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

World Journal of *Gastroenterology*

World J Gastroenterol 2013 June 28; 19(24): 3713-3914

Baishideng Publishing Group
www.wjgnet.com

WJG World Journal of Gastroenterology

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

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World Journal of Gastroenterology

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Weekly Volume 19 Number 24 June 28, 2013

CASE REPORT

World Journal of Gastroenterology
Volume 19 Number 24 June 28, 2013

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NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

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http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F, Lucky Plaza, 315-321 Lockhart Road, Wanchai, Hong Kong, China
Fax: +852-65557188
Telephone: +852-31779906
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

PUBLICATION DATE
June 28, 2013

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

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

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3713

World J Gastroenterol 2013 June 28; 19(24): 3713-3722
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Green tea and the risk of gastric cancer: Epidemiological evidence

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Received: January 10, 2013 Revised: April 14, 2013
Accepted: May 9, 2013
Published online: June 28, 2013

Abstract

Gastric cancer (GC) is one of the leading causes of cancer death in the world. Numerous efforts are being made to find chemoprotective agents able to reduce its risk. Amongst these, green tea has been reported to have a protective effect against stomach cancer. This article aims to critically evaluate all epidemiological studies reporting an association between green tea consumption and GC risk. MEDLINE, EBSCOHOST and Google Scholar were used to search for clinical trials of green tea and its correlation to stomach cancer. Studies include cohort and case-control studies. Outcome of interests are inverse association, no association, and positive association. Seventeen epidemiologic studies were reviewed. Eleven studies were conducted in Japan, five in China, and one with Japanese descendent in Hawaii. Ten case-control studies and seven cohort studies were included. The relative risks or odds ratio of GC for the highest level of green tea consumption was compared. Seven studies suggested no association, eight an inverse association, and one a positive association. One study had shown a significantly lowered GC risk when tea was served warm to cold. Another study also showed a significantly risk with lukewarm tea. All studies that analyzed men and women separately have suggested a reduced risk in women than in men, albeit no significant difference. This review demonstrates that there is insufficient information to support green tea consumption reduces the risk of GC. More studies on the subject matter are warranted.

Key words: Gastric cancer; Green tea; Epidemiology; Case-control study; Cohort study

Core tip: Gastric cancer (GC) is one of the leading causes of cancer death in the world. Numerous efforts are being made to find chemoprotective agents able to reduce its risk. This review demonstrates that there is insufficient information to support green tea consumption reduces the risk of GC. More studies on the subject matter are warranted.

Hou IC, Amarnani S, Chong MT, Bishayee A. Green tea and the risk of gastric cancer: Epidemiological evidence. *World J Gastroenterol* 2013; 19(24): 3713-3722 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3713.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3713

INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and second leading cause of death from cancer throughout the world. A 2011 world analysis showed that 989600 new GC cases and 738000 deaths were estimated to have

occurred in 2008[1]. The incidence of GC varies up to 10-fold across the world with the greatest percentage in China, followed by South Korea, South American countries and Japan[1]. These variations may be due to differences in environmental and lifestyle factors.

An excessive intake of protein, fat, salt or meat increases the risk of stomach cancer. Contrarily, dietary fiber, vegetables, fruit, and soy have been shown to play an important role in prevention of GC[2]. Tea, one of the most commonly consumed beverages in the world, has been reported in pre-clinical and epidemiological studies to provide protective effects against GC[3]. Green tea and its constituents have been shown to exhibit multiple health benefits[4-6]. Green tea and its bioactive constituents inhibit tumorigenesis in many animal models, including those for cancer of the skin, lung, oral cavity, esophagus, stomach, small intestine, colon, liver, pancreas, bladder, breast, and prostate[7-11].

Polyphenols, which include flavanols, flavandiols, flavonoids, and phenolic acids, constitute the most interesting group of green tea leaf components. Most green tea polyphenols (GTPs) are flavonoids. Flavonoids are phenol derivatives synthesized in substantial amounts (0.5%-1.5%) and variety (more than 4000 flavonoids identified), and widely distributed among plants[12]. The main flavonoids present in green tea are catechins. Catechins are colorless, astringent, water soluble compounds, and are readily oxidizable. Since green tea does not undergo fermentation, it contains greater amounts of various catechins than in black or oolong tea. Traces of catechins are also found in grapes, wine, and chocolate[13]. The four kinds of catechins found mainly in green tea include: (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin, (-)-epicatechin, and (-)-epigallocatechin-3-gallate (EGCG). Out of the above, EGCG accounts for more than 40% of the total catechin content. Figure 1 shows the chemical structure of the four major catechins present in green tea. EGCG is the most abundant polyphenol in green tea and has gained most attention as the active constituent responsible for the anticarcinogenic activity of this tea. One cup of brewed green tea (2.5 g of green tea leaves/200 mL of water) may contain 90 mg of EGCG[14]. In black tea, the catechins compounds such as theaflavins and thearubigins predominate. Black and green tea both contains similar amounts of flavonoids, however they differ in their chemical structure; green tea contains more catechins (simple flavonoids). Conversely, the oxidation undergone by black tea processing converts these simple flavonoids into theaflavins and thearubigins[12].

The first green tea clinical trial with cancer patients as a phase I study using green tea capsules was performed in 1997 and later published in 2001[15]. The purpose of this study was to determine the maximum-tolerated dose, toxicity, and pharmacology of oral green tea extract. A total of 49 cancer patients with solid tumors were studies. There were two treatment arms in this two years study: 0.05-5.05 g/m^2 once daily dose and 1.0-2.2 g/m^2 three-times-daily for 6 mo. The maximum tolerated dose was 4.2 g/m^2 once daily or 1.0 g/m^2 three times a day. This study recommended that 1.0 g/m^2 for three times daily should be considered for future clinical trials and that doses studied can be taken safely for at least 6 mo[15]. Thereafter, several phase I studies on healthy volunteers have also been conducted to define the basic pharmacokinetic parameters and safety profile for oral consumption of various types of green tea preparations[16,17]. In 2003, a phase I study investigated polyphenon E™ (a defined, decaffeinated GTP mixture) in healthy individuals[18]. The study concluded that greater oral bioavailability of EGCG can be achieved by taking the polyphenon E™ capsules on an empty stomach after an overnight fast. Recent phase I studies have concluded that the consumption of green tea appears to be relatively safe (8-16 cups of green tea once a day or in divided doses twice a day for 4 wk)[3]. However, up to 2 g orally twice per day was observed to be well tolerated in patients with stage 0 to Ⅱ chronic lymphocytic leukemia[19]. Bettuzzi *et al*[20] had reported that 600 mg of daily catechin extract had a statistically significant protective effect in patients with high-grade prostate intraepithelial neoplasia. EGCG delivered in the capsule form (200 mg/d for 12 wk) has also been reported to be effective in patients with human papilloma virus-infected cervical lesions[21].

Although many clinical trials have been conducted to explore the safety and efficacy of green tea extract in cancer patients, similar studies in GC patients have not yet been executed. Over the last three decades, a number of epidemiological studies were conducted to investigate the association between green tea consumption and stomach cancer risk in human subjects[22-27]. Nonetheless, the epidemiologic studies have not yielded clear conclusions concerning the protective effects of tea consumption against cancer formation in humans. The aim of this systematic and up-to-date review was to critically evaluate all epidemiological studies published so far to report an association between green tea consumption and GC risk.

SEARCH STRATEGY AND METHODS

Database and search strategy

Research articles presented in this review includes epidemiological studies of green tea consumption in relation to GC risks. Epidemiological studies were retrieved through three computerized literature search engines: PubMed (Medline), American University of Health Science's literature database EBSCOHOST, and Google Scholar. All 5490 journals in PubMed and 32 publications in EBSCOHOST are utilized in the search. Only articles published between 1990 and 2012 were retrieved. The major descriptor key words used for the search were stomach cancer, GC, green tea, *Camellia sinensis*, EGCG, polyphenol, catechin and the minor descriptor key words used were tumor, cancer, lesion, polyp, adenocarcinoma. The identifiers used were risk, prevention, treatment, prolong disease. In addition, relevant publications retrieved from reference lists were manually searched to be included in this review. Abstracts were reviewed and studies retrieved in full.

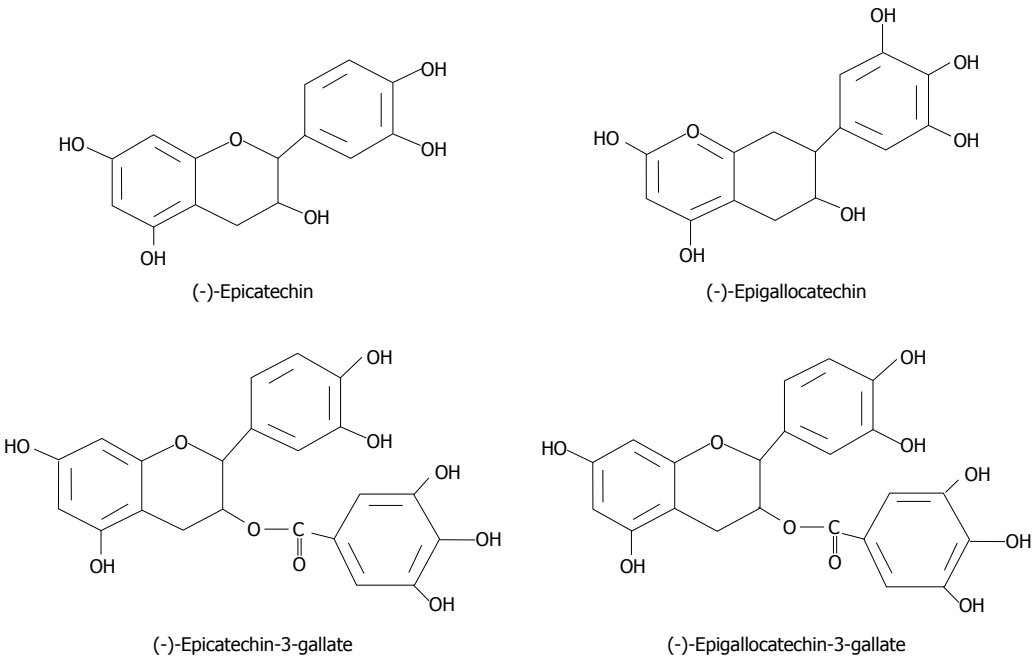

Figure 1 Structures of green tea catechins.

Inclusion criteria

Only case-control studies, cohort studies, and prospective studies reporting association between green tea and stomach cancer risk were included. Articles specifying the number of GC cases and controls for case-control studies, odds ratio (OR) or relative risk (RR) and its corresponding 95%CI for highest versus non/lowest levels of tea intake are presented in this review.

Exclusion criteria

In vitro cell culture and animal studies were excluded. Papers that failed to report the number of individuals (cases and controls) involved in the study and the level of green tea consumption (amount or frequency of green tea consumed) were also excluded. Articles written in foreign languages, as well as abstracts, were excluded since the complete publications were not available for this review work.

Data extraction

All articles were read in full. From those studies finally selected, the following data were extracted: study design, study name (first author, publication year), region and study period (years), population size, green tea consumption level, OR or RR with 95%CI of the highest consumption level, and adjustments.

GREEN TEA AND GC RISK

This review included a total of 17 epidemiological studies (10 case-control studies[28-37] and 7 cohort studies[38-44] published between 1988 and 2010. Figure 2 displays a flow diagram of the procedure used to identify the studies included in this review. There were 210 papers relevant to the words used for the search. Through the steps of title screening, 125 studies were excluded (16 duplicate articles, 31 articles not in English, and 78 were not epidemiologic studies). Abstracts from 85 articles were reviewed and an additional 67 studies were excluded (33 were not epidemiologic studies, 34 were not conducted in humans), resulting in 18 articles for full publication review. Of these, 7 were excluded (5 were not green tea, 2 did not report usable data). Of the remaining 11 articles, six articles were identified from the reference lists and are included in this review. As a result, 17 articles were found to meet the inclusion criteria described above.

Eleven studies were conducted among the Japanese population in Japan[28-30,33,35,39-44], five studies were conducted among the Chinese population in China[31,32,34,36,37], and others were conducted among the Japanese-born population in Hawaii, United States[38]. All of the Chinese studies were case-control studies. RR, OR, 95%CI of the highest green tea consumption levels were included. Table 1 represents the main characteristics of the studies included in this review.

Three hospital-based case control studies were carried out in Japan[29,33] and China[37]. In all investigations, all cases were confirmed histologically and controls were free of gastrointestinal diseases. Information on green tea consumption, as well as lifestyle habits and family history, was obtained through a self-administered questionnaire before the final diagnosis. Adjustments were made for age, gender, place of residence[29], age, gender, year and season at first hospital visit, smoking and alcohol drinking status, physical exercise, intake of coffee, black tea, fruit, rice and beef[33], or age, gender, education level, and smoking status[37]. The RRs or ORs were calculated using, as reference category, subjects who drank less than 1 cup/d, or non-drinking subjects[33,37]. Although Kato *et al*[29] did not find an association between green tea

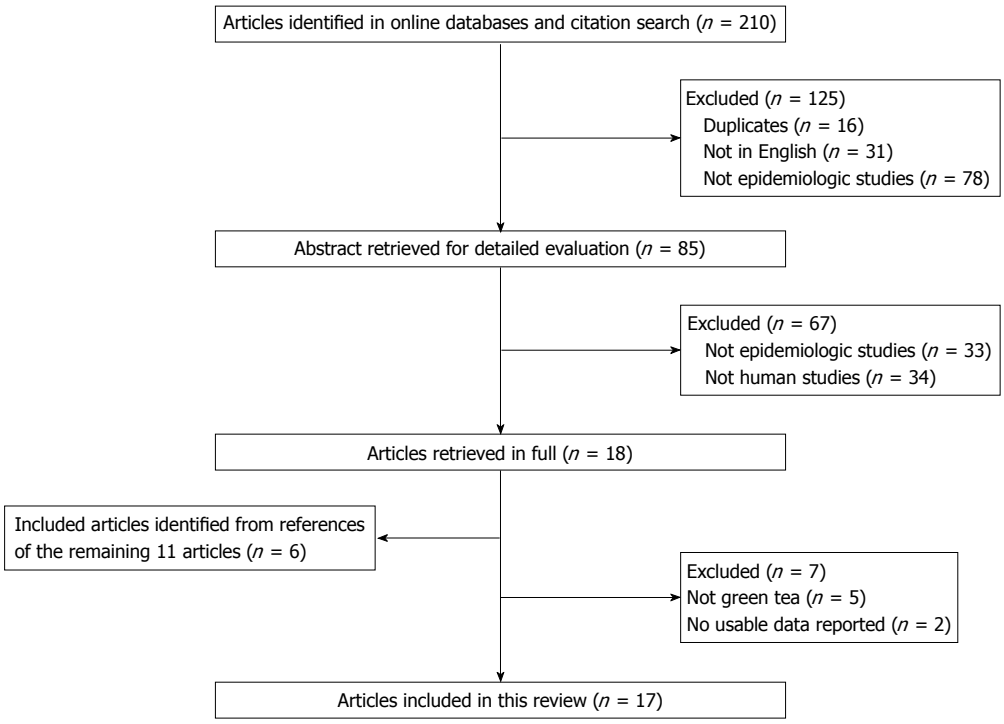

Figure 2 Flow diagram of identification of relevant studies.

consumption and GC risk, Inoue *et al*[33] and Deandrea *et al*[37] showed that the frequent consumption of green tea (≥ 7 cups/d and ≥ 750 g/year, respectively) decreased the risk of GC. Furthermore, Deandrea *et al*[37] observed a significant decrease in GC risks amongst drinkers of green tea at lukewarm temperature.

Five population-based case control studies were conducted in Japan[35] and China[31,32,34,37]. Cancer cases were confirmed pathologically[34,37], histologically[31,32] or by other methods[35]. In some epidemiological studies, controls were randomly selected[32,34,36] and matched according to age and gender[31,32,35], or study area[35]. Information was either obtained through interview[31,32,34,37] or administration of questionnaires[35]. Adjustments were made for age, education, birthplace, alcohol and cigarette usage, fresh fruit, vegetable and preserved fruit intake[31]; age, income, education among women and further adjusted for smoking and alcohol drinking among men[32]; age, gender, body mass index, cigarette smoking, and alcohol drinking[34]; and age gender, education, income, body mass index, smoking and alcohol drinking, very hot food eating habit, *Helicobacter pylori* (*H. pylori*) infection, stomach disease, family history of GC[35]. The RRs or ORs were calculated using, as reference category, non-tea drinkers[31,32,34,36] or low consumption of green tea[35]. Four of these five studies reported an inverse association between green tea consumption and the risk of GC[31,32,34,36]. The investigation conducted in Japan did not report any association[35]. Yu *et al*[31] reported that drinking warm or cold tea was associated with significant decreased GC risk compared with non-drinkers. This coincides with the findings of Deandrea *et al*[37] that there is a significant inverse association with consuming lukewarm green tea and GC risk.

Two population-hospital-based case control studies were conducted in Japan[28,30]. In both studies, cases were histologically diagnosed and hospital controls were recruited. Information from cases and hospital controls, as well as population controls, on the frequency and amount of green tea consumption was obtained by interview. Interviews were performed by the public health nurses and hospital staff[28] or by the authors and colleagues[30]. In both studies, the interview on cases and hospital controls were conducted before diagnostic procedures. The results were adjusted for age, gender, smoking, intake of mandarin oranges and other fruits[28]; and age, gender, residence, and smoking status[30]. The RRs for both studies were compared intermediate and high consumption with low consumption of green tea. Kono *et al*[28] observed a significantly decreased GC risk with high consumption of green tea (10 or more cups/d) compared to both hospital and population controls. However, Hoshiyama *et al*[30] observed a minimal to positive association in hospital and population controls.

All of the seven cohort studies were carried out among the Japanese population[38-44]. However, one was conducted in Hawaii, United States[38]. Information on the frequency and amount of green tea consumed and on other lifestyle factors was obtained by a self-administered postal questionnaire. In two of the seven cohort studies, the validity of the food frequency questionnaire was evaluated[42,44], and in two of the seven cohort studies the questionnaire was checked by interviewers[40,41]. In the study by Galanis *et al*[38] of 11907 randomly selected Japanese residents of Hawaii, 108 participants developed GC (follow-up period of 14.8 years on average). The study by Nakachi *et al*[39] on 8552 residents in Saitama Prefecture,

Table 1 Characteristics of epidemiological studies on green tea consumption and stomach cancer risk

Ref.	Region and observation period	Study population[1]	Green tea consumption levels[2]	RR or OR (95%CI)[3]	Direction of association	Adjustments
Case-control studies						
Kono *et al*[28], 1988	Kyushu, Japan, 1979-1982	139 GC cases 2574 HC 278 PC	≤ 4 cups/d 5-9 cups/d ≥ 10 cups/d	HC: 0.5 (0.3-1.1) PC: 0.3 (0.1-0.7)	Inverse (significant) with high consumption	Age, gender, cigarette smoking, mandarin oranges and other fruits
Kato *et al*[29], 1990	Aichi, Japan, 1985-1989	427 GC cases 3014 HC	1-4 cup/d ≥ 5 cups/d	Males: 1.01 (0.70-1.47) Females: 0.81 (0.51-1.27)	None	Age, gender, residence (metropolitan area in Aichi prefecture, other areas of Aichi prefecture, and other prefectures)
Hoyoshima *et al*[30], 1992	Saitama, Japan, 1984-1990	294 GC cases 202 HC 294 PC	≤ 4 cups/d 5-7 cups/d ≥ 8 cups/d	HC: 1.3 (0.8-2.1) PC: 0.8 (0.5-1.3)	Minimal to positive	Age, gender, residence, cigarette smoking
Yu *et al*[31], 1995	Shanghai, China, 1991-1993	711 GC cases 711 matched PC	Non drinkers Drinkers	Temperature: 0.71 (0.54-0.93) Boiling hot: 1.18 (0.75-1.86) Hot: 0.63 (0.46-0.87) Warm/cold: 0.51 (0.29-0.91)	Inverse (significant with warm/ cold green tea)	Age, education, birthplace, alcohol drinking, cigarette smoking, intake of fresh fruit, vegetables and preserved fruit
Ji *et al*[32], 1996	Shanghai, China, 1988-1989	1124 GC cases 1451 matched PC	Non drinkers Males: ≤ 1200 g/yr ≤ 2000 g/yr ≤ 3000 g/yr Females: ≤ 1200 g/yr > 1200 g/yr	Males: 0.76 (0.55-1.27) Females: 0.81(0.46-1.43)	Inverse	Age, income, and education among women; further adjusted for smoking and alcohol drinking among men
Inoue *et al*[33], 1998	Nagoya, Japan, 1990-1995	893 GC cases 21128 HC	Rarely ≥ 7 cups/ d	0.69 (0.48-1.00)	Inverse	Age, gender, year and season at first hospital visit, habitual smoking, habitual alcohol drinking, regular physical exercise, intake of coffee, black tea, fruit, rice and beef
Setiawan *et al*[34], 2001	Yangzhong, China, 1995	133 GC cases 433 PC	Non drinkers 1-21 cups/wk > 21 cups/wk	0.39 (0.15-1.01)	Inverse (significant)	Age, gender, body mass index, cigarette smoking, alcohol drinking
Hoshiyama *et al*[35], 2004	Japan, 1988-1990	157 GC cases 285 PC	< 1 cup/d 1-2 cups/d 3-4 cups/d 5-9 cups/d ≥ 10 cups/d	1.20 (0.6-2.5)	None	Age, gender, cigarette smoking, *H. pylori* infection, history of peptic ulcer, family history of stomach cancer, educational level, consumption of rice, miso soup, green-yellow vegetables, white vegetables, fruits, preference for salty foods
Mu *et al*[36], 2005	Taixing, China, 2000	206 GC cases 415 PC	Non-drinkers > 250 g/mo	0.39 (0.17-0.91)	Inverse	Age, gender, education, income, body mass index, cigarette smoking, alcohol drinking, very hot food eating habit, *H. pylori* infection, stomach disease, family history of stomach cancer
Deandrea *et al*[37], 2010	Harbin, China, 1987-1989	266 GC cases 533 HC	Non-drinkers < 750 g/ yr ≥ 750 g/yr	Temperature: 0.87 (0.60-1.25) Hot: 1.27 (0.85-1.90) Lukewarm: 0.19 (0.07-0.49)	Inverse (significant with lukewarm green tea)	Age, gender, education level, cigarette smoking
Prospective cohort studies						
Galanis *et al*[38], 1998	Hawaii, Unites States, 1975-1994	108 GC cases 11907 Japanese residents	Non-drinkers 1 cup/d > 2 cups/d	1.5 (0.9-2.3) Males: 1. 6 (0.9-2.9) Females: 1.3 (0.6-2.6)	Positive	Age, gender, years of education and Japanese place of birth
Nakachi *et al*[39], 2000	Saitama, Japan	488 GC cases 8552 adults	≤ 3 cups/d 4-9 cups/d ≥ 10 cups/d	0.59 (0.35-0.98) Males: 0.54 (0.22-1.34) Females: 0.57 (0.34-0.98)	Inverse	Cigarette smoking, alcohol drinking, intake of green and yellow vegetables, intake of rice
Tsubono *et al*[40], 2001	Miyagi, Japan, 1984-1992	419 GC cases 26311 adults	< 1 cups/d 1-2 cups/d 3-4 cups/d ≥ 5 cups/d	1.2 (0.9-1.6) Males: 1.5 (1.0-2.1) Females: 0.8 (0.5-1.3)	None	Age, gender, type of health insurance, history of peptic ulcer, cigarette smoking, alcohol consumption, consumption of rice, black tea, coffee, meat, green or yellow vegetables, pickled vegetables, other vegetables, fruits and bean-paste soup
Nagano *et al*[41], 2001	Hiroshima Nagasaki, Japan, 1979-1981	901 GC cases 37639 adults	0-1 cups/d 2-4 cups/d ≥ 5 cups/d	0.95 (0.76-1.2)	None	Age, gender, city of residence, radiation exposure, cigarette smoking, alcohol drinking, body mass index, education level

Hoshiyama *et al*[42], 2002	Japan (nationwide), 1988-1997	359 GC deaths 72851 adults	< 1 cups/d 1-2 cups/d 3-4 cups/d 5-9 cups/d ⩾ 10 cups/d	Men: 1.0 (0.5-2.0) Women: 0.7 (0.3-2.0)	None	Age, smoking, history of peptic ulcer, family history, consumption of rice, miso soup, green-yellow vegetables, fruits and preference for salty foods
Fujino *et al*[43], 2002	Japan (nationwide), 1988-1990	379 GC deaths 328030 adults	Everyday ⩽ 3 times/d > 3 times/d	Males: 1.11 (0.75-1.63) Females: 1.43 (0.78-2.62)	None	Age, gender, cigarette smoking, alcohol drinking, diet, sporting activities, medical history, education level
Sasazuki *et al*[44], 2004	Japan (nationwide) Cohort Ⅰ: 1990-2001 Cohort Ⅱ: 1993-1999	892 GC cases 72943 adults	< 1 cups/d 1-2 cups/d 3-4 cups/d ⩾ 5 cups/d	Males: 0.97 (0.77-1.22) Females: 0.70 (0.47-1.05)	None	Age, area, cigarette smoking, consumption of fruit, green-yellow vegetables, fish gut, miso soup, rice, black tea and coffee

[1]Number of gastric cancer (GC) cases observed over the study period; [2]Frequency of green tea consumption reported in each study; [3]Relative risk (RR) or odds ratio (OR) for the highest green tea (GT) consumption level studies. HC: Hospital controls; PC: Population controls; *H. pylori*: *Helicobacter pylori*.

Table 2 Summary of findings: Number of epidemiological studies with its reported direction of association between green tea consumption and gastric cancer risk

Study design	Direction of association			
	Negative/inverse	None	Positive	Total
Case-control studies	7	3	0	10
Cohort studies	1	5	1	7
Total	8	8	1	17

Japan found 488 deaths from GC (follow-up period of 11 years). In the study by Tsubono *et al*[40] on 26311 residents of the Miyagi Prefecture, 419 participants developed GC (follow-up period of 9 years). Nagano *et al*[41] examined 37639 atomic bomb survivors, and of those 901 participants developed GC (follow-up period of 15 years). In the study by Hoshiyama *et al*[42] on 72851 Japanese residents, 359 subjects died of GC (follow-up period of 7 years). In the study by Fujino *et al*[43] on 328030 Japanese residents, 345 subjects died of cancer (follow-up period of 7 years). Finally, in the study conducted by Sasazuki *et al*[44] of 72943 Japanese residents, 892 participants developed GC (follow-up period of (follow-up period of 10 years). In all cohorts studies, some adjustments were made: age[38-44], gender[38-41,44], years of education[38,41,43], cigarette smoking (for men only)[38-44], alcohol consumption (for men only)[39-41,43], place of birth[38], history of peptic ulcer[40,42], body mass index[41], coffee consumption and several other foodstuffs[39,40,42,43]. RRs of the highest green tea consumption levels were included instead for all consumption levels. In all studies, some adjustments were made: gender, age, years of education, cigarette smoking, alcohol consumption, place of birth, family history of GC, body mass index, coffee consumption and several other foodstuffs. The RRs were calculated using, as reference category, either non-drinkers[38] or the lowest level of green tea consumption[39-44]. In the first cohort study, Galanis *et al*[38] reported a non-significant increased risk of GC associated with green tea consumption. In contrast, Nakachi *et al*[39] reported a slight inverse association between green tea consumption and GC risks. The other five cohort studies found no association between green tea consumption and GC incidence[40-44].

CRITICAL ANALYSIS OF EPIDEMIOLOGICAL FINDINGS

Green tea contains polyphenols, powerful antioxidants that act as chemopreventive agents. EGCG is considered the major polyphenol that constitute to green tea's preventive constitute. Significant amounts of *in vitro* studies with human cancer cells as well as *in vivo* studies with animal models and have investigated the mechanism of EGCG for its anticarcinogenic properties. EGCG blocks multistage carcinogenesis by modulating a wide spectrum of signal transduction pathways, including mitogen-activated protein kinases, Janus kinase-signal transducer and activator of transcription, phosphoinositide 3-kinase/protein kinase B, Notch, nuclear factor-κB and Wnt/β-catenin, involved in cell proliferation, transformation, apoptosis, inflammation, invasion and metastasis[45]. Accumulative studies show another green tea catechin ECG can also interfere with multiple cell signaling pathways and has multiple cellular targets which are likely to interact in concert to reduce the risk of cancer[46]. Several clinical studies with human subjects have also demonstrated that consumption of green tea as well as EGCG exert beneficial effects[45]. However, epidemiologic studies are somewhat limited with mixed results. Of the 17 epidemiological studies investigated in this review, eight have no association[29,30,35,40-44], eight have inverse association[28,31-34,36,37,39], and one has positive association[38]. Table 2 provides a summary of findings. A trend observed is that decreased risk was associated with an increased green tea consumption level. The studies conducted in China showed a stronger reduction in GC among green tea drinkers than those conducted in Japan. A few authors have argued that the relative lack of subjects in Japan who do not drink green tea may have resulted in an insufficient number of non-drinkers, and this might be an explanation for the weaker association among Japanese studies[36,41,47]. In terms of study design, prospective/cohort studies are considered to be more reliable than case-controlled studies because of the large population size. However, the value of a study depends not only on the type of design, but also on the overall quality. This review identified seven cohort studies which examined the association between cancer risk and green tea consumption.

Of these, the smallest study observed an inverse association[39], whereas the five largest studies observed no association[40-44]. One study found a non-significant positive association[38].

A negative association is stronger in case-control studies than in cohort studies. Seven of the ten case-control studies have suggested an association of green tea consumption and reduced GC risk[28,31-34,36,37], whereas only one of the seven cohort studies showed a reduced GC risk[39]. These discrepancies may be partly associated with the limitations of case-control design: recall, information, selection, and confounding biases. Cancer patients may recall their dietary habits differently from healthy controls, and healthy controls are rarely representative of the population as a whole and tend to report a healthy dietary habit[36]. In retrospective studies like case control studies, the decreased consumption of green tea after abdominal symptoms due to GC may have biased the patient's recall of past consumption, resulting in underestimating the patient's true intake on green tea. Moreover, present dietary habits might influence the accuracy of recalling past dietary habits. In case-control studies, there may be a problem with the reliability of information, because information that exposes the past history is collected after cancer is diagnosed. In all studies included in this review, selection and confounding bias was minimized (*i.e.,* cases and controls were drawn from the same population; adjustment for certain factors). However, in hospital-based case control studies, the controls were not free of diseases. Furthermore, self-selection bias could not be excluded because information was obtained by questionnaire survey. Only one cohort study (reporting no association between green tea consumption and GC risk) showed limitations due to selection bias[30].

The non-significant findings regarding the effects of green tea consumption on GC risk in this review contradicts the results of previous experimental studies on this topic using *in vivo* animal models and *in vitro* cancer cell lines. The experimental studies have suggested that GTPs might have a protective effect against GC due to apoptosis-inducing, antimutagenic, anti-inflammatory, and antioxidant activities. The reason for this discrepancy between the results of experimental studies and this review is unclear. However, there might be a few possible explanations. First is the difference in causative factors between the cancers in humans and animals. It is possible that green tea may be only effective against GC in certain animal species. Second, in *in vivo* studies, the doses of green tea used in animal models are much higher in comparison to human consumption. Lastly, in the context of GC, there might be a difference in the biological activities of polyphenols as an independent compound rather than green tea taken as a whole. Despite the protective role of an independent compound against the development of cancer, it is possible that the adverse effect of green tea taken as a whole is due to the interactions and complex biological mechanisms of its multiple constituents.

In this review, eight studies have analyzed men and women separately, provided RRs or ORs for each gender[18,22,28-30,32-34]. In these eight studies, men were considered to show no relation at all. Women, however, seemed to show lower risk, though there was no statistically significant difference. It should also be noted that cigarette smoking and alcohol consumption are important confounding factors. For example, in a case-control study by Tsubono *et al*[40], a protective effect of green tea consumption was observed in women, mostly non-smokers, with an OR of 0.8 and a 90%CI of 0.5-1.3. However, no protective effect was found in men, who were mostly smokers (OR = 1.5, 95%CI: 1.0-2.1). In Asia, tea drinking is commonly associated with cigarette smoking in men. Although the study by Tsubono *et al*[40] and other studies included in this review tried to correct for smoking, the interaction between these two factors is difficult to assess.

It is noted by Yu *et al*[31] that among green tea drinkers, the risk of developing GC did not depend on the age when routine tea drinking started. This implied that green tea may disrupt gastric carcinogenesis at the intermediate and late stages. In addition to finding a negative association between green tea drinking and GC risk, Yu *et al*[31] noted that a lowered risk is observed when the tea was served warm/cold (boiling hot, OR = 1.18, 95%CI: 0.75-1.86; warm/cold, OR = 0.51, 95%CI: 0.29-0.91). This observation was further supported by another case-control study by Deandrea *et al*[37] (hot, OR = 1.27, 95%CI: 0.85-1.90; lukewarm, OR = 0.19, 95%CI: 0.07-0.49).

In one of the cohort studies, Galanis *et al*[38] suggested that tea might have a mutagenic effect (OR = 1.5, 95%CI: 0.9-2.3). However, the number of cases in this study was very small, and that may have resulted in the exaggerated risk estimates.

Overall, these data do not seem to suggest a protective effect of green tea on GC. The inconsistent results between the epidemiologic studies may be due to variables, such as differences in tea preparation and consumption, the methods of tea production, the bioavailability of tea compounds, and genetic variation in how the human body responds to tea consumption.

All research findings from tumor cell cultures, animal models, and epidemiological studies have shown the effects of green tea and tea polyphenols in GC prevention. However, this should be confirmed in clinical trials in order to gain more knowledge of the relationship between green tea consumption and GC risks. This review shows that the overall evidence for protective effects of green tea against cancer is inconclusive. Therefore, further epidemiologic studies and clinical trials are warranted. Adequate sample sizes, better descriptions of populations and/or clear definitions of green tea consumption may be required for conclusive studies. It is also important to consider the type of tea or its preparation (*e.g.,* short time *vs* long brewing time and hot tea *vs* iced tea) due to the marked impact of these factors on polyphenol content and concentration. It is also important to draw attention on the need of further in-depth studies on the nature and mechanisms of the active green tea compounds, on the bioavailability of the different catechins in human and

appropriate dose level to act as functional food. Further epidemiological research, designed specifically to study the effect of green tea on GC, is needed. Because many green tea drinkers brew more than one cup of tea from each batch of dried leaves, in future studies, green tea consumption should be assessed in terms of the amount of active ingredient consumed in a given period. Nevertheless, the *in vitro*, animal, and epidemiologic studies on the topic of green tea consumption and GC risks offered insightful results worthy of future research on potential cancer prevention in human.

This review has several limitations. First of all, this review only analyzed studies of the Japanese and Chinese population. Green tea is consumed mainly in Asian countries, such as China, Japan, and South Korea, it is not appropriate to generalize the findings in the study and apply it to all populations. The findings and explanations should be explored in further clinical research on a more diverse population. Second, as with most literature reviews, the results should be interpreted with caution because the highest consumption of green tea, lengths of follow-up, and questionnaire were not uniform. Although all publications included in this review adjusted for the consumption of dietary items other than green tea as much as possible, the possibility of residual confounding factors cannot be excluded. Lastly, 15 of the 17 studies investigated did not incorporate *H. pylori* infection, a strong risk factor for GC, as a confounding factor. The subjects with chronic gastritis caused by *H. pylori* infection might have limited their consumption of foods and beverages, including green tea. On the other hand, several studies have indicated positive interactions between green tea and *H. pylori*. EGCG, one of the green tea catechins, has been showed to possess significant protective effect against *H. pylori*-induced cytotoxicity in gastric epithelial cells *via* interference of the toll-like receptor 4 signaling induced by *H. pylori*[48]. Green tea or GTPs have exhibited bactericidal and/or bacteriostatic effects against *H. pylori*[49,50]. Various components of green tea have been reported to inhibit *H. pylori* infection as well as *H. pylori*-induced gastritis and gastric epithelial cell proliferation in animal models[51-53]. Chronic atrophic gastritis (CAG) represents a precancerous lesion of the stomach, and *H. pylori* infection is known to increase the risk of CAG. Shibata *et al*[54] conducted a cross-sectional study on 636 subjects livening in a farming village in Japan to examine the relationship among green tea consumption, *H. pylori* infection, and CAG. *H. pylori* infection was positively associated with the risk of CAG (OR = 3.73; 95%CI: 2.59-5.36). High green tea consumption (> 10 cups/d) was negatively associated with the risk of CAG, even after adjustment for *H. pylori* and lifestyle factors associated with green tea consumption (OR = 0.63; 95%CI: 0.43-0.93). The results support the hypothesis that green tea consumption prevents gastric preneoplasia. Hence, future epidemiological studies on green tea and GC risk should consider multivariate analysis on relationship among *H. pylori* infection sustained by cytotoxin-associated gene A-positive strains, other risk factors, and green tea consumption.

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P- Reviewers Franceschi F, Shibata T, Yen HH
S- Editor Huang XZ **L- Editor** A **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3723

World J Gastroenterol 2013 June 28; 19(24): 3723-3725
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

FIELD OF VISION

Hepatocellular carcinoma and food contamination: Aflatoxins and ochratoxin A as great prompter

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Received: February 26, 2013 Revised: April 18, 2013
Accepted: May 8, 2013
Published online: June 28, 2013

Abstract

Prolonged exposure to mycotoxins in the diet is related to cancer, among other diseases. Hepatocellular carcinoma (HCC) accounts for 70%-90% of primary liver cancers and is the third leading cause of cancer-related deaths worldwide. Secondary metabolites, like aflatoxins and ochratoxin A (OTA), produced by some fungi species stocked in an inappropriate manner are considered an important way to increase HCC incidence. Future epidemiologic studies of HCC should focus on good practices in food preparation, food storage and the consumption of OTA-containing foods.

Key words: Ochratoxin A; Aflatoxins; Mycotoxin; Food contamination; Hepatocellular carcinoma; Liver cancer

Core tip: This manuscript is a short commentary about the importance of considering mycotoxins produced by fungi, as important carcinogens and etiological agent able to induce hepatocellular carcinoma.

Felizardo RJF, Câmara NOS. Hepatocellular carcinoma and food contamination: Aflatoxins and ochratoxin A as great prompter. *World J Gastroenterol* 2013; 19(24): 3723-3725 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3723.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3723

COMMENTARY ON HOT TOPICS

Food contamination is a public health problem that is monitored worldwide by the Food and Agriculture Organization of the United Nations and by the World Health Organization. Care in food preparation and storage are extremely important to avoid ingestion of various microorganisms and their toxins. High temperatures and humidity during harvest, storage and processing of grains, nuts and other crops, are appropriate conditions for fungal and mold development, especially when food is stocked in an inappropriate manner. Species of *Aspergillus* and *Penicillium* are the major producers of aflatoxins and ochratoxins, my cotoxins that have been classified as potent carcinogens by the International Agency for Research on Cancer and that are well known for liver toxicity.

Hepatocellular carcinoma (HCC) is a major health problem with a rising incidence in Western countries[1], though most HCC cases (> 80%) occur in sub-Saharan Africa or Eastern Asia. China alone accounts for more than 50% of the world's cases[2]. HCC accounts for 70%-90% of primary liver cancers, making it the third leading cause of cancer-related deaths worldwide[3-5]. Hepatitis B virus, hepatitis C virus infections and alcohol intake are widely recognized as the main causes of HCC[6,7].

