol* 1997; **20**: 175-179

11 **Green BT**, Branch MS. Menetrier's disease treated with octreotide long-acting release. *Gastrointest Endosc* 2004; **60**: 1028-1029

12 **Gadour MO**, Salman AH, El Samman el Tel W, Tadros NM. Menetrier's disease: an excellent response to octreotide. A case report from the Middle East. *Trop Gastroenterol* 2005; **26**: 129-131

13 **Rothenberg M**, Pai R, Stuart K. Successful use of octreotide to treat Ménétrier's disease: a rare cause of abdominal pain, weight loss, edema, and hypoalbuminemia. *Dig Dis Sci* 2009; **54**: 1403-1407

14 **Nalle SC**, Turner JR. Menetrier's disease therapy: rebooting mucosal signaling. *Sci Transl Med* 2009; **1**: 8ps10

15 **Dempsey PJ**, Goldenring JR, Soroka CJ, Modlin IM, McClure RW, Lind CD, Ahlquist DA, Pittelkow MR, Lee DC, Sandgren EP. Possible role of transforming growth factor alpha in the pathogenesis of Ménétrier's disease: supportive evidence form humans and transgenic mice. *Gastroenterology* 1992; **103**: 1950-1963

16 **Takagi H**, Jhappan C, Sharp R, Merlino G. Hypertrophic gastropathy resembling Ménétrier's disease in transgenic mice overexpressing transforming growth factor alpha in the stomach. *J Clin Invest* 1992; **90**: 1161-1167

17 **Burdick JS**, Chung E, Tanner G, Sun M, Paciga JE, Cheng JQ, Washington K, Goldenring JR, Coffey RJ. Treatment of Ménétrier's disease with a monoclonal antibody against the epidermal growth factor receptor. *N Engl J Med* 2000; **343**: 1697-1701

18 **Settle SH**, Washington K, Lind C, Itzkowitz S, Fiske WH, Burdick JS, Jerome WG, Ray M, Weinstein W, Coffey RJ. Chronic treatment of Ménétrier's disease with Erbitux: clinical efficacy and insight into pathophysiology. *Clin Gastroenterol Hepatol* 2005; **3**: 654-659

19 **Pinski J**, Halmos G, Schally AV. Somatostatin analog RC-160 and bombesin/gastrin-releasing peptide antagonist RC-3095 inhibit the growth of androgen-independent DU-145 human prostate cancer line in nude mice. *Cancer Lett* 1993; **71**: 189-196

20 **Lahlou H**, Guillermet J, Hortala M, Vernejoul F, Pyronnet S, Bousquet C, Susini C. Molecular signaling of somatostatin receptors. *Ann N Y Acad Sci* 2004; **1014**: 121-131

21 **Watt HL**, Kharmate GD, Kumar U. Somatostatin receptors 1 and 5 heterodimerize with epidermal growth factor receptor: agonist-dependent modulation of the downstream MAPK signalling pathway in breast cancer cells. *Cell Signal* 2009; **21**: 428-439

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CASE REPORT

Infliximab stopped severe gastrointestinal bleeding in Crohn's disease

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Abstract

To report the result of rapid ulcer healing by infliximab in Crohn's patients with severe enterocolic bleeding. During 2005 and 2010, inflammatory bowel disease database of King Chulalongkorn Memorial and Samitivej hospitals were reviewed. There were seven Crohn's disease (CD) patients (4 women and 3 men; mean age 52 ± 10.4 years; range: 11-86 years). Two of the seven patients developed severe gastrointestinal bleeding (GIB) as a flare up of CD whereas the other five patients presented with GIB as their first symptom for CD. Their mean hemoglobin level dropped from 12 ± 1.3 g/dL to 8.7 ± 1.3 g/dL in a 3-d period. Median packed red blood cells units needed for resuscitation was 4 units. Because of uncontrolled bleeding, surgical resection was considered. However, due to the poor surgical candidacy of these patients (n = 3) and /or possible development of short bowel syndrome (n = 6), surgery was not pursued. Likewise angiographic embolization was not considered in any due to the risk of large infarction. All severe GIBs successfully stopped by one or two doses of intravenous infliximab. Our data suggests that infliximab is an alternative therapy for CD with severe GIB when surgery has limitation or patient is a high risk.

Key words: Crohn's disease; Gastrointestinal bleeding; Complications; Infliximab; Biologic agents

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INTRODUCTION

Although severe gastrointestinal bleeding (GIB) is an uncommon complication of inflammatory bowel disease (IBD), severe GIB occurs in 0.1% of ulcerative colitis[1] and 1.2%-1.3% of Crohn's disease (CD)[1,2]. This in turn sometimes progresses to a potential life-threatening condition. Approximately one third of CD patients developed GIB as a flare up and another one fourth of CD patients presented with GIB as an initial symptom[3]. Bleeding sources were mostly found in the colon (50%-85%) and the small bowel (15%-50%). Unfortunately, one third of CD related GIBs were severe and surgery was required because of refractory bleeding especially after failed conventional medical and endoscopic treatment[1,3]. Therefore treatment for severe hemorrhage in IBD remains a challenge. Recently, there has been only a handful of case reports of severe CD related GIB controlled with tumor

necrosis factor (TNF)-α antibody (infliximab). We report the largest number (n = 7) of CD patients presenting with severe GIB who were successfully treated with infliximab without the need for surgery.

CASE REPORT

There were seven CD patients (4 women and 3 men; mean age 52 ± 10.4 years; range: 11-86 years). Two of the seven patients developed severe GIB as a flare up of CD whereas the other five patients presented with GIB as their first symptom for CD (Tables 1 and 2).

In a group with flared CD (n = 2), one patient was diagnosed as colonic CD for 2 mo. She was steroid dependent who required oral prednisolone 35 mg/d and azathioprine 1.5 mg/kg per day. She was admitted because of severe bleeding per rectum and developed orthostatic hypotension. She required 4 units of pack red cell for resuscitation during those 3 d of hospitalization. Another patient was diagnosed as ileocolonic CD for 7 mo. She had been taking budesonide 9 mg/d and mesalamine 2 g/d to control her CD before admission. She developed acute abdominal pain, fever and severe hematochezia. Her hemoglobin (Hb) dropped from 12 to 10 g/dL within 2 d. A unit of pack red cell was required to maintain hemoglobin level.