Aflatoxins (AFT) are secondary metabolites produced by some *Aspergillus* species that contaminate food during storage, production and processing. Due to their high toxicity and mutagenic, teratogenic and carcinogenic effects[8], they have long been suggested as possible an

etiologic agent of HCC. Mycotoxin poisoning occurring in the presence of hepatitis B virus infection is related to an increased risk of HCC development. Aflatoxins are metabolized by hepatic enzymes, generating reactive epoxide species that are able to form a covalent bond with guanine[9]. The resulting adducts can promote cellular and macromolecule damage, including producing a characteristic mutation in the p53 tumor-suppressor gene[10], and have already been described as biomarkers for aflatoxin contamination[11,12].

Aflatoxin appears to act with chronic HBV infection as a cofactor for HCC, further increasing the risk for disease. Individuals who carried the HBV and excreted AFT metabolites were related to have an increased risk of HCC[13].

Another potential contributor to the high incidence of HCC in Asia and sub-Saharan Africa is dietary exposure to ochratoxin A (OTA).

OTA is an isocoumarin-derived mycotoxin that is most commonly produced by *Aspergillusochraceus*[14] growing on stored barley, corn or wheat. OTA can be found in a wide range of human foods such as cereals, beer, wine, cocoa, coffee, dried vine fruit and spices, as well as in some meat products and milk. It is a potent,thermostable, immunosuppressive and carcinogenic toxin[15], and its effects are attributable to its ability to interfere with protein synthesis. After being absorbed, OTA is metabolized by the liver and excreted as both bile and in the renal proximal tubules[16]. In experimental animals, OTA induces tumors in the kidney as well as in the liver[15,17]. Although several lines of evidence derived from animal experiments implicate OTA in hepatic carcinogenesis[18,19], no epidemiological data are available to evaluate such relationship. Biomarkers such as GSH (reduced glutathione)-conjugates, NAC (*N*-acetylcysteine)-conjugates and DNA-OTA adducts have been detected as products of OTA metabolism by the liver[20,21], but no association between HCC and OTA has been reported.

OTA is frequently related to Balkan nephropathy, which is characterized by a discrete form of tubulointerstitial nephropathy with insidious presentation and slow progression and high levels of OTA in the urine[16]. Not only humans but also some ruminant and non-ruminant animals can be contaminated by OTA. Metabolic studies show that OTA can persist in the body of pigs; the hazard thus arises due to contamination of animal feed and constitutes an additional source of OTA contamination in human food[22].

Future epidemiologic studies of HCC should focus on good practices in food preparation, food storage and the consumption of OTA-containing foods such as cereals and milk by liver-diseased patients. The discussion of these new possible causes of HCC should be extensively investigated and not be ignored.

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P- Reviewer Jin B **S- Editor** Gou SX
L- Editor A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3726

World J Gastroenterol 2013 June 28; 19(24): 3726-3746
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

Small-bowel capsule endoscopy: A ten-point contemporary review

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Received: March 29, 2013 Revised: May 27, 2013
Accepted: June 1, 2013
Published online: June 28, 2013

Abstract

The introduction of capsule endoscopy (CE) in clinical practice increased the interest for the study of the small-bowel. Consequently, in about 10 years, an impressive quantity of literature on indications, diagnostic yield (DY), safety profile and technical evolution of CE has been published as well as several reviews. At present time, there are 5 small-bowel capsule enteroscopy (SBCE) models in the worldwide market. Head-to-head trials have showed in the great majority of studies comparable results in terms of DY, image quality and completion rate. CE meta-analyses formed the basis of national/international guidelines; these guidelines place CE in a prime position for the diagnostic work-up of patients with obscure gastrointestinal bleeding, known and/or suspected Crohn's disease and possible small-bowel neoplasia. A 2-L polyethylene glycol-based purge, administered the day before the procedure, is the most widely practiced preparation regimen. Whether this regimen can be further improved (*i.e.*, by further decreasing its volume, changing the timing of administration, coupling it with prokinetics and/or other factors) or if it can really affect the DY, is still under discussion. Faecal calprotectin has been used in SBCE studies in two settings: in patients taking non-steroidal anti-inflammatory drugs, to evaluate the type and extent of mucosal damage and, more importantly from a clinical point of view, in patients with known or suspected Crohn's disease for assessment of inflammation activity. Although there is still a lot of debate around the exact reasons of SBCE poor performance in various small-bowel segments, it is worth to remember that the capsule progress is non-steerable, hence more rapid in the proximal than in lower segments of the small-bowel. Capsule aspiration, a relatively unexpected complication, has been reported with increasing frequency. This is probably related with the increase in the mean age of patients undergoing CE. CE video review is a time-consuming procedure. Therefore, several attempts have been made to develop technical software features, in order to make CE video analysis easier and shorter (without jeopardizing its accuracy). Suspected Blood Indicator, QuickView and Fujinon Intelligent Chromo Endoscopy are some of the software tools that have been checked in various clinical studies to date.

Key words: Capsule endoscopy; Calprotectin; Meta-analysis; Review; Preparation; Reading software; Complication; Indications

Core tip: This innovative, concise and "unique" review (structured as Q and A with several tables that make this paper very easy to read and hopefully enjoyable), keeps narrative text to the necessary minimum, in order to guide the reader to consult the wealth of information included in tabulated form. These tables are the outcome of the authors' personal endeavor to compile in a detailed, yet easy to refer way, informa-

tion that has often been overlooked by the plethora of similar reviews and/or info on contentious issues in capsule enteroscopy. We believe that this document can be used as reference for study, in reference lists of future manuscript and as important guide for future clinical research on the field.

Koulaouzidis A, Rondonotti E, Karargyris A. Small-bowel capsule endoscopy: A ten-point contemporary review. *World J Gastroenterol* 2013; 19(24): 3726-3746 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3726.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3726

INTRODUCTION

An early conceptual abstract on capsule endoscopy (CE), entitled "an endorobot for flexible endoscopy, a feasibility study", was published in 1994[1]. Then, in 1997 two groups of pioneers, initially working independently in Israel and London, joined forces to achieve wireless endoscope[2]. Three years later, in the Digestive Disease Week meeting of the millennium and almost concurrently in *Nature*[3], Professor Swain presented the world's first wireless capsule endoscope.

Indeed, the brainchild of Iddan[4] has revolutionised the field of gastrointestinal (GI) diagnostics, turning into reality the concept of painless and wireless endoscopy. Furthermore, the introduction of CE in clinical practice increased the interest for the study of the small-bowel. Consequently, in about 10 years, an impressive quantity of literature on indications, diagnostic yield (DY), safety profile and technical evolution of CE has been published as well as several reviews. Therefore, we aim to focus readers' attention on contemporary and contentious issues, often missed from similar reviews on the field. We herein present (in a comprehensive yet user-friendly manner) a systematic review of the current literature in a form of question-and-answer. We expect CE readers, of all experience levels, will find this review useful source of further reading and reference.

WHICH ARE THE DIFFERENCES AMONG THE CURRENT COMMERCIALLY AVAILABLE CAPSULES?

Since 2001, the year of approval by the Food and Drug Administration of the first video capsule with the prophetic, yet slightly unfortunate, brand name mouth-to-anus (M$_2$A®; Given®Imaging, Yoqneam, Israel), a total of more than 2000000 capsules have been ingested worldwide[5]. Furthermore, over the last decade, technology has improved in the field of CE as competition has become quite stiff. At present time, there are 5 small-bowel capsule enteroscopy (SBCE) models in the market worldwide (Table 1)[5,6]. Although similar in size and shape, they differ on several technical aspects. Of the 5 SBCE, four are in widespread use, although most of the published literature studies are with PillCam®. Nevertheless, head-to-head trials have showed in the great majority of studies comparable results in terms of DY, image quality and completion rate (Table 2)[7-11].

DO HIGH-GRADE EVIDENCE SUPPORT THE USE OF CE IN CLINICAL PRACTICE?

In recent years, many authors[12-14] reviewed systematically the validity of SBCE in clinical practice. Out of this evidence base, it clearly emerges that in daily practice the leading indications for CE are: Obscure gastrointestinal bleeding (OGIB accounts for 60%-70% of all SBCE examinations world-wide), and Crohn's disease (CD; known and/or suspected). Other clinical indications, although less common, are coeliac disease, small-bowel polyposis syndromes and clinical suspicion of small-bowel neoplasia[15,16]. Therefore, we decided to summarize (Table 3)[17-32], the results of the more robust - from a methodological point of view - publications which addressed the role of CE in the field of small-bowel coeliac disease. These meta-analyses have formed the basis of national/international guidelines, which place CE in a prime position for the diagnostic work-up of patients with OGIB, known and/or suspected CD and possible small-bowel neoplasia[33-36].

WHICH IS THE BEST PREPARATION REGIMEN FOR SMALL-BOWEL CAPSULE ENDOSCOPY?

This certainly is one of the most contentious issues in CE. Since the introduction of CE in clinical practice, it was clear that small-bowel cleanliness is one of the key factors (as in fact is often the case for endoscopic examinations) to guarantee high diagnostic performance. Thus far, several studies have been performed in order to test whether the administration of different purgatives and/or prokinetics would impact on small-bowel cleanliness. It is noteworthy that these studies are rather heterogeneous in terms of type of laxatives administered, dosages and/or administration schedule (Table 3)[22,25,30]. Furthermore, in some studies laxatives and prokinetics were administered concurrently, which is probably a further source of bias. Essentially, the current evidence base suggests that a preparation regimen based on laxatives [more specifically polyethylene glycol (PEG)] is more effective -than fasting alone- in improving the small-bowel mucosa visualization. Among the PEG-based laxatives, a low volume schedule seems to be at least equally effective than high volume regimens[25,30]. Therefore, a 2-L PEG-based purge, administered the day before the procedure, is the most widely practiced preparation regimen. Whether this regimen can be further improved (*i.e.*, by further decreasing its volume, changing the timing of administration, coupling it with prokinetics and/or other pharmaceutical factors) or if it can really affect the DY, is still under discussion[37].

Table 1 Available types of small-bowel capsule endoscopes and operating characteristics

Capsule device	Company	Country	Field of view (°)	Lens	LEDs	Image sensor	Transmission	Frames per second (fps)	Dimensions (mm)	Weight (g)	Battery life (h)	Real-time imager	FDA approval	Reviewing software	Optical enhancements
PillCam®SB2	Given®Imaging, Yokneam	Israel	156	Multi-element	4	CMOS	Radiofrequency	2-4[1]	11 × 26	3.45	9->11.5[2]	Yes	Yes	Rapid®v7	Blue-mode FICE 1,2,3
MiroCam®v2	IntroMedic® Co., Seoul	South Korea	170	N/A	4	CMOS	EFP	3	ø11 × 24	3.2	12	Yes	Yes	MiroView®v2	ALICE colour-mode
EndoCapsule®	Olympus© Co., Tokyo	Japan	145	N/A	4	CCD	Radiofrequency	2	ø11 × 26	3.45	10	Yes	Yes	OLYMPUS® WS-1	Contrast imaging
OMOM® (SmartCapsule)	Chongding Jinshan Science and Technology Co., Beijing	China	140	N/A	4	CCD	Radiofrequency	2 (variable)	13 × 27.9	6	8	Yes	No	OMOM® workstation	N/A
CapsoCam®SV1	CapsoVision® Inc., Saratoga	United States	360	N/A	16	N/A	On-board EPROM flash memory (USB)	16 (4 per camera)	11 × 31	N/A	15	No	No	CapsoView®	N/A

[1]PillCam®SB2 (L) captures 2 fps - PillCam®SB2-4 captures 4 fps; [2]PillCam®SB2 (L) battery life > 11.5 h - PillCam®SB2-4 battery life 8 h. LED: Light emitting diode; N/A: Not available; CMOS: Complementary metal-oxide-semiconductor; CCD: Charge-coupled device; EFP: Electric field propagation; EPROM: Erasable programmable read-only memory; USB: Universal Serial Bus; FDA: Food and Drug Administration; FICE: Fujinon Intelligent Chromo Endoscopy; ALICE: A Large Ion Collider Experiment.

IS THERE A ROLE FOR FAECAL TESTING (CALPROTECTIN) AS "SELECTION TOOL" FOR CAPSULE ENDOSCOPY

Due to its high DY and its negative predictive value (NPV), CE has shown considerable cost-effectiveness[38]. However, CE still remains less widely available and likely more expensive, when compared to other diagnostic modalities for the small-bowel[39]. Furthermore, although CE is generally considered overall a safe modality, it can lead to severe complications (capsule retention in some patients' subgroups is reported as high as 15%[13-15,40]. Consequently, any tool or methods that allows selection of candidates, hence a more targeted and/or smooth "delivery" of SBCE, is a welcome approach. However, any pre-CE selection tool should be easy to perform, safe, inexpensive and fast[41]. In light of all these issues, faecal inflammation tests [of which, faecal calprotectin (FC) is the more widely available] have been proposed. In fact, FC has been used in SBCE studies in two settings: in patients taking non-steroidal anti-inflammatory drugs, to evaluate the type and extent of mucosal damage (Table 4)[41-44] and, more importantly from a clinical point of view, in patients with known or suspected CD for assessment of inflammation activity (Table 4)[45-48]. In these patients, although there is no clear agreement on a cut-off level, FC seems to be a cost-effective "screening test", able to identify those with higher possibility to present small-bowel lesions.

HAS CE THE SAME DIAGNOSTIC CAPABILITY ALONG THE SMALL BOWEL?

There are several papers, mostly case presentations and/or case series, reporting patients in whom CE failed to identify small-bowel lesions which were subsequently diagnosed by other modalities[49-52]. Such missed lesions (including neoplastic pathology) were occasionally large and often located in the proximal small-bowel[50,51]. Although there is still a lot of debate about the reasons of poor SBCE performance[53], it is worth remembering that for any non-steerable capsule progress is more rapid in the proximal than in lower segments of the small-bowel[53]; furthermore, opaque bile secretions and/or intra-luminal content might consequently hamper/prevent detailed mucosa visualization. Table 5 summarises all studies reporting the number of exams in which one of the few small-bowel landmarks, the ampulla of Vater (AoV), was visible during CE[54-66]. Hence, this evidence base provides an indirect confirmation of the limitations of SBCE in evaluating the proximal small-bowel. Interestingly, even in earlier studies[54] which have not been confirmed since by other investigators, the AoV was missed in > 50% of SBCE examinations. This is obviously an important drawback, especially when SBCE is used as surveillance tool, in patients with small-bowel polyposis syndromes.

Table 2 Head-to-head trials of small-bowel capsule endoscopy systems

Ref.	Country	Centre	Objective(s)	Study type	Design	CE type	Outcome(s)	Conclusion
Hartmann *et al*[7]	Germany	Single centre	Head-to-head evaluation of technical performance and DY of two CE systems (PillCam®SB *vs* EndoCapsule®)	Prospective	►OGIB pts; ►Pts randomized to undergo 2 CEs using different CE in random order	►PillCam®SB (Given®Imaging, Yoqneam, Israel); ►Pts randomized to undergo 2 CEs using different CE in random order	►Pts enrolled: 40; ►CR: PillCam®SB 33/40 (82%); EndoCapsule® 40/40 (100%); P = NS; ►Overall DY: PillCam®SB 26/50 (52%); EndoCapsule® 29/50 (58%); P = NS; ►DY (SB P2): PillCam®SB 22/50 (44%), EndoCapsule® 25/50 (50%), P = NS; ►In all discordant SB P2findings (not detected by the PillCam®SB but detected by EndoCapsule®), PillCam®SB examinations were incomplete	►Statistically non-significant trend for EndoCapsule® to detect more bleeding sources in pts with suspected small-bowel bleeding than PillCam®SB; ►This is (likely) due to the longer recording time with EndoCapsule®
Cave *et al*[8]	United States	Multi-centre (4 centres)	Comparison of performance (DY in pts with OGIB): EndoCapsule® *vs* PillCam®SB	Prospective	►OGIB pts; ►EndoCapsule® and PillCam®SB swallowed by each participant 40 min apart; ►Ingestion of CEs in randomized order; ►Head-to-head comparison of CEs	►EndoCapsule® (Olympus© America, Allentown, PA); ►PillCam®SB (Given®Imaging, Yoqneam, Israel)	►Pts with OGIB (transfused or with haematocrit < 31%) (males) or < 28% (females): 63; ►Available data 51/63; 9 pts excluded for technical reasons + 3 pts for protocol violation; ►24 videos read as normal, 14 as abnormal (from both CEs). Disagreement occurred in 13; ►No adverse events reported for either CE. Overall agreement: 38/51 (74.5%), κ = 0.48, P = 0.008; Limitations: Although ingestion randomized, videos reading not blind (different shape of the image margin)	►Both devices are safe and have comparable DY within the previously reported range; ►Subjective difference in image quality favouring the EndoCapsule®; ►Lack of electromechanical interference between 2 different CE
Kim *et al*[9]	South Korea	Single centre	Head-to-head evaluation of technical performance DY and of two capsule systems (PillCam®SB *vs* MiroCam®)	Prospective	►Pts referred to CE for various indications; ►Each pt was randomly assigned to swallow 1 of 2 CEs, the second CE was swallowed once fluoroscopy indicated that first CE had reached the SB	►MiroCam® (IntroMedic Co. Ltd., Seoul, South Korea); ►PillCam®SB (Given®Imaging, Yoqneam, Israel)	►Pts enrolled: 24; ►Mean operating time: MiroCam® 702 min; PillCam®SB 446 min, P < 0.001; ►CR: MiroCam® 20/24 (83%); PillCam®SB 14/24 (59%), P = 0.031; ►DY: MiroCam® 11/24 (45.8%); PillCam®SB 10/24 (41.7%), P = 1.0; ►DY (additive of both capsules): 12/24 (50%); ►Concordance of findings among the two capsule systems 87.5%, κ = 0.74	►MiroCam shows a longer operating time and a higher CR; ►Nevertheless, the 2 capsule systems showed comparable efficiency; ►Sequential capsule endoscopy with the MiroCam and PillCam SB produced slight (but NS) increase in DY
Pioche *et al*[10]	France	Multi-centre	Head-to-head evaluation of the diagnostic concordance (κ value): PillCam®SB SB2 *vs* MiroCam®	Prospective	►OGIB pts; ►Each pt ingested 2 CEs at a 1 h interval in a random order; ►Videos read in a random order by 2 experienced (> 200 CEs) readers; ►Image-by-image review of cases of disagreement between the readers was performed by 3 expert readers	►MiroCam®; (IntroMedic Co. Ltd., Seoul, South Korea); ►PillCamSB2 (Given®Imaging, Yoqneam, Israel)	►83 pts; drop-outs explained (10 technical issues), 73 pts/videos analysed; ►31 concordant (-) ve cases (42.4%) and 30 concordant (+) ve cases (41.1%); ►Satisfactory diagnostic concordance between the 2 systems (κ = 0.66); ►DY similar among the 2 CE systems(PillCam®SB 2 *vs* MiroCam®: 46.6% *vs* 56.2%, respectively; P = 0.02); ►SBTT longer with MiroCam® *vs* PillCam®SB (mean SBTT: 268 *vs* 234 min, P < 0.05); ►Reading time longer with MiroCam® *vs* PillCam®SB (mean reading time 40 *vs* 23 min, P < 0.05); ►(+) ve diagnosis obtained in 46.6% *vs* 56.2% of pts with PillCam®SB2 *vs* MiroCam®, respectively; ►PillCam®SB2 *vs* MiroCam®CEs identified 78.6% *vs* 95.2% of (+) ve cases, respectively, P = 0.02	►MiroCam® showed a slightly higher DY, difference not statistically significant; ►The 2 CE systems showed comparable efficiency for the diagnosis of OGIB
Dolak *et al*[11]	Austria	Single centre	Head-to-head comparison (MiroCam® *vs* EndoCapsule®) of: CR of SB examinations, DY in SB disease	Prospective	►Pts referred to CE for various indications; ►Each pt was randomly assigned to swallow either MiroCam® first, followed by the EndoCapsule® 2 h later, or vice versa; ►All videos analysed by two investigators independently	►MiroCam® (IntroMedic Co. Ltd., Seoul, South Korea); ►EndoCapsule® (Olympus America, Allentown, PA)	►Pts enrolled: 50; ►CR: MiroCam® 48/50 (96%) *vs* EndoCapsule® 45/50 (90%); P = 0.38; ►DY in SB: MiroCam® 25/50 (50%) *vs* EndoCapsule® 24/50 (48%); P > 0.99; ►Concordance of findings among the two CE systems: 68%; κ = 0.50	►The two capsule endoscopy systems were not statistically different with regards to CR and DY; ►Moderate concordance, mainly caused by missed pathological findings (which affected both devices), needs consideration in clinical practice

DY: Diagnostic yield; CE: Capsule endoscopy; OGIB: Obscure gastrointestinal bleeding; pts: Patients; CR: Completion rate; NS: Not significant (statistically); SB: Small-bowel; P2: Refers to grading of angioectasias; SBTT: Small-bowel transit time.

Table 3 Available meta-analyses and systematic reviews in the field of small-bowel capsule endoscopy

Ref.	Title	Search (start - end date)	Type	Subject	Data extractors	Total titles found	Titles entered meta-analysis	Individuals included	Outcome/conclusion
Liao *et al*[13]	Indications, detection, completion, and retention rates of SBCE: A systematic review	2000 - Jan 2009	Systematic review of evidence base	Indications, DR, CR and RR of SBCE	2	227	227	22753 Pts; 22840 CE	►Most common indications: OGIB (66.0%); investigation of clinical symptoms (10.6%); definite/suspected CD (10.4%); ►Pooled DRs for overall, OGIB, CD, neoplasia: 59.4%, 60.5%, 55.3%, 55.9%, respectively; ►Commonest cause for OGIB: angiodysplasia (50.0%); ►Pooled CRs (overall): 83.5%; breakdown 83.6% (OGIB), 85.4% (clinical symptoms), 84.2% (CD); ►Pooled RRs (overall): 1.4%; breakdown 1.2% (OGIB), 2.6% (clinical symptoms), 2.1% (CD); ►Hence, most common indication for SBCE is OGIB, with high DR and low RR; ►A relatively high RR is associated with definite/suspected CD and neoplasms
Marmo *et al*[17]	Meta-analysis: Capsule enteroscopy *vs* conventional modalities in diagnosis of SB diseases	Jan 1966 - Mar 2005	Meta-analysis of diagnostic test accuracy	DY/safety of SBCE *vs* alternative modalities (PE, SBBaR or enteroclysis) in SB disease	2	187	17	526 pts (289 OGIB and 237 CD)	►17 studies (526 patients) met inclusion criteria; ►Overall, the rate difference for SB disease (*i.e.*, the absolute pooled difference in the rate of positive findings) of SBCE *vs* alternative modalities was 41% (95%CI: 35.6-45.9); ►For OGIB, 37% (95%CI: 29.6-44.1) for Crohn's disease 45% (95%CI: 30.9-58.0); ►Incomplete SBCE occurred in 13%, more often in OGIB (17%) than in pts with CD (8%) ($P < 0.006$); ►Adverse events: 29 pts (6%); ►Capsule retention more frequent in pts with CD (3% *vs* 1%, OR 4.37)
Triester *et al*[18]	A meta-analysis of the yield of CE compared to other diagnostic modalities in patients with OGIB	N/A - April 2005	Meta-analysis of diagnostic test accuracy	IY (yield of CE-yield of comparative modality) and 95%CI of CE over comparative modalities	2	80	14	396 CE-PE; 88 CE-SBBaR	►14 studies ($n = 396$) compared DY CE *vs* PE in OGIB, 63% *vs* 28%, respectively (IY = 35%, $P < 0.00001$, 95%CI: 26%-43%); ►For clinically significant findings ($n = 376$) DY was 56% (CE) *vs* 26% (PE), IY = 30%, $P < 0.00001$, 95%CI: 21%-38%; ►3 studies ($n = 88$) compared DY of CE *vs* SBBaR, 67% *vs* 8%, respectively (IY = 59%, $P < 0.00001$, 95%CI: 48%-70%); ►For clinically significant findings DY was 42% (CE) *vs* 6% (SBBaR); IY = 36%, $P < 0.00001$, 95%CI: 25%-48%; ►NNT to yield one additional clinically significant finding with CE over either modality: 3 (95%CI: 2-4); ►1 study compared DY (significant findings) of CE *vs* intraoperative enteroscopy ($n = 42$, IY = 0%, $P = 1.0$, 95%CI: -16%-16%); ►1 study compared DY (significant findings) of CE *vs* CT enteroclysis ($n = 8$, IY = 38%, $P = 0.08$, 95%CI: -4%-79%); ►1 study compared DY (significant findings) of CE *vs* mesenteric angiogram ($n = 17$, IY = -6%, $P = 0.73$, 95%CI: -39%-28%); ►1 study compared DY (significant findings) of CE *vs* SB MRI ($n = 14$, IY = 36%, $P = 0.007$, 95%CI: 10%-62%); ►CE-DY *vs* PE (vascular lesions): 36% *vs* 20% (IY = 16%, $P < 0.00001$, 95%CI: 9%-23%); ►CE-DY *vs* PE (inflammatory lesions): 11% *vs* 2% (IY = 9%, $P = 0.0001$, 95%CI: 5%-13%); ►CE-DY *vs* PE (tumours or "other" findings): no difference
Leighton *et al*[19]	Capsule endoscopy: A meta-analysis for use with OGIB and CD	N/A - April 2005	Meta-analysis of diagnostic test accuracy	DY and safety of SBCE *vs* alternative modalities (PE, SBBaR or enteroclysis) in SB disease	2	80	20	537 pts	►CE superior to PE/SB radiography for diagnosing SB pathology in pts with OGIB (yield comparable to intraoperative endoscopy); ►Incremental yield of CE over PE/SB radiography is > 30% for clinically significant findings, due to visualization of additional vascular, inflammatory lesions by CE; ►CE was also superior to SB radiography, C + IL, CT enterography, PE for diagnosing non-stricturing SBCD; ►Marked improvement in yield with the use of CE over all other methods in pts who had established CD and were evaluated for SB recurrence; ►Unknown whether these results will translate into improved pt outcomes with the use of CE *vs* alternate methods
Leighton *et al*[19]	Capsule endoscopy: A meta-analysis for use with OGIB and CD	N/A - April 2005	Meta-analysis of diagnostic test accuracy	DY and safety of SBCE *vs* alternative modalities (PE, SBBaR or enteroclysis) in SB disease	2	80	20	537 pts	►CE superior to PE/SB radiography for diagnosing SB pathology in pts with OGIB (yield comparable to intraoperative endoscopy); ►Incremental yield of CE over PE/SB radiography is > 30% for clinically significant findings, due to visualization of additional vascular, inflammatory lesions by CE; ►CE was also superior to SB radiography, C + IL, CT enterography, PE for diagnosing non-stricturing SBCD; ►Marked improvement in yield with the use of CE over all other methods in pts who had established CD and were evaluated for SB recurrence; ►Unknown whether these results will translate into improved pt outcomes with the use of CE *vs* alternate methods
Triester *et al*[20]	A meta-analysis of the yield of CE compared to other diagnostic modalities in patients with non-stricturing SB Crohn's disease	N/A - Aug 2005	Meta-analysis of diagnostic test accuracy		2	82	9	250 pts	►9 studies ($n = 250$) compared DY CE *vs* SBBaR in CD: 63% *vs* 23%, respectively (IY = 40%, $P < 0.001$, 95%CI: 28%-51%); ►4 studies ($n = 114$) compared DY CE *vs* C + IL in CD: 61% *vs* 46%, respectively (IY = 15%, $P = 0.02$, 95%CI: 2%-27%); ►3 studies ($n = 93$) compared DY CE *vs* CT enterography/enteroclysis: 69% *vs* 30%, respectively (IY = 38%, $P = 0.001$, 95%CI: 15%-60%); ►2 studies compared DY CE *vs* PE: (IY = 38%, $P < 0.001$, 95%CI: 26%-50%); ►1 study compared DY CE *vs* SBMRI (IY = 22%, $P = 0.16$, 95%CI: -9%-53%); Sub-analysis (pts with suspected CD): no difference in DY CE *vs* SBBaR ($P = 0.09$), C + IL ($P = 0.48$), CT enterography ($P = 0.07$) or PE ($P = 0.51$); Sub-analysis (pts with established CD): difference in DY CE *vs* SBBaR ($P < 0.001$ C + IL ($P = 0.002$), CT enterography ($P < 0.001$) and PE ($P < 0.001$)

Pasha *et al*[21]	DBE and CE have comparable DY in SB disease: A meta-analysis	N/A - Dec 2006	Meta-analysis of diagnostic test accuracy	Comparison of DY of CE *vs* DBE	2	113	11	397 pts	►Pooled DY CE *vs* DBE: 60% *vs* 57% (IYW = 3%, 95%CI: -4%-10%, P = 0.42, FEM); ►Pooled DY CE *vs* DBE (vascular findings, 10 studies): 24% *vs* 24% (IYW = 0%, 95%CI: -5%-6%, P = 0.88, REM); ►Pooled DY CE *vs* DBE (inflammatory findings, 9 studies): 18% *vs* 16% (IYW = 0%, 95%CI: -5%-6%, P = 0.89, FEM); ►Pooled DY CE *vs* DBE (polyps/tumours, 9 studies): 11% *vs* 11% (IYW = -1%, 95%CI: -5%-4%, P = 0.76, FEM); ►SB disease: CE *vs* DBE have comparable DY, including OGIB, CE should be the initial diagnostic test for determining the insertion route of DBE
Niv[22]	Efficiency of bowel preparation for capsule endoscopy examination: A meta-analysis	N/A - July 2007	Meta-analysis of RCTs and cohort studies	Purgative use *vs* fasting alone for SBCE	1	6	8	130 bowel prep; 107 fasting	►237 pts, 130 with and 107 without preparation; ►Seven out of 8 studies included a comparison of GTT, SBTT and CR; ►SBCE CR: 76% in pts with preparation *vs* 68% without prep (difference did not reach statistical significance); ►No statistically significant difference between CEs performed with or without preparation in GTT (pooled effect size, -0.054; 95%CI: -0.418-0.308) or SBTT (pooled effect size, -0.327; 95%CI: -1.419 - -0.765)
El-Matary *et al*[23]	Diagnostic characteristics of given video capsule endoscopy in diagnosis of celiac disease: A meta-analysis		Meta-analysis of diagnostic test accuracy	Coeliac and CE	2	N/A	3	107 pts	►3 studies (n = 107, 63 pts with CD/44 without) met inclusion criteria; ►Pooled SBCE (overall) Sens and Spec: 83% (95%CI: 71%-90%) and 98% (95%CI: 88%-99.6%), respectively; ►No major complications reported; ►Costs mentioned only in 1 study. Overall, diagnostic characteristics of SBCE, could not justify the routine use of SBCE as alternative to biopsy
Chen *et al*[24]	A meta-analysis of the yield of CE compared to DBE in pts with SB diseases	N/A - Feb 2007	Meta-analysis of diagnostic test accuracy	Comparison of DY of CE *vs* DBE	2	163	8	277 pts	►8 studies (n = 277 pts) prospectively compared the yield of CE and DBE were included; ►No difference between the yield of CE and DBE (170/277 *vs* 156/277, OR 1.21, 95%CI: 0.64-2.29); ►Sub analysis: yield of CE significantly higher than that of DBE without combination of oral+anal insertion approaches (137/219 *vs* 110/219, OR 1.67, 95%CI: 1.14-2.44, P < 0.01), but not superior to the yield of DBE with combination of the two insertion approaches (26/48 *vs* 37/48, OR 0.33, 95%CI: 0.05-2.21, P < 0.05); ►Focused meta-analysis of the fully published articles concerning OGIB showed similar results wherein the yield of CE was significantly higher than that of DBE without combination of oral + anal insertion approaches (118/191 *vs* 96/191, fixed model: OR 1.61, 95%CI: 1.07-2.43, P < 0.05) and the yield of CE was significantly lower than that of DBE by oral+ anal combinatory approaches (11/24 *vs* 21/24, fixed model: OR 0.12, 95%CI: 0.03-0.52, P < 0.01)
Rokkas *et al*[25]	Does purgative preparation influence the diagnostic yield of small bowel video capsule endoscopy? A meta-analysis	N/A - Feb 2008	Meta-analysis of RCTs and cohort studies	Purgative use *vs* fasting alone for SBCE	2	194	12	718 pts purgative; 444 controls	►12 eligible studies (6 prospective/6 retrospective), including 16 sets of data; ►Significant difference in DY between pts prepared with purgatives (n = 263) *vs* pts prepared with clear liquids (n = 213): OR = 1.813 (95%CI: 1.251-2.628, P = 0.002); ►Significant difference in SBVQ between pts prepared with purgatives (n = 404) *vs* pts prepared with clear liquids (n = 249): OR = 2.113 (95%CI: 1.252-3.566, P = 0.005); There was no statistically significant difference regarding CR rate. Purgatives did not affect VCE GTT or VCE SBTT
Dionisio *et al*[26]	CE has a significantly higher DY in patients with suspected and established small-bowel CD: A meta-analysis	2000 - May 2009	Meta-analysis of diagnostic test accuracy	DY of CE *vs* modalities in patients with suspected/ established CD	2	291	12	428 pts	►8 studies (n = 236 pts) compared CE *vs* C + IL, 4 (n = 119 pts) CE *vs* CTE, 2 (n = 102 pts) *vs* PE, 4 (n = 123 pts) *vs* MRE; ►For suspected CD, several comparisons met statistical significance; Yields in this subgroup were: CE *vs* SBR: 52% *vs* 16% (IYw = 32%, P < 0.0001, 95%CI: 16%-48%), CE *vs* CTE: 68% *vs* 21% (IYw = 47%, P < 0.00001, 95%CI: 31%-63%), CE *vs* C + IL: 47% *vs* 25% (IYw = 22%, P = 0.009, 95%CI: 5%-39%); ►For established CD, statistically significant yields for CE *vs* an alternate diagnostic modality in patients were seen: CE *vs* PE: 66 *vs* 9% (IYw = 57%, P < 0.00001, 95%CI: 43-71%), CE *vs* SBR: 71 *vs* 36% (IYw = 38%, P < 0.00001, 95%CI: 22%-54%), CE *vs* CTE: 71 *vs* 39% (IYw = 32%, $P \leqslant 0.0001$, 95%CI: 16%-47%)
Wu *et al*[27]	Systematic review and meta-analysis of RCTs of Simethicone for GI endoscopic visibility	N/A- Nov 2009	Meta-analysis of RCTs	Simethicone and CE	2	128	4	121 pts	►Adequate or excellent/good SB mucosa visualization in pts receiving Simethicone *vs* those who did not (66.1% *vs* 37.2%); ►Pooled OR = 2.84 (95%CI: 1.74-4.65, P = 0.00); no significant heterogeneity (P = 0.16, I^2 = 38.8%) or publication bias (P = 0.251); ►Sens analysis: studies stratified by factors such as bowel preparation (purgative *vs* fasting): Significant results for bowel preparation + fasting (OR = 4.43, 95%CI: 1.82-10.76, P = 0.00) with P = 0.78, I^2 = 0.0%, No significant results for bowel preparation + purgative (OR = 1.59, 95%CI: 0.78-3.27, P = 0.203) with P = 0.20, I^2 = 38.9%
Rokkas *et al*[25]	Does purgative preparation influence the diagnostic yield of small bowel video capsule endoscopy? A meta-analysis	N/A - Feb 2008	Meta-analysis of RCTs and cohort studies	Purgative use *vs* fasting alone for SBCE	2	194	12	718 pts purgative; 444 controls	►12 eligible studies (6 prospective/6 retrospective), including 16 sets of data; ►Significant difference in DY between pts prepared with purgatives (n = 263) *vs* pts prepared with clear liquids (n = 213): OR = 1.813 (95%CI: 1.251-2.628, P = 0.002); ►Significant difference in SBVQ between pts prepared with purgatives (n = 404) *vs* pts prepared with clear liquids (n = 249): OR = 2.113 (95%CI: 1.252-3.566, P = 0.005); There was no statistically significant difference regarding CR rate. Purgatives did not affect VCE GTT or VCE SBTT

Dionisio *et al*[26]	CE has a significantly higher DY in patients with suspected and established small-bowel CD: A meta-analysis	2000 - May 2009	Meta-analysis of diagnostic test accuracy	DY of CE *vs* modalities in patients with suspected/ established CD	2	291	12	428 pts	►8 studies (n = 236 pts) compared CE *vs* C + IL, 4 (n = 119 pts) CE *vs* CTE, 2 (n = 102 pts) *vs* PE, 4 (n = 123 pts) *vs* MRE; ►For suspected CD, several comparisons met statistical significance; Yields in this subgroup were: CE *vs* SBR: 52% *vs* 16% (IYw = 32%, P < 0.0001, 95%CI: 16%-48%), CE *vs* CTE: 68% *vs* 21% (IYw = 47%, P < 0.00001, 95%CI: 31%-63%), CE *vs* C + IL: 47% *vs* 25% (IYw = 22%, P = 0.009, 95%CI: 5%-39%); ►For established CD, statistically significant yields for CE *vs* an alternate diagnostic modality in patients were seen: CE *vs* PE: 66 *vs* 9% (IYw = 57%, P < 0.00001, 95%CI: 43-71%), CE *vs* SBR: 71 *vs* 36% (IYw = 38%, P < 0.00001, 95%CI: 22%-54%), CE *vs* CTE: 71 *vs* 39% (IYw = 32%, $P \leqslant 0.0001$, 95%CI: 16%-47%)
Wu *et al*[27]	Systematic review and meta-analysis of RCTs of Simethicone for GI endoscopic visibility	N/A - Nov 2009	Meta-analysis of RCTs	Simethicone and CE	2	128	4	121 pts	►Adequate or excellent/ good SB mucosa visualization in pts receiving Simethicone *vs* those who did not (66.1% *vs* 37.2%); ►Pooled OR = 2.84 (95%CI: 1.74-4.65, P = 0.00); no significant heterogeneity (P = 0.16, I^2 = 38.8%) or publication bias (P = 0.251); ►Sens analysis: studies stratified by factors such as bowel preparation (purgative *vs* fasting): Significant results for bowel preparation + fasting (OR = 4.43, 95%CI: 1.82-10.76, P = 0.00) with P = 0.78, I^2 = 0.0%, No significant results for bowel preparation + purgative (OR = 1.59, 95%CI: 0.78-3.27, P = 0.203) with P = 0.20, I^2 = 38.9%
Cohen *et al*[28]	Use of CE in diagnosis and management of pediatric patients, based on meta-analysis	Jan 2001 - May 2010	Systematic review of evidence base	Systematic compilation of data on indications and outcomes of CE in paediatric patients	2	N/A	15	740 examinations; 723 pts	►Most common indication for CE (in pts < 18 yr): suspicion or evaluation of IBD (overall 54%), Breakdown: suspected CD (34%), known CD (16%), UC (1%), indeterminate colitis (3%) ►CR and RR: 86.2% (95%CI: 81.5-90.3%) and 2.6% (95%CI: 1.5-4.0%), respectively; ►CE RR (gastric and SB): 0.5% and 1.9%, respectively, similar to those of adults, by indication; ►CE with positive findings: 65.4% (95%CI: 54.8%-75.2%); ►CE resulting in new diagnosis: 69.4% (95%CI: 46.9%-87.9%); CE leading to change in therapy: 68.3% (95%CI: 43.6%-88.5%)
Teshima *et al*[29]	DBE and CE for OGIB: An updated meta-analysis	N/A - June 2010	Meta-analysis of diagnostic test accuracy	OGIB; CE or DBE	2	147	10	651 CE; 642 DBE	►Pooled DY for CE: 62% (95%CI: 47.3%-76.1%) ►Pooled DY for DBE 56% (95%CI: 48.9%-62.1%); OR for CE *vs* DBE of 1.39 (95%CI: 0.88-2.20; P = 0.16); Subgroup analyses ►DBE-DY after (+)ve CE: 75.0% (95%CI: 60.1%-90.0%) ►DBE-DY after (-)ve CE: 27.5% (95%CI: 16.7%-37.8%) ►DBE-OR (for successful diagnosis after (+)ve CE) compared with DBE: 1.79 (95%CI: 1.09-2.96, P = 0.02) ►In OGIB CE and DBE have similar DY, DBE-DY significantly higher when performed in pts with prior positive CE
Belsey *et al*[30]	Meta-analysis: efficacy of SB preparation for SBCE	Jan 2000 - Dec 2010	Meta-analysis of RCTs	Purgative use *vs* fasting alone for SBCE	2	33	8	291 PEG; 133 NaP; 322 fasting	►8 studies, using PEG or NaP-based bowel cleansing regimens; ►Any form of purgative significantly better visibility than fasting alone (OR = 2.31; 95%CI: 1.46-3.63, P < 0.0001); ►Similar results on DY (OR = 1.88; 95%CI: 1.24-2.84; P = 0.023); Subgroup analyses (per cleansing regimen used): ►PEG-based regimens showed benefit (OR = 3.11; 95%CI: 1.96-4.94, P < 0.0001); ►NaP-based regimens no significant difference from fasting alone (OR = 1.32; 95%CI: 0.59-2.96, P < 0.0001); ►Use of purgatives (alongside fasting) is recommended in SBCE; PEG-based regimens offer a clear advantage over NaP; ►Lower volume PEG regimens as efficacious as higher volumes traditionally used for colonoscopy preparation
Rokkas *et al*[31]	The role of video CE in the diagnosis of coeliac disease: A meta-analysis	N/A - April 2011	Meta-analysis of diagnostic test accuracy	Coeliac and CE	2	461	6	166 pts	►Pooled CE Sens: 89% (95%CI: 82%-94%) and Spec: 95% (95%CI: 89%-98%), AuROC: 0.9584; ►Although not as accurate as pathology, CE a reasonable alternative method of diagnosing coeliac disease
Koulaouzidis *et al*[32]	Diagnostic yield of SBCE in patients with IDA: A systematic review	Jan 2001 - Nov 2011	Systematic review of evidence base	IDA and CE	2	1225	24	1960 pts	►Pooled SBCE-DY in IDA: 47% (95%CI: 42%-52%), with significant heterogeneity among included studies (I^2 = 78.8%, P < 0.0001); ►Pooled SBCE-DY (subgroup 1: 4 studies focused solely on IDA pts): 66.6% (95%CI: 61.0%-72.3%, I^2 = 44.3%) ►Pooled SBCE-DY (subgroup 2: 20 studies not focusing only on IDA pts): 44% (95%CI: 39%-48%, I^2 = 64.9%); ►SBCE in subgoup 1: more vascular (31% *vs* 22.6%, P = 0.007), inflammatory (17.8% *vs* 11.3%, P = 0.009), neoplastic (7.95% *vs* 2.25%, P < 0.0001) lesions detected

CE: Capsule endoscopy; N/A: Not available or not applicable; Sens: Sensitivity; Spec: Specificity; AuROC: Area under Receiver operation characteristics curve; DBE: Double-balloon enteroscopy; OGIB: Obscure gastrointestinal bleeding; DY: Diagnostic Yield; pts: Patients; IY: Incremental yield; GTT: Gastric transit time; SBTT: Small bowel transit time; SBCE: Small-bowel capsule enteroscopy; OR: Odds ratio; RR: Relative risk; C + IL: Colonoscopy with ileoscopy; PE: Push enteroscopy; SBCD: Small bowel Crohn's disease; CT: Computed tomography; IDA: Iron deficiency anemia; FEM: Fixed effect model.