In patients who presented with hematochezia as their first CD symptom (n = 5), three of the five patients had had abdominal pain and watery diarrhea for 10-14 d prior to the present of hematochezia. The other two presented initially with hematochezia without prior warning gastrointestinal (GI) symptoms. All of those denied the use of non-steroidal anti-inflammatory drugs (NSAIDs) prior to the presentation. Skin signs and symptoms that suggestive of Behçet's disease were not recognized in any.

The average baseline Hb was 12 ± 1.3 g/dL in all patients. Coagulogram and platelets count were normal. The average C-reactive protein level was high (mean 14 ± 18 mg/L; normal 0-6). Endoscopy and ileo-colonoscopy were performed as the initial investigations. One patient with suspected proximal ileal bleeding underwent a double balloon enteroscopy. Endoscopic findings showed multiple discrete deep ulcers with either active oozing or visible vessel in all seven patients. Of these, two patients with visible vessel found on the ulcer underwent endoscopic hemostasis with hemoclipping. However, recurrent hematochezia developed in both and repeat endoscopy failed to indentify other source of bleeding despite the inactive status of previously clipped vessels. Bleeding sources located in the small bowel and mainly in the ileum without colonic source in five patients, while the other had pure colonic lesion. One patient had ulcers in both ileum and colon. Biopsies from Ileum and colon were done in all patients and they revealed acute and chronic inflammation. No granuloma was identified. All specimens were negative for inclusion body and *Mycobacterium tuberculosis* (by polymerase chain reaction).

Despite, an intravenous dexamethasone 5 mg was given at every 6 h for 3-5 d, all patients still had persistent hematochezia. Their mean Hb level dropped from 12 ± 1.3 g/dL to 8.7 ± 1.3 g/dL in a 3-d period. Median packed red blood cells units needed for resuscitation was 4 units. Because of uncontrolled bleeding, surgical resection was considered. Due to the poor surgical candidacy of these patients (n = 3) and/or possible development of short bowel syndrome (n = 6), surgery was not pursued. Likewise angiographic embolization was not considered in any due to the risk of large infarction from multiple areas of embolization. Then infliximab (5 mg/kg) was infused instead. Infliximab rapidly stopped bleeding definitely within 24 h in 6 patients. Another patient developed recurrent bleeding after 3 d of the first dose of infliximab. Subsequently, bleeding ceased promptly after the second dose of infliximab that administered at day tenth. Median doses of infliximab were two. All underwent a follow-up ileo-colonoscopy that revealed a significant improvement of ileal and colonic ulceration (Figures 1 and 2). At the 30-d follow up, no patients reported recurrent bleeding.

DISCUSSION

In our case series, we identified CD patients with severe GIB presented as either the first manifestation or a flare up of disease. None of our patients had histories or findings suggestive of NSAIDs induced ulcers or Behçet' s disease. The common location of ulcers in our series involved ileum, ileocolic region, colon and a combination of all areas. The most common endoscopic findings were extensive multiple deep ulcers with or without active oozing. Infliximab was used as a last resort for controlling bleeding after failure of standard treatments. In fact, surgical treatment was considered in all cases, but it was not opted as mentioned earlier. Almost all patients responded promptly within 24 h after a single dose of infliximab. Only one patient with ileocolonic CD needed the second dose to achieve definite hemostasis after the recurrent bleeding.

Management of severe GIB in CD is problematic since there are multiple lesions with the possibility of bleeding from multiple sites. Endoscopy should be attempted in all patients, but only a quarter of patients that the bleeding sites could be precisely identified[3]. Medical therapies such as steroids, azathioprine and mesalamine also have been reported to control bleeding, but the prompt response is uncertain. Surgical resection is also a crucial therapy[4-6]. Recurrent bleeding was significantly lower in surgically treated patients (5.7%) compared with medically treated patients (38.5%). However, there is a significant rate of post operative and perioperative mortality at 6.9%[7]. In addition, the risk of developing short bowel syndrome after resection should be considered because these patients may have an extensive small bowel involvement[8-10]. Radiological intervention, one of the alternative treatments for small bowel bleeding[11], can accidentally contribute to small bowel infarction after multiple area of embolization. From those five series reported on GIB related to CD (n = 101), an angiographic embolization

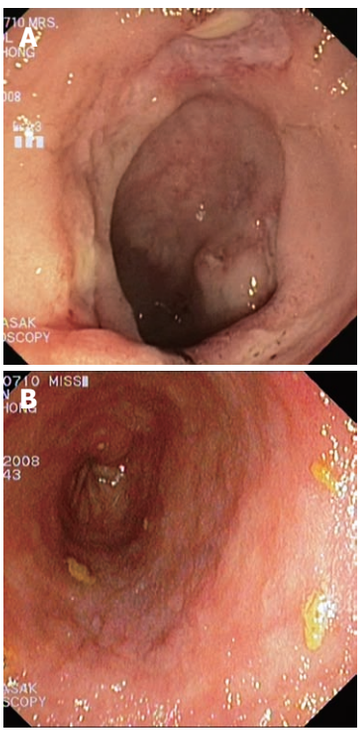

Figure 1 Deep ileal ulcer (A) and completely healed ileal ulcer 6 wk after infliximab (B).

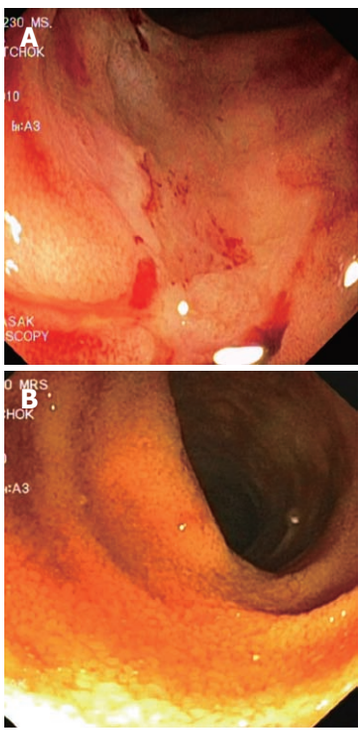

Figure 2 Ileal ulcer with hemoclips (A) and healing ileal ulcer 6 wk after infliximab (B). Note the clips still presented.

Table 1 Clinical characteristics and outcomes of infliximab treatment in 2 Crohn's disease patients with severe gastrointestinal bleeding as a flare-up disease

No.	Age (yr)	Sex	Duration of CD	Location	Current treatment	Presenting symptom	Dropped rate of Hb (g/dL)	PRBC (unit)	Characteristic of lesion	Infliximab therapy	Bleeding controlled in	Follow-up (mo)
1	11	F	2 mo	Colon	Prednisolone 35 mg/d azathioprine	GIB (1 d)	from 11 to 8 in 3 d	4	Multiple deep colonic ulcers without oozing	Infliximab 5 mg/kg (single dose)	1 d	12
2	19	F	7 mo	Ileocolon	Budesonide 9 mg/d 5-ASA	GIB (1 d)	from 12 to 10 in 3 d	1	Multiple ileal and colonic ulcers with oozing	Infliximab 5 mg/kg (d0, d10)	10 d	10

CD: Crohn's disease; GIB: Gastrointestinal bleeding; Hb: Hemoglobin; 5-ASA: 5-aminosalicylates; PRBC: Packed red blood cell; F: Female.

Table 2 Clinical characteristics and outcomes of infliximab treatment in 5 Crohn's disease patients with severe gastrointestinal bleeding as a first presentation

No.	Age (yr)	Sex	Presenting symptom	Dropped rate of Hb (g/dL)	PRBC (unit)	Location	Characteristic of lesion	Infliximab therapy	Bleeding controlled in	Follow-up (mo)
1	59	M	Diarrhea and abdominal pain (10 d) GIB (1 d)	from 10 to 8 in 3 d	6	Ileum	Multiple ileal ulcers with oozing and one visible vessel	Infliximab 5 mg/kg (d0, week 2)	1 d	24
2	86	M	GIB (1 d)	from 12 to 8.5 in 4 d	7	Ileum	Multiple ileal ulcers with oozing	Infliximab 5 mg/kg (d0, week 2)	1 d	36
3	71	F	Diarrhea and abdominal pain (10 d) GIB	from 13 to 10 in 3 d	3	ileum	Multiple ileal ulcers with oozing	Infliximab 5 mg/kg (d0, week 2)	1 d	12
4	50 yr	F	Diarrhea and abdominal pain (14 d) GIB (1 d)	from 14 to 10 in 3 d	2	Ileum and jejunum	Multiple ileal and jejunum ulcers with oozing	Infliximab 5 mg/kg (d0, week 2)	1 d	36
5	71 yr	M	1st episode GIB from ileal ulcer (1 mo) Recurrent GIB (1 d)	from 11 to 6.5 in 5 d	6	Ileum	Multiple ileal ulcers with oozing and one visible vessel	Infliximab 5 mg/kg (single dose)	1 d	24

CD: Crohn's disease; GIB: Gastrointestinal bleeding; Hb: Hemoglobin; 5-ASA: 5-aminosalicylates; PRBC: Packed red blood cell; M: Male; F: Female.

Table 3 Successful control severe lower gastrointestinal bleeding in Crohn's disease with infliximab

Study	Sex	Age (yr)	Location	Duration of disease	Current treatment	Presenting symptom	PRBC (unit)	Characteristic of lesion	Infliximab therapy	Bleeding controlled in	Follow-up (mo)
Belaihe *et al*[26], 2002	F	28	Ileocolon CD	3 yr	Budesonide azathioprine	Lower GIB	5	Multiple deep ulcers at colon without bleeding stigmata	Infliximab 5 mg/kg (d0, week 2, week 6)	14 d	5
	F	59	colon CD	9 yr	Prednisolone, metronidazole, ciprofloxacin	Lower GIB	4	Multiple deep ulcers at colon without bleeding stigmata	Infliximab 5 mg/kg (single dose)	4 d	4
Papi *et al*[7], 2003	M	50	Ileocolon CD S/P resection and ileocolonic anastomosis due to bleeding	9 mo	Prednisolone azathioprine	Lower GIB with hypovolumic shock	NA	Deep ulcers at ileocolon anastomosis without bleeding stigmata	Infliximab 5 mg/kg (d0, week 2, week 6)	NA	12
	M	68	Ileum CD S/P ileal resection due to stricture	24 yr	Mesalamine	Melena	4	Large ulcer at ileocolon anastomosis without bleeding stigmata	Infliximab 5 mg/kg (d0, week 2, week 6)	NA	3
Tsujikawa *et al*[25], 2004	M	31	Ileocolon CD S/P ileocolectomy due to ulcer bleeding	12 yr	Salazosulfapyrimidine	Lower GIB	NA	Multiple ulcers at ileocolon anastomosis and ileum without bleeding stigmata	Infliximab 5 mg/kg (d0, week 2, week 6)	NA	4
Ando *et al*[22], 2009	F	16	Colonic CD	1 yr	Mesalamine prednisolone	Lower GIB with hypovolumic shock	6	Multiple deep ulcers at colon with diffuse mucosal inflammation without bleeding stigmata	Infliximab 5 mg/kg (d0, week 2, week 6)	3 d	12
Meyer *et al*[24], 2009	F	19	Ileocolonic CD	6 yr	Mesalamine prednisolone	Lower GIB with hypovolumic shock	4	Multiple ulcers at terminal ileum without bleeding stigmata	Infliximab 5 mg/kg (d0, week 2, week 6)	NA	6
Julián Gómez *et al*[23], 2010	M	44	Ileocolon CD S/P total colectomy with ileostomy due to toxic megacolon	NA	NA	Postop small bowel resection due to obstruction bleeding	10	Multiple deep ulcers at small bowel without bleeding stigmata	Infliximab 5 mg/kg (d0, week 2, week 6) and maintenance dose	5 d	3
Alcalde Vargas *et al*[21], 2011	M	27	Ileocolon CD	2 yr	Mesalamine	Massive lower GIB	8	Multiple ulcers entire colon and abundant dark red blood at terminal ileum	Infliximab 5 mg/kg (single dose)	4 d	NA
	F	36	Colon and perianal CD	NA	Amoxicillin-clavulanate metronidazole	Massive lower GIB	12	Multiple deep ulcers entire colon and blood clots	Infliximab 5 mg/kg (single dose)	6 d	NA
	M	24	Colon CD	1 mo	Prednisolone	Massive lower GIB	5	Multiple deep ulcers entire colon and spontaneous bleeding mucosa	Infliximab 5 mg/kg (single dose)	4 d	NA