Table 4 Studies evaluating the clinical application of faecal calprotectin in the setting of small-bowel capsule endoscopy

Ref.	Country	Centre	Study type	Design	Participants	FC	CE	Objective(s)	Outcome(s)
Goldstein *et al*[41]	United States	Multi-centre	Prospec-tive	Double-blind, triple-dummy, placebo controlled	334 healthy subjects	N/A	M2A®; Given® Imaging, Yokneam, Israel	Evaluate incidence of SB injury and correlation with FC in healthy subjects on celecoxib or ibuprofen + omeprazole	►Mean increase in FC higher in subjects on ibuprofen+omeprazole compared with celecoxib alone ($P < 0.001$); ►No correlation between FC and SB mucosal breaks
Hawkey *et al*[42]	Germany, United Kingdom	Multi-centre	Prospec-tive	Double-blind, double-dummy, placebo controlled	139 healthy subjects	Phical Calprotectin Test Kit NovaTec Immunodiag-nostica, GmbH Dietzenbac, Germany	M2A®; Given® Imaging, Yokneam, Israel	Investigate SB injury lumiracoxib reduces *vs* naproxen + omeprazole	►More SB mucosal breaks on naproxen+omeprazole (77.8% *vs* 40.4%, $P < 0.001$); ►Furthermore, higher FC *vs* placebo (96.8 *vs* 14.5 μg/g, $P < 0.001$); ►27.7% on lumiracoxib had SB mucosal breaks (*vs* placebo, $P = 0.196$; *vs* naproxen, $P < 0.001$) ►No increase in FC (-5.7 μg/g; *vs* placebo, $P = 0.377$; *vs* naproxen, $P < 0.001$)
Smecuol *et al*[43]	Argentina, Spain, Canada	Multi-centre	Prospec-tive	Non-blinded study	20 healthy subjects	Calprest® Eurospital SpA, Trieste, Italy	M2A®; Given® Imaging, Yokneam, Israel	Determine SB damage by low-dose ASA (on a short-term basis)	►Short-term administration of low-dose ASA associated with mucosal abnormalities of the SB mucosa; ►Median baseline FC (6.05 μg/g; range: 1.9-79.2 μg/g) increased significantly after ASA use
Werlin *et al*[44]	United States, Israel, United Kingdom	Multi-centre	Prospec-tive	N/A	42 pts with CF* (aged 10-36 yr); 29 had pancreatic insufficiency	Calprest® Eurospital SpA, Trieste, Italy	PillCam®SB; Given® Imaging, Yokneam, Israel	Examine the SB of pts with CF without overt evidence of GI disease using CE	►Varying degrees of diffuse areas of inflammatory findings in the SB: oedema, erythema, mucosal breaks and frank ulcerations; ►No adverse events recorded; FC markedly high in pts with pancreatic insufficiency, 258 μg/g (normal < 50)
Koulaouzidis *et al*[45]	United Kingdom	Single centre	Retro-spective	Chart review	70 pts with suspected CD and (-) ve bi-directional endoscopy	CALPRO NovaTec Immunodiag-nostica GmbH, Dietzenbac, Germany	(1) PillCam® SB; Given® Imaging, Yokneam, Israel; (2) MiroCam®; IntroMedic Co., Seoul, South Korea	Value of FC as selection tool for further investigation of the SB with SBCE, in a cohort of pts with suspected CD	►FC = 50-100 μg/g: normal SBCE, despite symptoms suggestive of IBD; ►FC > 100 μg/g: good predictor of positive SBCE; ►FC > 200 μg/g: associated with higher SBCE DY (65%); confirmed CD in 50%; ►Measurement of FC prior SBCE: useful tool to select patients for referral. If FC < 100 μg/g: SBCE is not indicated (NPV 1.0)
Jensen *et al*[46]	Denmark	Single centre	Prospec-tive	Blinded study	83 pts from GI OPD clinics with suspected CD	Calprotectin ELISA, BÜHLMANN Laboratories AG, Basel, Switzerland	PillCam®SB; Given® Imaging, Yokneam, Israel	Determine FC levels in CD restricted to SB compared to colonic CD, in pts on first diagnostic work-up; Assess the Sens and Spec of FC in suspected CD	►In pts with SB or colonic CD FC is equal: median 890 μg/g *vs* 830 mg/kg, respectively ($P = 1.0$); ►FC cut-off = 50 μg/g: 92% and 94% Sens for SB and colonic CD, respectively; ►Overall, Sens and Spec for FC: 95% and 56%; ►CD was ruled out with NPV of 92%; ►In suspected CD, FC is effective marker to r/o CD and select patients for endoscopy

Koulaouzidis *et al*[47]	United Kingdom	Single centre	Retro-spective	Chart review	49 pts; known or suspected CD	CALPRO NovaTec Immuno-diagnos-tica GmbH, Dietzenbac, Germany	PillCam®; Given® Imaging, Yokneam, Israel; MiroCam®; IntroMedic Co., Seoul, South Korea	Assess performance of 2 SBCE inflammation scoring systems (LS and CECDAI) correlating them with FC; Define threshold levels for CECDAI	►LS performs better than CECDAI in describing SB inflammation, especially at FC < 100 μg/g ►CECDAI levels of 3.8 and 5.8 correspond to LS thresholds of 135 and 790, respectively
Sipponen *et al*[48]	Finland	Single centre	Pro-spective	Blinded study	84 pts; known or suspected CD	Calprest® Eurospital SpA, Trieste, Italy	PillCam®; Given® Imaging, Yokneam, Israel; MiroCam®; IntroMedic Co., Seoul, South Korea	Study the role of FC and S100A12 in predicting SB inflammatory lesions	►CE abnormal in 35/84 (42%) pts: 14 CD, 8 NSAID-enteropathy, 8 angioectasias, 4 polyps/tumours, 1 ischemic stricture ►Median FC/S100A12: 22 μg/g (range: 2-342 μg/g)/0.048 μg/g (range: 0.003-1.215 μg/g) ►FC significantly higher in CD pts (median 91, range: 2-312) compared with pts with normal CE or other abnormalities (P = 0.008) ►Faecal S100A12 (0.087 μg/g, range: 0.008-0.896 μg/g): no difference between the groups (P = 0.166) ►Sens, Spec, PPV, NPV in detecting SB inflammation; FC (cut-off 50 μg/g): 59%, 71%, 42%, 83%; S100A12 (cut-off 0.06 μg/g): 59%, 66%, 38%, 82%, respectively

CF: Cystic Fibrosis; CD: Crohn's disease; GI: Gastrointestinal; OPD: Out-Patient Department; SB: Small-bowel; FC: Faecal calprotectin; ASA: Acetyl-salycylic acid; CE: Capsule endoscopy/e; Pts: Patients; Sens: Sensitivity; Spec: Specificity; SBCE: Small-bowel capsule endoscopy; LS: Lewis score; CECDAI: Capsule endoscopy Crohn's disease activity index; NPV: Negative predictive value; PPV: Positive predictive value; N/A: Not available or not applicable; NSAID: Non-steroidal anti-inflammatory drug.

Table 5 Studies looking at the identification rate of the ampulla in capsule endoscopy

Ref.	CE	Type of CE model; Company	AoV seen, *n* (%)	Reviewers	Reviewing speed (fps)	Frames AoV visible[2]	Comments
Wijeratne *et al*[53][1]	138	NS	9 (6.0)	1	NS	NS	4 FAP patients (AoV not seen)
Kong *et al*[54]	110	M2A®; Given®Imaging Ltd.	48 (43.6)	2	15	3.5 ± 2.5	
Clarke *et al*[55]	125	M2A®; Given®Imaging Ltd.	13 (10.4)	2	5	NS	
Iaquinto *et al*[56]	23	PillCam®SB; Given®Imaging Ltd.	0 (0.0)	2	NS	N/A	FAP patients (11/23 had duodenal polyps)
Metzger *et al*[57]	20	PillCam®SB1; Given®Imaging Ltd.	1 (5.0)	NS	NS	NS	Repeat examinations
		PillCam®SB2; Given®Imaging Ltd.	5 (25.0)	NS	NS	NS	
Katsinelos *et al*[58]	14	NS	0 (0.0)	1	NS	N/A	FAP patients
Nakamura *et al*[59]	96	PillCam®SB1; Given®Imaging Ltd.	18 (18.0)	2	10	NS	
Karagiannis *et al*[60]	10	PillCam®Colon; Given®Imaging Ltd.	6 (60.0)	NS	NS	NS	Two-headed PillCam®
Lee *et al*[61][1]	30	PillCam®SB; Given®Imaging Ltd.	13 (43.3)	NS	NS	NS	
	30	PillCam®SB2; Given®Imaging Ltd.	15 (50.0)	NS	NS	NS	
	50	PillCam®SB1; Given®Imaging Ltd.	0 (0.0)	2	NS	N/A	
Selby *et al*[62]	50	PillCam®SB2; Given®Imaging Ltd.	9 (18.0)	2	NS	NS	
	8	PillCam®ESO1; Given®Imaging Ltd.	0 (0.0)	2	NS	N/A	Two-headed PillCam®
	12	PillCam®ESO2; Given®Imaging Ltd.	1 (8.0)	2	NS	NS	Two-headed PillCam®
Koulaouzidis *et al*[63]	11	PillCam®ESO1; Given®Imaging Ltd.	4 (36.4)	1	7	NS	Two-headed PillCam®
	7	PillCam®ESO2; Given®Imaging Ltd.	1 (14.3)	1	9	NS	Two-headed PillCam®
Park *et al*[64]	30	PillCam®SB; Given®Imaging Ltd.	13 (43.3)	6	7	3.1 ± 1.1	
	30	PillCam®SB2; Given®Imaging Ltd.	15 (50.0)	6	9	3.1 ± 1.5	
	262	PillCam®SB1; Given®Imaging Ltd.	28 (10.7)	1	6	36.35 ± 73.24	
Koulaouzidis *et al*[65]	148	PillCam®SB2; Given®Imaging Ltd.	13 (8.8)	1	6	42.46 ± 69.3	
	209	MiroCam®; IntroMedic Ltd.	18 (8.6)	1	6	87.20 ± 248.4	
Friedrich *et al*[66]	25	CapsoCam®SV1; Capsovision Ltd.	22 (71)	3	NS	3.1 ± 1.8	

[1]Published only as abstracts; [2]mean ± SD. CE: Capsule endoscopy; NS: Not stated; N/A: Not available or not applicable; AoV: Ampulla of Vater; fps: Frames per second; FAP: Familial adenomatous polyposis syndrome.

CAPSULE ENDOSCOPE ASPIRATION; HOW COMMON IS THIS?

Capsule enteroscopy is generally considered safe, having an overall complication rate of about 1%-3%[13,14]. Undoubtedly, the most feared complication of CE is capsule retention in the small bowel (overall retention rate 1.5%-2%), which seems directly related with the clinical indication for SBCE[13,14,40]. Interestingly enough, other possible complications - which were postulated at the time of CE introduction (*i.e.*, retention inside colonic diverticula, interaction with pacemakers, *etc.*) to represent

potential hurdles for the method, were shown to be very infrequent and/or without clinically relevant consequences[67-71]. Conversely, capsule aspiration - an unexpected complication - has been reported with increasing frequency (Table 6)[72-93]. Overall, this is probably related to the increase in the mean age of patients undergoing CE. In fact, capsule aspiration occurs in 1 out of 800-1000 procedures[88], mostly in elderly male patients with co-morbidities and/or swallowing disorders. In the majority of cases capsule aspiration resolves quickly, because patients expectorate the capsule. However, in selected cases, emergency bronchoscopy is required. Thus far, only one fatality-directly associated with capsule aspiration- has been reported[90].

CAN WE SHORTEN OUR READING TIME IN CAPSULE ENDOSCOPY?

Few will disagree with the notion that CE is a time-consuming procedure. In fact, although capsule administration and swallowing requires only a couple of minutes, SBCE transit through the small bowel, although variable, on overage lasts about 2-5 h[94]. This results in 14400-72000 frames, depending on capsule frame rate (Table 1). This large amount of visual information requires careful evaluation by the CE reader. In addition, any small-bowel lesion may only be visible in just a few or even in a single frame[95]. Therefore, focused and undivided attention is required for the entire duration of each CE video evaluation. In light of all that, several attempts have been made to develop technical software features, in order to make CE video analysis easier and shorter (without jeopardising its accuracy). The first software feature designed for this purpose was the Suspected Blood Indicator (SBI), an automatic system able to pick up, in a completely automatic fashion, frames containing several red pixels and, therefore (theoretically), to detect blood and or other red-coloured lesions. Nevertheless, the accuracy profile of this tool (Table 7) is suboptimal and, at present time[96-102], it can be used only as supportive tool[102].

Given®Imaging Ltd. has also introduced another software tool, which aims specifically at shortening the CE reading time, the QuickView. This sampling tool is able to select one frame every X CE frames (the sampling rate can be set by the reader) and therefore present, with the click of a tag-button, a shortened CE video which can be reviewed in a few minutes. Although the sampling method of the QuickView system is only quantitative, it has showed a promising sensitivity and specificity in identifying small-bowel lesions (Table 8), and reveals promising potential when coupled with other image enhancing systems[104-112]. Olympus has similar software function (express mode) and we are aware of a single relevant study with very similar results[113].

In the last few years, Given®Imaging Ltd., through a collaboration with Fujinon Inc., Japan introduced the electronic chromo-endoscopy (Fujinon Intelligent Chromo Endoscopy, FICE) in the field of capsule enteroscopy. Data available thus far, show that application of FICE in SBCE videos, leads to improved image quality and definition of the surface texture of small-bowel lesions (Table 9)[114-120]. Although this seems to facilitate the detectability of small-bowel findings, it is still under question whether it proves to be clinically significant[121]. Similar function from Olympus Inc., shows promising results[122].

WHAT'S NEW ON THE FIELD OF SMALL-BOWEL CAPSULE ENDOSCOPY?

As aforementioned, there are differences among different capsule models (Table 1). Since its introduction in clinical practice in 2001, CE technology has been significantly. For instance, battery life is longer, image capture frame rate has increased, angle of view is now wider, light control has been optimized, and many real time viewing systems are now available. Nevertheless, these impressive advancements, do not allow overcoming the main current limitation of CE, *i.e.*, uncontrolled propulsion; CE relies totally on natural bowel peristalsis, *i.e.*, it still remains a rather "passive" diagnostic technique.

Several research groups are working to design brand new capsules able to actively move or to be remotely manoeuvred through their descent in the small bowel[123]. These new capsules would allow not only recognizing a small bowel lesion but also, in a near future, to collect targeted tissue samples or to deliver drugs (Table 10)[124-141].

CONCLUSION

Since CE introduction in clinical practice in 2001, over 1500 papers, focused on SBCE, have been published (PubMed search 17/03/2012; keyword term: "small bowel capsule endoscopy"; available from: http://www.ncbi.nlm.nih.gov/pubmed/?term=small+bowel+capsule+endoscopy).

Out of those, < 20% are clinical trials; case reports and reviews account for about 40% of published evidence. As the amount of information has increased exponentially, and in fact continues to do so[12], it is often difficult for the busy clinician to retrieve and filter data or extract answers to questions arising from the daily clinical practice. In the present review, we opted to answer certain pertinent questions on contentious and important issues in CE through comprehensive tables. Essentially, we aim to present an easy-to-read review with all the necessary evidence to support opinions expressed herein.

The analysis of the publications listed in the tables clearly demonstrates how SBCE, although much "younger" than other endoscopic techniques, has found a definite role in the diagnostic work-up of certain patient-subgroups. Further success of this modality depends not only on continuous technological progress (*i.e.*, introduction of new capsule models, improved battery life and/or development of new reading software features)[142] but also on the search for new diagnostic strategies, aiming to select for SBCE those patients with higher potential for positive DY[32,45,81,111,117].

Table 6 Case reports of aspiration of capsule endoscopes

Ref.	Case (age/gender)	Comorbidities	CE model/ company	Swallowing difficulties	No. of attempts to swallow CE/ gagging or coughing	Aspiration time/where in bronchial tree CE seen	Capsule removal (if employed)	Final diagnosis
Schneider *et al*[72]	64/male	Mechanical MV on phenprocoumon, BMI 15.5	M2A®; Given®Imaging Ltd.	No Hx of dysphagia	4/gagging and spitting capsule - last attempt recurrent coughing (aspiration presumed)	2 min/trachea-bronchi	Spontaneous resolution	NS
Fleischer *et al*[73]	76/male	HHT	M2A®; Given®Imaging Ltd.	No Hx of dysphagia	1/lodged in his throat - no respiratory difficulty, could talk, vital signs normal	60 min/ cricopharyngeus	Endoscopy-Roth net; 6 d post-dilation, patient ingested capsule with no problem	Spasticity, prominence of cricopharyngeus; endoscopy and oesophageal dilation 1 wk later
Sinn *et al*[74]	69/female	On phenprocoumon	M2A®; Given®Imaging Ltd.	No Hx of dysphagia	1/coughed several times	50 s/bifurcation of the trachea	Spontaneous resolution	NS
Tabib *et al*[75]	87/female	Recent onset IDA, CHF, IHD, AF, bladder cancer, CRF	M2A®; Given®Imaging Ltd.	No Hx of dysphagia, pre-CE barium meal	2/choking, dyspnoea, CE felt lodged in the throat	NS/right main-stem bronchus - bronchus intermedius	Rigid bronchoscopy	NS
Buchkremer *et al*[76]	74/male	Recent diagnosis of coeliac disease; past Hx of ankylosing spondylitis	M2A®; Given®Imaging Ltd.	No Hx of dysphagia	NS/dyspnoea started after CE ingestion	NS/right main-stem bronchus	Flexible bronchoscopy	NS
Rondonotti *et al*[77]	NS	NS	M2A®; Given®Imaging Ltd.	NS	NS/coughed several times	NS/NS	Spontaneous resolution	NS
Nathan *et al*[78]	93/male	No significant past medical Hx	M2A®; Given®Imaging Ltd.	No Hx of dysphagia	1/coughed hours post-ingestion	Approximately 8 h/ bronchial tree	Spontaneous resolution	NS
Shiff *et al*[79]	75/male	NS	M2A®; Given®Imaging Ltd.	No Hx of dysphagia	2/some coughing	NS/bronchi	Spontaneous resolution Eventually, CE endoscopic placement	NS
Sepehr *et al*[80]	67/male	HTN, DM, CVA	NS	Hx of dysphagia (intermittent)	1/coughing, tachypnoea, and tachycardia	NS/trachea	Endoscopy-Roth net	NS
Koulaouzidis *et al*[81]	76/male	NS	PillCam®SB; Given®Imaging Ltd.	No Hx of dysphagia	1/coughed weakly	15 s/trachea	Spontaneous resolution	NS
Guy *et al*[82]	90/male	Ischaemic CVA	NS	No Hx of dysphagia	NS/no symptoms	NS/bronchial tree	Rigid bronchoscopy - stone retrieval basket	NS
Leeds *et al*[83]	85/male	NS	NS	No Hx of dysphagia	NS/difficulty swallowing CE, slightly painful	8 h/lobar bronchus	Spontaneous resolution	NS
Bredenoord *et al*[84]	65/male	Sigmoid colectomy for diverticulae; Ileal carcinoid resected	NS	Hx of dysphagia	Lengthy swallowing attempt/ coughing noted	NS/right main bronchus	Spontaneous resolution, eventually, CE was swallowed on same session	Normal small-bowel
Choi *et al*[85]	75/male	Prior CVA	PillCam®SB; Given®Imaging Ltd.	No Hx of dysphagia	NS/coughed several times	2 h/left main bronchus	Flexible Bronchoscopy-Roth net and bronchial wall irrigation to induce cough	NS, patient declined further investigations
Depriest *et al*[86]	90/male	IHD, AF, PVD (warfarin + clopidogrel)	PillCam®SB; Given®Imaging Ltd.	No Hx of dysphagia	NS/some cough	NS/left main bronchus, then right main bronchus	Chest percussive therapy + postural drainage; Flexible bronchoscopy + extraction basket + Roth net	NS
Depriest *et al*[86]	90/male	IHD, AF, PVD (warfarin + clopidogrel)	PillCam®SB; Given®Imaging Ltd.	No Hx of dysphagia	NS/some cough	NS/left main bronchus, then right main bronchus	Chest percussive therapy + postural drainage; flexible bronchoscopy + extraction basket + Roth net	NS

Kurtz *et al*[87]	73/male	Renal cell cancer, MV (bovine), hyperlipidaemia, melaena	NS	No Hx of dysphagia	Sips of water, 1st attempt, 2 min later non-productive cough (20 s)	Level of carina; then right main stem bronchus	Bronchoscopy-retrieval basket (multiple spontaneous ejections from trachea prior bronchoscopy)	NS
Lucendo *et al*[88]	80/male	Advanced PD, DM, walking + speech difficulties	PillCam®SB; Given®Imaging Ltd.	No Hx of dysphagia	Several attempts/persistent coughing and some dyspnoea	20 s/tracheobronchial tree	Spontaneous resolution	Oesophageal ulcer + ileal ulcer
Pezzoli *et al*[89]	82/male	Unexplained anemia, HTN	NS	No Hx of dysphagia	NS/asymptomatic (minimal cough)	3 d/in the right bronchus	Spontaneous resolution	NS
Parker *et al*[90]	77/female	Hysterectomy	NS	No Hx of dysphagia	Initial attempt unsuccessful/chocking episode, CE coughed-up	NS/NS	Spontaneous resolution, endoscopic placement with AdvanCE® device	Patient suffered intracranial bleed, eventually succumbed
Despott *et al*[91]	65/male	COPD, cirrhosis, pancreatitis	NS	No Hx of dysphagia	NS/asymptomatic	NS/right main bronchus	Rigid bronchoscopy-Roth net	Endoscopic placement with AdvanCE® device
	73/male	COPD	NS	NS	NS/brief coughing	NS/left main bronchus	Bronchoscopy-snare + Roth net	Endoscopic placement with AdvanCE® device
	81/male	NS	NS	NS	NS/fleeting choking sensation	NS/right main bronchus	Rigid bronchoscopy-crocodile grasping forceps	NS
Girdhar *et al*[92]	83/male	COPD, GORD	PillCam®SB; Given®Imaging Ltd.	No Hx of dysphagia	Difficult, requiring multiple sips of water/some cough, after 1 h mild shortness of breath	NS/left main bronchus	Flexible bronchoscopy + rat-tooth alligator forceps + stiff-wire basket with a pin-vise handle	NS
Poudel *et al*[93]	80/male	AF, IHD, CVA on anti-coagulants, anaemia + melaena	M2A®; Given®Imaging Ltd.	NS	NS	24 h/left main stem bronchus; then right bronchus	Flexible bronchoscopy + net + snare forceps + tripod; eventually, grasped with basket	NS

MV: Mitral valve; BMI: Body mass index; HHT: Hereditary haemorrhagic telangiectasia; IDA: Iron deficiency anaemia; CHF: Chronic heart failure; IHD: Ischaemic heart disease; AF: Atrial fibrillation; CRF: Chronic renal failure; Hx: History; Ns: Not stated; HTN: Hypertension; DM: Diabetes mellitus; CVA: Cerebrovascular accident; PVD: Peripheral vascular disease; PD: Parkinson's disease; COPD: Chronic obstructive pulmonary disease; GORD: Gastro-oesophageal reflux disease; CE: Capsule endoscopy.

Table 7 Studies looking at the clinical validity of Suspected Blood Indicator, feature of capsule endoscopy reading software, in small-bowel capsule endoscopy

Ref.	Country	Centre	Objective(s)	Study type	Design	CE type	Outcome(s)	Conclusions
Gross *et al*[96]	United States	Single centre	Accuracy of SBI to number of blood transfusions	Retrospective	►Gold standard for lesions detected by experienced CE reviewer	M2A; Given® Imaging Ltd.	►Gold standard: 72 pts; ►pts received blood transfusions ranging between 0-16 units; ►Overall: A total of 17 pts had positive SBI. Active bleeding in 16 pts, who were transfused an average of 8 units before the study; ►55 pts had a negative SBI and no active bleeding was seen on their capsule studies. In this group, the average number of PRBC transfused was 1 unit. There was one patient who had a false positive SBI with no active bleeding seen in the capsule study review	Pts receiving blood transfusions are more likely to have a positive SBI correlating with the localization of active bleeding
Liangpunsakul *et al*[97]	United States	Single centre	Assess accuracy of SBI	Retrospective	►Gold standard for lesions detected by experienced CE reviewer; ►Significant lesions considered AVMs, ulcers, erosions, active bleeding; ►Reviewing speed: 15fps	M2A; Given® Imaging Ltd.	►Gold standard: 109 lesions; ►SBI: 31 potential areas of blood; correctly identified lesions: 28; ►Overall: SBI (Sens, PPV, accuracy): 25.7%, 90%, 34.8%, respectively; ►For actively bleeding SB lesions only: SBI (Sens, PPV, accuracy): 81.2%, 81.3%, 83.3%, respectively	SBI has good Sens and PPV for actively bleeding SB lesions

D'Halluin *et al*[98]	France	Multi-centre (7 centres)	Assess Sens/Spec of SBI (in OGIB)	Retro-spective	►Gold standard for lesions detected by experienced CE reviewer, SBI tags marked by another investigator; ►Significant lesions considered Bleeding or having a bleeding potential: high (P2), low (P1), or absent (P0); ►Concordance: same time code in frames selected by expert reader and those tagged by SBI; ►Reviewing speed: NS	M2A; Given® Imaging Ltd.	►156 SBCE recordings evaluated: In 83 (normal): either no lesion (n = 71) or P0 lesion (n = 12); in 73 abnormal: P2 (n = 114) and P1 (n = 92) lesions; ►154 red tags analysed: SBI (Sens, Spec, PPV, NPV) for P2 or P1: 37%, 59%, 50%, 46%, respectively	►SBI-based detection of SB lesions (with bleeding potential) is of limited clinical value
Singnorelli *et al*[99]	Italy	Single centre	Assess Sens/Spec of SBI per lesion, overall, according to red findings (identified by the reader), and per patient	Retro-spective	►Gold standard for lesions detected by four experienced CE reviewers; ►Outcomes: Sens, Spec and accuracy calculated both per lesion/patient; ►Reviewing speed: NS	M2A; Given® Imaging Ltd.	►95 patients; 209 red findings; ►Overall Sens: 28%; ►Sens higher for identification of blood (61%) than for nonbleeding "red" findings, *e.g*., AVMs (26%); ►Per-patient Sens, Spec: 41%, 70%, respectively	►SBI has low Sens/Spec in per-lesion and per-patient SBCE evaluation; ►Complementary/rapid screening tool; ►Complete review of the recordings is still necessary
Ponferrada *et al*[100]	Spain	Single centre	Assess accuracy/performance of SBI	Prospec-tive	►Gold standard for lesions detected by experienced CE reviewers	M2A; Given® Imaging Ltd.	►57 consecutive patients; ►Indications: OGIB (64.9%), CD (14%), malabsorption (14%), suspicion of SB tumour (7.1%); ►SBI Sens, Spec, PPV, NPV: 58.3%, 75.5%, 38.8%, 87.2%, respectively	
Buscaglia *et al*[101]	United States	Single centre	Assess accuracy/performance of SBI according to CE indications	Retro-spective	►Gold standard for lesions detected by experienced CE reviewer; ►Significant lesions:AVMs, varices, venous ectasias, red spots, ulcers, erosions, blood, blood clots ►Concordant and discordant findings between CE reviewer and SBI; ►Reviewing speed: 8-15 fps	M2A; Given® Imaging Ltd.	►CE indications: OGIB (n = 112), suspected CD (n = 122), anaemia of unknown origin (n = 53), other (n = 4); ►221 lesions with bleeding potential; ►Overall: SBI (Sens, Spec, PPV, NPV): 56.4%, 33.5%, 24.0%, 67.3%, respectively; ►For actively bleeding lesions: SBI (Sens, PPV): 58.3%, 70%, respectively; ►For suspected CD: SBI (Sens, NPV): 64%, 80.4%, respectively; ►For OGIB: SBI Sens 58.3%; ►For anaemia: SBI Sens 41.3%;	►SBI performance characteristics suboptimal/insufficient to screen for SB lesions with bleeding potential; ►Even in pts with active intestinal bleeding, SBI Sens was only < 60%
Park *et al*[102]	South Korea	Single centre	Investigate whether SBI is affected by background colour and CE velocity	Experi-mental	►Paper-made phantom SB models in a variety of colours to simulate the background colours observed in CE; ►Red spots were attached inside them; ►CE manually passed through models; ►SBI red spots detection rate was evaluated based on colours of SB models and CE velocities (0.5, 1, 2 cm/s)	M2A; Given® Imaging Ltd.	►SBI red spots detection rate differed significantly per background colour of SB model, P < 0.001; ►SBI red spots detection rate highest for very pale magenta, burnt sienna, yellow background; ►SBI red spots detection rate lowest for dark brown, very pale yellow background ►SBI red spots detection rate decreases at rapid CE passage (1-2 cm/s) compared to slower (0.5 cm/s) for very pale yellow (P = 0.042), yellow (P = 0.001), very pale magenta (P = 0.002), burnt sienna (P = 0.001) background; ►Red spots detection rate no different according to velocity for light greyish pink (P = 0.643) or dark brown (P = 0.396) background	►SBI Sens affected by background colour and capsule passage velocity in the models

PRBC: Pack red blood cells; fps: Frames per second; SBI: Suspected Blood Indicator; CE: Capsule endoscopy; AVM: Arterio-venous malformations; SB: Small-bowel; Sens: Sensitivity; Spec: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; OGIB: Obscure gastrointestinal bleeding; CD: Crohn's disease.

Table 8 Studies looking at the clinical validity of QuickView, feature of capsule endoscopy reading software, in small-bowel capsule endoscopy

Ref.	QuickView sampling rate	QuickView reading frame mode/ reading speed (fps)	Average reading time (mean)	Comparison with/ reading frame mode/ reading speed used (fps)	Rapid® Reader version	Reviewers	Cases					QuickView		Lesions missed
							Total	OGIB	CD	Polyposis	Other	Sensitivity (%)	Specificity (%)	
Ponferrada *et al*[100]	NS	25, 15, 5	NS	Conventional/NS/15, 15, 5		2	57	37	8	N/A	12	96.5 (5 fps)	NS	NS
Schmelkin[104]	NS	NS	NS	NS	4.0	1	47	47	N/A	N/A	N/A	100	100	N/A
Appalaneni *et al*[105]	NS	Single frame, 25	3 min	NS	NS	2	50	NS	NS	NS	NS	NS	NS	2
Westerhof *et al*[106]	High (17)	NS	4.4 min (median)	Conventional/dual view/18	4.0	2	100	56	30	2	12	NS	NS	13
Shiotani *et al*[107]	High (17)	Single, 6	17.9 min	NS	5.0	3	44	NS	NS	NS	14	NS	NS	10
Hosoe *et al*[108]	Normal	NS	NS	NS	5.0	3	45	NS	NS	NS	14	NS	NS	NS
Saurin *et al*[109]	NS	NS	11.6 min	Conventional/NS/NS	5.0	12	106	106	N/A	N/A	N/A	89.2	Jul-84	8
Shiotani *et al*[110]	5, 15, 25, 35	Single, NS	NS	NS	6.5	4	87	NS	NS	NS	NS	NS	NS	NS
Koulaouzidis *et al*[111]	35 18	Dual view (WL + BM)	475 s (QuickView WL) 450 s (QuickView BM)	Conventional/ single or dual view/12-20	7.0	1	200	106	81	4	9	92.3 (QVWL P1 + P2) 91 (QVBM P1+P2)	96.3 (QVWL P1 + P2) 96 (QVBM P1 + P2)	
Kyriakos *et al*[112]	NS	NS, 3	16.3 min (6.7)	Conventional/NS/NS	5.0	2	100	55	22	3	20	NS	NS	12

NS: Not stated; NA: Not applicable; fps: Frames per second; QVWL; QuickView with white light; QVBM: QuickView with blue mode; OGIB: Obscure gastrointestinal bleeding; CD: Crohn's disease; P1, P2: Classification as per probability of bleeding.

Table 9 Studies looking at the clinical validity of Fujinon® intelligent chromoendoscopy enhancement/Blue mode, feature of capsule endoscopy reading software, in small-bowel capsule endoscopy

Ref.	Country	Centre	Study type	Objective(s)	Design	Images	FICE	CE	Outcome(s)
Imagawa *et al*[114]	Japan	Single centre	Retrospective	Assess whether visualization of SB lesions improves with FICE	►5 experienced readers compared CE-WL images to their FICE counterparts	►Angiectasis (n = 23); ►Erosion/ ulcers (n = 47); ►Tumour (n = 75)	FICE 1,2,3	PillCam®SB1; Given®Imaging Ltd.	►FICE 1: AVMs: improvement in 87% (20/23) cases; erosion/ulceration: improvement 53.3% (26/47) cases; tumour images: improvement 25.3% (19/75) cases; ►FICE 2: AVMs: improvement in 87% (20/23) cases; erosion/ulceration: improvement in 25.5% (12/47) cases; tumour images: improvement in 20.0% (15/75) cases; ►FICE 3: All images groups: only equivalence achieved in all cases; intra-observer agreement: good to satisfactory (5.4 or higher)
Imagawa *et al*[115]	Japan	Single centre	Prospective	Assess whether FICE improves detection rate of SB lesions in CE	►A CE reader reviewed CE-WL videos; ►Another reader, reviewed CE-FICE videos with FICE 1,2,3	50 pts	FICE 1,2,3	PillCam®SB1; Given®Imaging Ltd.	►Angioectasias detection: CE-WL: 17 AVMs; CE-FICE 1: 48 AVMs; CE-FICE 2: 45 AVMs; CE-FICE 3: 24 AVMs; significant CE-FICE 1 and 2 (P = 0.0003 and P < 0.0001, respectively) ►Detection rate for erosion, ulceration and tumour did not differ statistically between CE-WL and CE-FICE 1,2,3; ►Similar interpretation time (CE-WL: 36 ± 6.9 min; CE-FICE 1: 36 ± 6.4 min; FICE 2: 38 ± 5.8 min; FICE 3: 35 ± 6.7 min)

Gupta *et al*[116]	Belgium	Single centre	Retrospective	Assess potential benefit of FICE for SB lesion detection in patients with OGIB	CE videos analysed by 2 GI fellows with and without FICE 1,2,3; Reference standard: Senior consultant described findings as P0, P1 and P2 lesions	60 pts with OGIB	FICE 1,2,3	PillCam®SB1; Given®Imaging Ltd.	►Overall, 157 lesions diagnosed with CE-FICE *vs* 114 with CE-WL ($P = 0.15$); ►For P2 lesions; CE-FICE Sens/Spec: 94%/95% *vs* CE-WL Sens/Spec: 97%/96%, respectively; 5/55 AVMs better characterized with CE-FICE than CE-WL ►More P0 diagnosed by CE-FICE than CE-WL (39 *vs* 8, $P < 0.001$); ►Intra-class kappa correlations between fellows and reference: CE-FICE *vs* CE-WL for P2 lesions: 0.88 *vs* 0.92; CE-FICE *vs* CE-WL for P1 lesions: 0.61 *vs* 0.79
Krystallis *et al*[117]	United Kingdom	Single centre	Retrospective	Assess FICE and Blue mode visualisation of SB lesions in CE	►2 experienced reviewers CE-WL images to FICE/Blue mode counterparts	►Angioectasias ($n = 18$); ►Erosion/ulcers ($n = 60$); ►Villi oedema ($n = 17$); ►Cobblestone ($n = 11$); ►Blood lumen ($n = 15$); ►LICS/other ($n = 46$)	Blue mode; FICE 1,2,3	Pillcam®SB1/SB2; Given®Imaging Ltd.	►Total of 167 images, for all lesion categories: ►Blue mode *vs* WL: image improvement in 83%; $\kappa = 0.786$ ►FICE 1 *vs* WL: image improvement in 34%; $\kappa = 0.646$ ►FICE 2 *vs* WL: image improvement in 8.6%; $\kappa = 0.617$ ►FICE 3 *vs* WL: image improvement in 7.7%; $\kappa = 0.669$
Duque *et al*[118]	Portugal	Single centre	Prospective	Assess reproducibility and diagnostic accuracy of CE-FICE	►4 physicians reviewed 150 FICE images; ►2 experienced physicians analysed 20 CE; 1 interpreted CE-WL; the other, CE-FICE videos	20 patients with OGIB	Blue mode; FICE 1,2,3	PillCam®SB2; Given®Imaging Ltd.	►Concordance between the 4 gastroenterologists: 0.650; ►CE-WL identified 75 findings and the CE-FICE 95; ►CE-FICE did not miss any lesions identified by CE-WL and allowed the identification of a higher number of AVMs (35 *vs* 32) and erosions (41 *vs* 24)
Nakamura *et al*[119]	Japan	Single centre	Prospective	Assess preview of angioectasias by CE-FICE preview (compared to CE-WL)	►One experienced physician analysed CEs in QuickView mode; ►Mean reading time, sensitivity and specificity for angiodysplasia detection were evaluated including SBI	50 pts with angiodysplasia were randomly assigned to 2 equally sized groups of CE-WL reading and CE-FICE reading	SBI; Blue mode; FICE 1,2,3	PillCam®SB2; Given®Imaging Ltd.	►Mean reading time: 14min for both CE-WL and CE-FICE reading; ►The two previews for angiodysplasia were significantly superior to the function of SBI ($P < 0.01$); ►Sens and Spec of CE-WL: 80% and 100%, respectively; ►Sens and Spec of CE-FICE: 91% and 86%, respectively; ►FICE reading was superior in Sens, while it resulted in more false (+) ve lesion findings and lower Spec
Sakai *et al*[120]	Japan	Single centre	Prospective	►Assess whether CE-FICE improves detectability of SB lesions by CE trainees and if it contributes to reducing the bile-pigment effect; ►Evaluate whether poor bowel preparing affects the accuracy of lesion recognition by FICE	►4 gastroenterology trainees interpreted 12 CE videos with WL and FICE 1,2,3; ►Lesion detection rate under each of the three FICE settings was compared with that by conventional CE-WL	►60 AVMs; ►82 erosions/ulcers	FICE 1,2,3	PillCam®SB2; Given®Imaging Ltd.	►60 angioectasias; CE trainees identified: 26 by CE-WL, 40 by CE-FICE1, 38 by CE-FICE2, 31 by CE-FICE3; ►82 erosions/ulcerations, CE trainees identified: 38 by CE-WL, 62 by CE-FICE1, 60 CE- FICE2, 20 by CE-FICE3; ►CE-FICE 1 and 2 significantly improved detectability of angioectasias ($P = 0.0017$ and $P = 0.014$, respectively) and erosions/ulcers ($P = 0.0012$ and $P = 0.0094$, respectively); ►Detectability of SB lesions by CE-FICE1 was not affected ($P = 0.59$) by the presence of bile-pigments; ►Detectability of SB lesions by CE-WL ($P = 0.020$) and CE-FICE2 ($P = 0.0023$) was reduced by the presence of bile-pigments; ►In poor bowel visibility conditions, CE-FICE yielded a high rate of false-positive findings

FICE: Fujinon® Intelligent chromoendoscopy enhancement; CE: Capsule endoscopy; SB: Small bowel; WL: White light; OGIB: Obscure gastrointestinal bleeding; SBI: Suspected Blood Indicator; AVM: arterio-venous malformation; κ: Inter-observer agreement; LICS: Lesions of indeterminate clinical significance; Sens: Sensitivity; Spec: Specificity.

Table 10 Experimental and models in development for capsule-endoscopy the future?

Ref.	Project	Status	Active actuation	Magnetic propulsion	Therapeutic capabilities
Johannessen *et al*[124]	IDEAS: A miniature lab-in-a-pill multi-Sens or microsystem	Prototype	No	Yes	Yes
Karagozler *et al*[125]	Miniature endoscopy capsule robot using biomimetic micro-patterned adhesives	Prototype	Yes	No	No
Quirini *et al*[126]	An approach to capsular endoscopy with active motion	Prototype	Yes	No	No
Valdastri *et al*[127]	Wireless therapeutic endoscopic capsule: *in vivo* experiment	Prototype	No	Yes	Yes
Glass *et al*[128]	A legged anchoring mechanism for capsule endoscopes using micro-patterned adhesives	Prototype	Yes	No	No
Valdastri *et al*[129]	An endoscopic capsule robot: a meso-scale engineering case study	Concept	Yes	No	No
Tortora *et al*[130]	Propeller-based wireless device for active capsular endoscopy in the gastric district	Prototype	Yes	No	No
Valdastri *et al*[131]	A magnetic internal mechanism for precise orientation of the camera in wireless endoluminal applications	Prototype	No	Yes	No
Ciuti *et al*[132]	Robotic magnetic steering and locomotion of capsule endoscope for diagnostic and surgical endoluminal procedures	Prototype	No	Yes	Yes
Bourbakis *et al*[133]	Design of new-generation robotic capsules for therapeutic and diagnostic endoscopy	Concept	Yes	No	Yes
Gao *et al*[134]	Design and fabrication of a magnetic propulsion system for self-propelled capsule endoscope	Concept	No	Yes	No
Simi *et al*[135]	Design, fabrication, and testing of a capsule with hybrid locomotion for gastrointestinal tract exploration	Concept	No	Yes	No
Morita *et al*[136]	A further step beyond wireless capsule endoscopy	Concept	No	Yes	No
Yang *et al*[137]	Autonomous locomotion of capsule endoscope in gastrointestinal	Concept	Yes	No	No
Filip *et al*[138]	Electronic stool (e-Stool): A novel self-stabilizing video capsule endoscope for reliable non-invasive colonic imaging	Prototype	Yes	No	No
Yim *et al*[139]	Design and rolling locomotion of a magnetically actuated soft capsule endoscope	Prototype	Yes	No	No
Kong *et al*[140]	A robotic biopsy device for capsule endoscopy	Prototype	Yes	No	Yes
Woods *et al*[141]	Wireless capsule endoscope for targeted drug delivery: Mechanics and design considerations	Prototype	Yes	No	Yes

Certain issues (*i.e.*, best small-bowel preparation for CE[143,144], occurrence of some potentially life-threatening complications, visualisation quality of the proximal small-bowel) remain open and they will surely be the target of further clinical studies and technical improvements.

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P- Reviewer Driscoll D **S- Editor** Wen LL
L- Editor A **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3747

World J Gastroenterol 2013 June 28; 19(24): 3747-3760
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Predominant mucosal expression of 5-HT$_{4(+h)}$ receptor splice variants in pig stomach and colon

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Supported by Grant G.0061.08 from the Fund for Scientific Research Flanders
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Received: August 17, 2012 Revised: December 3, 2012
Accepted: December 15, 2012
Published online: June 28, 2013

Abstract

AIM: To investigate cellular 5-HT$_{4(-h/+h)}$ receptor distribution, particularly in the epithelial layer, by laser microdissection and polymerase chain reaction (PCR) in porcine gastrointestinal (GI) tissues.

METHODS: A stepwise approach was used to evaluate RNA quality and to study cell-specific 5-HT$_4$ receptor mRNA expression in the porcine gastric fundus and colon descendens. After freezing, staining and laser microdissection and pressure catapulting (LMPC), RNA quality was evaluated by the Experion automated electrophoresis system. 5-HT$_4$ receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expressions were examined by endpoint reverse transcription (RT)-PCR in mucosal and muscle-myenteric plexus (MMP) tissue fractions, in mucosal and MMP parts of hematoxylin and eosin (HE) stained tissue sections and in microdissected patches of the epithelial and circular smooth muscle cell layer in these sections. Pig gastric fundus tissue sections were also stained immunohistochemically (IHC) for enterochromaffin cells (EC cells; MAB352); these cells were isolated by LMPC and examined by endpoint RT-PCR.

RESULTS: After HE staining, the epithelial and circular smooth muscle cell layer of pig colon descendens and the epithelial cell layer of gastric fundus were identified morphologically and isolated by LMPC. EC cells of pig gastric fundus were successfully stained by IHC and isolated by LMPC. Freezing, HE and IHC staining, and LMPC had no influence on RNA quality. 5-HT$_4$ receptor and GAPDH mRNA expressions were detected in mucosa and MMP tissue fractions, and in mucosal and MMP parts of HE stained tissue sections of pig colon descendens and gastric fundus. In the mucosa tissue fractions of both GI regions, the expression of h-exon containing receptor [5-HT$_{4(+h)}$ receptor] mRNA was significantly higher ($P < 0.01$) compared to 5-HT$_{4(-h)}$ receptor expression, and a similar trend was obtained in the mucosal part of HE stained tissue sections. Large microdissected patches of the epithelial and circular smooth muscle cell layer of pig colon descendens and of the epithelial cell layer of pig gastric fundus, also showed 5-HT$_4$ receptor and GAPDH mRNA expression. No 5-HT$_4$ receptor mRNA expression was detected in gastric LMPC-isolated EC cells from IHC stained tissues, which cells were positive for GAPDH.

CONCLUSION: Porcine GI mucosa predominantly expresses 5-HT$_{4(+h)}$ receptor splice variants, suggesting their contribution to the 5-HT$_4$ receptor-mediated mucosal effects of 5-HT.

Key words: 5-hydroxytryptamine 4 receptors; Pig; Gastric fundus; Colon descendens; Epithelium; Smooth muscle; Laser microdissection and pressure catapulting

Priem EKV, De Maeyer JH, Vandewoestyne M, Deforce D, Lefebvre RA. Predominant mucosal expression of 5-HT$_{4(+h)}$ receptor splice variants in pig stomach and colon. *World J Gastroenterol* 2013; 19(24): 3747-3760 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3747.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3747

INTRODUCTION

The 5-HT$_4$ receptor is a G-protein coupled receptor (GPCR) that activates the adenylyl cyclase/cyclic adenosine monophosphate/protein kinase A pathway in response to serotonin (5-HT). The 5-HT$_4$ receptor is expressed on excitatory motor neurons in the gut, facilitating acetylcholine release, which stimulates gastrointestinal (GI) motility[1-3]. This presynaptic facilitation is thought to be the principal mechanism for the prokinetic action of 5-HT$_4$ receptor agonists, explaining their therapeutic use in GI dysmotility-related disorders, such as chronic constipation, gastroparesis and gastroesophageal reflux disease[4]. The selective 5-HT$_4$ receptor agonist prucalopride is now used in patients with chronic laxative-resistant constipation; indeed, it facilitates acetylcholine release from cholinergic neurons towards human colonic circular[5], as well as longitudinal[6], smooth muscle. The non-selective 5-HT$_4$ receptor agonist cisapride was, until it was withdrawn because of non-specific cardiac side effects, used for increasing gastric emptying in patients with gastroparesis[7]. In addition, prucalopride accelerates gastric emptying in humans[8], corresponding with its facilitating effect on acetylcholine release from cholinergic nerves towards human gastric circular muscle[9]. Our group has previously shown that the pig is a good model for human 5-HT$_4$ receptors on GI cholinergic neurons; the presence of facilitatory 5-HT$_4$ receptors on cholinergic neurons innervating pig gastric circular[10] and longitudinal[11] muscle and colonic circular muscle[12] was illustrated in functional assays.

However, apart from cholinergic neurons, other locations for the 5-HT$_4$ receptor in the colon and stomach have been proposed. In the human colon, 5-HT$_4$ receptors were reported to be present on circular smooth muscle cells, inducing relaxation[13]. A functional study by Borman *et al*[14] in 1996 reported that 5-HT-induced secretion in the human sigmoid colon is mediated *via* 5-HT$_{2A}$ receptors; however, in the ascending colon, a combination of 5-HT$_{2A}$ and 5-HT$_4$ receptors appears to be involved. Nevertheless, a recent study showed the presence of mRNA of several 5-HT$_4$ receptor splice variants in the mucosal layer of the human sigmoid colon[15]; 5-HT$_4$ receptor mRNA was also reported in the pig colonic mucosa[16]. In the rat colon, it has been suggested that 5-HT-induced mucosal ion transport and Cl^- secretion is mediated by 5-HT$_4$ receptors[17-20]. Immunohistochemical and functional assays showed the presence of 5-HT$_4$ receptors in mouse colonic epithelial cells, including enterochromaffin (EC) cells and goblet cells, inducing mucosal 5-HT release and Cl^- secretion[20]. The presence of 5-HT$_4$ receptor transcripts, detected by reverse transcription polymerase chain reaction (RT-PCR), has also been reported in the gastric mucosa of humans[21,22] and pigs[16], but cellular distribution within the epithelial layer has not yet been investigated.

More detailed information on the expression and localization of GPCRs, with special attention to the 5-HT$_4$ receptor, is needed in human enteric neuronal subpopulations, mast cells and epithelial cells, to provide a better understanding of function and activity of 5-HT$_4$ receptors in the GI wall, which may offer new therapeutic perspectives[23]. To date, the majority of information on 5-HT$_4$ receptor distribution is based on functional studies[12] or on 5-HT$_4$ receptor expression studies using homogenates of tissues[15,16,21,22]. However, homogenates of tissues limit the potential of expression studies: important cell-specific transcript information is lost because of the heterogeneity of tissues, such as GI tissues. Techniques have been developed to enable collection of particular cells from mixed populations, which generally involve either fluorescence activated cell sorting (FACS) purification of dissociated cells or laser-assisted microdissection. In contrast to FACS, microdissection can be applied to most tissues[24] and laser microdissection has already been used in previously reported gene expression studies to investigate site-specific gene expression. In the laser microdissected enteric ganglia of the human intestine, 5-HT$_{3A}$ receptor mRNA expression was described[25] and in microdissected human colonic mucosal epithelium, transcripts encoding 5-HT$_{3A}$, 5-HT$_{3C}$, 5-HT$_{3D}$ and 5-HT$_{3E}$ subunits were detected[26]. In different species, 5-HT$_4$ receptors show splice variation in the intracellular C-terminus starting after the common amino acid structure L358. In humans, nine splice variants have been described (Figure 1A). In pig, at least another nine different splice variants, not described in humans, have been reported (Figure 1A), as well as unique splice variation, with variants composed of duplicated exons[16]. Splice variants in the extracellular loops of GPCRs are rare[27]; however, the 5-HT$_4$ receptor can have an extra insertion of 14 amino acids in the second extracellular loop, encoded by the h-exon (Figure 1A). In humans, this H variant has been described in combination with the b-terminal exon [5-HT$_{4(hb)}$][28]. When comparing the pharmacology of the 5-HT$_{4(hb)}$ splice variant, when transiently expressed in cells being CV-1 (simian) in origin, and carrying the SV40 genetic material (COS)-7 cells, with that of the 5-HT$_{4(b)}$ and 5-HT$_{4(a)}$ splice variant, it showed a smaller fraction of receptors coupled to G-proteins and the 5-HT$_4$ receptor antagonist GR113808 behaved as a partial agonist[28]. In the human GI tract, the h exon could be amplified in combination with the b exon only from the lower esophageal sphincter; however, h exon-carrying 5-HT$_4$ transcripts were also obtained from other parts of the GI tract, suggesting that the h-exon might be expressed in combination with other C-terminal exons[28]. In pigs, De Maeyer *et al*[16] showed that the 5-HT$_{4(h)}$ splice variant also exists in combination with C-terminal

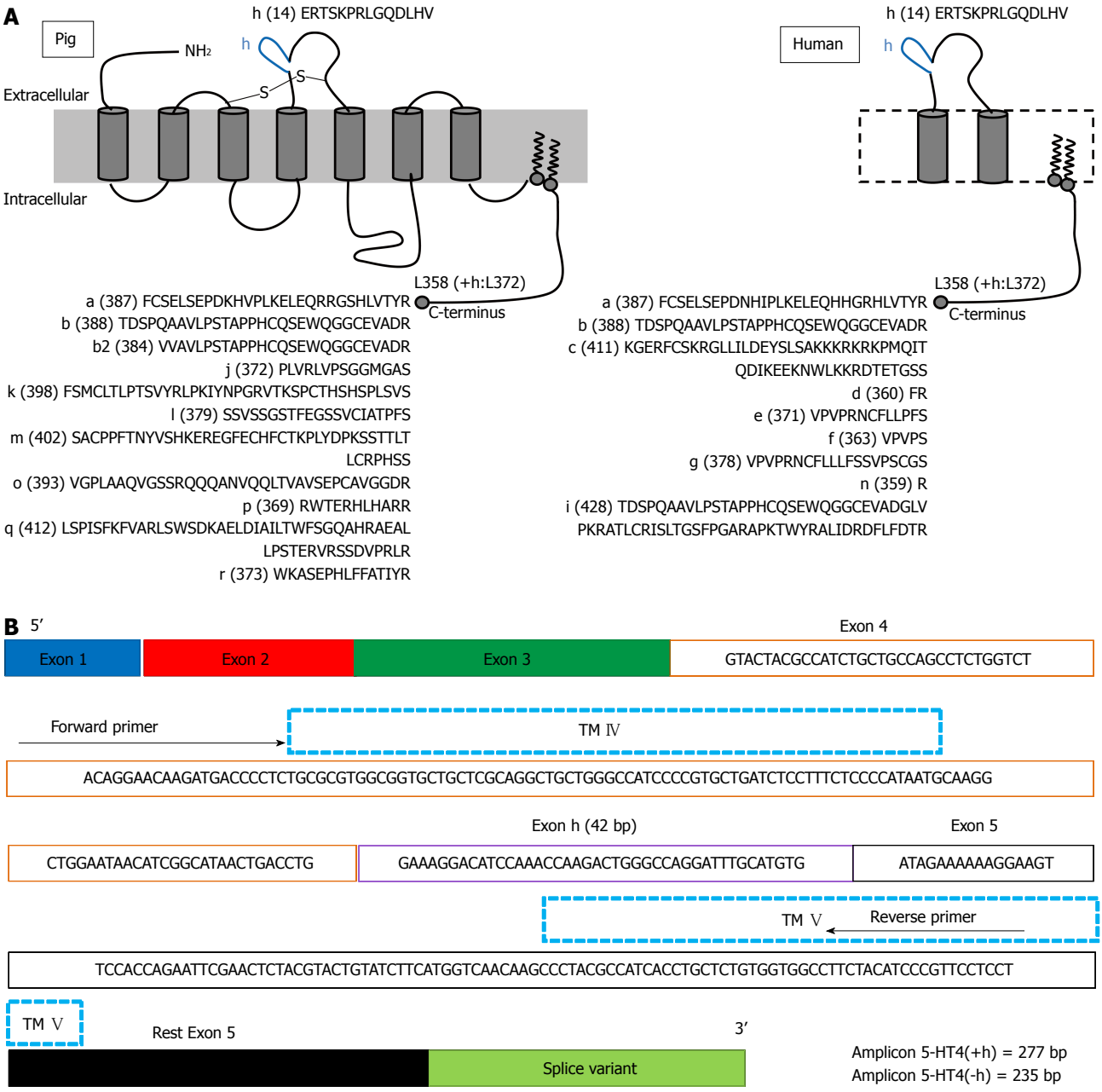

Figure 1 Schematic representation. A: The porcine and human 5-HT$_4$ receptor splice variants; B: cDNA of the porcine 5-HT$_{4(hb)}$ receptor based on gene browsing (transcript ID: ENSSSCT00000015770 on http://www.ensembl.org/Sus_scrofa/Gene). The 5-HT$_4$ receptor variants have identical sequences up to Leu^{358}, or Leu^{372} when exon h is included, and differ by the length and composition of their C-terminal domain. The presence of the h sequence of 14 amino acids in the second extracellular loop depends on the all or none inclusion of exon h between exon 4 and 5. The positions of the boundaries in between exons, the transmembrane (TM) domains Ⅳ and Ⅴ and the positions of primers used in this study are indicated. The primers will detect all 5-HT$_4$ receptor splice variants. Amplification will result in a 277 bp amplicon, when containing the 42 bp h-exon or a 235 bp amplicon, not containing the h-exon.

exons other than 5-HT$_{4(b)}$, namely 5-HT$_{4(ha)}$, 5-HT$_{4(hm)}$ and 5-HT$_{4(hr)}$. H-exon containing 5-HT$_4$ transcripts were also found along the porcine GI tract, with predominant expression in the mucosal layer. Therefore, the aim of the present study was to develop and validate an experimental protocol for the assessment of 5-HT$_4$ receptor distribution (with and without the h exon) at the cellular level in laser microdissected porcine GI tissues, paying special attention to the mucosal layer of pig colon descendens and gastric fundus.

MATERIALS AND METHODS

Tissue preparation and tissue processing

Young male pigs (10-12 wk, 15-25 kg-breed Line 36) were obtained from Rattlerow Seghers, Lokeren Belgium. The Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University approved all the experimental procedures.

The pigs were anaesthetized with an intramuscular injection of 5 mL Zoletil 100 (containing 50 mg/mL ti-

letamine and 50 mg/mL zolazepam; Virbac Belgium SA, Heverlee, Belgium). After exsanguination, the stomach and the colon descendens, prelevated 10 cm above the anus to the transverse colon, were removed and thoroughly washed in ice cold aerated (5% CO_2/95% O_2) phosphate buffered saline (PBS) at pH 7.4 (Life Technologies Europe, Ghent, Belgium). The gastric fundus was cut open along the lesser curvature and small pieces of tissue were cut in the direction of the circular muscle layer from the ventral side. The colon descendens was opened along the mesenteric border, fat tissue was removed and tissues were cut in the direction of the circular muscle layer.

Freezing tissue fractions for direct RNA processing: The GI tissues were divided by blunt dissection into a mucosal-submucosal (mucosa) fraction and a muscular-myenteric plexus (MMP) fraction. The fractions were cut into small pieces, put in an RNase-free vial (Life Technologies Europe), rapidly frozen in liquid N_2 and stored at -80 ℃. After frozen tissue homogenization and before RNA extraction, MMP samples were treated with proteinase K (Qiagen, Antwerp, Belgium) to increase the total RNA output. Proteinase K removes proteins such as the contractile proteins, connective tissue and collagen, which define a fibrous tissue such as the smooth muscle layer (Rneasy fibrous tissue handbook, Qiagen). RNA from mucosa and MMP fractions was extracted using the RNeasy Mini Kit (Qiagen) according to manufacturer's guidelines and RNA samples were stored at -80 ℃.

Freezing tissues for section preparation and laser microdissection: Whole tissues, containing the mucosal and the smooth muscle layers were cut into full-thickness small pieces with a sterile scalpel, placed in tissue embedding medium PELCO CryO-Z-T (Pelco International, CA, United States), rapidly frozen in liquid N_2 containing cold isopentane and stored at -80 ℃. The frozen tissue samples were cut into 8 μm-thick sections using a cryostat (Leica CM 1950; Leica Microsystems, Diegem, Belgium) with disposable RNase-free knives. Sections of 8 μm thickness are considered to represent a monolayer of cells[29,30]. The sections were placed on chilled (-20 ℃) nuclease free polyethylene naphthalate-covered membrane slides (Carl Zeiss, Oberkochen, Germany) and immediately stored at -80 ℃ until the staining procedure. The membrane slides used for immunohistochemistry were extra coated with poly-L-Lysine (Sigma, Bornem, Belgium), which was diluted with 0.1% diethylpyrocarbonate (DEPC)-treated water. All materials (pincers, brushes) were treated with RNase ZAP (Sigma) and glassware and pincers were heated for 6 h at 200 ℃, to remove all exogenous RNases.

To distinguish morphologically the different layers of the tissue sections for laser microdissection, the frozen tissue sections were stained with hematoxylin and eosin (Sigma) in RNase-free conditions. Hematoxylin and eosin (HE) staining started with fixing the slides in 70% ethanol for 1 min, followed by dipping the slides for 15 s in DEPC-treated water to remove PELCO CryO-Z-T embedding medium. Hematoxylin staining was carried out by placing the slides for 1 min in the hematoxylin solution (0.1%), followed by dipping the slides for 15 s in DEPC-treated water and 15 s in 70% ethanol. Slides were then placed for 1 min in eosin solution (0.25%), followed by dehydrating the slides for 15 s in the following order: DEPC-treated water, 70% ethanol, 100% ethanol. The staining procedure was finished with a 3 min xylene treatment and the slides were air dried for 10 min at room temperature, before scraping off the whole tissue section, or the mucosal and MMP part of the tissue section separately, or applying laser microdissection. Staining solutions based on ethanol and xylene were pre-cooled at -20 ℃; aqueous solutions were pre-cooled at 4 ℃. All solutions were diluted with 0.1% DEPC-treated water, kept in 50 mL RNase-free conical tubes (Life Technologies Europe) and kept on ice during the staining procedure.

Immunohistochemistry: To distinguish and isolate EC cells using the laser microdissection and pressure catapulting (LMPC) technique, visualization with cell-specific antibodies of these cells is required. To extract intact RNA of the cell samples, an immunohistochemically (IHC) protocol under RNase-free conditions was developed according to the staining procedure reported by Brown *et al*[24]. Cryosections were rinsed for 15 s with cold (4 ℃) PBS (pH 7.4; Life Technologies Europe) and then fixed for 5 min in ice-cold (-20 ℃) acetone. Acetone was removed by a cold PBS rinse (15 s) and slides were incubated for 30 min at 4 ℃ with blocking buffer (0.25% Triton X-100, 1% bovine serum albumin, 10% goat serum) supplemented with 1 mol/L NaCl. Then, sections were briefly rinsed with cold PBS and incubated overnight at 4 ℃ with the rat anti-serotonin primary antibody MAB352 (Milipore, Overijse, Belgium), used as a marker for EC cells. MAB352 was diluted 1:200 in PBS supplemented with 1 mol/L NaCl. Unbound primary antibody was removed by rinsing three times with cold PBS supplemented with 1 mol/L NaCl. Sections were then incubated with chicken anti-rat secondary antibody Alexa Fluor 488 (Life Technologies Europe) diluted 1:100 in PBS with 1 mol/L NaCl for 2 h at 4 ℃. Unbound secondary antibody was removed by rinsing three times with cold PBS with 1 mol/L NaCl and excess NaCl was removed by a PBS rinse (5 s). Sections were dehydrated in 70% and then 100% ethanol (3 min each) and air dried for 10 min at room temperature before laser microdissection.

Laser microdissection and pressure catapulting

LMPC was performed using the laser microdissection system from PALM Technologies (Carl Zeiss) containing a PALM Microbeam, RoboStage and a PALM RoboMover (PALM RoboSoftware version 4). Under direct microscopic visualization, LMPC permits the procurement of histologically or immunohistologically defined

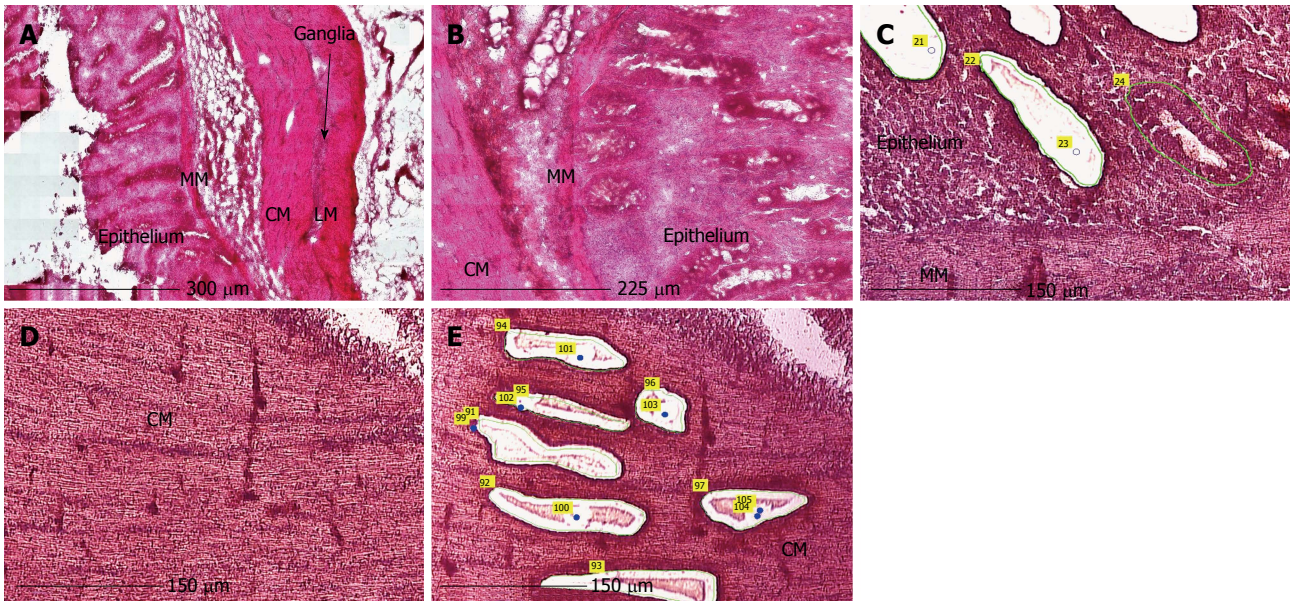

Figure 2 Photomicrographs of hematoxylin and eosin stained tissue sections of colon descendens: epithelium, muscularis mucosae, circular muscle layer, longitudinal muscle layer and ganglion. A: Overview of all layers in colon descendens; B: Detail of the epithelium; C: Epithelium with large patches microdissected by LMPC; D: Details of CM; E: CM with large patches of smooth muscle cells microdissected by LMPC. MM: Muscularis mucosae; CM: Circular muscle layer; LM: Longitudinal muscle layer; LMPC: Laser microdissection and pressure catapulting.

tissue and cell samples (Figure 2). Approximately 15 large patches of cells from the epithelium or circular smooth muscle layer in HE stained sections, or 70 EC cells in IHC stained sections were laser-dissected and pressure-catapulted in 50 μL RLT lysis buffer (RNeasy kit, Qiagen). The cell collecting time was limited to 2 h per slide and after 2 h of cell sampling, the remaining tissue on the membrane slide was scraped off and RNA was extracted to determine if RNA integrity was preserved after 2 h. The samples were homogenized by vortexing, centrifuged and then placed at -80 ℃ for later use. Seven EC cell collections were pooled into one sample with a final volume of 350 μL, resulting in a collection of approximately 500 cells per sample. Total RNA from the cell samples was extracted using the RNeasy Micro kit (Qiagen), according to the manufacturer's instructions.

Endpoint RT-PCR

Quantification of RNA was determined using a Nanodrop ND-1000 spectrophotometer (Isogen Life Science, Temse, Belgium) and the quality of RNA extracted from tissue fractions and tissue sections was assessed using the Experion automated electrophoresis system (BioRad, Nazareth Eke, Belgium).

cDNA of tissue fractions was prepared from 1 μg total RNA, whereas cDNA of whole tissue sections, parts of tissue sections and LMPC samples was prepared from the maximal input of total RNA as possible, as the amount of total RNA was less than 1 μg. The production of cDNA from sample RNA by RT was carried out according to manufacturer's instructions, using SuperScript Ⅲ Reverse Transcriptase SuperMix (Life Technologies Europe) containing random hexamers and oligo (dT)$_{20}$. The obtained cDNA was stored at -20 ℃ before PCR. cDNA amplification reactions were carried out using the AccuPrime Pfx SuperMix (Life Technologies Europe). The template cDNA of mucosa and MMP tissue fractions for amplification was diluted 1:10. Expression of the 5-HT$_4$ receptor within the samples was analysed using 5-HT$_4$ receptor-specific primers spanning exon-intron-exon junctions: an exon 4-specific forward primer and an exon 5-specific reverse primer (Figure 1B). These primers will detect alternative splicing of the h-exon because the h-exon is located between exon 4 and exon 5. The quality of cDNA produced was assessed by amplifying cDNA for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To amplify cDNA of tissue fractions and tissue sections, PCR reactions were performed using the following protocol: 5 min at 95 ℃, followed by 36 cycles with annealing temperature of 54 ℃. The LMPC samples had a low RNA output; therefore, PCR reactions to amplify the cDNA for both 5-HT$_4$ receptor and GAPDH, were performed using two rounds of PCR[15], according to the following protocol: a first reaction of 5 min at 95 ℃ followed by 36 cycles at 54 ℃ annealing temperature, followed by a second reaction using 1.5 μL of the product of the first reaction as a template for a second round of PCR (5 min at 95 ℃ followed by 36 cycles) with the same primers, but with a higher annealing temperature of 56 ℃ to increase specificity. RT and endpoint PCR reactions were processed on a C1000 Thermal Cycler (BioRad). PCR products were separated by 2% agarose gel electrophoresis and visualised by ethidium bromide staining. The primers (Eurogentec, Seraing, Belgium) used were published previously by De Maeyer *et al*[16]: 5-HT$_4$R forward primer (5'-ACAGGAACAAGATGACCCCT-3'); 5-HT$_4$R reverse primer (5'-AGGAGGAACGGGATGTAGAA-3'); GAPDH forward

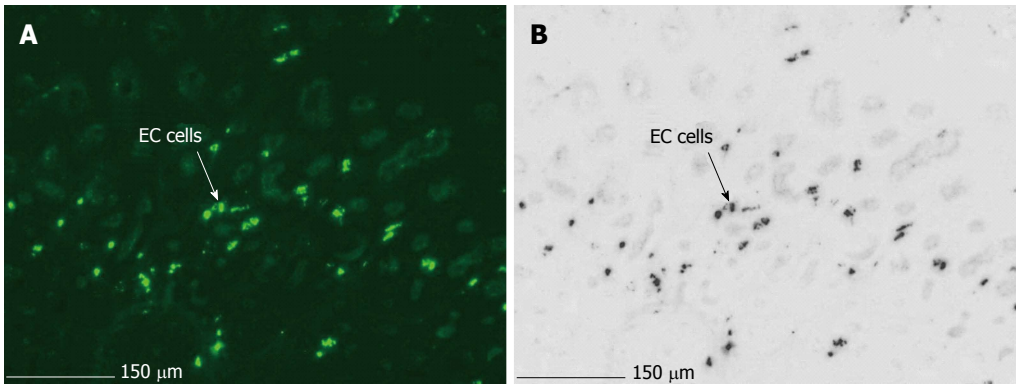

Figure 3 Photomicrographs of immunohistologically stained 8 μm sections of pig gastric fundus showing. A: MAB352 (1:200) immunofluorescent enterochromaffin cells (EC) in the epithelium (× 20); B: Invert color visualization of MAB352 (1:200) immunofluorescent EC cells.

primer (5'-ACCACAGTCCATGCCATCAC-3'); GAPDH reverse primer (5'-TCCACCCTGTTGCTGTA-3').

Statistical analysis

Semi-quantification of PCR products was determined by the intensity of PCR bands on the agarose gels using Image J 1.45 software. Band intensity was expressed as relative absorbance units, and the background of the image was determined and subtracted from the gel image. The ratio between the 5-HT$_4$ receptor and GAPDH RNA was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Data presented are mean ± SE of the mean for animals (n). Statistical analyses were performed using Graphpad Prism software v.5.01 (United States). Differences in intensity were determined by an unpaired t test; $P < 0.05$ was considered statistically significant.

RESULTS

Evaluating cell-specific visualization and RNA integrity

The main difficulties, when using LMPC to analyse gene expression of a specific cell type, are efficient and selective isolation of the desired cells, and obtaining RNA of good quality. Therefore, optimization of the LMPC experimental design was needed for the pig GI tissues, which are highly heterogeneous and rich in endogenous RNase and other enzymes. First, to select the desired cells from the heterogeneous GI tissues, a good visualization of the tissue layers and cells under direct microscopy was necessary. This requires that the morphology of the tissue be preserved, because fractures and air bubbles within the specimen will hamper the view. Tissues for section preparation and LMPC were therefore frozen in liquid N_2 containing isopentane. After HE staining, the different layers of colon descendens (Figure 2) and gastric fundus (not shown) could be identified based on their morphological characteristics. After cell-specific IHC staining, MAB352-immunoreactive EC cells (Figure 3) were present in the crypts, villi and epithelial lining of the mucous membrane of the gastric fundus and were isolated by LMPC.

To evaluate the impact of the different protocol steps on RNA integrity, a systematic approach was followed by evaluating RNA yield and quality after each protocol step. RNA quality was assessed by comparing 28S and 18S and pre-18S ribosomal peaks to a set of degradation standards using the Experion automated electrophoresis system, where the RNA quality indicator (RQI) returns a number between 10 (intact RNA) and 1 (highly degraded RNA)[31]. The analysis showed that RNA quality was not affected after tissue fraction collection (Figure 4A), HE staining (Figure 4B), LMPC (Figure 4C) or after IHC staining (Figure 4D). However, electropherograms of RNA collected by LMPC could not be analysed systematically because the amount of RNA collected was too low. In Figure 4C, RNA quality from large microdissected patches of either epithelial or smooth muscle cells is shown, indicating that RNA was remained mostly intact, but the small ribosomal RNA peaks (18S/28S) indicate a low amount of RNA.