CD: Crohn's disease; GIB: Gastrointestinal bleeding; PRBC: Packed red blood cell; M: Male; F: Female; NA: Not available.

was attempted only in one patient[1-3,12,13]. Unfortunately, that such patient subsequently necessitated surgery due to small bowel infarction after an ileocecal artery embolization[3]. Angiography with intra-arterial vasopressin infusion in case where embolization is not possible has previously been proven to be successful in two CDs related GIB[14,15]. However, many side effects and complications could develop from this technique including hypertension, coronary vasoconstriction, cardiac arrhythmia, and bowel infarction. To decrease the risk for bowel infarction, it is advisable to use superselective angiographic embolization[16]. Although the risk of bowel infarction may be decreased, this serious complication cannot be ignored. In experienced centers, bowel infarction still developed in 5%-24% of lower GIB patients who treated with superselective mesenteric arterial embolization[17,18].

The pathogenesis of hemorrhagic type CD remains unclear. One possible hypothesis is transmural inflammations leading to mucosal ulcers erode to blood vessels. On endoscopic examinations, all of our patients had diffuse deep ulcers and majority of them (86%) had active oozing. Since severe hemorrhage usually develops from ulcers eroding into blood vessels, any treatment that can rapidly heal the mucosa is an ideal therapeutic tool to control and prevent recurrent hemorrhage. Anti-TNF-α (infliximab) has been shown to induce rapid mucosal healing[19,20]. Therefore, infliximab has a possible role in treating severe hemorrhagic CD. Moreover, the identification for precise bleeding site is not required since infliximab can systematically heal multiple small bowel ulcers.

To date, eleven CDs related GIB treated with infliximab from the seven series has been reported (Table

3)[7,21-26]. Severe hematochezia was presented in eight flare-up CD patients and the other three presented with hematochezia as their initial CD manifestation. Four patients previously had undergone for surgical treatment including ileocolectomy and total colectomy[7,23,25,26]. Colon and ileocolon were the most common locations of GIB. Ileocolonoscopy showed multiple discrete deep ulcers in all. Majority of patients had more than one potential site of bleeding[7,21-26]. The high risk bleeding stigmata was found in only one patient that presented with diffuse spontaneous mucosal bleeding[21]. One patient underwent a total colectomy and small bowel resection, but the bleeding recurred[23]. No patient underwent angiographic therapy. Infliximab was administered as a last resource for uncontrolled bleeding. Most of patients responded to the first dose of infliximab. Only one patient required the second dose of infliximab on day fourteenth to control recurrent bleeding[26]. Six patients received three doses of infliximab and another five received only a single dose of infliximab. Maintenance with infliximab was considered only in one patient[23]. Surgery was not pursued in any[4,15-20].

To our knowledge, we report the largest cases series of severe GIB in CD in which infliximab had been used. Infliximab was able to control hemostasis as a result of rapid ulcer healing. Definite hemostasis was achieved after the first or second dose of infliximab. Nevertheless, more further prospective studies are required to confirm the utilization of infliximab for severe GIB in CD.

In conclusion, infliximab may be a good alternative treatment to control severe bleeding related to small bowel and colonic ulcers in active CD especially in patients with high risk for surgery and/or high risk to develop a short bowel syndrome.

REFERENCES

1 **Pardi DS**, Loftus EV, Tremaine WJ, Sandborn WJ, Alexander GL, Balm RK, Gostout CJ. Acute major gastrointestinal hemorrhage in inflammatory bowel disease. *Gastrointest Endosc* 1999; **49**: 153-157

2 **Robert JR**, Sachar DB, Greenstein AJ. Severe gastrointestinal hemorrhage in Crohn's disease. *Ann Surg* 1991; **213**: 207-211

3 **Belaiche J**, Louis E, D'Haens G, Cabooter M, Naegels S, De Vos M, Fontaine F, Schurmans P, Baert F, De Reuck M, Fiasse R, Holvoet J, Schmit A, Van Outryve M. Acute lower gastrointestinal bleeding in Crohn's disease: characteristics of a unique series of 34 patients. Belgian IBD Research Group. *Am J Gastroenterol* 1999; **94**: 2177-2181

4 **Lazarev M**, Ullman T, Schraut WH, Kip KE, Saul M, Regueiro M. Small bowel resection rates in Crohn's disease and the indication for surgery over time: experience from a large tertiary care center. *Inflamm Bowel Dis* 2010; **16**: 830-835

5 **Wolff BG**. Crohn's disease: the role of surgical treatment. *Mayo Clin Proc* 1986; **61**: 292-295

6 **Dolgin SE**. Surgical management of upper gastrointestinal and small bowel Crohn's disease. *Semin Pediatr Surg* 2007; **16**: 172-177

7 **Papi C**, Gili L, Tarquini M, Antonelli G, Capurso L. Infliximab for severe recurrent Crohn's disease presenting with massive gastrointestinal hemorrhage. *J Clin Gastroenterol* 2003; **36**: 238-241

8 **Thompson JS**, Iyer KR, DiBaise JK, Young RL, Brown CR, Langnas AN. Short bowel syndrome and Crohn's disease. *J Gastrointest Surg* 2003; **7**: 1069-1072

9 **Kristensen M**, Lenz K, Nielsen OV, Jarnum S. Short bowel syndrome following resection for Crohn's disease. *Scand J Gastroenterol* 1974; **9**: 559-565