Expression of the 5-HT$_4$ receptor in porcine tissue fractions

Tissue samples from pig colon descendens and gastric fundus were dissected into the mucosa fraction and the MMP fraction before freezing in liquid N_2. After RNA extraction and endpoint PCR analysis of these fractions, 5-HT$_4$ receptor expression was detected in mucosa as well as MMP fractions of the colon descendens and gastric fundus (Figure 5). All tissue samples were positive for GAPDH, confirming the integrity of the samples and completed PCR reactions. In all samples, 5-HT$_4$ receptors containing the h-exon [277 bp; 5-HT$_{4(+h)}$ receptor] as well as 5-HT$_4$ receptors without the h-exon [235 bp; 5-HT$_{4(-h)}$ receptor] were present (Figure 5). However, a third band, corresponding to a fragment of more than 300 bp was also observed. Therefore, we isolated this unknown PCR band using a QIA quick Gel extraction kit (Qiagen) and determined its DNA sequence with an ABI3130XL sequencer (Life Technologies Europe). After sequence analysis, we aligned the unknown sequence with the 5-HT$_{4(+h)}$ and 5-HT$_{4(-h)}$ receptor sequences and observed that the unknown band contained the same sequence and

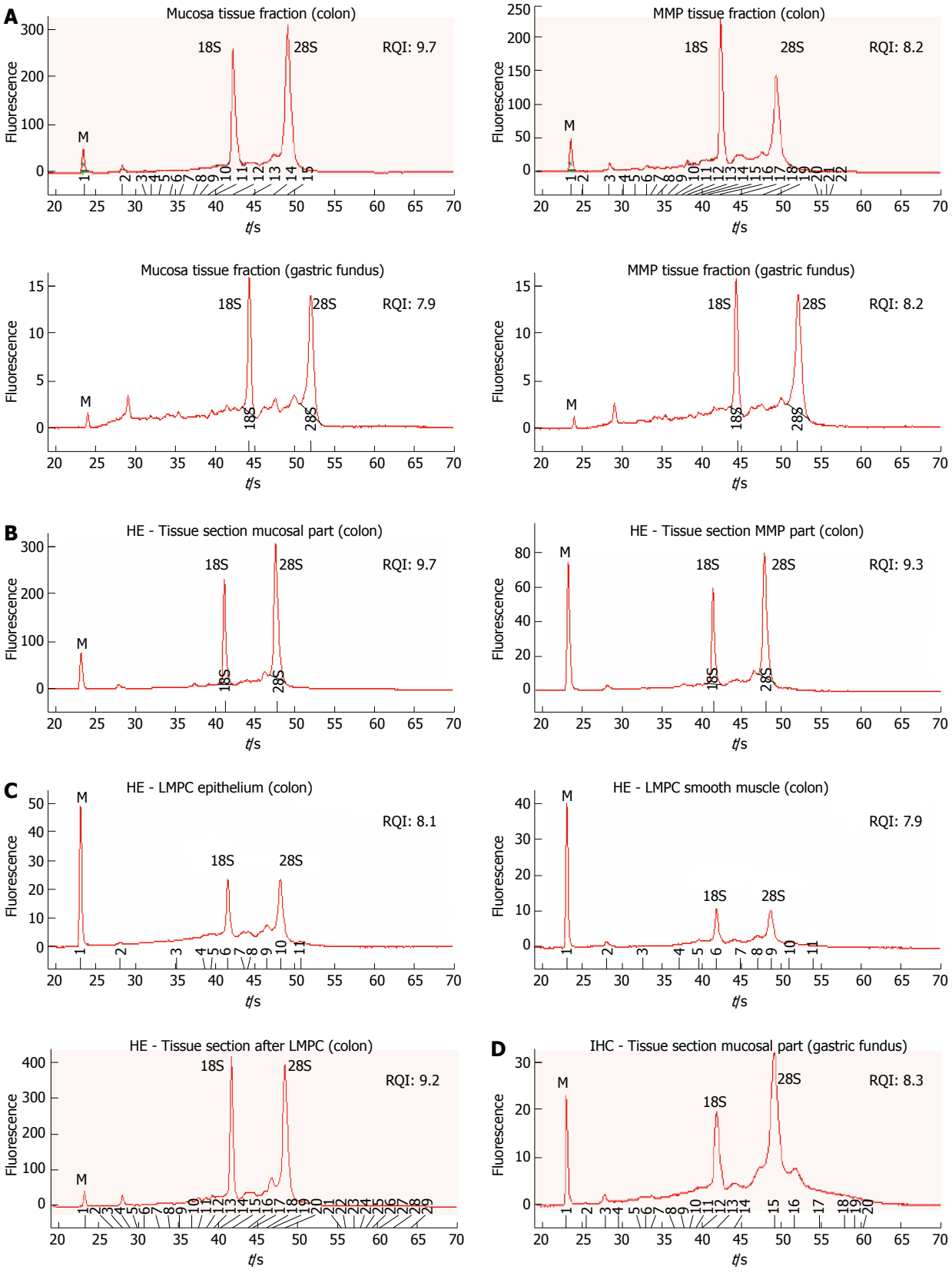

Figure 4 Representative experion electropherograms of collected RNA. A: Mucosa and MMP tissue fractions of colon descendens and gastric fundus; B: The mucosal part and the MMP part of HE stained tissue sections of colon descendens; C: Large patches of epithelial cells and smooth muscle cells obtained by LMPC from HE stained tissue sections and the whole HE stained tissue section scraped off after LMPC in colon descendens; D: The mucosal part of an IHC stained tissue section of gastric fundus. Electropherograms show fluorescence (ordinate) *vs* time (abscissa) with RQI values. Positions of 18S and 28S ribosomal RNA and marker (M) peaks are indicated. MMP: Muscle-myenteric plexus; HE: Hematoxylin and eosin; IHC: Immunohistochemically; RQI: RNA quality indicator.

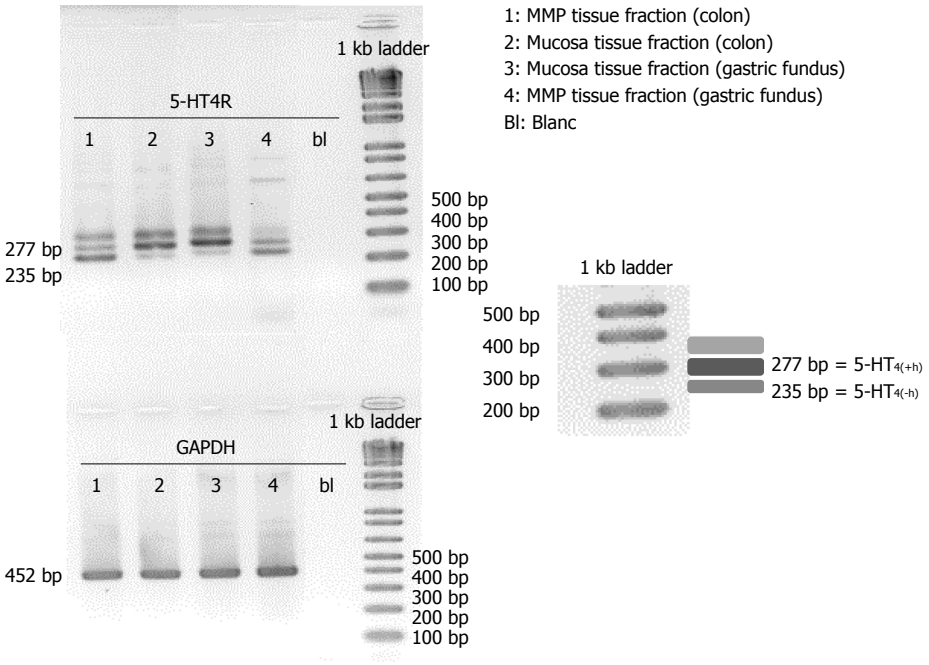

Figure 5 Invert color image of endpoint polymerase chain reaction analysis of the 5-HT$_4$ receptor and of the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene expressed in mucosa and muscle-myenteric plexus tissue fractions of pig colon descendens and gastric fundus. Dominant expression of 5-HT$_{4(+h)}$ receptor in the mucosa of the colon descendens and gastric fundus is observed. Part of the ladder is increased to indicate the size of the expected polymerase chain reaction (PCR) products. A third unknown upper band is shown above the 277 bp band, due to dimerization of the PCR product with other PCR fragments after the PCR reaction. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MMP: Muscle-myenteric plexus.

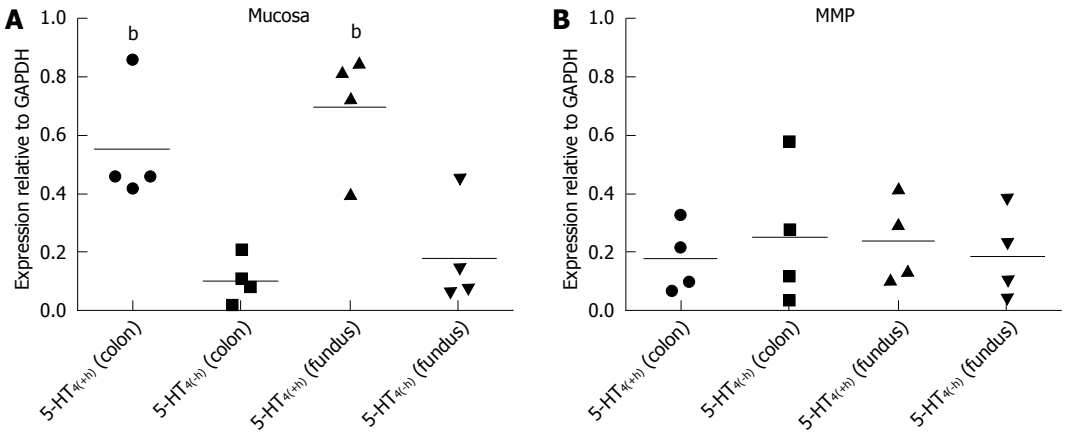

Figure 6 Expression of 5-HT$_{4(+h)}$ and 5-HT$_{4(-h)}$ receptors in the mucosa (A) and muscle-myenteric plexus (B) fractions of colon descendens and gastric fundus. Data are given as ratio relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The line indicates the mean of $n = 4$ for each region-fraction. $^bP < 0.01$ *vs* values for 5-HT$_{4(-h)}$ receptors in colon descendens or gastric fundus. MMP: Muscle-myenteric plexus.

length as the 5-HT$_{4(+h)}$ receptor, but chromatogram details suggested the presence of additional nucleotides in the tail of the sequence, possibly caused by the formation of a heteroduplex with a 5-HT$_{4(+h)}$ strand and a 5-HT$_{4(-h)}$ strand, or formation of a triplex with other PCR fragments, resulting in a different electrophoretic separation.

Mucosa fractions of the colon descendens and gastric fundus contained relatively more h-exon containing 5-HT$_4$ receptors. Semi-quantification by expressing the intensity of the bands compared with the intensity of GAPDH and statistical analysis (Figure 6), confirmed the significantly ($P < 0.01$) more pronounced expression of 5-HT$_{4(+h)}$ receptor within the mucosa fractions (the ratio *vs* GAPDH was 0.55 ± 0.10 in the colon descendens and 0.69 ± 0.13 in the gastric fundus; $n = 4$) compared to the expression of the 5-HT$_{4(-h)}$ receptor (colon descendens: 0.12 ± 0.03; gastric fundus: 0.21 ± 0.10; $n = 4$). Within the MMP fraction of the colon descendens and gastric fundus there was no difference in expression of the 5-HT$_{4(+h)}$ receptor (colon descendens: 0.18 ± 0.06; and gastric fundus: 0.18 ± 0.05; $n = 4$) and 5-HT$_{4(-h)}$ receptor (colon descendens: 0.26 ± 0.12; gastric fundus: 0.17 ± 0.09; $n = 4$).

Expression of the 5-HT$_4$ receptor in porcine HE stained tissue sections

Whole tissue sections, and the mucosal or MMP part of

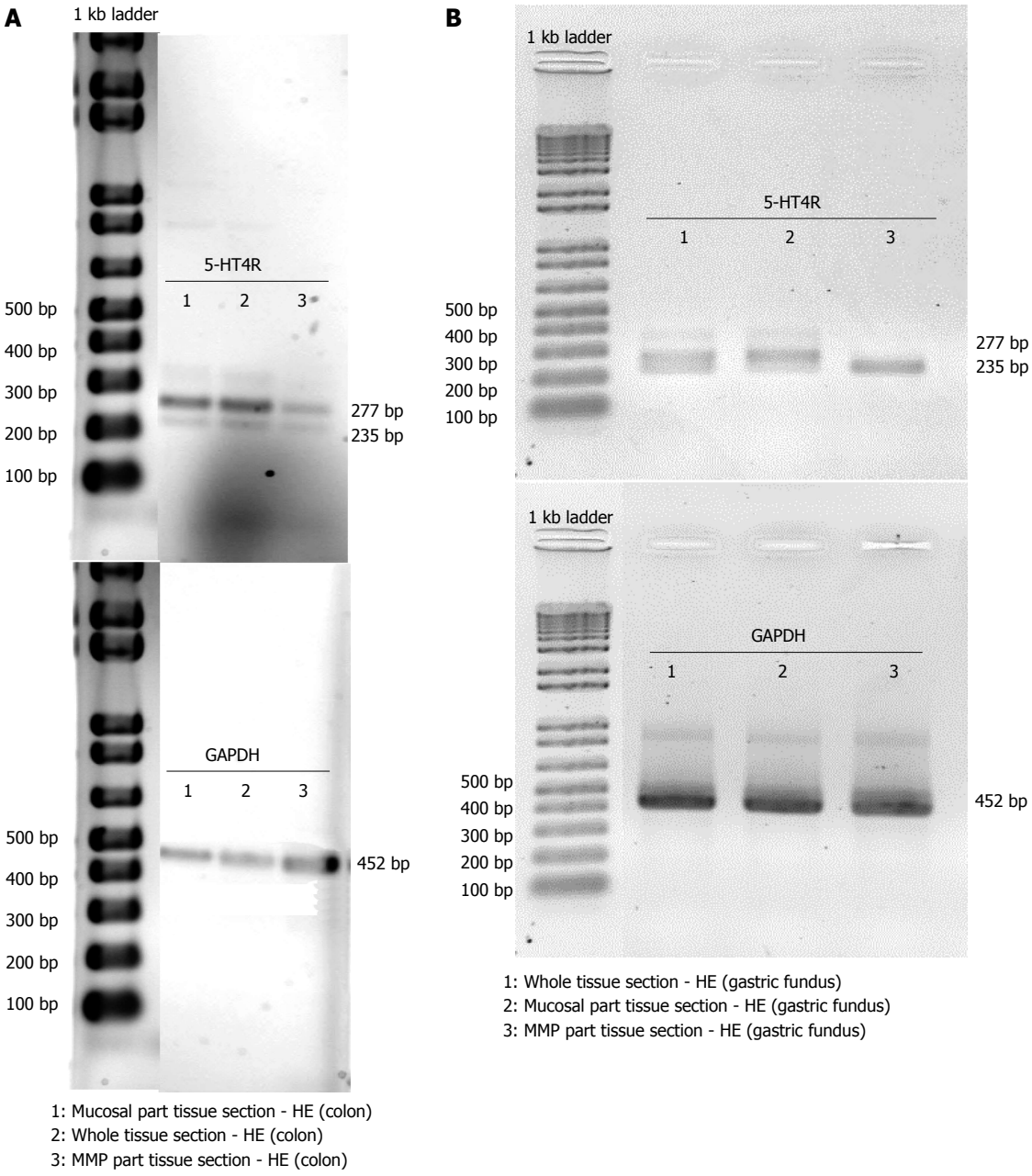

Figure 7 Invert color image of endpoint polymerase chain reaction analysis. 5-HT$_4$ receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression is shown in a hematoxylin and eosin (HE) stained whole tissue section, and in the mucosal as well as the muscle-myenteric plexus (MMP) part of a HE stained tissue section of colon descendens (A) and gastric fundus (B). The size of the expected polymerase chain reaction products is indicated.

tissue sections of the colon descendens and gastric fundus were scraped off a membrane slide and 5-HT$_4$ receptor expression was analyzed.

Colon descendens: 5-HT$_4$ receptor and GAPDH expressions were detected in the whole tissue sections, and in the mucosal and MMP parts of tissue sections of the colon descendens (Figure 7A). After semi-quantification, the values for 5-HT$_4$ receptor expression were 5-HT$_{4(+h)}$R 1.21 ± 0.66 and 5-HT$_{4(-h)}$R 0.63 ± 0.45 within whole tissue sections (n = 3); 5-HT$_{4(+h)}$R 0.86 ± 0.39, 5-HT$_{4(-h)}$R 0.28 ± 0.14 within the mucosal part of tissue sections (n = 3); and 5-HT$_{4(+h)}$R 0.47 ± 0.05, 5-HT$_{4(-h)}$R 0.24 ± 0.11 within the MMP part of tissue sections (n = 3). The tendency for more pronounced expression of the h-exon containing splice variant did not reach significance.

Gastric fundus: 5-HT$_4$ receptor and GAPDH expression was also detected in the whole tissue sections, and mucosal and MMP parts of tissue sections of the gastric fundus (Figure 7B). After semi-quantification, expression values were 0.51 and 0.19 for 5-HT$_{4(+h)}$R, 0.23 and 0.11 for 5-HT$_{4(-h)}$R in whole tissue sections, 1.13 and 0.24 for 5-HT$_{4(+h)}$R and 0.31 and 0.05 for 5-HT$_{4(-h)}$R in the mucosal part of tissue sections. In the two samples with the MMP part of tissue sections, the 5-HT$_{4(-h)}$ receptor was found in both (0.24 and 0.45), while the 5-HT$_{4(+h)}$ receptor was only found in one of the samples (0.53) (Figure 7B

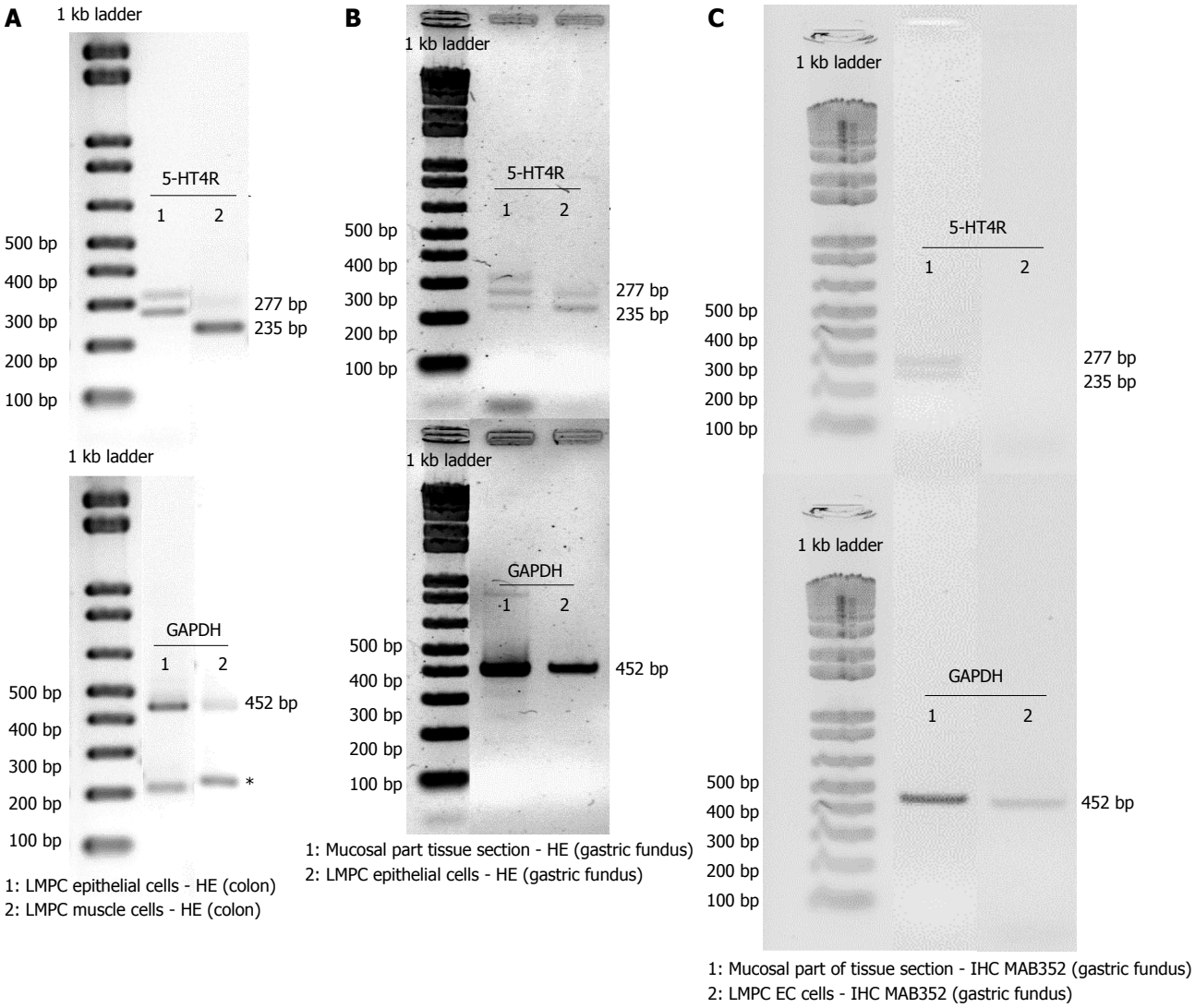

Figure 8 Invert color representation of the double round of endpoint polymerase chain reaction analysis. A, B: 5-HT$_4$ receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression is shown in large patches of epithelium cells and smooth muscle cells obtained by laser microdissection and pressure catapulting (LMPC) from hematoxylin and eosin (HE) stained tissue sections of colon descendens (A) and gastric fundus (B, only epithelial cells were obtained), and in enterochromaffin cells obtained by LMPC from MAB352 immunohistochemical (IHC) stained tissue sections of gastric fundus (C). For comparison, the result obtained in the mucosal part of the HE or IHC stained tissue section of gastric fundus is also shown in B and C. The size of the expected polymerase chain reaction products and presence of non-specific amplification are indicated (asterisk).

lane 3, showing the result with only the 5-HT$_{4(-h)}$ receptor detected).

Expression of the 5-HT$_4$ receptor in LMPC-isolated cell populations from HE stained porcine tissue sections

After HE staining, large microdissected patches of cells were taken from the epithelium and the circular smooth muscle layer of the colon descendens and from the epithelium of the gastric fundus. Low RNA yields meant that only after a second round of PCR, with the same 5-HT$_4$ receptor specific-primers, was 5-HT$_4$ receptor expression detected in large microdissected patches of epithelial cells (Figure 8A and B) or smooth muscle cells (Figure 8A). Non-specific amplification was occasionally observed because of the high number of cycles (Figure 8A). In the colon descendens (Figure 8A), higher expression of the 5-HT$_{4(+h)}$ receptor was observed compared with the 5-HT$_{4(-h)}$ receptor within the LMPC-isolated epithelial cells [5-HT$_{4(+h)}$R 1.03 and 0.85, 5-HT$_{4(-h)}$R 0.24 and 0.11], while the opposite occurred for the smooth muscle cells [5-HT$_{4(+h)}$R 1.14 and 0.91, 5-HT$_{4(-h)}$R 5.07 and 4.03]. In the gastric fundus epithelial cells (Figure 8B), the expression appeared similar for the 5-HT$_{4(+h)}$R (0.45 and 0.20) and 5-HT$_{4(-h)}$R (0.67 and 0.24).

Expression of the 5-HT$_4$ receptor in LMPC-isolated EC cells from IHC stained porcine tissue sections

After IHC staining, EC cells (MAB352; Figure 8C), were isolated by LMPC from gastric fundus tissue sections. After a second round of PCR with the same 5-HT$_4$ receptor specific-primers, no 5-HT$_4$ receptor expression was detected in LMPC-isolated EC cells, although the cells were positive for the *GAPDH* gene, confirming the integrity of the samples and completed PCR reactions at

least for GAPDH. Additionally, in the mucosal part (Figure 8C) of IHC stained tissue sections, scraped off after LMPC of EC cells, 5-HT$_4$ receptor and GAPDH expressions were detected.

DISCUSSION

The aim of this study was to investigate the 5-HT$_4$ receptor distribution in the pig GI tract by confining gene expression analysis to site-specific regions of interest, with special attention being paid to the mucosal layer of the pig colon descendens and gastric fundus by isolating epithelial cells using the LMPC technique. A stepwise approach was used by first studying mucosal and MMP tissue fractions, then tissue sections where different cell layers were discerned morphologically by HE staining, and finally tissue sections stained for a particular cell type by IHC. The impact of the freezing method and staining method on the RNA quality was evaluated in mucosa and MMP tissue fractions (Figure 4A), and mucosal and MMP parts of HE stained tissue sections (Figure 4B) of the pig colon descendens and gastric fundus. The major advantages of LMPC are the isolation of biological material without direct user contact, thereby avoiding contamination, and the preservation of cellular integrity[32]. The main obstacles, when using LMPC to analyse 5-HT$_4$ receptor mRNA expression in different cell types, are firstly to recognize the cells of interest and secondly to obtain RNA of good quality. To recognize the cells of interest while preserving RNA integrity, an HE protocol and a cell-specific IHC protocol were developed in RNase-free conditions. Developing a suitable IHC staining procedure was more complex compared with the HE staining, because a standardized IHC procedure requires a long overnight antibody incubation to obtain good antibody labeling; however, long incubation in aqueous buffers activates endogenous RNases, resulting in RNA degradation[33]. Our attempts to develop a fast IHC protocol resulted in diminished visualization because of the short antibody labeling time. Therefore, our IHC staining protocol was based on the report published by Brown *et al*[24], where overnight antibody incubation and RNA integrity could be maintained with the addition of 1 mol/L NaCl to all aqueous solutions, resulting in superior protection of RNA. It is not yet clear why a saline solution preserves RNA, although we can speculate that normal saline protects the integrity of cell membranes, which may prevent the release of intracellular RNases[34]. Our results confirm the preservation of RNA after HE staining (Figure 4B) as well as after overnight IHC staining (Figure 4D). EC cells in the mucosal layer of the pig gastric fundus were visualized by IHC staining with the MAB352 antibody against 5-HT (Figure 3). Indeed, Penkova *et al*[35] showed that the MAB352 antibody is selective for EC cells in the human gastric mucosa, as verified by electron microscopy.

In this study, 5-HT$_4$ receptor expression was detected in both mucosa and MMP tissue fractions (Figures 5 and 6), and in mucosal and MMP parts of HE stained tissue sections (Figure 7) of the colon descendens, as well as of the gastric fundus, confirming previously reported data by De Maeyer *et al*[16]. Expressions of both a variant with the h-exon [5HT$_{4(+h)}$ receptor, 277 bp] and one without the h-exon [5HT$_{4(-h)}$ receptor, 235 bp] were observed. This was made possible by analysis of the 5-HT$_4$ receptor mRNA expression profile using forward and reverse primers based on exon 4 and 5, which were designed to amplify part of the common receptor region encoded by exon 4 and 5, which flank exon h (Figure 1B). The study was not designed to discriminate the C-terminal tail associated with the h-exon; the 5HT$_{4(+h)}$ receptor detected might indeed be associated with several C-terminal splice variants, because the h-exon has already been associated with the a, m and r C-terminal in pigs[16]. Semi-quantitative analysis revealed that in the mucosa tissue fractions of colon descendens and gastric fundus, the expression level of the 5-HT$_{4(+h)}$ receptor was significantly higher compared with the 5-HT$_{4(-h)}$ receptor, and a similar trend was obtained in the mucosal part of HE stained tissue sections. While evaluation of 5-HT$_4$ receptor RNA expression in human GI full-thickness tissue samples showed similar levels in the stomach compared with more distal levels[36], expression in human gastric mucosal specimens was much less pronounced than in mucosal specimens of more distal regions of the GI tract[21,22]. In the pig gastric fundus mucosa, however, the expression of the 5-HT$_4$ receptor was similar to that in the mucosa of the colon descendens, with a clear-cut preponderance of the h-exon-containing receptor. The predominant mucosal location of h-exon containing 5-HT$_4$ receptor splice variants might correspond to the preferential involvement of this type of 5-HT$_4$ receptor splice variant in the mucosal effects of 5-HT$_4$ receptor activation, such as goblet cell degranulation, chloride secretion and control of 5-HT release[21].

Both the mucosal and the MMP part of the GI tract contain several cell types on which the presence of 5-HT$_4$ receptors has been suggested, at least in some regions in some species, such as EC cells, smooth muscle cells of the muscularis mucosae and submucosal intrinsic neurons in the mucosal part; and myenteric cholinergic neurons, smooth muscle cells and interstitial cells of Cajal in the MMP part[37-41]. To obtain more information on the cell-specific distribution of the 5-HT$_4$ receptors, cell layers or particular cell types were isolated by LMPC. In the colon descendens, patches of the epithelial cell layer obtained by LMPC showed expression of 5-HT$_4$ receptors, predominantly the 5HT$_{4(+h)}$ receptor. Possible cell types involved might be EC cells and goblet cells, which were recently shown to express 5-HT$_4$ receptors in the mouse intestine[21]. In mouse, application of 5-HT$_4$ receptor agonists led to mucosal 5-HT release and mucus secretion in a tetrodotoxin-insensitive manner, indicating direct activation of stimulatory 5-HT$_4$ receptors on the EC cells and goblet cells[21]. In the porcine and human small intestine however, analysis of 5-HT release suggested the presence of inhibitory 5-HT$_4$ receptors on the EC cells[42]. Relaxant 5-HT$_4$ receptors have been proposed on circular smooth muscle in the human colon on the basis

of functional data[13]; therefore, patches of cells were also obtained from the circular muscle layer of the pig colon descendens, which revealed 5-HT$_4$ receptor expression. However, some authors were not able to confirm the presence of relaxant 5-HT$_4$ receptors in human colonic circular muscle strips[43]; we were also unable to obtain evidence for muscular 5-HT$_4$ receptors in pig colonic circular muscle strips[12]. Thus, we can not exclude the possibility that the 5-HT$_4$ receptor expression observed in LMPC-isolated cell patches from the pig colonic circular muscle layer represents the 5-HT$_4$ receptors on intercalated interstitial cells of Cajal. In addition, in the pig gastric fundus, LMPC-isolated cell patches of the epithelial cell layer showed 5-HT$_4$ receptor expression. The human gastric mucosa was shown to contain a considerable number of EC cells scattered within the lining epithelium[35]; therefore, we stained EC cells in porcine gastric mucosa immunohistochemically and isolated them by LMPC. Although the 5-HT$_4$ receptor was still detected in the full mucosal part of these IHC stained sections, 5-HT$_4$ receptor expression was not detected in the isolated EC cells. The EC cells showed expression of GAPDH; therefore, this might be related to the small amount of 5-HT$_4$ receptor RNA obtained, even when pooling 500 LMPC-isolated cells. In the gastric fundus, LMPC was not used to obtain cell patches from the muscle layer, as there are no functional data suggesting 5-HT$_4$ receptors to be present on muscle cells in the stomach.

In conclusion, this study, using endpoint RT-PCR, confirmed the presence of 5-HT$_4$ receptors in the mucosa and in the MMP part of porcine gastric fundus and colon descendens, and showed that the mucosa predominantly expresses h-exon-containing 5-HT$_4$ receptors. The mucosal h-exon-containing 5-HT$_4$ receptors might form additional sites of action for 5-HT$_4$ receptor agonists. 5-HT$_4$ receptors were detected in LMPC-isolated epithelial cell patches in the gastric fundus and colon descendens, and in circular muscle cell patches in the colon descendens. No 5-HT$_4$ receptor expression was detected in gastric LMPC-isolated EC cells stained by immunohistochemistry; the expression of 5-HT$_4$ receptors in individual cell types might be too low to detect by LMPC and endpoint PCR.

ACKNOWLEDGMENTS

The authors thank Mrs. An Neesen, Sandra Soetaert and Trees Lepez for their technical help.

COMMENTS

Background

5-HT$_4$ receptors are distributed throughout the gastrointestinal (GI) tract. They are expressed on excitatory motor neurons, promoting the stimulatory effect of these neurons on GI motility. This explains the therapeutic use of 5-HT$_4$ receptor agonists in conditions with impaired GI motility, such as constipation. Several reports indicate that 5-HT$_4$ receptors are also expressed in the GI mucosa. 5-HT$_4$ receptors are G-protein coupled receptors containing seven transmembrane domains. The intracellular tail shows splice variation, with different lengths of the intracellular amino acid sequence. Exceptionally among G-protein coupled receptors, 5-HT$_4$ receptors can also have an extra insertion of 14 amino acids in the second extracellular loop, encoded by the h-exon (42 base pairs). Previous distribution studies of 5-HT$_4$ receptors in the GI tract did not consider the all or none presence of the h-sequence.

Research frontiers

Using homogenates of tissues limits the potential to study cell-specific expression: important cell-specific transcript information is lost because of cell heterogeneity of tissues such as GI tissues. Techniques have been developed to enable collection of particular cells from mixed populations, which generally involve either fluorescence activated cell sorting (FACS) purification of dissociated cells or laser-assisted microdissection. In contrast to FACS, microdissection can be applied to most tissues.

Innovations and breakthroughs

The major advantages of laser microdissection and pressure catapulting (LMPC) are the isolation of biological material without direct user contact, thereby avoiding contamination, and the preservation of cellular integrity. The main concerns when using LMPC to analyse 5-HT$_4$ receptor mRNA expression of a specific cell type is the efficient and selective isolation of the right cells, and obtaining RNA of good quality. To address these issues, and to optimize a LMPC experimental design for GI tissue that is highly heterogeneous, rich in endogenous RNase and enzymes, a systematic approach was required to evaluate the impact on RNA integrity of different critical steps. Therefore, RNA yield and quality were determined using the Experion automated electrophoresis system after tissue collection, hematoxylin and eosin (HE) staining or immunohistochemistry (IHC) staining, and LMPC by analysing RNA extracted from mucosal and muscular myenteric plexus (MMP) tissue fractions, and from scraped off tissue sections after staining and after LMPC. Developing a suitable IHC staining procedure was more complex compared with the HE staining, because a standard IHC procedure requires a long overnight antibody incubation to obtain good antibody labeling, but long incubation in aqueous buffers activates endogenous RNases, resulting in RNA degradation. The authors attempted to develop a fast IHC protocol, which resulted in diminished visualization because the short antibody labeling time. Therefore, the authors used an overnight antibody incubation for the IHC staining protocol whereby RNA integrity was maintained by the addition of 1 mol/L NaCl to all aqueous solutions, resulting in superior protection of RNA. It is not yet clear why a saline solution preserves RNA, although the authors speculate that normal saline protects the integrity of cell membranes, preventing the release of intracellular RNases.

Applications

This study, using endpoint reverse transcription-polymerase chain reaction (PCR) and LMPC, confirms the presence of 5-HT$_4$ receptors in the mucosal and MMP parts of the porcine gastric fundus and colon descendens, and shows that the mucosa predominantly expresses h-exon containing 5-HT$_4$ receptors. The mucosal h-exon-containing 5-HT$_4$ receptors might be preferentially involved in the mucosal response to 5-HT$_4$ receptor activation and might form potential drug targets for 5-HT$_{4(+h)}$ receptor-selective agonists.

Terminology

5-HT$_4$ receptors: The 5-HT$_4$ receptor is a G-protein coupled receptor that activates the adenylyl cyclase/cyclic adenosine monophosphate/protein kinase A pathway in response to serotonin (5-hydroxytryptamine; 5-HT). The 5-HT$_4$ receptor is expressed on excitatory motor neurons in the gut, facilitating acetylcholine release, which stimulates GI motility; LMPC: Under direct microscopic visualization, LMPC permits sampling of histologically or immunohistologically defined tissue and cell samples. The LMPC system uses a focused pulsed nitrogen UV-A laser beam (wavelength 355 nm) whose source is positioned below the material and the high energy generated beam is focused through a microscope ocular lens onto the biological material on the slide.The RoboMover stage is used to move the sample through the laser beam path to allow the user to control the size and shape of the area to be cut. The beam is then defocused and this energy is used to catapult the membrane and corresponding biological material from the slide. When the laser beam strikes the material, it is blasted off the glass surface and catapulted into the cap of the vial.

Peer review

This manuscript describes the expression of the +h 5-HT$_4$ receptor variant in porcine stomach and colon. The significance of this work is the use of LMPC and end-point PCR to allow the positive identification of the specific HTR$_4$ variant (+ exon h) in discernible functional cell types and tissue regions relevant to serotoninergic responses. This study also discusses several technique modifications and improvements and artifacts in detail, which might be helpful for wider application of similar interests.

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doi:10.3748/wjg.v19.i24.3761

World J Gastroenterol 2013 June 28; 19(24): 3761-3769
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Assessment of vascular invasion in gastric cancer: A comparative study

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Supported by Grants from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
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Received: January 30, 2013 Revised: April 3, 2013
Accepted: April 13, 2013
Published online: June 28, 2013

Abstract

AIM: To evaluate and compare detection of lymphatic and blood vessel invasion (LVI and BVI) by hematoxylin-eosin (HE) and immunohistochemistry (IHC) in gastric cancer specimens, and to correlate with lymph node status.

METHODS: IHC using D2-40 (a lymphatic endothelial marker) and CD34 (a pan-endothelial marker) was performed to study LVI and BVI in surgical specimens from a consecutive series of 95 primary gastric cancer cases. The results of the IHC study were compared with the detection by HE using McNemar test and kappa index. The morphologic features of the tumors and the presence of LVI and BVI were related to the presence of lymph node metastasis. A χ^2 test was performed to obtain associations between LVI and BVI and other prognostic factors for gastric cancer.

RESULTS: The detection rate of LVI was considerably higher than that of BVI. The IHC study identified eight false-positive cases and 13 false-negative cases for LVI, and 24 false-positive cases and 10 false-negative cases for BVI. The average Kappa value determined was moderate for LVI (κ = 0.50) and low for BVI (κ = 0.20). Both LVI and BVI were statistically associated with the presence of lymph node metastasis (HE: P = 0.001, P = 0.013, and IHC: P = 0.001, P = 0.019). The morphologic features associated with LVI were location of the tumor in the distal third of the stomach (P = 0.039), Borrmann's macroscopic type (P = 0.001), organ invasion (P = 0.03) and the depth of tumor invasion (P = 0.001). The presence of BVI was related only to the depth of tumor invasion (P = 0.003).

CONCLUSION: The immunohistochemical identification of lymphatic and blood vessels is useful for increasing the accuracy of the diagnosis of vessel invasion and for predicting lymph node metastasis.

Key words: Gastric cancer; Tumour-node-metastesis staging; Lymph node metastasis; Predictive factor; Lymphatic vessel invasion; Blood vessel invasion; Immunohistochemistry; CD34; D2-40

Core tip: The presence of lymphatic vessel invasion in gastric cancer is the strongest risk factor for lymph node metastasis and is known as an independent prog-

nostic factor. The subjective evaluation of vessel invasion performed with conventional hematoxylin-eosin staining can lead to inaccurate false-positive and false-negative results. This study shows that the immunohistochemical identification of lymphatic and blood vessels is useful for increasing the accuracy of the diagnosis of lymphatic and blood vessel invasion and for predicting lymph node metastasis in gastric cancer.

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INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and the second most common cause of cancer deaths in the world[1]. A steady decline in the incidence and mortality rates of gastric carcinoma has been observed worldwide over the past several decades, but there is a significant variation in incidence between the populations at the greatest and least risk[2]. In areas without endoscopic screening for GC, especially in developing countries, GC presents as an advanced disease and has a high frequency of nodal involvement[3]. Surgery is the only effective intervention for a cure or for long-term survival and nodal status is one of the most important independent predictors of patient survival[4].

The depth of invasion is an independent prognostic factor for gastric carcinoma and is associated with patient survival[5,6]. Early GC is limited to the mucosa and submucosa and is associated with a better prognosis. In Japan, where asymptomatic patients are screened, there is a high incidence of early diagnosis, ranging from 30% to 50%, in contrast with the smaller fraction of 16%-24% in Western countries[2]. Minimizing the number of invasive procedures used in cancer treatment is critical for improving the patient's quality of life. Minimally invasive treatments, such as endoscopic mucosal resection, may be possible only in highly selective cases of early GC[7-9].

Lymph node metastasis is one of the most important prognostic factors in patients with GC[2,10]. Studies have estimated that the lymph nodes will be involved in 3%-5% of cases of gastric adenocarcinoma limited to the mucosa, in 11%-25% of cases limited to the submucosa, in 50% of T2 tumors and in 83% of T3 tumors[11]. Hence the accurate assessment of potential lymph node metastasis is an important issue for the appropriate treatment of early GC.

The histologic identification of lymphatic vessel invasion (LVI) by tumor cells has long been recognized as a potential prognostic indicator and a predictor of patient outcomes in various malignancies[12-18]. One of the earliest steps in the metastatic cascade is (lympho)vascular invasion, *i.e.*, the penetration of tumor cells into lymph and/or blood vessels in and around the primary tumor[19-21]. Therefore, tumor cell emboli in the lymph and blood vessels are considered to be the morphological correlates of metastases to loco-regional lymph nodes and to distant hematogenous sites, respectively. Consistent with the distribution of lymphatic vessels in the gastric wall, LVI is most frequently observed in the muscularis mucosa layer and in the superficial submucosa[22,23].

Usually, LVI and blood vessel invasion (BVI) are identified based on conventional hematoxylin-eosin (HE) staining, and the diagnosis is made based on the presence of tumor emboli within the vascular channels lined by a single layer of endothelial cells, with or without red blood cells[14,19,20]. However, if the cancer cells completely obliterate the lumen, it is not possible to diagnose vascular invasion. Additionally, retraction artifacts that isolate tumor aggregates *via* tissue shrinkage during fixation are sometimes confused with true tumor emboli in lymphatic vessels. Besides, using that criterion, vascular invasion detected on HE sections does not always allow for a distinction between BVI and LVI[14].

Recently, interest in vascular invasion has increased because of the development of specific markers for the lymphatic endothelium used in immunohistochemistry (IHC), such as Prox-1, which is a transcription factor; Lyve-1, which is a hyaluronan receptor; podoplanin, which is a glomerular podocyte membrane protein and D2-40[21]. It has been demonstrated that D2-40 is the best marker for the lymphatic endothelium[24]. Used in combination with panendothelial markers such as CD34 or CD31, D2-40 permits the differentiation between BVI and LVI and the study of both processes in GC metastasis[25].

There have been numerous studies regarding LVI and BVI in GC. However, most of them have not defined the criteria used to determine the presence or absence of lymphatic and vascular invasion. Additionally, many large retrospective series of GC cases have extracted the reporting of (lympho)vascular invasion from the patients' medical records, without histological reviews by central pathologists for consistency and without immunohistochemical studies[6,9,15,26]. Uncertain criteria for the diagnosis of (lympho)vascular invasion may affect the clinical assessment of prognosis and may change the course of therapy for the patients[27-30].

The aim of this study was to evaluate, in a consecutive series of patients with GC, a technique that uses a combined immunohistochemical expression profile to detect LVI and BVI and compare this technique to routine HE assessment. In addition, we analyzed the relationship between lymph node metastasis and clinicopathological findings, especially those of LVI and BVI re-evaluated by IHC staining.

MATERIALS AND METHODS

This study was reviewed and approved by the university's research ethics committee (COEP-UFMG). Ninety-five consecutive cases of GC, diagnosed and treated between

Table 1 Clinicopathological characteristics of 95 patients with gastric cancer *n* (%)

Clinicopathological data	
Sex	
Male	62 (65.3)
Female	33 (34.7)
Curvature	
Small curvature	54 (56.8)
Large curvature	10 (10.5)
Small and large	14 (14.7)
Not evaluated	17 (17.9)
Primary tumor	
pT1a	9 (9.5)
pT1b	12 (12.6)
pT2a	12 (12.6)
pT2b	8 (8.4)
pT3	51 (53.7)
pT4	3 (3.2)
Regional lymph nodes	
pN0	28 (29.5)
pN1	36 (37.9)
pN2	13 (13.7)
pN3	12 (12.6)
pNx	6 (6.3)
Distant metastasis	
pMx	87 (91.6)
pM1	8 (8.4)
Laurén classification	
Intestinal	45 (47.4)
Diffuse	25 (26.3)
Mixed/indeterminate	25 (26.3)
WHO classification	
Adenocarcinoma NOS	48 (50.5)
Tubular	5 (5.3)
Papillary	5 (5.3)
Mucinous	4 (4.2)
Signet-ring cell	22 (23.2)
Undifferentiated	9 (9.5)
Others	2 (2.2)
Ming classification	
Expansive	40 (42.1)
Infiltrative	40 (42.1)
Not evaluated	15 (15.8)

NOS: Not otherwise specified; WHO: World Health Organization; pT: Primary tumor; pN: Regional lymph nodes; pM: Distant metastasis.

2000 and 2006 and identified from the pathology archives, were selected for study. All patients underwent curative gastrectomy with standard lymphadenectomy at the Clinical Hospital of the Federal University of Minas Gerais. None of the patients had received preoperative radiation therapy or chemotherapy. In total, 57 patients underwent distal gastrectomy, 33 had total gastrectomy and five had partial gastrectomy.

All surgical specimens of the primary tumors and regional lymph nodes had been processed and examined histologically by routine HE staining, according to the institutional protocol[31]. The definitions of stages and the criteria for histological classification followed the World Health Organization classification[2] and the Japanese classification for GC[32]. The resected primary tumors and regional lymph nodes were reviewed histologically by two pathologists using HE staining.

IHC for CD34 and D2-40 was then performed on the corresponding paraffin blocks. Serial 4-μm-thick sections were deparaffinized in xylene and rehydrated. A hydrogen peroxide quench, using 3% H_2O_2 with methyl alcohol, was performed for 20 min. A 15-min incubation with a serum-free protein blocking agent was then performed. Antigen retrieval was performed using EDTA buffer at pH 9.0 (CD34), a citrate buffer at pH 6.0 (D2-40) and a steam cooker for 20 min, with a bench cool down period of 15 min. A 30-min incubation in the primary antibodies (Biogenex CD34 monoclonal mouse antihuman, clone QBEND/10, at 1:10 dilution; Dako D2-40 monoclonal mouse antihuman, clone D2-40, at 1:30 dilution) was performed at room temperature. Secondary detection consisted of a 30-min incubation using Labeled Streptavidin-Biotin link (Dako LSAB®+ System), and the staining was visualized by a 5-min incubation with diaminobenzidine tetrahydrochloride. The slides were counterstained with Harris' hematoxylin and cover-slipped. A normal stomach was used for the control slide for each immunohistochemical stain. For the negative control, all of the reagents except for the primary antibody were used.

All slides were assessed simultaneously by two investigators (Gresta LT and Cabral MMDA) on a double-observation microscope, without knowledge of the clinicopathological data. The slides (HE, CD34 and D2-40) were screened for (lympho)vascular invasion using strict criteria[14]. With HE, invasion was considered only if the tumor cells were within an endothelium-lined, vessel-like structure. With IHC, vessels with endothelial cells stained by CD34, but not by D2-40, were recognized as blood vessels, and vessels with endothelial cells stained by both CD34 and D2-40 were recognized as lymphatic vessels. Every vessel with tumor cell invasion on one of the three consecutive sections was identified in the other slides and was classified as a blood vessel or lymphatic invasion, based on the immunohistochemical staining profile.

Statistical analysis

The statistical calculations were performed using SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, United States). The McNemar test was used to determine the significance of intergroup differences. $P \leqslant 0.05$ was considered to be statistically significant. The estimation of the agreement rate between the two methods was obtained using the Kappa statistic (κ). A χ^2 test was applied for the analysis of associations between categorical variables.

RESULTS

The clinical and pathological characteristics of the 95 patients with GC are summarized in Table 1. Lymphatic vessels were identified by immunostaining as D2-40-positive and CD34-positive (Figure 1A-C). Blood vessels were identified by immunostaining as D2-40-negative and CD34-positive (Figure 1D-F).

Lymphatic and BVI (HE and IHC)

Histological HE staining revealed LVI from the primary

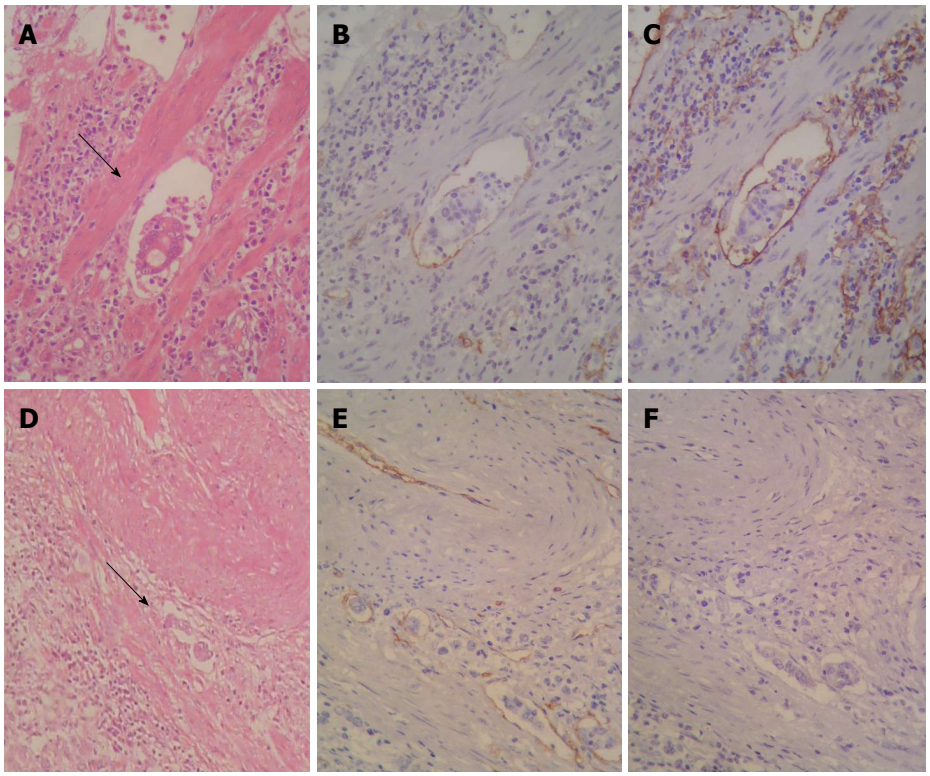

Figure 1 Sequential sections stained with hematoxylin and eosin and Immunohistochemistry showing neoplastic cell emboli within a space surrounded by the endothelial lining (arrows). A: Lymphatic vessel invasion (LVI)-hematoxylin and eosin (HE, × 400); B: LVI CD34 (× 400); C: LVI D2-40 (× 400); D: Blood vessel invasion (BVI) (HE, × 100); E: BVI CD34 (× 200); F: BVI D2-40 (× 200).

Table 2 Diagnostic agreement between methods of detection for lymphatic and blood vessel invasion (n = 95)

Variables		HE	IHC	*P* value	κ
LVI	Positive	61	66	0.38	0.50
	Negative	34	29		
BVI	Positive	38	26	0.02	0.20
	Negative	57	69		

BVI: Blood vessel invasion; LVI: Lymphatic vessel invasion; HE: Hematoxylin and eosin; IHC: Immunohistochemistry.

tumor in 61 of the 95 patients (64.2%). In 53 of those cases, LVI detected by HE staining was confirmed with D2-40 staining. In contrast, LVI was newly detected in 13 of 34 patients who had been diagnosed as free of LVI by HE staining. Figure 2 shows examples of false-positive and false-negative for LVI.

The specimens examined using HE staining showed a false-negative BVI rate of 12.6% (12/95) and a false-positive rate of 25.2% (24/95). The positive rate of BVI determined by HE staining was 40% (38/95); however, BVI was confirmed by CD34 in only 27.4% of the cases (26/95).

Figure 3 shows the prevalence of LVI and BVI with conventional HE staining and IHC in 95 primary tumors. Table 2 shows the average kappa values for both methods determined separately for LVI and BVI. The agreement was fair for BVI (κ = 0.20) and medium for LVI (κ = 0.50).

Table 3 Correlation between lymphatic and blood vessel invasion and lymph node status (n = 89) n (%)

Vascular invasion		Lymph node metastasis		*P* value
		Negative	Positive	
LVI-HE	Negative	23 (71.8)	9 (28.2)	
	Positive	5 (8.8)	52 (91.2)	0.001
LVI-IHC	Negative	20 (74.0)	7 (26.0)	
	Positive	8 (13.0)	54 (87.0)	0.001
BVI-HE	Negative	22 (41.5)	31 (58.5)	
	Positive	6 (16.6)	30 (83.4)	0.013
BVI-IHC	Negative	25 (38.5)	40 (61.5)	
	Positive	3 (12.5)	21 (87.5)	0.019

IHC: Immunohistochemistry; HE: Hematoxylin and eosin; BVI: Blood vessel invasion; LVI: Lymphatic vessel invasion.

Correlation of LVI and BVI with other prognostic factors

The LVI and BVI diagnosed by both HE and IHC were significantly correlated with lymph node metastasis, as shown in Table 3.

Table 4 shows other clinical-pathologic variables that were significantly correlated with LVI and BVI when detected by IHC.

DISCUSSION

To improve the detection of vascular invasion in GC, which is normally performed by routine HE staining, and to distinguish LVI from BVI, we introduced an IHC method using the combination of two markers: one spe-

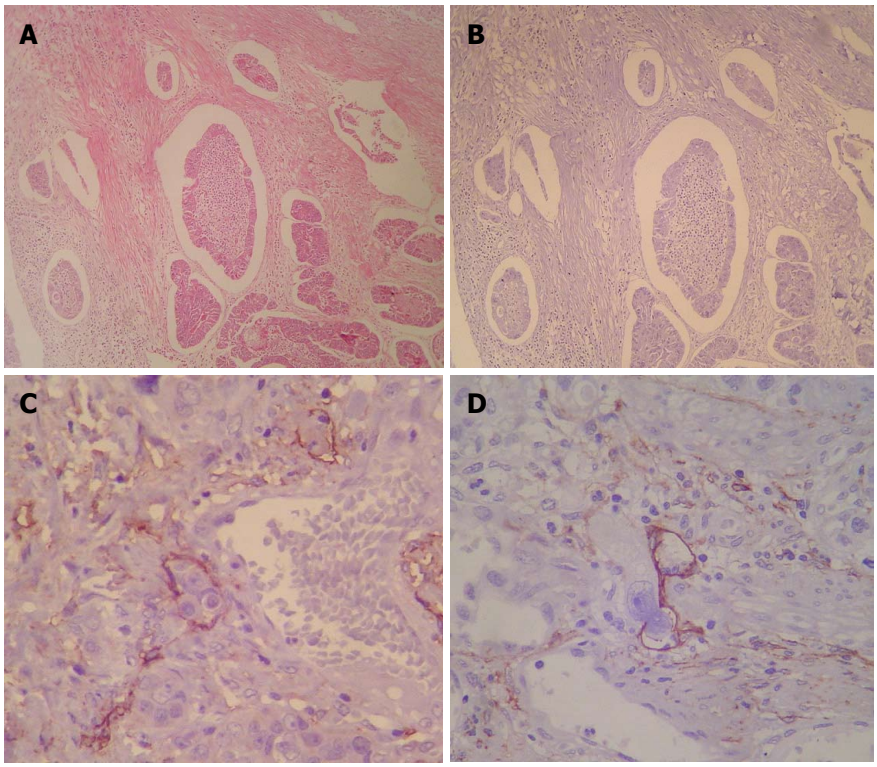

Figure 2 Example of a patient diagnosed for lymphatic vessel invasion by routine histological examination. A: Example of a patient diagnosed as positive for lymphatic vessel invasion (LVI) by routine histological examination; B: As false-positive for lymphatic vessel invasion by D2-40 (× 100); C, D: Examples of patients diagnosed as free of LVI by routine histological examination. False-negatives for LVI detected by D2-40 (× 400).

Table 4 Correlation of lymphatic and blood vessel invasion detected by immunohistochemistry with other prognostic factors (n = 95) n (%)

Data	LVI			BVI		
	Negative	Positive	*P* value	Negative	Positive	*P* value
Tumor location						
Distal third	15 (27.3)	40 (72.7)	0.0391	42 (76.3)	13 (23.7)	0.281
Other locations	14 (35.0)	26 (65.0)		29 (72.5)	11 (27.5)	
Curvature						
Small curvature	19 (35.2)	35 (64.8)	0.122	41 (76.0)	13 (24.0)	0.131
Large curvature	3 (30.0)	7 (70.0)		8 (80.0)	2 (20.0)	
Small and large	1 (7.2)	13 (92.8)		7 (50.0)	7 (50.0)	
Macroscopy						
Borrmann Ⅰ	5 (71.4)	2 (28.6)	0.0011	7 (100.0)	0 (0.0)	0.24
Borrmann Ⅱ	4 (13.3)	26 (86.7)		21 (70.0)	9 (30.0)	
Borrmann Ⅲ	5 (18.5)	22 (81.5)		17 (62.9)	10 (37.1)	
Borrmann Ⅳ	0 (0.0)	12 (100.0)		7 (58.3)	5 (41.7)	
Organ invasion						
Negative	26 (45.6)	31 (54.4)	0.0031	46 (80.7)	11 (19.3)	0.297
Duodenum	1 (4.3)	22 (95.7)		15 (65.2)	8 (34.8)	
Esophagus	1 (12.5)	7 (87.5)		6 (75.0)	2 (25.0)	
Both E + D	0 (0.0)	3 (100.0)		1 (33.3)	2 (66.7)	
Other	1 (25.0)	3 (75.0)		3 (75.0)	1 (25.0)	
Tumor depth						
Early	17 (80.9)	4 (19.1)	0.0011	21 (100.0)	0 (0.0)	0.0031
Advanced	12 (16.2)	62 (83.8)		50 (67.5)	24 (32.5)	
Laurén histology						
Intestinal	12 (26.6)	33 (73.3)	0.228	36 (80.0)	9 (20.0)	0.332
Diffuse	11 (44.0)	14 (56.0)		19 (76.0)	6 (24.0)	
Mixed/not classified	6 (24.0)	19 (76.0)		16 (64.0)	9 (36.0)	

BVI: Blood vessel invasion; LVI: Lymphatic vessel invasion; E + D: Esophagus + duodenum.

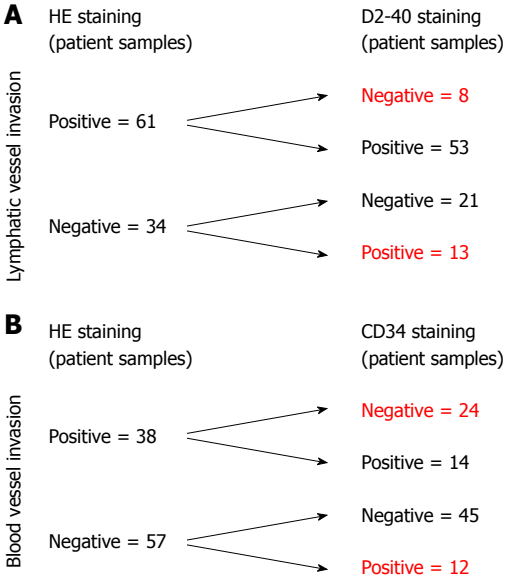

Figure 3 Diagnostic comparison in 95 patients with gastric cancer. A: Diagnostic comparison of lymphatic vessel invasion; B: Diagnostic comparison of blood vessel invasion. HE: Hematoxylin and eosin.

cific to the lymphatic vessel endothelium (D2-40) and the other pan-endothelial (CD34). In addition to the effective detection of LVI and BVI, this method also enabled the correct evaluation of the predictive value of vascular invasion in GC for the occurrence of lymph node metastasis. As we expected, the identification of LVI and BVI was also correlated with other prognostic factors important for GC, such as tumor depth and organ invasion, which have been detected in other studies.

In this study, the group of 95 patients generally reflected the profile of GC described in the literature with regard to age, gender, location of tumor and Laurén histological type. The patients with diffuse-type carcinoma were significantly younger than those with the intestinal-type ($P = 0.04$). This peculiar feature of diffuse-type neoplasms is well established and reflects differences in pathogenesis that are generally linked to genetic factors, whereas intestinal-type neoplasms are more influenced by environmental factors, such as diet and infection with *Helicobacter pylori*.

Data from the literature indicate that most diagnoses in developing countries occur late, when the disease is already in the advanced stage[31]. In our study, 53.7% of the GCs were in-depth stage pT3 tumors. Additionally, more than half of our sample (64.2%) had lymph node metastasis at the time of diagnosis.

The prevalence of LVI and BVI in GC has been determined in various studies to vary from 7.2% to 86%[9,15,28,33-35]. This wide variation in results could be explained by the different methods used to evaluate vascular invasion, *i.e.*, HE only or usage of IHC staining with endothelium-specific markers. Three consistent instances of this variation include the studies of Arigami *et al*[36], of Sako *et al*[22] and of Yonemura *et al*[23], who reported higher rates of detection of LVI with the IHC method when compared to routine staining with HE. All three studies strengthened the role of IHC in the analysis of vascular invasion in GC[36].

We observed that the difference between HE and IHC when detecting LVI was not statistically significant ($P = 0.38$). However, there were 8 cases of false-positive and 13 cases of false-negative that were isolated only after IHC. Thus, the Kappa coefficient was considered to be only moderate for LVI ($\kappa = 0.50$). The evaluation of LVI by only HE is subject to these misconceptions because of the inability to distinguish retraction artifacts around glands or cell groups from true vascular invasion. Occasionally, neoplastic cells occupy the vascular lumen completely, which makes their identification impossible without specific marking of the lymphatic endothelium. Additionally, false-positive results can occur when BVI is misinterpreted as LVI with HE staining only[36].

The correlation between LVI and the presence of metastasis was statistically significant when assessed by both methods ($P = 0.001$). This finding agrees with published data, in which LVI of the primary tumor was found to be crucial for the occurrence of lymph node metastasis[37]. Therefore, it is possible to infer that LVI is more widely found in patients with lymph node metastases than in those in which the examined lymph nodes are negative.

We found 24 cases of false-positives for BVI with HE and 10 false-negatives identified by IHC. Therefore, the detection of BVI was more accurate and significantly less frequent with IHC than with HE ($P = 0.02$). This result produced a very low Kappa coefficient ($\kappa = 0.20$) because of the identification of a large number of cases as false-positives. The false-positive results obtained could be explained as cases of LVI that were inadvertently interpreted as BVI, as it is not possible to distinguish between blood vessels and lymphatic vessels in all cases using only HE[14].

Our results show that BVI evaluated by both methods is positively correlated with the presence of lymph node metastasis, in contrast to what has been demonstrated by some previous studies[7]. It is interesting to note that although the previous studies have examined large numbers of cases, they performed retrospective review studies that included only cases of early GC, which explains the low occurrence of lymph node metastasis and BVI. Our study, in contrast, analyzed BVI and lymph node metastasis not only in early GC but also in advanced cases of GC, which resulted in the statistical significance described in Table 4.

The presence of lymph node metastasis is considered to be the most important prognostic factor in GC, and it is related to the presence of vascular invasion[37,38]. Retrospective studies have shown that the presence of LVI and BVI detected by the IHC method is related to tumor recurrence in patients with and without lymph node metastasis and is also related to a low survival rate[16,36,39]. In this regard, our study revealed the importance of LVI and BVI as predictive factors, even in the absence of lymph node involvement.

The early GC concept applies to those tumors with more superficial infiltration of the gastric wall. It is thought that cases of early GC are less likely to show invasion of blood and lymph vessels. Our data show that, compared with advanced GC, early GC exhibits less lymph node involvement ($P = 0.001$), less LVI ($P = 0.001$) and less BVI ($P = 0.007$) detected by IHC. However, studies of lymphatic network density in the normal gastric wall have found that the concentration of lymphatic vessels is considerably greater in the muscularis mucosa, which can be infiltrated in early GC[22].

The risk of lymph node metastasis in early GC is only 3.2% for the intramucosal and is approximately 19.2% when invasion reaches the submucosa[40]. Our results agree with these findings. We found two cases of early GC (11.2%) with invasion of the submucosa and lymph node metastasis. Conversely, in 59 cases of advanced GC (83.0%), the lymph nodes were positive for metastasis.

At present, non invasive imaging methods to properly evaluate the likelihood of lymph node metastasis in GC do not exist. Thus, lymph node staging in early GC still relies on the assessment of specific tumor characteristics that are related to increased lymph node metastasis, *i.e.*, depth of tumor infiltration in the gastric wall, tumor size greater than 2.0 cm, Laurén histological classification and LVI. It is noteworthy that, among these factors, the presence of lymphatic vessel involvement is the most significant isolated predictive factor for the occurrence of lymph node metastasis[7]. Thus, it is essential to include LVI and BVI evaluation by IHC in routine pathologic protocols of GC surgical specimens.

Gastrectomy with lymphadenectomy is indicated in poorly differentiated intramucosal carcinomas, with dimensions larger than 20 mm or in submucosal carcinomas. However, these criteria are quite strict and may result in unnecessary surgery. Gotoda *et al*[41] proposed more expanded criteria for the endoscopic treatment of early GC that combined histological type, LVI and BVI, ulceration and tumor size, thereby enabling the expansion of the universe of patients with early GC who could potentially be eligible for endoscopic resection, even with submucosal invasion.

The meta-analysis published by Kwee *et al*[40] revealed several variables significantly associated with the presence of lymph node metastasis in early GC. Most of these predictive factors may be perfectly evaluated through preoperative exams, endoscopy with biopsy and non-invasive imaging methods, such as computed tomography and endoscopic ultrasound. However, the presence or absence of LVI and BVI can only be judged by a histopathological study after tumor resection.

LVI and BVI must be systematically analyzed as the histological parameters with the greatest prognostic significance and as decisive factors in the choice of complementary adjuvant therapy. Therefore, we suggest that more sensitive and more specific methods be incorporated into the routine protocols for histopathological examination of GC.

Our results show that the application of IHC using two combined markers (CD34 and D2-40) provides a more accurate detection of LVI and BVI when compared to routine staining with HE. These findings may be of great value in clinical practice, especially in cases in which it is not possible to determine the precise lymph node status because of an insufficient number of lymph nodes or because lymphadenectomy was not performed.

ACKNOWLEDGMENTS

The authors would like to pay tribute to professor Ana Margarida MF Nogueira in recognition of her great contribuition to our work and to gastrointestinal pathology.

COMMENTS

Background

Gastric cancer (GC) is the fourth most common cancer and the second most common cause of cancer deaths in the world. Lymph node metastasis is one of the most important prognostic factors in patients with GC. The presence of lymphatic vessel invasion in GC is the strongest risk factor for lymph node metastasis and is known as an independent prognostic factor.

Research frontiers

Usually, lymphatic and blood vessel invasion (BVI) are identified based on conventional hematoxylin-eosin staining, and the diagnosis is made based on the presence of tumor emboli within the vascular channels lined by a single layer of endothelial cells, with or without red blood cells. However, this subjective evaluation can lead to inaccurate false-positive and false-negative results.

Innovations and breakthroughs

Recently, interest in vascular invasion has increased because of the development of specific markers for the lymphatic endothelium used in immunohistochemistry (IHC), such as Prox-1, which is a transcription factor; Lyve-1, which is a hyaluronan receptor; podoplanin, which is a glomerular podocyte membrane protein and D2-40. It has been demonstrated that D2-40 is the best marker for the lymphatic endothelium. Used in combination with panendothelial markers such as CD34 or CD31, D2-40 permits the differentiation between lymphatic and BVI and the study of both processes in GC metastasis.

Applications

Uncertain criteria for the diagnosis of (lympho)vascular invasion may affect the clinical assessment of prognosis and may change the course of therapy for the patients. This study shows that the immunohistochemical identification of lymphatic and blood vessels is useful for increasing the accuracy of the diagnosis of lymphatic and BVI and for predicting lymph node metastasis in GC.

Terminology

Blood and lymphatic vessels show different functions and phenotypic expression. IHC is a procedure which identifies specific antigens expressed by the cells or tissues, making it possible to determine their origin. Vascular invasion is the presence of tumor cells within the lumen of blood or lymphatic vascular spaces, which can be transported into the bloodstream, producing metastasis.

Peer review

Authors demonstrated that the IHC of two different endothelial markers D2-40 and CD34 is useful for accurate diagnosis of vessel invasion and for predicting lymph node metastasis.

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P- Reviewers Langley RR, Pan WS, Shen LZ, Shibata MA
S- Editor Gou SX **L- Editor** A **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3770

World J Gastroenterol 2013 June 28; 19(24): 3770-3780
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Interaction of 14-3-3σ with KCMF1 suppresses the proliferation and colony formation of human colon cancer stem cells

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Author contributions: Zou J and Yu XF contributed equally to this work; Zou J designed the research; Zou J, Mi L, Yu XF and Dong J performed the majority of the experiments; Zou J, Mi L and Dong J analysed the data; Zou J and Yu XF wrote the paper.

Supported by The Medical Guidance Projects of Shanghai Science Committee, No. 10411961800; National Natural Science Foundation of China, No. 81101617

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Received: December 17, 2012 Revised: February 14, 2013
Accepted: March 21, 2013
Published online: June 28, 2013

Abstract

AIM: To investigate the biological function of 14-3-3σ protein and to look for proteins that interact with 14-3-3σ protein in colon cancer stem cells.

METHODS: Reverse transcription polymerase chain reaction was performed to amplify the *14-3-3σ* gene from the mRNA of colon cancer stem cells. The gene was then cloned into the pGEM-T vector. After being sequenced, the target gene *14-3-3σ* was cut from the pGEM-T vector and cloned into the pGBKT7 yeast expression plasmid. Then, the bait plasmid pGBKT7-14-3-3σ was transformed into the yeast strain AH109. After the expression of the pGBKT7-14-3-3σ fusion protein in the AH109 yeast strain was accomplished, a yeast two-hybrid screening assay was performed by mating AH109 with Y187 that contained a HeLa cDNA library plasmid. The interaction between the 14-3-3σ protein and the proteins obtained from positive colonies was further confirmed by repeating the yeast two-hybrid screen. After extracting and sequencing the plasmids from the positive colonies, we performed a bioinformatics analysis. A coimmunoprecipitation assay was performed to confirm the interaction between 14-3-3σ and the proteins obtained from the positive colonies. Finally, we constructed 14-3-3σ and potassium channel modulatory factor 1 (KCMF1) siRNA expression plasmids and transfected them into colon cancer stem cells.

RESULTS: The bait plasmid pGBKT7-14-3-3σ was constructed successfully, and the 14-3-3σ protein had no toxic or autonomous activation effect on the yeast. Nineteen true-positive colonies were selected and sequenced, and their full-length sequences were obtained. We searched for homologous DNA sequences for these sequences from GenBank. Among the positive colonies, four coding genes with known functions were obtained, including *KCMF1*, quinone oxidoreductase (*NQO2*), hydroxyisobutyrate dehydrogenase (*HIBADH*) and *14-3-3σ*. For the subsequent coimmunoprecipitation assay, the plasmids PCDEF-Flag-14-3-3σ, PCDEF-Myc-KCMF1, PCDEF-Myc-NQO2 and PCDEF-Myc-HIBADH were successfully constructed, and the sequences were further confirmed by DNA sequencing. The Fugene 6 reagent was used to transfect the plasmids, and fluorescence-activated cell sorting analysis showed the transfection efficiency was 97.8% after 48 h. The HEK 293FT cells showed the stable expression of the PCDEF-Flag-14-3-3σ, PCDEF-Myc-KCMF1, PCDEF-Myc-NQO2 and PCDEF-Myc-HIBADH plasmids. After anti-Myc antibody immunoprecipitation with Myc-KCMF1, Myc-NQO2 and Myc-HIBADH from cell lysates, the presence of Flag-14-3-3σ protein in the immunoprecipitated complex was determined by western blot analysis. The knock-down expression of the 14-3-3σ and KCMF1 proteins significantly inhibited cell proliferation and colony formation of SW1116csc.

CONCLUSION: Genes of the proteins that interacted

with 14-3-3σ were successfully screened from a HeLa cDNA library. KCMF1 and 14-3-3σ protein may affect the proliferation and colony formation of human colon cancer stem cells.

Key words: 14-3-3σ protein; Interacting proteins; Yeast two-hybrid system; Colon cancer stem cells

Core tip: Of the 14-3-3 proteins, tumor-suppressor activity has most clearly been defined for 14-3-3σ. In the study, we constructed *14-3-3σ* bait gene and expressed as a fusion to the GAL4 DNA-binding domain successfully. Using the yeast two-hybrid system, we found novel binding proteins from the HeLa cDNA library which closely interact with 14-3-3σ. Our results also suggest that 14-3-3σ may interact with potassium channel modulatory factor 1 (KCMF1) protein. The knock-down expression of 14-3-3σ and KCMF1 proteins significantly inhibited proliferation and colony formation of SW1116csc cells. So, 14-3-3σ and other proteins may be involved in proliferation and colony formation of human colon cancer stem cells.

Zou J, Mi L, Yu XF, Dong J. Interaction of 14-3-3σ with KCMF1 suppresses the proliferation and colony formation of human colon cancer stem cells. *World J Gastroenterol* 2013; 19(24): 3770-3780 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3770.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3770

INTRODUCTION

The 14-3-3 proteins are among the most abundant proteins within the cell, having been initially identified in 1967 as a family of acidic proteins within the mammalian brain. This family of highly conserved proteins consisting of seven isotypes in human cells (β, γ, ε, η, σ, τ, ξ) plays crucial roles in regulating multiple cellular processes, including the maintenance of cell cycle checkpoints and DNA repair, the prevention of apoptosis, the onset of cell differentiation and senescence, and the coordination of cell adhesion and motility. All 14-3-3 proteins bind to phosphoserine/phosphothreonine-containing peptide motifs corresponding to the sequences RSXpSXP or RXXXpSXP[1]. Many 14-3-3-binding proteins contain sequences that closely match these motifs, although a number of ligands bind to 14-3-3 in a phospho-independent manner using alternative sequences that do not closely resemble these motifs. Pozuelo Rubio *et al*[2] recently used 14-3-3-affinity chromatography and mass spectrometry to identify more than 200 14-3-3-binding ligands. All of these ligands lost their ability to bind to 14-3-3 upon dephosphorylation by the serine/threonine phosphatase PP2A *in vitro*. Some of the proteins bound to the 14-3-3-affinity column contained sequences that closely matched the optimal binding motifs, while others diverged significantly from the consensus sequences, despite their apparent phospho-specific binding.

Among the 14-3-3 proteins, 14-3-3σ is the isoform most directly linked to cancer[3]. There are several lines of evidence indicating that 14-3-3σ acts as a tumour suppressor gene and that its inactivation is crucial in tumorigenesis. The downregulation of 14-3-3σ by CpG methylation is detected in adenocarcinoma of the breast (96%), squamous cell carcinoma of the vulva (60%), lung cancer (83%), hepatocellular carcinoma (89%), ovarian carcinoma (60%), endometrioid endometrial adenocarcinoma (74%), gastric adenocarcinoma (43%), basal cell carcinoma (68.3%), squamous cell carcinoma of the bladder, neuroendocrine tumours (85%), and prostate cancer (45%)[4-10]. In our former proteomic study on human colon cancer stem cells, we found the expression of 14-3-3σ was obviously increased in colon cancer stem cells compared with differentiated cancer cells[11]. 14-3-3σ may be involved in the course of self-renewal, proliferation and differentiation of colon cancer stem cells.

To better understand the role of 14-3-3σ in the tumorigenesis, self-renewal, and differentiation of stem cells, we used the yeast two-hybrid system to find novel binding proteins that interact with 14-3-3σ. The bait gene, *14-3-3σ*, was expressed as a fusion to the GAL4 DNA-binding domain, while the HeLa cDNA library was expressed as a fusion to the GAL4 activation domain. When the bait and library fusion proteins interact, the DNA-binding domain and activation domain are brought into proximity, thus activating the transcription of the reporter genes. Using this system, we found novel binding proteins from the HeLa cDNA library that closely interact with 14-3-3σ. This investigation provides functional clues for further exploration into novel cancer-related proteins for the treatment of colon cancer.

MATERIALS AND METHODS

Bacteria, yeast strains and plasmids

All yeast strains and plasmids for the yeast two-hybrid experiments were obtained from Clontech (Palo Alto, CA, United States) as components of the MATCHMAKER two-hybrid system 3. The *Escherichia coli* (*E. coli*) strain DH5α was used to clone every shuttle plasmid. The pGBKT7 DNA binding domain (DNA-BD) cloning plasmid, pGADT7 AD cloning plasmid, pGBKT7-53 control plasmid, pGADT7, pGBKT7-Lam control plasmid and pCL1 plasmid were obtained from Clontech Ltd. (K1612-1). The HeLa MATCHMAKER cDNA library was also obtained from Clontech Ltd.

Chemical agents and culture media

Taq DNA polymerase, T4 DNA ligase, EcoRI and BglII restriction endonuclease were purchased from Takara Company, Japan. Lithium acetate, semi-sulphate adenine, acrylamide and *N*,N'-bis-acrylamide were purchased from Sigma Company United States. Tryptone and yeast ex-

tracts were purchased from the Oxoid Company, United States. X-α-gal and the culture media YPDA, SD/-Trp, SD/-Leu, SD/-Trp/-Leu, SD/-Trp/-Leu/-His, SD/-Trp/-Leu/-His/-Ade were purchased from Clontech, United States.

Total RNA isolation human colon cancer stem cells

Human colon cancer stem cells (SW1116csc) were isolated and maintained in serum-free DMEM/F12 medium supplemented with human recombinant epidermal growth factor (20 μg/L; Invitrogen), human recombinant basic fibroblast growth factor (20 μg/L; Invitrogen), L-glutamine (2 mmol/L), insulin (4 U/L), penicillin G (1×10^5 U/L), and streptomycin (100 mg/L). Total RNA was isolated with TRIzol reagent (Invitrogen, United States) according to the manufacturer's instructions. The total RNA recovered from the DNase I digestion was measured at 260 and 280 nm with a spectrophotometer (Ultraspec 2000, Pharmacia Biotech), with the 260 nm reading used to estimate the concentration of total RNA. The 260/280-nm ratios and a 1% agarose-formaldehyde gel stained with ethidium bromide were used to confirm the RNA quality of the samples.

Construction of bait plasmid and expression of the 14-3-3σ protein

To make the bait plasmid, reverse transcription polymerase chain reaction (RT-PCR) was performed to amplify the *14-3-3σ* gene from SW1116csc cells. The sequences of the primers contained *Eco*RI and *Bgl*II restriction enzyme sites. The PCR conditions were as follows: 94 ℃ for 45 s, 60 ℃ for 45 s, 72 ℃ for 1 min, for 35 cycles. Ten nanograms of the 747 bp PCR product were cloned into the pGEM-T vector. The primary structure of the insert was confirmed by direct sequencing. The fragment encoding 14-3-3σ was released from the pGEM-T-14-3-3σ by digestion with *Eco*RI and *Bgl*II and was then ligated into pGBKT7. Vector pGBKT7-expressing proteins were fused with amino acids 1-147 of the GAL4 DNA-BD, and pGADT7-expressing proteins were fused with amino acids 768-881 of the GAL4 activation domain. The plasmid pGBKT7-14-3-3σ, containing the full-length *14-3-3σ* gene, could directly express the DNA binding domain, c-Myc and 14-3-3σ fusion protein. The plasmid was transformed into the yeast strain AH109 with the lithium acetate method.

Toxicity and autonomous activation assays

The purified bait plasmid was transformed into the AH109 strain and was then cultured on SD/-Trp/agar plates for detection. Approximately 2 mm of AH109 colonies, transformed by pGBKT7-14-3-3σ and pGBKT7, were incubated in 3 mLYPDA liquid medium at 30 ℃ for 16 h with shaking. The absorbance values at 600 nm (A_{600}) in different groups were compared. Additionally, transformants containing the pGBKT7-14-3-3σ and pGBKT7 plasmids were transferred to SD/-Trp/X-a-gal, SD/-Trp/-his/X-a-gal and SD/-Ade/-Trp/X-a-gal plates at 30 ℃ for 5 d. In parallel, AH109 cells transformed by pCL1 and pGBKT7-Lam served as the positive and negative controls, respectively.