10 **Slater G**, Aufses AH. Small bowel length in Crohn's disease. *Am J Gastroenterol* 1991; **86**: 1037-1040

11 **Korzenik JR**. Massive Lower Gastrointestinal Hemorrhage in Crohn's Disease. *Curr Treat Options Gastroenterol* 2000; **3**: 211-216

12 **Cirocco WC**, Reilly JC, Rusin LC. Life-threatening hemorrhage and exsanguination from Crohn's disease. Report of four cases. *Dis Colon Rectum* 1995; **38**: 85-95

13 **Driver CP**, Anderson DN, Keenan RA. Massive intestinal bleeding in association with Crohn's disease. *J R Coll Surg Edinb* 1996; **41**: 152-154

14 **Mellor JA**, Chandler GN, Chapman AH, Irving HC. Massive gastrointestinal bleeding in Crohn's disease: successful control by intra-arterial vasopressin infusion. *Gut* 1982; **23**: 872-874

15 **Alla VM**, Ojili V, Gorthi J, Csordas A, Yellapu RK. Revisiting the past: intra-arterial vasopressin for severe gastrointestinal bleeding in Crohn's disease. *J Crohns Colitis* 2010; **4**: 479-482

16 **Kazama Y**, Watanabe T, Akahane M, Yoshioka N, Ohtomo K, Nagawa H. Crohn's disease with life-threatening hemorrhage from terminal ileum: successful control by superselective arterial embolization. *J Gastroenterol* 2005; **40**: 1155-1157

17 **Kuo WT**, Lee DE, Saad WE, Patel N, Sahler LG, Waldman DL. Superselective microcoil embolization for the treatment of lower gastrointestinal hemorrhage. *J Vasc Interv Radiol* 2003; **14**: 1503-1509

18 **Bandi R**, Shetty PC, Sharma RP, Burke TH, Burke MW, Kastan D. Superselective arterial embolization for the treatment of lower gastrointestinal hemorrhage. *J Vasc Interv Radiol* 2001; **12**: 1399-1405

19 **Colombel JF**, Sandborn WJ, Reinisch W, Mantzaris GJ, Kornbluth A, Rachmilewitz D, Lichtiger S, D'Haens G, Diamond RH, Broussard DL, Tang KL, van der Woude CJ, Rutgeerts P. Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med* 2010; **362**: 1383-1395

20 **D'haens G**, Van Deventer S, Van Hogezand R, Chalmers D, Kothe C, Baert F, Braakman T, Schaible T, Geboes K, Rutgeerts P. Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: A European multicenter trial. *Gastroenterology* 1999; **116**: 1029-1034

21 **Alcalde Vargas A**, Justiniano JM, Carnerero EL, Salado CT, Domingo IG, Galán JL. [Utility of infliximab therapy in severe enterorrhagia associated with Crohn's disease. Report of three cases]. *Gastroenterol Hepatol* 2011; **34**: 24-28

22 **Ando Y**, Matsushita M, Kawamata S, Shimatani M, Fujii T, Okazaki K. Infliximab for severe gastrointestinal bleeding in Crohn's disease. *Inflamm Bowel Dis* 2009; **15**: 483-484

23 **Julián Gómez L**, Atienza R, Barrio J, Gil P, Gómez de la Cuesta S, Pinto P, Alcaide N, Caro Patón A. [Infliximab treatment of severe bleeding complicating Crohn's disease]. *Rev Esp Enferm Dig* 2010; **102**: 57-58

24 **Meyer MM**, Levine EJ. Acute hemorrhagic Crohn's disease controlled with infliximab. *Inflamm Bowel Dis* 2009; **15**: 1456-1457

25 **Tsujikawa T**, Nezu R, Andoh A, Saotome T, Araki Y, Ishizuka Y, Sasaki M, Koyama S, Fujiyama Y. Inflixmab as a possible treatment for the hemorrhagic type of Crohn's disease. *J Gastroenterol* 2004; **39**: 284-287

26 **Belaiche J**, Louis E. Severe lower gastrointestinal bleeding in Crohn's disease: successful control with infliximab. *Am J Gastroenterol* 2002; **97**: 3210-3211

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Dual therapy for third-line *Helicobacter pylori* eradication and urea breath test prediction

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Abstract

We evaluated the efficacy and tolerability of a dual therapy with rabeprazole and amoxicillin (AMX) as an empiric third-line rescue therapy. In patients with failure of first-line treatment with a proton pump inhibitor (PPI)-AMX-clarithromycin regimen and second-line treatment with the PPI-AMX-metronidazole regimen, a third-line eradication regimen with rabeprazole (10 mg q.i.d.) and AMX (500 mg q.i.d.) was prescribed for 2 wk. Eradication was confirmed by the results of the ^{13}C-urea breath test (UBT) at 12 wk after the therapy. A total of 46 patients were included; however, two were lost to follow-up. The eradication rates as determined by per-protocol and intention-to-treat analyses were 65.9% and 63.0%,

respectively. The pretreatment UBT results in the subjects showing eradication failure; those patients showing successful eradication comprised 32.9 ± 28.8 permil and 14.8 ± 12.8 permil, respectively. The pretreatment UBT results in the subjects with eradication failure were significantly higher than those in the patients with successful eradication (P = 0.019). A low pretreatment UBT result (≤ 28.5 permil) predicted the success of the eradication therapy with a positive predictive value of 81.3% and a sensitivity of 89.7%. Adverse effects were reported in 18.2% of the patients, mainly diarrhea and stomatitis. Dual therapy with rabeprazole and AMX appears to serve as a potential empirical third-line strategy for patients with low values on pretreatment UBT.