Screening of the HeLa cDNA library by the yeast two-hybrid system

The screening of the HeLa cDNA library was performed as follows. One large (2-3 mm), fresh (< 2 mo old) colony of AH109 (bait) was inoculated into 50 mL SD/-Trp and incubated and shaken at 250-270 r/min at 30 ℃ overnight (16-24 h). Then, the cells were pelleted by centrifuging the entire 50-mL culture at 1000 r/min for 5 min. After the supernatant was decanted, the cell pellet was resuspended in the residual liquid by vortexing. A HeLa cDNA library was cloned into pACT2 and the yeast reporter strain Y187. The entire AH109 (bait) culture and 1 mL of the HeLa cDNA library were combined and cultured in a 2-L sterile flask; 45 mL of 2 × YPDA/Kan was added and swirled gently. After a 20-h mating period, the cells were pelleted, re-suspended and spread on 50 large (150 mm) plates containing 100 mL SD/-Ade/-His/-Leu/-Trp. After 6-15 d, the yeast colonies were transferred onto plates containing X-α-gal to evaluate the expression of the MEL1 reporter gene (blue colonies).

Isolation and transformation of yeast plasmids

Approximately 1×10^6 colonies were screened, and positive clones were identified. The yeast plasmids were isolated from the positive colonies with the lyticase method and transformed into super-competent *E. coli* DH5α by electroporation. The transformants were plated on ampicillin SOB selection media and grown under selection conditions. Subsequently, the pACT2-cDNA constructs were re-isolated following the standard protocol, analysed by restriction digestion and sequenced.

Bioinformatics analysis

After the positive colonies were sequenced, the sequences were blasted against sequences in GenBank to analyse the function of the genes (http://www.ncbi.nlm.nih.gov/blast). Other bioinformatics analyses, including their molecular weight, theoretical PI, estimated half-life, secondary structure prediction, and so on, were respectively performed with different software tools (www.expasy.ch/tools/protparam.html; www.cmpharm.ucsf.edu/nomi/nnpredict.html; http://www.ch.embnet.org/software/COILS_form.html; www.expasy.org/tools/protscale.html; http://www.ch.embnet.org/software/TMPRED_form.html; http://www.cbs.dtu.dk/services/SignalP/).

Plasmid constructs and transfection

The plasmids in positive yeast clones were isolated from the colonies by the lyticase method. The *14-3-3σ*, potassium channel modulatory factor 1 (*KCMF1*), quinone oxidoreductase (*NQO2*) and hydroxyisobutyrate dehydrogenase (*HIBADH*) genes were PCR amplified with

Table 1 Different plasmids transfected into HEK 293FT cells

Plasmid	1 Sample 1 KCMF1	2 Negative Control-1	3 Sample 2 NQO2	4 Negative Control-2	5 Sample 3 HIBADH	6 Negative Control-3	7 Negative Control-4	8 Positive	Transfection control	
									Positive	Negative
Flag-mfrP53	-	-	-	-	-	-	-	+	pEGFP	293FT
Myc-Large T	-	-	-	-	-	-	-	+		
Flag-14-3-3	+	-	+	-	+	-	+	-		
Myc-HIBADH	-	-	-	-	+	+	-	-		
Myc-NQO2	-	-	+	+	-	-	-	-		
Myc-KCMF1	+	+	-	-	-	-	-	-		
VECTOR-Myc	-	-	-	-	-	-	-	-		
VECTOR-Flag	-	+	-	+	-	+	-	-		

KCMF1: Potassium channel modulatory factor 1; NQO2: Quinone oxidoreductase; HIBADH: Hydroxyisobutyrate dehydrogenase.

specific primers, and the products were characterised by restriction digest using SfiI. After PCDEF-Flag and PCDEF-Myc plasmids were digested with the SfiI restriction enzyme, 14-3-3σ, KCMF1, NQO2 and HIBADH cDNA were cloned into them. The correct plasmids were named PCDEF-Flag-14-3-3σ, PCDEF-Myc-KCMF1, PCDEF-Myc-NQO2 and PCDEF-Myc-HIBADH. The plasmid sequences were analysed by DNA sequencing. HEK 293FT cells (5×10^5 cells) were cultured in a 6-cm dish until 60% confluence was reached. Various types of plasmids were transfected using the Fugene 6 Transfection Kit (Roche) according to the manufacturer's instructions (Table 1). Briefly, 1.5 μL Fugene 6 was diluted with 100 μL Opti-MEMI (Invitrogen), mixed, and incubated at room temperature for 5 min. A 0.5 μg quantity of plasmid was added to the Fugene 6/Opti-MEM combination at a 3 (DNA):1 (Fugene) ratio, then mixed and incubated at room temperature for 45 min. The mixture of Fugene 6 and plasmid DNA was then added to cells cultured in 0.5 mL fresh Opti-MEM and incubated at 37 ℃ in 5% CO_2. After 14-16 h, the transfection reagent was replaced with fresh culture medium; 48 h later, the transfected cells were harvested and fluorescence-activated cell sorting (FACS) was performed.

Fluorescence-activated cell sorting

FACS of enhanced green fluorescent protein (EGFP) positive cells was performed on a FACS Aria (Becton Dickinson). The cells were washed twice with Ca^{2+}- and Mg^{2+}-free HBSS and then incubated with 50 U papain (Worthington) and 100 U DNase I (Sigma) in PIPES at 37 ℃ for 10 min with gentle shaking. The samples were spun, resuspended in 2 mL DMEM/F12/N2 and dissociated by sequentially titrating with three serially narrowed glass Pasteur pipettes. The papain was inactivated with DMEM/F12/N2 plus 20% FBS, and the cells were pelleted. The pelleted cells were resuspended in HBSS and then passed over a 40 μm cell strainer. The cells were resuspended at a final concentration of (3-6) × 10^6 cells/mL with 1.0 μg 7-amino-actinomycin D (7-AAD). The cells were analysed by forward and side scatter for EGFP fluorescence through a 530 ± 30 nm bandpass filter and for 7-AAD fluorescence through a 695 ± 40 nm bandpass filter. The EGFP-positive cells were sorted at 2000-5000 events/s with a purification mode algorithm. Untransfected cells were used as a control to set the background fluorescence; a false-positive rate of 0.1% was accepted to ensure an adequate yield.

Coimmunoprecipitation and Western blotting

The HEK 293FT cell line was grown in accordance with the ATCC recommendations. Forty-eight hours after plasmid transfection, the cells were lysed in ice-cold 1% Triton X-100 buffer containing a cocktail of protease inhibitors. The lysates were cleared by centrifugation at 12000 *g* for 15 min at 4 ℃. Coimmunoprecipitation assays using cleared cell lysates were performed at 4 ℃ for 2 h with the appropriate antibody. Immune complexes were precipitated with protein G Sepharose beads for an additional 1 h, washed three times with cold lysis buffer, resuspended in 16 Laemmli sample buffer, boiled for 5 min, subjected to SDS-PAGE and transferred to NC filters. The NC filters were blocked for 1 h at 4 ℃ in 5% nonfat milk in TBS (50 mmol/L Tris, 150 mmol/L NaCl) containing 0.1% Tween-20 (Sigma). They were then incubated for 2 h with primary antibodies (1:1000 dilution) in the blocking solution. After extensive washes in TBS 0.1% Tween-20, the filters were incubated for 1 h with HRP-conjugated anti-mouse antibody (Serotech) diluted 1:5000 in TBS 5% nonfat milk solution. After final washes in TBS 0.1% Tween, Western blottings were developed with the ECL kit from Amersham Biosciences.

siRNA plasmid constructs and transfection

Selection of the siRNA sequence was based on the siRNA Target Finder and Design Tool available at the Ambion Inc. web site and related reference. The siRNAs targeting human 14-3-3σ and KCMF1 mRNA common sequence 5'-CCCAGAAGAUGGACUUCUA-3' and 5'-CGCGUGUCGAAGACUAUUU-3' were synthesised and purified by Shanghai Sangon Corporation. The sense strand of the pU-siRNA inserts was 5'-GATCCACCTCACCAAGGCCAGCACTTCAAGAGAGCTGGCCTTGGTGAGGTTTTTTTTGGAAGTCGACA-3'; it was inserted into *BamH*I-*Hind*III linearised pRNAT-U6.1/neo vector (Ambion Inc., Austin, TX, United States). The inserted

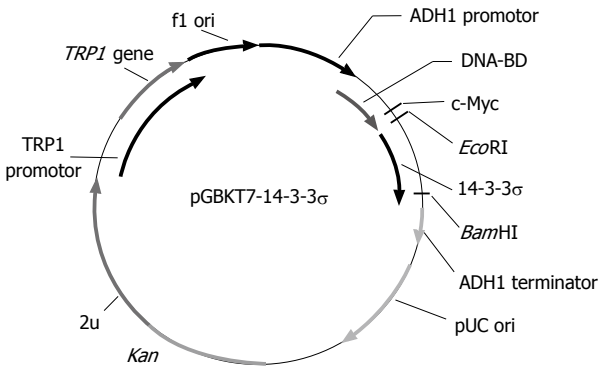

Figure 1 Map of bait plasmid pGBKT7-14-3-3σ.

sequences were verified by DNA sequencing. The control siRNA vector (pU-siCONT; Ambion Inc.) was constructed by the insertion of a sequence that expressed a hairpin siRNA with no significant homology to any known sequences in the human, mouse or rat genomes. The control insert sequence was: 5'-GATCCACTACCGTTGTTATAGGTGTTCAAGAGACACCTATAACAACGGTAGTTTTTTGGAAA-3'. One microgram per well of pU-14-3-3σ-siRNA, pU-KCMF1-siRNA expression plasmid or control plasmid (pU-siCONT) were transfected into SW1116csc. Forty-eight hours later, transfection-positive cells were observed under a fluorescence microscope.

Cell proliferation assay and colony formation in soft agar

SW1116csc and siRNA-transfected SW1116csc cells were seeded at a density of 1×10^4 in 35-mm Petri dishes. The cultured cells stained with trypan blue were observed and counted in triplicate over 6 wk. The cells were disassociated, suspended in medium containing 0.3% agar, and plated onto a bottom layer containing 0.6% agar. The cells were plated at a density of 3×10^4 cells/6-cm dish, and the number of colonies that were > 0.5 mm in diameter was counted 14 d later.

RESULTS

RNA quality characterisation

Total RNA was extracted from 1×10^8 SW1116csc cells and yielded approximately 10 μg of high purity total RNA. The absorbance ratios of the RNA at 260/280 and 230/260 nm were 2.03 and 2.00, respectively, indicating that the RNA was of the highest quality and was therefore useful for the following experiments.

Construction of the pGBKT7-14-3-3σ plasmid

The bait plasmid pGBKT7-14-3-3σ was constructed with complete E6 cDNA successfully, and the results of sequencing analysis suggested that the cDNA was in-frame, no artefacts were added to the E6 sequence and the restriction sites were correct (Figure 1).

Toxicity and autonomous activation assays

Two to three millimetre AH109 colonies, transformed by pGBKT7-14-3-3σ and pGBKT7, were incubated in 3 mL YPDA liquid medium. The A_{600} nm values in the AH109-pGBKT7-14-3-3σ and AH109-pGBKT7 groups were 0.98 and 0.99, respectively, which suggested that the pGBKT7-14-3-3σ plasmid was not toxic to yeast and had no effect on the growth of the yeast. Furthermore, the AH109-pGBKT7-14-3-3σ clones were white and were detected on the SD/-Trp/-His/X-α-gal and SD/-Ade/-Trp/X-α-gal plates (Figure 2). Therefore, the 14-3-3σ protein was believed to have no autonomous activation effect.

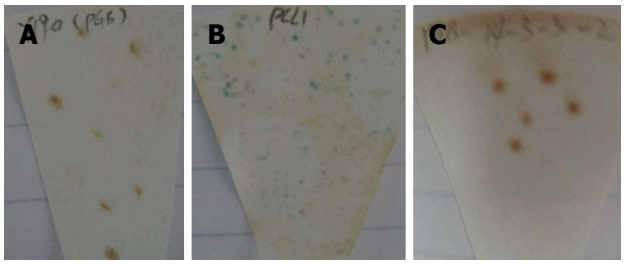

Figure 2 X-Gal filter assay analysis result. A: Negative control plasmid pGBKT7; B: Positive control plasmid pCL1; C: pGBKT7-14-3-3σ plasmid.

Screening for positive clones

Diploids were detected under an inverted microscope 20 h after co-incubation, which indicated that yeast mating was successful. Nineteen positive colonies grew on the SD/-Ade/-His/-Leu/-Trp/X-α-gal agar medium and restreaked three times. Finally, 19 putative positive yeast colonies were obtained.

Gene sequencing and analysis

Nineteen positive yeast colonies were selected, and each purified plasmid DNA was directly transferred to competent DH5α cells by electroporation. The transformants containing only the pGADT7-HeLa cDNA library plasmids were obtained by plating on LB/Amp agar medium. Sequencing analysis was performed, and 4 genes that interacted with *14-3-3σ*, including *KCMF1, NQO2, HIBADH* and *14-3-3σ*, were identified (Table 2).

Verification of protein interactions between X and Y in yeast

The diploids were detected under an inverted microscope, and blue colonies were grown on the SD/-Ade/-His/-Leu/-Trp/X-α-gal agar medium.

Plasmid constructs and transfection efficience

The PCDEF-Flag-14-3-3σ, PCDEF-Myc-KCMF1, PCDEF-Myc-NQO2 and PCDEF-Myc-HIBADH plasmids were successfully constructed. The sequences of the plasmids were further confirmed by DNA sequencing. The plasmids were transfected into HEK 293FT cells with the Fugene 6 reagent (Figure 3). After 48 h, FACS assay was performed and showed that the transfection efficiency was 97.8% (Figure 4).

Table 2 Positive hit of the screening HeLa cDNA library

Bait	Prey library	Identical colonies	Positive clone name	Positive gene identified	NCBI accession number	Gene code match	
pGB-14-3-3	Human HeLa MATCHMAKER cDNA library	19	Sigma 12	SFN	NM_006142.3	Yes	Full Lengh
			Sigma 13				
			Sigma 30				
			Sigma 4	HIBADH	NM_152740.3	Yes	Full Lengh
			Sigma 15				
			Sigma 21	NQO2	NM_000904.3	Yes	Full Lengh
			Sigma 32				
			Sigma 23				
			Sigma 33				Full Lengh
			Sigma 28	KCMF1	NM_020122.4	Yes	

KCMF1: Potassium channel modulatory factor 1; NQO2: Quinone oxidoreductase; HIBADH: Hydroxyisobutyrate dehydrogenase.

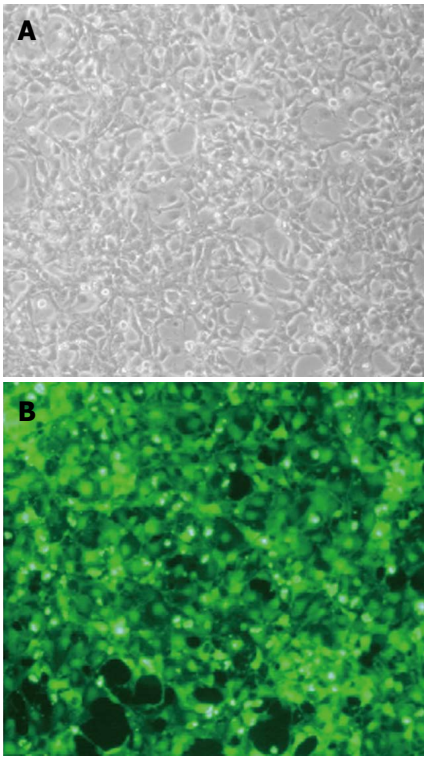

Figure 3 Plasmids transfected with the Fugene 6 transfection reagent. A: HEK 293FT cells in light phase 48 h after transfection with plasmids; B: HEK 293FT cells in fluorescent phase 48 h after transfection with plasmids.

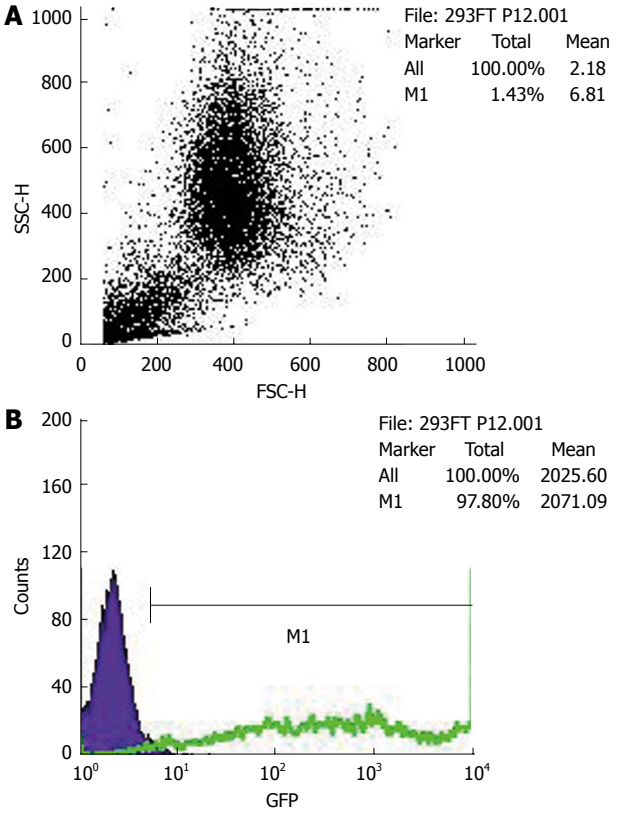

Figure 4 Fluorescence-activated cell sorting assay. It demonstrates the method for calculating green fluorescent protein (GFP)-positive cells with fluorescence-activated cell sorting. The debris is gated out, and the target populations are identified and positively or negatively selected. The dot plots represent 10000 events, with the side scatter and forward scatter plots (A) and the fluorescence intensity of GFP (B). Figure B shows a histogram demonstrating the GFP-positive cell populations. The histogram plots the fluorescence of GFP-positive cells on region M1 against counts on the Y-axis.

Confirmation of interaction by coimmunoprecipitation

Three interactions detected in this two-hybrid screen were further confirmed in HEK 293FT cells by specific coimmunoprecipitation of Flag-tagged bait proteins with the three Myc-tagged prey proteins. HEK 293FT cells stably expressed the PCDEF-Flag-14-3-3σ, PCDEF-Myc-KCMF1, PCDEF-Myc-NQO2 and PCDEF-Myc-HIBADH plasmids (Figure 5A). An anti-Myc antibody was used to immunoprecipitate Myc-KCMF1, Myc-NQO2 and Myc-HIBADH from cell lysates. The presence of Flag-14-3-3σ protein in the immunoprecipitated complex was determined by western blot analysis (Figure 5B). These coimmunoprecipitations confirm that several of the novel interactions identified in the present two-hybrid screen are reproducible in the context of mammalian cells and therefore validate the results obtained by the two-hybrid assay.

Inhibition of cell proliferation and colony formation of SW1116csc by siRNA plasmid transfection

After pU-14-3-3σ-siRNA or pU-KCMF1-siRNA expression plasmid transfection, the knock-down of the 14-3-3σ and KCMF1 proteins in SW1116csc cells was observed (Figure 6A). We tested for differences in the

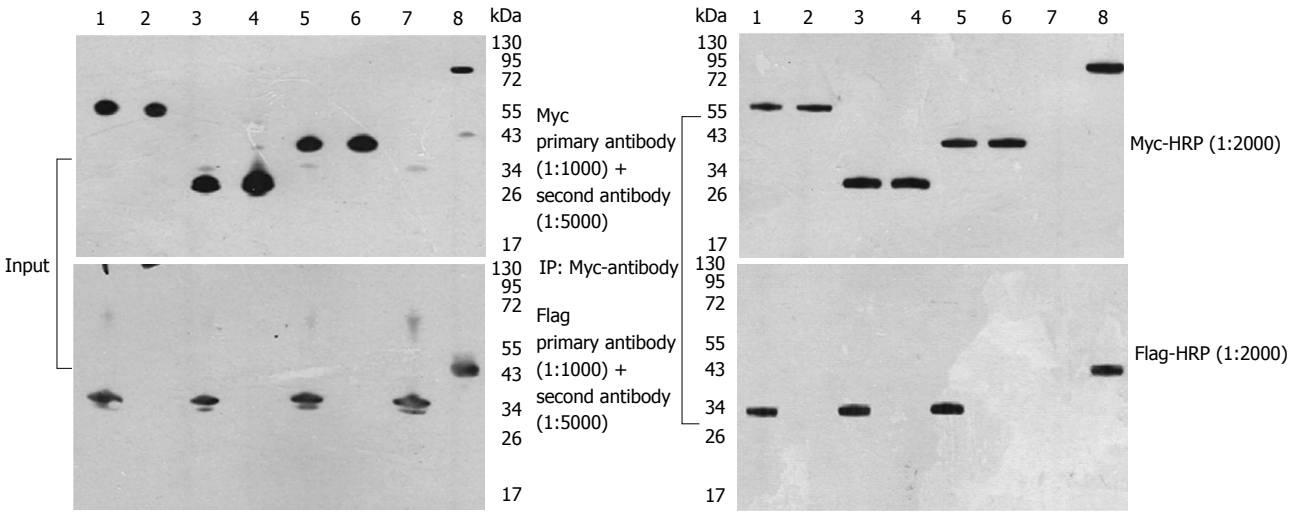

Figure 5 Expression and interaction of 14-3-3σ, potassium channel modulatory factor 1, quinone oxidoreductase, hydroxyisobutyrate dehydrogenase and 14-3-3σ plasmids in HEK 293FT cells. A: Expression of the 14-3-3σ, potassium channel modulatory factor 1 (KCMF1), quinone oxidoreductase (NQO2), hydroxyisobutyrate dehydrogenase (HIBADH) plasmids. HEK 293FT cells were transfected with different plasmids. Forty-eight hours post-transfection, the cells were lysed, and Western blotting analysis was performed with an anti-Myc or anti-Flag antibody (1:1000); B: Interaction of 14-3-3σ and KCMF1, NQO2 or HIBADH. HEK 293FT cells were transfected with different plasmids. Forty-eight hours post-transfection, the cells were lysed and immunoprecipitations were performed with an anti-Myc antibody (1:2000). After the proteins were resolved by SDS-PAGE, immunoblot analysis was performed with antibodies against Flag protein (1:2000). 1: Sample 1 KCMF1; 2: Negative Control-1; 3: Sample 2 NQO2; 4: Negative Control-2; 5: Sample 3 HIBADH; 6: Negative Control-3; 7: Negative Control-4; 8: Positive.

proliferation rate between SW1116csc and siRNA-transfected SW1116csc. The cells were examined from week 1 to week 7 after seeding. As shown in Figure 6B, there was a difference in the growth rate between the transfected and control cells. The transfected cells grew slowly and showed growth inhibition after week 4. The self-renewing capacity of the transfected cells was also examined with the colony formation assay. When plated at a density of 100 cells/well, 14-3-3σ-siRNA, KCMF1-siRNA and 14-3-3σ + KCMF1-siRNA transfected cells generated a lower mean number of tumour spheres (57.4 ± 3.6, 55.3 ± 3.3 and 23.7 ± 2.8, respectively) compared to the SW1116csc cells (71.4 ± 4.1) (Figure 6C).

DISCUSSION

Protein-protein interactions occur in a wide variety of biological processes and essentially control cell fate from division to death. Yeast two-hybrid assays represent a versatile tool to study protein interactions *in vivo*. The yeast two-hybrid system 3, based on the system originally designed by Fields and Song, takes advantage of the properties of the GAL4 protein of the yeast *Saccharomyces cerevisiae*. The GAL4-based assay uses the yeast transcription factor GAL4 for the detection of protein interactions by transcriptional activation. GAL4 possesses a characteristic phenomenon in which the transactivation function can be restored when the factor's DNA-binding domain (DBD) and its transcription-AD are brought together by two interacting heterologous proteins. The GAL4-yeast two-hybrid assay uses two expression vectors, one with DBD and the other with AD. The GAL4-DBD fuses to protein "X" and GAL4-AD fuses to protein "Y" to form the bait and the target of the interaction trap, respectively. A selection of host cells with different reporter genes and different growth selection markers provides a means to detect and confirm protein-protein interactions and has significantly fewer false positives.

To investigate the role of 14-3-3σ in tumorigenesis, the yeast two-hybrid system 3 was used to screen the proteins interacting with 14-3-3σ. In this study, the bait plasmid pGBKT7-14-3-3σ was transformed into the yeast strain AH109. To further confirm the expression of 14-3-3σ protein in the AH109 yeast strain, we performed Western blotting analysis and observed strong 14-3-3σ expression. After the bait plasmid pGBKT7-14-3-3σ yeast strain AH109 was mated with the HeLa cDNA library yeast strain Y187, resulting diploid yeast cells were plated on QDO media containing X-a-gal. Nineteen true positives were confirmed and obtained. Through sequencing analysis of isolated library plasmids, we obtained the sequences of four types of genes with known functions.

In addition to their well-known pro-proliferative and anti-apoptotic effects, 14-3-3 proteins have also been found to suppress cell growth and cell-cycle progression, especially after DNA damage, indicating functions in tumour suppression[12-15]. Of the 14-3-3 proteins, the tumour-suppressor activity has most clearly been defined for 14-3-3σ[16-19]. 14-3-3σ is unique among the 14-3-3 proteins in that it is expressed primarily in epithelial cells and forms homodimers almost exclusively[20]. Further insight as to why the loss of 14-3-3σ might facilitate tumor formation comes from the discoveries by Wilker *et al*[21] reported that 14-3-3σ is a crucial regulator of translation during mitosis and that 14-3-3σ function is required for proper mitotic exit and cytokinesis. In eukaryotic cells, most mRNA translation occurs *via* a cap-dependent

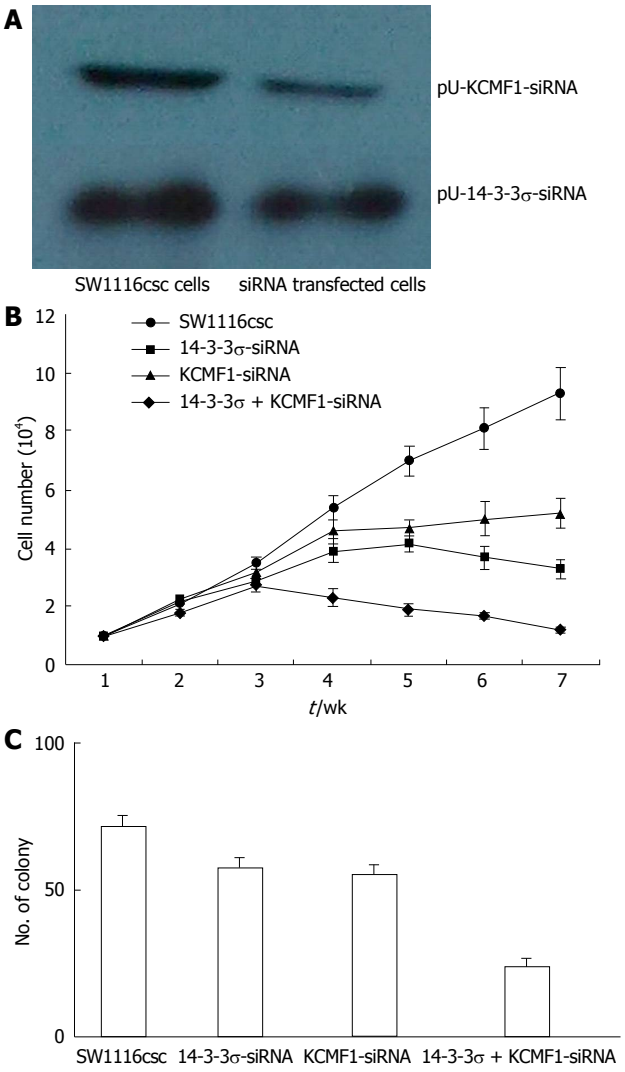

Figure 6 Effects of 14-3-3σ and potassium channel modulatory factor 1 protein expression on cell proliferation and colony formation of colon cancer stem cells. A: Expression of 14-3-3σ and potassium channel modulatory factor 1 (KCMF1) proteins in SW1116csc and siRNA transfected cells; B: Growth curves of SW1116csc, 14-3-3σ siRNA transfected, KCMF1 siRNA transfected and 14-3-3σ + KCMF1 siRNA transfected cells. The mean ± SD are shown; C: Colony formation after the incubation of 100 separate cells for 14 d. The mean ± SD are shown.

mechanism in which ribosome recruitment begins with the binding of eukaryotic initiation factors, such as eIF4B, to a modified guanosine residue (known as a "cap") at the 5' end of the mRNA. However, some mRNAs contain internal ribosome entry sites and are translated in a cap-independent manner. During mitosis, cap-dependent translation is suppressed and cap-independent translation is stimulated, allowing for the translation of key cell-cycle regulators such as cell division cycle 2-like 1. Experiments by Wilker *et al*[21] showed that 14-3-3σ is needed for the mitotic switch from cap-dependent to cap-independent translation and that 14-3-3σ appears to mediate this switch by binding to eIF4B and perhaps other factors involved in cap-dependent translation. When cells are depleted of 14-3-3σ, cap-dependent translation is not suppressed and cytokinesis is impaired, resulting in the generation of binucleated cells, a phenotype observed in the early stages of tumour formation.

14-3-3 acts as an adaptor or "chaperone molecule", which is able to move freely from the cytoplasm to the nucleus and vice-versa[22]. 14-3-3 proteins are mainly cytoplasmic molecules; they can form homodimers or heterodimers, and interact with various cellular proteins. 14-3-3 proteins are phosphoserine-binding proteins that bind the consensus motifs RSXpSXP and RXY/FXpSXP. These consensus motifs are present in almost all of the 14-3-3 binding proteins[1]. More than a hundred small molecules interact with 14-3-3 in a phosphorylation-dependent manner. These proteins include protein kinases (murine leukaemia viral oncogene homologue-RAF1, MEK kinase, PI3 kinase and Grb10), receptor proteins (insulin-like growth factor 1 and glucocorticoid receptors), enzymes (serotonin N-acetyltransferase, tyrosine and tryptophan hydroxylase), structural and cytoskeletal proteins (vimentins and keratins), scaffolding molecules (calmodulin), proteins involved in cell cycle control (cdc25, p53, p27 and wee1) proteins involved in transcriptional control (histone acetyltransferase, and TATA box binding proteins), and proteins involved in apoptosis (BAD)[1,23]. However, a few proteins interact with 14-3-3 in a phosphorylation-independent manner such as *Bax*. Recently, using direct proteomic analysis, researchers have identified a large number of polypeptides (> 200) that can associate with 14-3-3 proteins. These polypeptides are involved in numerous cell functions, including fatty acid synthesis, reductive metabolism, iron and other metabolisms, DNA/chromatin interactions including transcription factors, RNA binding, protein synthesis, protein folding and processing, proteolysis, protease inhibitors, ubiquitin metabolism, cellular signaling and apoptosis, actin dynamics, cellular trafficking and transporters, signaling kinases, cell division, nuclear proteins, oncogenic signaling, and cytoskeletal proteins[2,24]. A study demonstrated that some of the 14-3-3 binding proteins are involved in the regulation of the cytoskeleton, GTPase functions, membrane signaling, and cell fate determination[25]. In this study, we found that 14-3-3σ could interact with the proteins KCMF1, NQO2, HIBADH and 14-3-3σ.

The function of NQO2 is not clearly understood. NQO2 is expressed selectively in the kidneys, skeletal muscles, liver, heart, and lungs, suggesting a tissue-specific action of the enzyme. Some studies have suggested that greater NQO2 expression may activate certain types of chemicals in the brain, leading to oxidative stress and neuronal damage[26]. Other studies have implied that NQO2 can protect against quinone-induced skin carcinogenesis[27]. Recently, new evidence has shown for the first time that NQO2 catalyses the reduction of electrophilic oestrogen quinones and thereby acts as a detoxification enzyme. Gaikwad *et al*[28] successfully demonstrated that oestrogen-3,4-quinone binds to NQO2 and established that oestrogen quinines are endogenous biological substrates of NQO2. Moreover, they demonstrated that NQO2 is faster at reducing oestrogen quinones than its

homologue NQO1. Such findings reveal a possible relationship between breast cancer and NQO2, although no studies to date have addressed this issue. In addition, NQO2 can stabilise the p53 protein[29], a known breast tumor suppressor gene product. *p53* is recognised as a highly penetrant breast cancer susceptibility gene, and loss of both p53 and breast cancer type 1 susceptibility protein (BRCA1) results in the rapid and efficient formation of mammary carcinomas[30]. Interestingly, the expression of 14-3-3σ is coordinately upregulated by the cellular tumour antigen p53 and BRCA1 and contributes to the DNA-damage-induced cell-cycle checkpoint mediated by these tumour suppressors[31]. It is logical to assume that 14-3-3σ binds to and sequesters NQO2 in the cytoplasm, thus enabling DNA damage to be repaired before the cell cycle progresses. In this study, we found that 14-3-3σ could interact with NQO2 directly and further confirmed the important function of 14-3-3σ protein in DNA repair and cell cycle progress.

KCMF1 encodes a zinc-finger protein with hitherto barely characterised function. KCMF1 was mentioned in 2001 at NCBI as an expressed sequence tag clone potentially involved in the regulation of potassium channels. A partial expressed sequence tag sequence of KCMF1 was identified as a differentially regulated gene during kidney tubulogenesis *in vitro* and designated as developing branching tubulogenesis 91 (Debt91)[32]. In addition, KCMF1 was shown to be downregulated in Ewing's sarcoma cell lines after the overexpression of CD99 and upregulated through fibroblast growth factor (FGF) receptor signalling pathways in gastric cancer cells and was consequently named basic FGF induced in gastric cancer[33]. Kreppel *et al*[34] showed that the nuclear zinc-finger protein KCMF1 was overexpressed in epithelial cancers and especially in human and mouse pancreatic cancer. KCMF1 enhanced proliferation, migration and invasion. The downregulation of KCMF1 *in vivo* reduced preneoplastic changes in the transforming growth factor-α transgenic pancreatic cancer model. One study showed that 14-3-3σ was highly expressed in pancreatic adenocarcinoma[35]. Our results suggest that 14-3-3σ may also interact with KCMF1. The knock-down of 14-3-3σ and KCMF1 proteins expression significantly inhibited cell proliferation and colony formation of human colon cancer stem cells. Further study is required to understand how the interaction between 14-3-3σ and KCMF1 proteins affects cell proliferation and colony formation of SW1116csc.

In summary, we constructed a *14-3-3σ* bait gene and successfully expressed it as a fusion with the GAL4 DNA-binding domain. Using the yeast two-hybrid system, we found novel binding proteins (KCMF1, NQO2, HIBADH and 14-3-3σ) from the HeLa cDNA library that closely interact with 14-3-3σ. The knock-down of 14-3-3σ and KCMF1 protein expression significantly inhibited cell proliferation and colony formation of colon cancer stem cells.

COMMENTS

Background

The cancer stem cell (CSC) hypothesis is currently at the center of a rapidly evolving field, involving a change of perspective on the development and treatment of cancers. However, research has been hampered by the lack of distinct molecular markers of CSCs. Among all 14-3-3 proteins, 14-3-3σ is the isoform most directly linked to cancer. There are several lines of evidence indicating that *14-3-3σ* acts as a tumor suppressor gene and that its inactivation is crucial in tumorigenesis.

Research frontiers

All 14-3-3 proteins bind to phosphoserine/phosphothreonine-containing peptide motifs corresponding to the sequences RSXpSXP or RXXXpSXP. Many 14-3-3-binding proteins contain sequences that closely match these motifs, although a number of ligands bind to 14-3-3 in a phospho-independent manner using alternative sequences that do not closely resemble these motifs. The interaction of 14-3-3σ with other proteins may be involved in proliferation and colony formation of human colon cancer stem cells.

Innovations and breakthroughs

The authors constructed *14-3-3σ* bait gene and expressed as a fusion to the GAL4 DNA-binding domain successfully. Using the yeast two-hybrid system, we found novel binding proteins [potassium channel modulatory factor 1 (KCMF1), quinone oxidoreductase, hydroxyisobutyrate dehydrogenase and 14-3-3σ] from the HeLa cDNA library which closely interact with 14-3-3σ. The knock-down expression of 14-3-3σ and KCMF1 proteins significantly inhibited cell proliferation and colony formation of colon cancer stem cells.

Applications

The study of CSCs has important implications for future cancer treatment and therapies. The CSC hypothesis states that if the CSCs were eliminated, the tumor would simply regress due to differentiation and cell death. By selectively targeting CSCs relative proteins, it may be possible to treat patients with aggressive, non-resectable tumors and prevent the tumor from metastasizing.

Terminology

14-3-3 proteins are among the most abundant proteins within the cell, having been initially identified in 1967 as a family of acidic proteins within the mammalian brain. This family of highly conserved proteins consisting of seven isotypes in human cells (β, γ, ε, η, σ, τ, ξ) plays crucial roles in regulating multiple cellular processes including the maintenance of cell cycle checkpoints and DNA repair, the prevention of apoptosis, the onset of cell differentiation and senescence, and the coordination of cell adhesion and motility.

Peer review

The paper presents an original work about colon CSCs, and shows an original pathway for colon cancer management.

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P- Reviewer Torigoe T **S- Editor** Zhai HH **L- Editor** A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3781

World J Gastroenterol 2013 June 28; 19(24): 3781-3791
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Glycyrrhizic acid attenuates CCl_4-induced hepatocyte apoptosis in rats *via* a p53-mediated pathway

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Supported by Leading Academic Discipline Project of State Administration of Traditional Chinese Medicine of China
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Received: February 24, 2013 Revised: May 14, 2013
Accepted: May 18, 2013
Published online: June 28, 2013

Abstract

AIM: To investigate the effect of glycyrrhizic acid (GA) on carbon tetrachloride (CCl_4)-induced hepatocyte apoptosis in rats *via* a p53-dependent mitochondrial pathway.

METHODS: Forty-five male Sprague-Dawley rats were randomly and equally divided into three groups, the control group, the CCl_4 group, and the GA treatment group. To induce liver fibrosis in this model, rats were given a subcutaneous injection of a 40% solution of CCl_4 in olive oil at a dose of 0.3 mL/100 g body weight biweekly for 8 wk, while controls received the same isovolumetric dose of olive oil by hypodermic injection, with an initial double-dose injection. In the GA group, rats were also treated with a 40% solution of CCl_4 plus 0.2% GA solution in double distilled water by the intraperitoneal injection of 3 mL per rat three times a week from the first week following previously published methods, with modifications. Controls were given the same isovolumetric dose of double distilled water. Liver function parameters, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined. Pathologic changes in the liver were detected by hematoxylin and eosin staining. Collagen fibers were evaluated by Sirius red staining. Hepatocyte apoptosis was investigated using the terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end labeling (TUNEL) assay and the cleaved caspase-3 immunohistochemistry assay. The expression levels of p53 and apoptosis-related proteins were evaluated by immunohistochemistry or Western blotting analysis.