Key words: *Helicobacter pylori*; Amoxicillin; Dual therapy; Eradication; Urea breath test

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TO THE EDITOR

Eradication of *Helicobacter pylori* (*H. pylori*) has been reported as an effective strategy in the treatment of peptic ulcers and gastric mucosa-associated lymphoid tissue lymphomas and also prevents the recurrence of gastric cancer after endoscopic resection[1-7]. The first-line regimen for the treatment of *H. pylori* infection in Japan is triple therapy with a proton pump inhibitor (PPI), amoxicillin (AMX) and clarithromycin (CLR) administered for 7 d. Failure of this first-line therapy against *H. pylori* infection has been reported in approximately 20% of infected patients[8,9]. With the increase in the frequency of CLR-resistant *H. pylori*, there is rising concern about the potential decline in the eradication rate of this infection[10]. Although therapy with PPI-AMX-metronidazole (MNZ) administered for 1 wk has been found to be effective as a second-line regimen in patients failing the first-line regimen, approximately 10% of patients fail to respond to even second-line treatment, necessitating the establishment of an alternative third-line strategy for the effective eradication of *H. pylori*[3,11].

Although *H. pylori* bacteria easily develop resistance to CLR and MNZ, *H. pylori* has been considered to seldom become resistant to AMX. AMX is the preferred antibiotic because it is bactericidal and resistance is rare; therefore, it can be used again after treatment failure[8]. A number of studies have suggested that good success rates for *H. pylori* eradication could be obtained with AMX and PPI dual therapy if the effective PPI dose and frequency of administration were increased[12]. The majority of patients who experience two eradication failures have the rapid metabolizer genotype of CYP2C19. Because omeprazole and lansoprazole are extensively metabolized by CYP2C19 in this genotype, their plasma concentrations will not attain levels sufficient to inhibit acid secretion, and therefore, antibiotics such as AMX will be less stable in the stomach, resulting in a lower eradication rate[13]. The PPI rabeprazole is a substitute of benzimidazole. CYP2C19 is less involved in the metabolism of rabeprazole than in that of omeprazole and lansoprazole[14]. Moreover, rabeprazole has a greater and more rapid acid-inhibitory effect than does omeprazole. Several reports on the pharmacokinetics and pharmacodynamic characteristics of PPIs have indicated that a sufficient plasma concentration of PPIs can be achieved in patients with the rapid metabolizer genotype of CYP2C19 by frequent PPI dosing[12,15]. Furuta *et al*[16] recently reported an excellent eradication rate of 87.8% following dual therapy with rabeprazole 4 times/day and AMX as a third-line rescue. However, their study was completed at only one or two centers. Our study was designed as a prospective, multicenter trial with the participation of 16 Japanese hospitals affiliated with the National Hospital Organization to investigate the efficacy of dual therapy with 4 times daily dosing of rabeprazole and AMX as empiric third-line rescue therapy.

A total of 46 patients (26 males, 20 females; age 60.7 ± 12.9 years, mean ± SD) referred to us between January 2009 and January 2012 were enrolled. Endoscopic examinations were conducted before treatment in all patients, and *H. pylori* positivity was confirmed by histology, stool antigen test, *H. pylori*-specific IgG antibodies or the ^{13}C-urea breath test. All patients had a history of two treatment failures (first-line treatment used: triple therapy with PPI- AMX-CLR for 7 d; second-line treatment used; triple therapy with PPI-AMX-MNZ for 7 d). The exclusion criteria in this study were (1) age < 18 years; (2) presence of clinically significant underlying disease (hepatic or renal disease, diabetes mellitus); (3) history of gastric surgery; and (4) allergy to any of the drugs used in the study. *H. pylori* eradication failure was defined as a positive ^{13}C-urea breath test (UBT) at the end of 12 wk after completion of treatment. The ^{13}C-urea used was 100 mg ^{13}C-labelled urea, produced by Otsuka pharmaceutical Co., LTD, Japan. The procedure was modified from the European standard protocol for the detection of *H. pylori*[17]. We

Table 1 Demographic characteristics of the patients and the results of eradication therapy

Characteristics	Total (n = 46)	Eradication success (n = 29)	Eradication failure (n = 15)	P value
Age (mean ± SD, yr)	60.7 ± 12.9	59.8 ± 13.4	60.8 ± 12.1	0.813
Sex (male/female)	26/20	15/14	10/5	0.530
Diagnosis (GU/DU/CG)	23/15/8	15/10/4	8/3/4	0.450
Pretreatment UBT	20.4 ± 21.2	14.8 ± 12.8	32.9 ± 28.8	0.019
Eradication rate (ITT) %	63.0			
Eradication rate (PP) %	65.9			

GU: Gastric ulcer; DU: Duodenal ulcer; CG: Chronic gastritis; UBT: Urea breath test; ITT: Intention-to-treat; PP: Per protocol.

chose 2.5 permil for cut-off level of the rise in the delta value of $^{13}CO_2$ at 15 min after the ingestion of ^{13}C-urea.

The treatment regimen was rabeprazole 10 mg q.i.d. and AMX 500 mg q.i.d. administered for 2 wk. Participants were requested to return at the conclusion of the therapy for an interview regarding any adverse events. Successful *H. pylori* eradication was defined as a negative UBT at the end of 12 wk after completion of treatment. Statistical analyses were performed using the chi-square, Fisher's exact and Student's t tests, as appropriate. P values of less than 0.05 were accepted as representing statistical significance. The study was conducted with the approval of the Ethics Committee of the National Hospital Organization Tokyo Medical Center, and informed consent was obtained from all patients prior to the examinations. The clinical trial registration number of the University Hospital Medical Information Network was R000003204.

Of the 46 patients enrolled, 2 dropped out of the study, leaving 44 patients in the per protocol (PP) set. *H. pylori* eradication was confirmed in 29 patients, representing an eradication rate of 63.0% [95% confidence intervals (CI): 47.6%-76.8%] by intention-to-treat (ITT) analysis and 65.9% (95% CI: 50.1%-79.5%) by PP analysis (Table 1). Patient compliance with the prescribed treatment was excellent. Adverse events were recorded in 8 patients (18.2%; 95% CI: 8.2%-32.7%). Six patients had mild diarrhea or soft stools but went on to complete the study. Two patients developed stomatitis.

Because the numerical results of the UBT are a function of the total urease activity within the stomach, they represent a quantitative index of the density of gastric *H. pylori* colonization[18]. As a low pretreatment UBT value could be one of the predictive factors for eradication success, the pretreatment UBT value was analyzed. The pretreatment UBT results in the subjects with eradication failure and in those with successful eradication were 32.9 ± 28.8 and 14.8 ± 12.8 (permil, mean ± SD), respectively. The results of the statistical analysis showed that the pretreatment UBT results in the subjects with eradication failure were significantly higher than those in the patients with successful eradication (P = 0.019, effect size 0.81). We plotted original receiver operator characteristic (ROC) curves for the pretreatment UBT results to establish the appropriate cutoff value. According to the ROC curves, the optimal cutoff value in our population was 28.5. When patients were assigned to two groups (UBT results ≤ 28.5 permil and > 28.5 permil), the eradication rates were 81.3% (26/32) and 25.0% (3/12), respectively (P = 0.001). A low pre-treatment UBT value (≤ 28.5 permil) predicted the success of the eradication therapy with a sensitivity of 89.7 %, specificity of 60.0 %, positive predictive value of 81.3%, negative predictive value of 75.0% and accuracy of 79.5%.

Currently, a standard third-line therapy still remains to be established. *H. pylori* isolates after two eradication failures are often resistant to both MNZ and CLR. The alternative candidates for third-line therapy are fluoroquinolones-AMX-PPI, rifabutin-AMX-PPI, and high-dose PPI/AMX therapy[2,19-21]. Gisbert *et al*[22] conducted a prospective multicenter study to evaluate the outcomes of treatment with a third-line levofloxacin-based regimen. The patients were treated for 10 d with a regimen consisting of omeprazole, levofloxacin and AMX. The eradication rates as determined by PP and ITT analyses were 66% and 60%, respectively. However, resistance to fluoroquinolones has been shown to be easily acquired, and in countries with a high rate of use of these drugs, the resistance rates are relatively high. González Carro *et al*[23] evaluated the efficacy of a third-line rifabutin-based triple therapy. The patients were treated with PPI, rifabutin and AMX for 10 d. The eradication rates as determined by PP and ITT analyses were 62.2% and 60.8%, respectively. However, it has been suggested that the use of rifabutin be reserved for the treatment of multidrug-resistant *Mycobacterium tuberculosis* strains[24].

Our results for the dual therapy with 4 times daily dosing of rabeprazole and AMX for 14 d, which yielded eradication rates in the PP and ITT analyses of 65.9% and 63.0%, were as successful as other empiric third-line therapy regimens. In particular, a low pretreatment UBT result (≤ 28.5 permil) predicted the success of the eradication therapy with a positive predictive value of 81.3%, sensitivity of 89.7% and specificity of 60.0%, so the dual therapy appeared to serve as a promising option for empiric third-line rescue therapy in patients with a low pretreatment UBT value.

We recently reported the resistant rates of *H. pylori* to AMX. The resistance rates to AMX (MIC ≥ 0.06 μg/mL) in the groups with no history of eradication treatment, a history of one treatment failure, and a history of two treatment failures were 13.6%, 26.5% and 49.5%, respectively. The MIC_{90} of AMX increased by 2-fold after each eradication failure[25]. Resistance to AMX in *H. pylori* was gradually induced after unsuccessful eradication. Because the AMX resistance rate after two treatment failures was relatively high, the eradication rate of the present study was lower than that of previous report by Furuta *et al*[16]. Therefore, antimicrobial susceptibility testing of *H. pylori* is desirable before the selection of a suitable third-line therapy, although the culture-based antibiotic susceptibil-

ity testing for *H. pylori* is expensive, time-consuming, and not always available on a routine basis[26]. There are several limitations to our study. First, our eradication study was single armed using the dual therapy, and different doses or superiority over quinolone-based therapy was not evaluated. Second, we did not examine the *in vitro* susceptibility in patients treated with the dual therapy. Thus, *in vitro* resistance to AMX was not elucidated. These issues should be re-evaluated in future studies.

Finally, although we did not achieve excellent eradication success, the dual therapy appeared to serve as a promising option for empiric third-line rescue therapy in patients with low pretreatment UBT values. The antimicrobial susceptibility testing of *H. pylori* is desirable before the selection of a suitable third-line therapy in patients with high pretreatment UBT values.

REFERENCES

1 **Fukase K**, Kato M, Kikuchi S, Inoue K, Uemura N, Okamoto S, Terao S, Amagai K, Hayashi S, Asaka M. Effect of eradication of Helicobacter pylori on incidence of metachronous gastric carcinoma after endoscopic resection of early gastric cancer: an open-label, randomised controlled trial. *Lancet* 2008; **372**: 392-397

2 **Suzuki H**, Nishizawa T, Muraoka H, Hibi T. Sitafloxacin and garenoxacin may overcome the antibiotic resistance of Helicobacter pylori with gyrA mutation. *Antimicrob Agents Chemother* 2009; **53**: 1720-1721

3 **Nishizawa T**, Suzuki H, Hibi T. Quinolone-Based Third-Line Therapy for Helicobacter pylori Eradication. *J Clin Biochem Nutr* 2009; **44**: 119-124

4 **Nishizawa T**, Suzuki H, Masaoka T, Minegishi Y, Iwasahi E, Hibi T. Helicobacter pylori eradication restored sonic hedgehog expression in the stomach. *Hepatogastroenterology* 2007; **54**: 697-700

5 **Suzuki H**, Nishizawa T, Hibi T. Therapeutic strategies for functional dyspepsia and the introduction of the Rome III classification. *J Gastroenterol* 2006; **41**: 513-523

6 **Suzuki H**, Nishizawa T, Hibi T. Can Helicobacter pylori-associated dyspepsia be categorized as functional dyspepsia? *J Gastroenterol Hepatol* 2011; **26** Suppl 3: 42-45

7 **Suzuki M**, Suzuki H, Minegishi Y, Ito K, Nishizawa T, Hibi T: H. pylori-Eradication Therapy Increases RUNX3 Expression in the Glandular Epithelial Cells in Enlarged-Fold Gastritis. *J Clin Biochem Nutr* 2010; **46:** 259-264

8 **Suzuki H**, Nishizawa T, Hibi T. Helicobacter pylori eradication therapy. *Future Microbiol* 2010; **5**: 639-648

9 **Hirata K**, Suzuki H, Nishizawa T, Tsugawa H, Muraoka H, Saito Y, Matsuzaki J, Hibi T. Contribution of efflux pumps to clarithromycin resistance in Helicobacter pylori. *J Gastroenterol Hepatol* 2010; **25** Suppl 1: S75-S79

10 **Sasaki M**, Ogasawara N, Utsumi K, Kawamura N, Kamiya T, Kataoka H, Tanida S, Mizoshita T, Kasugai K, Joh T. Changes in 12-Year First-Line Eradication Rate of Helicobacter pylori Based on Triple Therapy with Proton Pump Inhibitor, Amoxicillin and Clarithromycin. *J Clin Biochem Nutr* 2010; **47**: 53-58

11 **Nishizawa T**, Suzuki H, Masaoka T, Iwasaki E, Hibi T. A new eradication resistance index as a predictor of metronidazole-containing second-line treatment of Helicobacter pylori. *Digestion* 2007; **76**: 215-220

12 **Furuta T**, Shirai N, Xiao F, Takashita M, Sugimoto M, Kajimura M, Ohashi K, Ishizaki T. High-dose rabeprazole/amoxicillin therapy as the second-line regimen after failure to eradicate H. pylori by triple therapy with the usual doses of a proton pump inhibitor, clarithromycin and amoxicillin. *Hepatogastroenterology* 2003; **50**: 2274-2278

13 **Nishizawa T**, Suzuki H, Nakagawa I, Iwasaki E, Masaoka T, Hibi T. Gatifloxacin-based triple therapy as a third-line regimen for Helicobacter pylori eradication. *J Gastroenterol Hepatol* 2008; **23** Suppl 2: S167-S170

14 **Shirai N**, Furuta T, Moriyama Y, Okochi H, Kobayashi K, Takashima M, Xiao F, Kosuge K, Nakagawa K, Hanai H, Chiba K, Ohashi K, Ishizaki T. Effects of CYP2C19 genotypic differences in the metabolism of omeprazole and rabeprazole on intragastric pH. *Aliment Pharmacol Ther* 2001; **15**: 1929-1937

15 **Sugimoto M**, Furuta T, Shirai N, Kajimura M, Hishida A, Sakurai M, Ohashi K, Ishizaki T. Different dosage regimens of rabeprazole for nocturnal gastric acid inhibition in relation to cytochrome P450 2C19 genotype status. *Clin Pharmacol Ther* 2004; **76**: 290-301

16 **Furuta T**, Sugimoto M, Kodaira C, Nishino M, Yamade M, Uotani T, Ikuma M, Shirai N. The dual therapy with 4 times daily dosing of rabeprazole and amoxicillin as the 3rd rescue regimen for eradication of H. pylori. *Hepatogastroenterology* 2010; **57**: 1314-1319

17 **Logan RP**, Polson RJ, Misiewicz JJ, Rao G, Karim NQ, Newell D, Johnson P, Wadsworth J, Walker MM, Baron JH. Simplified single sample 13Carbon urea breath test for Helicobacter pylori: comparison with histology, culture, and ELISA serology. *Gut* 1991; **32**: 1461-1464

18 **Kobayashi D**, Eishi Y, Ohkusa T, Ishige T, Minami J, Yamada T, Takizawa T, Koike M. Gastric mucosal density of Helicobacter pylori estimated by real-time PCR compared with results of urea breath test and histological grading. *J Med Microbiol* 2002; **51**: 305-311

19 **Nishizawa T**, Suzuki H, Kurabayashi K, Masaoka T, Muraoka H, Mori M, Iwasaki E, Kobayashi I, Hibi T. Gatifloxacin resistance and mutations in gyra after unsuccessful Helicobacter pylori eradication in Japan. *Antimicrob Agents Chemother* 2006; **50**: 1538-1540

20 **Nishizawa T**, Suzuki H, Umezawa A, Muraoka H, Iwasaki E, Masaoka T, Kobayashi I, Hibi T. Rapid detection of point mutations conferring resistance to fluoroquinolone in gyrA of Helicobacter pylori by allele-specific PCR. *J Clin Microbiol* 2007; **45**: 303-305

21 **Suzuki S**, Suzuki H, Nishizawa T, Kaneko F, Ootani S, Muraoka H, Saito Y, Kobayashi I, Hibi T. Past rifampicin dosing determines rifabutin resistance of Helicobacter pylori. *Digestion* 2009; **79**: 1-4

22 **Gisbert JP**, Castro-Fernández M, Bermejo F, Pérez-Aisa A, Ducons J, Fernández-Bermejo M, Bory F, Cosme A, Benito LM, López-Rivas L, Lamas E, Pabón M, Olivares D. Third-line rescue therapy with levofloxacin after two H. pylori treatment failures. *Am J Gastroenterol* 2006; **101**: 243-247

23 **González Carro P**, Pérez Roldán F, De Pedro Esteban A, Legaz Huidobro ML, Soto Fernández S, Roncero Garcia Escribano O, Esteban López-Jamar JM, Pedraza Martin C, Ruíz Carrillo F. Efficacy of rifabutin-based triple therapy in Helicobacter pylori infected patients after two standard treatments. *J Gastroenterol Hepatol* 2007; **22**: 60-63

24 **Nishizawa T**, Suzuki H, Matsuzaki J, Muraoka H, Tsugawa H, Hirata K, Hibi T. Helicobacter pylori resistance to rifabutin in the last 7 years. *Antimicrob Agents Chemother* 2011; **55**: 5374-5375

25 **Nishizawa T**, Suzuki H, Tsugawa H, Muraoka H, Matsuzaki J, Hirata K, Ikeda F, Takahashi M, Hibi T. Enhancement of amoxicillin resistance after unsuccessful Helicobacter pylori eradication. *Antimicrob Agents Chemother* 2011; **55**: 3012-3014

26 **Gisbert JP**. "Rescue" regimens after Helicobacter pylori treatment failure. *World J Gastroenterol* 2008; **14**: 5385-5402

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Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

November 9-13, 2012
American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel Diseases
Hollywood, FL 33028, United States

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GENERAL INFORMATION

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ic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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