RESULTS: After 8 wk of treatment, GA significantly reduced serum activity of ALT (from 526.7 ± 57.2 to 342 ± 44.8, $P < 0.05$) and AST (from 640 ± 33.7 to 462.8 ± 30.6, $P < 0.05$), attenuated the changes in liver histopathology and reduced the staging score (from 3.53 ± 0.74 to 3.00 ± 0.76, $P < 0.05$) in CCl_4-treated rats. GA markedly reduced the positive area of Sirius red and the ratio of the hepatic fibrotic region (from 7.87% ± 0.66% to 3.68% ± 0.32%, $P < 0.05$) compared with the CCl_4 group. GA also decreased the expression level of cleaved caspase-3 compared to the CCl_4 group. TUNEL assay indicated that GA significantly diminished the number of TUNEL-positive cells compared with the CCl_4 group ($P < 0.05$). GA treatment clearly decreased the level of p53 ($P < 0.05$) detected by immunohistochemistry and Western blotting analysis. Compared with the CCl_4 group, we also found that GA reduced the Bax/Bcl-2 ratio ($P < 0.05$), the expression of cleaved caspase-3 ($P < 0.05$), cleaved caspase-9 ($P < 0.05$), and inhibited cytochrome C and second mitochondria-derived activator of caspases (Smac) release from mitochondria to cytoplasm, *i.e.*, GA reduced the expression

level of Smac, which inhibited c-IAP1 activity ($P < 0.05$), ultimately inhibiting the activity of caspase-3, according to Western blotting analysis. As a result, GA suppressed activation of the caspase cascades and prevented hepatocyte apoptosis.

CONCLUSION: GA can inhibit CCl_4-induced hepatocyte apoptosis *via* a p53-dependent mitochondrial pathway to retard the progress of liver fibrosis in rats.

Key words: P53; Apoptosis; Liver fibrosis; Glycyrrhizic acid; Mitochondria

Core tip: This study is the first to investigate the effects of glycyrrhizic acid (GA) on p53-dependent apoptosis in carbon tetrachloride (CCl_4)-induced hepatic injury. The results indicated that GA can attenuate hepatocyte apoptosis *via* a p53-mediated mitochondrial pathway and retard the progression of liver fibrosis induced by CCl_4 in rats.

Guo XL, Liang B, Wang XW, Fan FG, Jin J, Lan R, Yang JH, Wang XC, Jin L, Cao Q. Glycyrrhizic acid attenuates CCl_4-induced hepatocyte apoptosis in rats *via* a p53-mediated pathway. *World J Gastroenterol* 2013; 19(24): 3781-3791 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3781.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3781

INTRODUCTION

Liver fibrosis, induced by various pathological factors, is a common outcome in many chronic liver diseases, and is a serious threat to human health. It is known that the foundation of liver fibrosis is the imbalance between synthesis and degradation of extracellular matrix (including collagen, glycoproteins, polysaccharides, amines, *etc*.).

It has been shown that hepatocyte apoptosis can induce liver fibrosis[1-3]. Hepatocyte apoptosis is a major form of cell death which is primarily triggered by activation of the caspase family of cysteine proteases during the progression of chronic liver disease[4]. Many reports have shown that p53 is accumulated in hepatocytes in several fibrotic liver diseases[5-7]. The protein p53 can lead to apoptosis predominantly through p53-regulated genes such as *P21*, *PUMA*, *NOXA* and *Bax*[8]. The intensity of inflammation induces pro-apoptotic protein p53 with inhibition of anti-apoptotic Bcl-2 in non-alcoholic fatty liver disease[5]. Thioacetamide activates p53, increases caspase-3, Bax and Bad protein contents, and possibly causes the release of cytochrome C from mitochondria and the disintegration of membranes, eventually leading to apoptosis of cells in thioacetamide (TAA)-induced liver fibrosis and cirrhosis[9]. The pro-apoptotic protein, Bax, is a positive regulator and the anti-apoptotic protein, Bcl-xL, is a negative regulator that regulates the release of cytochrome C from mitochondria to the cytoplasm[10,11]. The presence of Bax protein is a direct result of the release of cytochrome C from mitochondria and activation of caspase-9[12]. Inhibitors of apoptosis proteins (IAPs), which regulate apoptosis through various factors, play a vital role in inhibition of the apoptotic process[13]. c-IAP1, c-IAP2 and Survivin, as key members of IAPs, can inhibit the activity of caspase-3 and -7, thus blocking cell apoptosis[14,15]. During the apoptotic process, second mitochondria-derived activator of caspases (Smac), released from mitochondria into the cytoplasm, bind and antagonize IAPs, subsequently reducing the inhibition of caspases by IAPs resulting in apoptosis[16-18]. p53 activation enhances X-IAP inhibition-induced cell death by promoting mitochondrial release of Smac[19]. Therefore, inhibiting p53-dependent hepatocyte apoptosis may be an effective therapeutic strategy for the treatment and prevention of hepatic fibrosis.

Chinese herbal medicine has been widely used to cure diseases for thousands of years in China, especially chronic liver diseases. In recent years, the efficacy of Chinese herbal medicine has been appraised by modern biological technology[20,21]. Glycyrrhizic acid (GA), also known as Glycyrrhizin[22], is the major bioactive component of licorice root extract. GA, a glycosylated saponin, which has one molecule of glycyrrhetinic acid and two molecules of glucuronic acid, has adrenal cortex hormone-like effects[23,24]. GA has numerous pharmacologic effects, such as anti-inflammatory, anti-viral, anti-tumor and hepatoprotective activities[25]. GA also exerts an anti-apoptotic effect through the inhibition of hepatocyte apoptosis[26,27]. Recent findings indicate that GA significantly inhibits hepatocyte apoptosis by down-regulating the expression of caspase-3 and inhibiting the release of cytochrome C from mitochondria into the cytoplasm[28].

It has been reported that carbon tetrachloride (CCl_4) can induce hepatocyte apoptosis and liver fibrosis in animal models[29-33]. The damage responses, induced by CCl_4 injection in rat and mouse models, are similar to liver cirrhosis in humans[34]. Thus, we presumed here that GA treatment started from the early stage of chronic liver disease could effectively attenuate hepatocyte apoptosis, consequently inhibit liver fibrosis and retard disease progression in rats. This study sought to investigate the effects of GA on p53-dependent apoptosis in CCl_4-induced hepatic injury.

MATERIALS AND METHODS

Materials

GA was purchased from Sigma (St Louis, MO, United States). Anti-caspase-3, anti-caspase-9, anti-c-IAP1, anti-cytochrome C, anti-Smac, anti-Bcl-2, anti-Bax and anti-COX-IV antibodies were purchased from Cell Signaling Technology (Beverly, MA, United States). Anti-GADPH and anti-p53 antibodies were bought from Abcam (Cambridge, United Kingdom), horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobin G antibodies were purchased from Cell Signaling Technology. The chemiluminescence reaction kit (ECL Plus) was purchased from Millipore (Billerica, MA, United States). Anti-cleaved-caspase-3 antibody and the mitochondria/cytoplasm fractionation kit were purchased from Beyotime Biotechnology (Haimen, Jiangsu Province, China).

Animal model of liver fibrosis and treatment

Male SD rats weighing 150-200 g were purchased from the Experimental Animal Center of Zhongshan Hospital, Fudan University. Rats were kept in a temperature-controlled room with an alternating 12-h dark and light cycle. Forty-five rats were randomly and equally divided into three groups, the control group, the CCl_4 group, and the GA treatment group. To induce liver fibrosis in this model, rats were given a subcutaneous injection of a 40% solution of CCl_4 (Wako Pure Chemical, Osaka, Japan) in olive oil at a dose of 0.3 mL/100 g body weight biweekly for 8 wk, while controls received the same isovolumetric dose of olive oil by hypodermic injection, with an initial double-dose injection. In the GA group, rats were also treated with a 40% solution of CCl_4 plus 0.2% GA solution in double distilled water by the intraperitoneal injection of 3 mL per rat three times a week from the first week following previously published methods[35,36], with modifications. Controls were given the same isovolumetric dose of double distilled water. Animals were sacrificed 24 h after the last injection. Blood was obtained from the left ventricular apex for measurements of aminotransferases and the samples were stored at -20 ℃. The liver was removed and rinsed with 0.9% saline, some liver sections were fixed in 10% buffered formaldehyde and embedded in paraffin for, and the remaining liver was stored at -70 ℃ for protein experiments.

Liver function

Blood was centrifuged at 3500 *g* at 4 ℃ for 10 min to separate the plasma. The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were detected using a Siemens Advia 1650 automatic analyzer.

Sirius-red and hematoxylin and eosin staining

The thick sections (5 μm) were stained with hematoxylin and eosin (HE) and Sirius-red. HE staining was performed to assess pathologic changes in the liver. The standard of pathological grade was according to consensus on evaluation of the diagnosis and severity of hepatic fibrosis[37]. Sirius-red staining was performed to detect hepatic fibrosis. The Sirius red-positive areas were assessed in four different fields for each section by Image J Software (National Institutes of Health, Bethesda, MD, United States) and were in accordance with the following expression (collagen area/total area-vascular lumen area) × 100[38].

Immunohistochemical staining

Liver tissue sections were subjected to dewaxing, hydration and thermal induction antigen retrieval. Slices were blocked and incubated with anti-p53 antibody (1:50) and anti-cleaved-caspase-3 antibody (1:100) which were diluted in TBS-5% bovine serum albumin (BSA) at 4 ℃ overnight. Negative-control antibody was species-matched. The following day, the slices were washed and incubated with secondary antibodies. The slices were then incubated with 3, 3'-diaminobenzidine tetrachloride for 5-10 min to develop the color, and staining was observed under light microscopy (Olympus, Japan).

Terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end labeling assay

The terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end labeling (TUNEL) assay (Roche, Germany) was performed in accordance with the manufacturer's protocol. Nuclei were redyed with 4,6-diamidino-2-phenylindole (DAPI) staining. Cells marked by TUNEL were evaluated using fluorescence microscopy (Olympus, Japan).

Protein preparation

Mitochondria were isolated with a tissue mitochondria isolation kit according to the manufacturer's instructions. During mitochondria preparation, all samples were placed on ice. Eighty mg liver tissue was cut into pieces, tissue mitochondria isolation reagent A with phenylmethylsulfonyl fluoride (PMSF) was added, and then homogenized in an ice bath approximately 10 times. The homogenate was centrifuged at 600 rpm at 4 ℃ for 5 min. The supernatant was then collected and centrifuged at 11000 *g* at 4 ℃ for 10 min. The supernatant contained the cytoplasmic protein, and the precipitate contained the mitochondria. The cytoplasmic and mitochondrial fractions of the lysate were estimated by Western blotting. Liver tissues were homogenized in RIPA Lysis Buffer with PMSF and then centrifuged at 12000 *g* for 15 min at 4 ℃, and the supernatant was the total protein.

Western blotting analysis

Proteins were separated by 10% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% BSA for 2 h, and then incubated overnight at 4 ℃ with rabbit anti-caspase-9, anti-caspase-3, anti-Smac, anti-cytochrome C, anti-c-IAP1, anti-Bcl-2, anti-Bax antibodies and mouse anti-p53, anti-GAPDH and anti-COXIV antibodies. The membranes were then incubated with HRP-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (1:5000, diluted) at room temperature for 2 h, and then washed again and detected by the enhanced chemiluminescence (ECL) reaction. The intensities of the bands were analyzed by Image J software.

Statistical analysis

Each experiment was repeated at least 3 times. Data were estimated using analysis of variance and all values are expressed as mean ± SD. A P value < 0.05 was considered significant. All analyses in the study were implemented by SPSS 11.5 software for Windows (Chicago, IL, United States).

RESULTS

Function of GA on serum parameters of hepatic fibrosis induced by CCl_4

The activities of ALT and AST were significantly increased in the CCl_4 treated group compared with those in the control group (P < 0.05). In the GA group, the activities of ALT and AST were markedly decreased compared

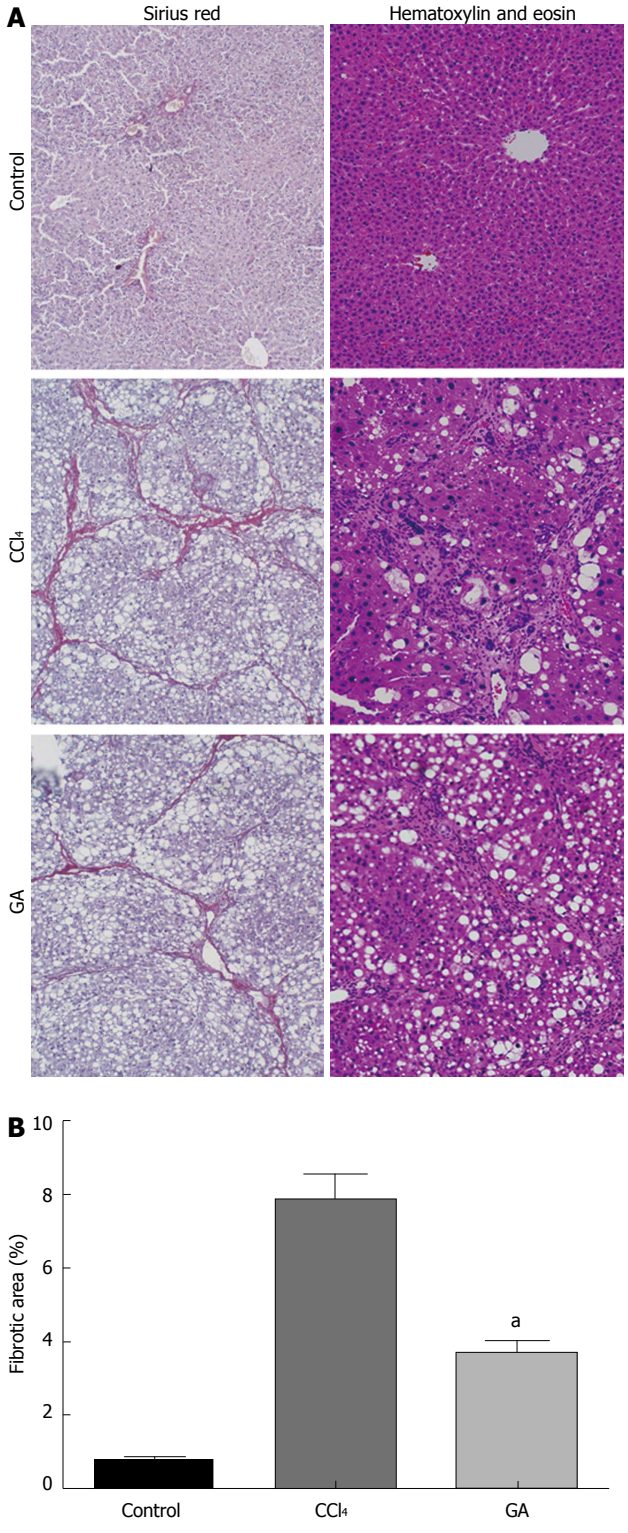

Figure 1 Histological examination of liver by hematoxylin and eosin and Sirius red staining. A: Histological examination. Rats were treated with carbon tetrachloride (CCl_4) and/or glycyrrhizic acid (GA). Liver tissue sections were stained with hematoxylin and eosin or Sirius red (original magnification, × 100); B: Quantitative analysis of liver fibrosis by Sirius red staining. Results are represented as fibrotic area (%), which signifies the proportion of area stained red/area of total area-vascular lumen. Values are mean ± SD. $^aP < 0.05$ *vs* CCl_4.

with those in rats with liver fibrosis not treated with GA ($P < 0.05$) (Table 1).

Table 1 Effect of glycyrrhizic acid on plasma alanine aminotransferase and aspartate aminotransferase activity in CCl_4-induced rats

Group	ALT (U/L)	AST (U/L)
Control	42.4 ± 6.0	70.2 ± 2.3
CCl_4	526.7 ± 57.2	640 ± 33.7
GA	342 ± 44.8[a]	462.8 ± 30.6[a]

$^aP < 0.05$ *vs* the carbon tetrachloride (CCl_4) group. GA: Glycyrrhizic acid; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Table 2 Histopathological semiquantitative scores in the liver

Group	*n*	0	+ 1	+ 2	+ 3	+ 4	Staging scores
Control	15	15	0	0	0	0	0
CCl_4	15	0	0	2	3	10	3.53 ± 0.74
GA	15	0	10	1	10	3	3.00 ± 0.76[a]

$^aP < 0.05$ *vs* the carbon tetrachloride (CCl_4) group. GA: Glycyrrhizic acid.

Role of GA in the improvement of liver fibrosis induced by CCl_4

After 8 wk of CCl_4 administration, liver histopathology was significantly changed in the CCl_4 group. The livers, in the control group, showed an integrated lobular structure with central venous and hepatic cord radiation (Figure 1). The staging score was 0 (Table 2). The positive area of Sirius red staining in the control group was around the central vein rather than in the hepatic parenchyma. There were numerous Steatosis and ballooning of hepatocytes in the GA and CCl_4 groups. In the CCl_4 group, the liver showed fibrous connective tissue proliferation, fiber interval formation which was associated with disorder of lobular structure in the portal area, and most rat livers appeared to have pseudo lobules (Figure 1). The score of hepatic fibrosis in the CCl_4 group increased to 3.53 ± 0.74 (Table 2). The positive areas of Sirius red staining in the CCl_4 group were in the boundaries of the hepatic lobules and the ratio of the hepatic fibrotic region was 7.87% ± 0.66%. In the GA group, livers appeared to have fibrous connective tissue proliferation, the formation of a few fiber intervals in the portal area, and the occasional pseudo lobule (Figure 1). The score was 3.00 ± 0.76 ($P < 0.05$) in the GA group (Table 2). The positive area of Sirius red staining in the GA group was decreased, and the ratio of the hepatic fibrotic region (3.68% ± 0.32%, $P < 0.05$) was reduced compared with the CCl_4 group (Figure 1).

Impact of GA on hepatic apoptosis induced by CCl_4

The expression level of cleaved caspase-3 was high in the livers of rats in the CCl_4 group. Interestingly, this level was reduced in the GA-treated group as detected by immunohistochemistry (Figure 2A). Under fluorescence microscopy, the TUNEL assays showed no stain and non-apoptotic nuclei in the normal liver tissue. High quantities of TUNEL cells were observed in the livers of the CCl_4 group and numerous condensed and fragmented nuclei.

In the GA-treated group, there were few TUNEL cells, and less DAPI staining was observed in the same slice. The merged images indicated that TUNEL-positive cells were different, as numerous fused cells were observed in the CCl_4 group, while a significant reduction in these cells was detected in the GA-treated group (Figure 2B). Overall, these findings indicated that GA reduced apoptosis in liver lesion progression.

Effect of GA on the level of proteins induced by CCl_4

The level of p53 was significantly higher in the livers of rats in the CCl_4 group than in the other two groups as detected by immunohistochemical staining, while in the GA group, the expression level of p53 was reduced (Figure 3A). This is consistent with the Western blotting analysis (Figure 3B) which showed that p53 was activated in the CCl_4 group and clearly reduced in the GA group.

We examined the impact of GA on Bcl-2 and Bax protein expression in CCl_4-induced liver injury by Western blot analysis. As shown in Figure 4A, the expression level of the anti-apoptotic protein, Bcl-2, was decreased, while the expression of the pro-apoptotic protein, Bax, was increased in mitochondrial fraction of CCl_4-induced hepatic injury, and the Bax/Bcl-2 ratio was elevated in the CCl_4 group. In contrast, GA reversed the expression levels of Bcl-2 and Bax, and improved the Bax/Bcl-2 ratio. Both pro- and anti-apoptotic Bcl-2 proteins regulate cytochrome C from mitochondria to cytoplasm. The cytoplasmic fraction in the control group contained a negligible amount of cytochrome C. However, cytochrome C accumulated in the cytoplasm of liver tissue in the CCl_4 group, and GA inhibited the release of cytochrome C (Figure 4B). Caspase activation plays an important role in apoptosis, and caspase-3 cleavage is a typical feature of apoptosis[39]. In the current study, we found that there was increased cleavage of caspase-9 (37 kDa) and caspase-3 (17 kDa) in the CCl_4 group, suggesting severe apoptosis. Intriguingly, the levels of caspase-9 and caspase-3 cleavage diminished in the GA group (Figure 4C and D).

We also found that the cytoplasmic fraction in the control group contained a negligible amount of Smac. However, Smac accumulated in the cytoplasm of livers in rats exposed to CCl_4. GA treatment significantly inhibited the release of Smac induced by CCl_4 (Figure 4E). The expression level of c-IAP1 corresponded to the decreased expression of Smac in the GA-treated group compared with the CCl_4 group (Figure 4F), and the consequence was in accordance with the view that Smac has an antergic effect on c-IAP1 activity which can inhibit the activity of caspases[16-18]. These results indicated that GA could prevent CCl_4-induced apoptosis by suppressing the activation of upstream caspase-3. GA treatment ameliorated CCl_4-induced hepatic injury, and indicated the involvement of the p53 pathway in CCl_4-induced hepatocyte apoptosis.

DISCUSSION

Liver fibrosis is a common outcome in many chronic liver diseases. Liver fibrosis and cirrhosis, as shown in recent studies, are reversible processes[40,41]. However, there have been few effective therapies for the treatment of hepatic fibrosis in recent years[42]. There is an urgent need to investigate the effect of innocuous anti-fibrotic agents[43]. CCl_4-induced liver injury is one of the best-characterized models of hepatotoxicity, and can be used in the clinic to examine anti-hepatotoxic and/or hepatoprotective drugs[44].

GA, used in the treatment and control of chronic viral hepatitis, is now routinely used in Japan, due to its well-recognized transaminase-lowering effect in clinical applications[25,45,46]. Neominophagen C is a Japanese preparation containing 0.2% glycyrrhizin, 0.1% cysteine, and 2% glyceine, and mainly acts as an anti-inflammatory or cytoprotective drug rather than an antiviral. It can improve mortality in patients with subacute liver failure and ameliorate liver function in patients with subacute hepatic failure, chronic hepatitis, and cirrhosis[47].

Apoptosis is one of the events involved in the process of liver fibrosis. Thus, factors that affect apoptosis may be used to modulate liver fibrosis[33]. A line of evidence has shown that loss of p53 function is a common and considerable occurrence in the development of many human malignancies. In unstressed cells, expression of p53 is regulated and maintained at a low level through the ubiquitin/proteasome pathway[48]. Endogenous p53 activation in hepatocytes causes spontaneous liver fibrosis in double minute 2-knockout mice[3]. It also appears to modulate ethanol-induced hepatocyte apoptosis, since it was completely abrogated in mice with a *p53* null background[49]. Mitochondria react to different cytotoxic stimuli, are central death regulators and play a vital role in p53-dependent death, in other words, the p53-dependent signal induces cell death through the mitochondrial pathway[50,51]. When the death signal is conducted to the mitochondria, the cell membrane permeability is increased and apoptosis-related proteins are released[52].

Many reports have demonstrated that drugs can ameliorate CCl_4-mediated hepatic apoptosis in rats, such as branched-chain amino acids[32] and the water-soluble extract of *Salvia miltiorrhiza*[33]. GA has an anti-apoptotic effect through the inhibition of hepatic apoptosis[26,27]. It significantly inhibited hepatocyte apoptosis by down-regulating the expression of caspase-3 and inhibiting the release of cytochrome C from mitochondria into the cytoplasm[28]. GA can alter Kaposi sarcoma-associated herpesvirus latency by triggering p53-mediated apoptosis[53]. Here we demonstrated that intervention with GA from the early stage of chronic liver disease effectively attenuated p53-dependent hepatocyte apoptosis and liver fibrosis, thus retarding disease progression in rats.

Apoptosis and necrosis contribute to the process of liver fibrosis[29,33]. Whether necrotic liver injury or apoptosis is dominant in CCl_4-induced liver injury models remains controversial. A previous study showed that CCl_4 can induce acute hepatocellular damage which is characterized by necrotic cell death[54], while another study indicated that a substantial number of hepatocytes undergo apoptosis in the acute stage after CCl_4 adminis-

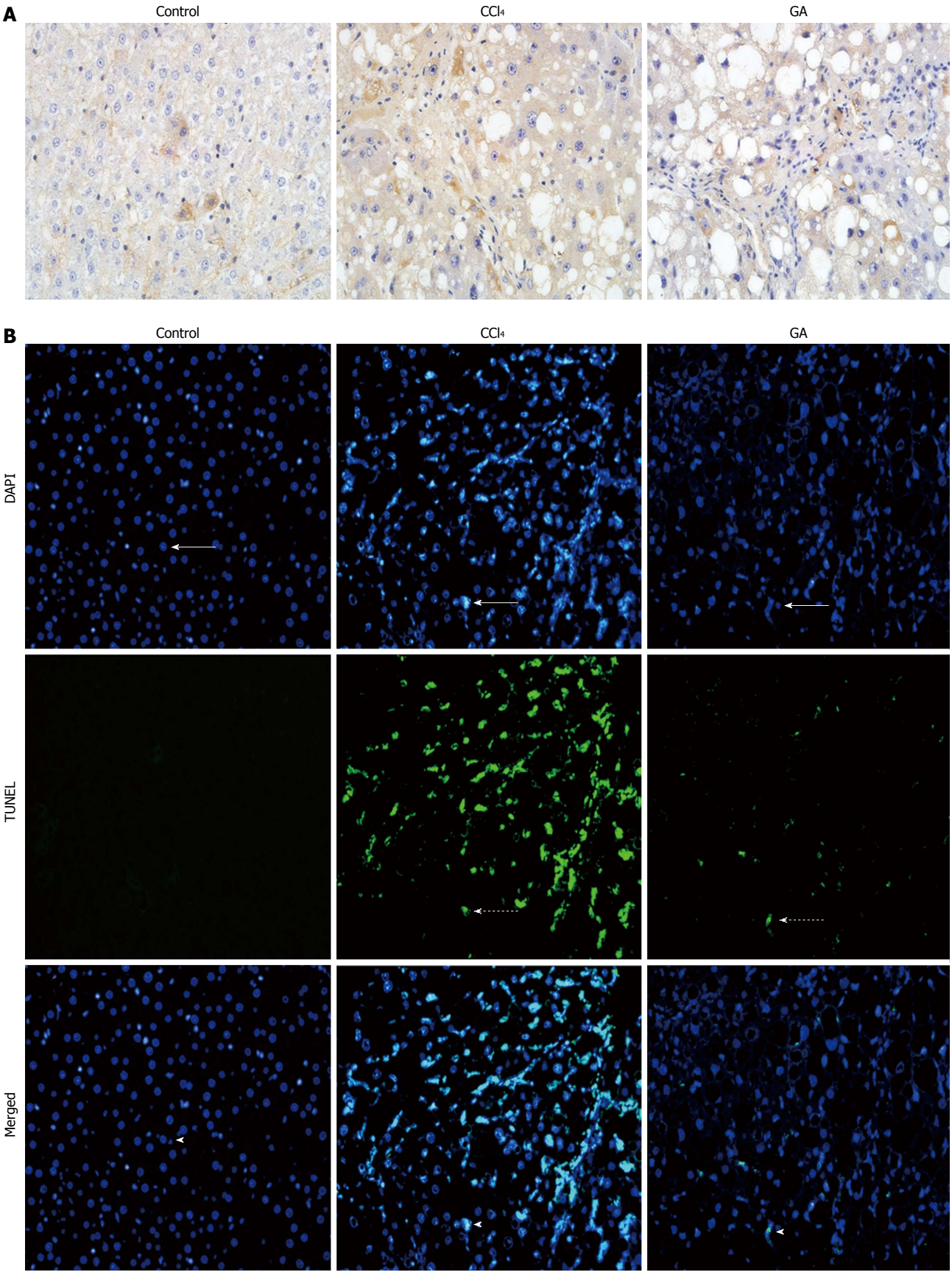

Figure 2 Impact of glycyrrhizic acid treatment on hepatic apoptosis induced by carbon tetrachloride in rats. A: Liver tissue sections from the different groups were subjected to immunohistochemistry to determine the expression level of cleaved caspase-3 (original magnification, × 400); B: Fluorescence microscopy image showing terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end labeling (TUNEL) stain (dashed arrows), and the same tissue slices were respectively counterstained with 4'6-diamidino-2-phenylindole (DAPI) to localize the nuclei (arrows). Images of combined with DAPI, indicated TUNEL-positive cells (arrow heads) (original magnification, × 200). GA: Glycyrrhizic acid; CCl_4: Carbon tetrachloride.

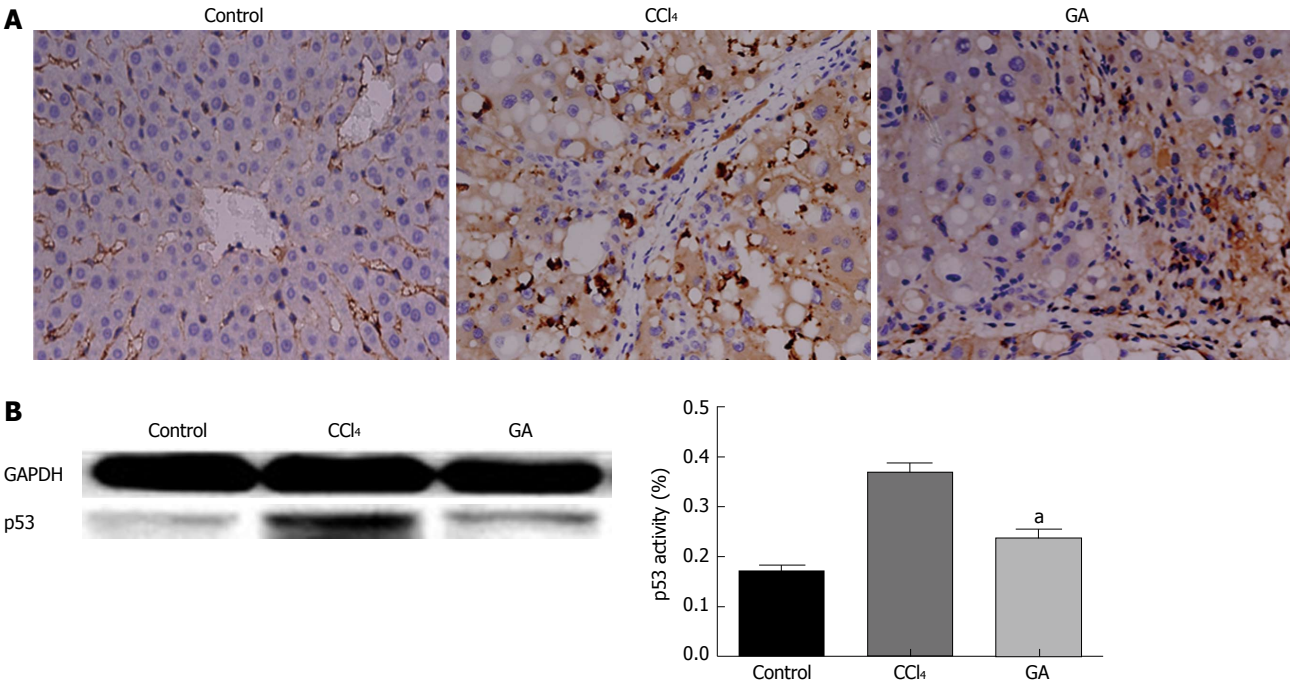

Figure 3 Effect of glycyrrhizic acid treatment on the expression level of p53 in the livers of rats injured by carbon tetrachloride. A: Liver tissue slices from the different groups were subjected to immunohistochemistry (original magnification, × 400). B: Total protein fractions prepared from livers were analyzed by Western blotting to assess the expression level of p53 and GAPDH to confirm the same sample loading. The results of Western blotting analysis were similar in at least three replicate independent experiments. All values are presented as mean ± SD. Statistical significance was defined as follows: $^aP < 0.05$ *vs* the carbon tetrachloride (CCl_4) group. GA: Glycyrrhizic acid.

tration[29]. In the present study, we found both apoptosis and necrosis occurred in the CCl_4-induced chronic liver injury model. These results were consistent with other reports[32,33]. Discrepancies may be attributed to the time points of observation.

Steatosis and ballooning of hepatocytes are the earliest, most frequent, and most striking pathological changes observed in CCl_4-induced liver injury[29,55,56], and we found this pathological change using H and E staining. According to immunohistochemical staining, p53 expression level was significantly increased in the CCl_4 group compared with the GA group. Western blot analysis showed that p53 was sharply up-regulated in the CCl_4 group compared to the GA group. This indicated that p53 was activated after CCl_4 administration, however, GA reduced the expression level of p53.

To date, TUNEL assay[27], cleaved caspase-3 immunohistochemical staining[57] and serum CK18 fragment[58] have been identified as the markers of apoptosis. In the study we first detected DNA fragmentation of hepatocytes using the TUNEL assay. TUNEL-positive cells in the CCl_4 group were significantly increased compared with the GA group. GA reduced the number of TUNEL-labeled cells[27]. However, the TUNEL assay is not a specific marker of apoptosis, thus we performed cleaved caspase-3 immunohistochemical staining. The results coincided with those from the TUNEL assay. Apoptosis, a form of cell death, is principally caused by activation of the caspase family of cysteine proteases[4]. In accordance with Western blotting analysis, accompanied by the reduction in p53, the expression level of Bcl-2 was sharply decreased and the expression level of Bax was obviously increased in the mitochondrial fraction of the CCl_4 group, and the Bax/Bcl-2 ratio was elevated, while this tendency was reversed in the GA-treated group. Our results demonstrated that GA suppressed p53 activity, resulting in an increase in Bcl-2 and a decrease in Bax. In addition, GA inhibited the release of cytochrome C into the cytoplasm from mitochondria, and then inactivated caspase-9 and caspase-3. GA also reduced the expression of Smac, which was released from mitochondria, and bound to and antagonized c-IAP1, subsequently increased the inhibitory effect of c-IAP1 on caspase-3 and finally suppressed hepatocyte apoptosis. The degree of hepatic injury was associated with a substantial number of hepatocytes undergoing apoptosis[27]. The results also demonstrated that hepatic injury in the CCl_4 group was more serious than that in the GA group on the basis of histological observation, Sirius red staining assay, serum transaminase and TUNEL analyses. To our knowledge, these findings were to report that the effects of GA on p53-mediated activity in hepatocyte apoptosis in the liver of CCl_4-treated rats. Whether other mechanisms or pathways are involved in liver fibrosis requires further exploration.

In summary, our findings showed that GA exerted anti-apoptotic effects *via* a p53-dependent mitochondrial pathway (Figure 5). GA protected against CCl_4-induced hepatocyte apoptosis by regulating the Bcl-2 family of proteins, expression of Smac and caspase cleavage. These anti-apoptotic effects were related to decreases in the expression of pro-apoptotic proteins in the cytoplasm

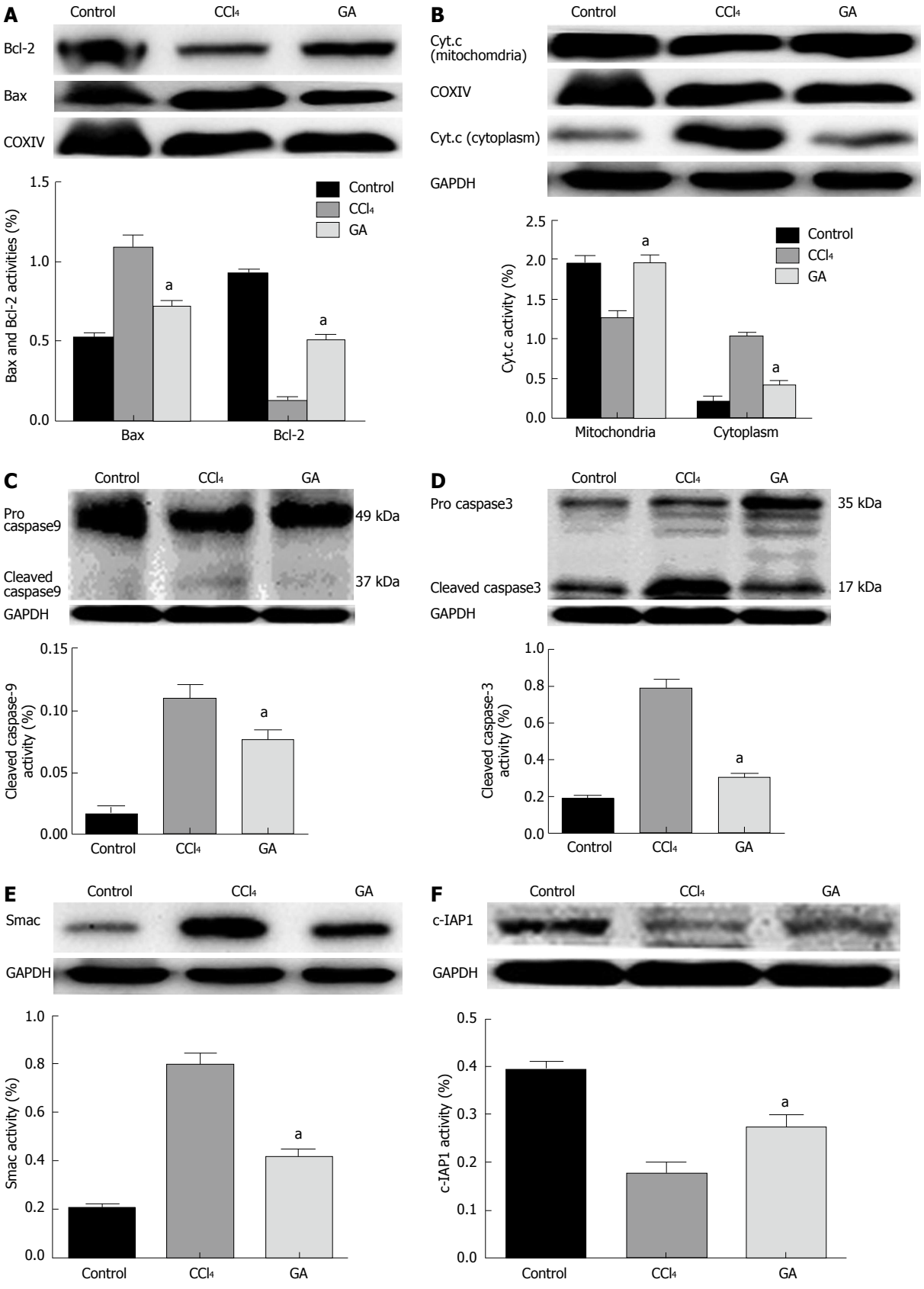

Figure 4 Impact of glycyrrhizic acid on CCl_4-treated hepatocyte apoptosis signal cascades. Protein extracts from livers in the different groups were subjected to Western blotting. A: Expression levels of Bax and Bcl-2 in the mitochondria; B: Expression levels of cytochrome C (Cyt.c) in the cytoplasm and mitochondria; C: Expression level of caspase-9 in the total protein; D: Expression level of caspase-3 in the total protein; E: Expression level of Smac in the cytoplasm; F: Expression level of c-IAP1 the total protein. In all these experiments glyceraldehyde-3-phosphate dehydrogenase (GAPDH), COXIV were used to ensure equal sample loading. The Western blotting results represent three independent tests. The bar graph represents the value of in the different proteins *via* the density of bands from at least three independent tests. All values are presented as mean ± SD. Statistical significant was defined as follows: $^aP < 0.05$ *vs* the CCl_4 group. GA: Glycyrrhizic acid; CCl_4: Carbon tetrachloride.

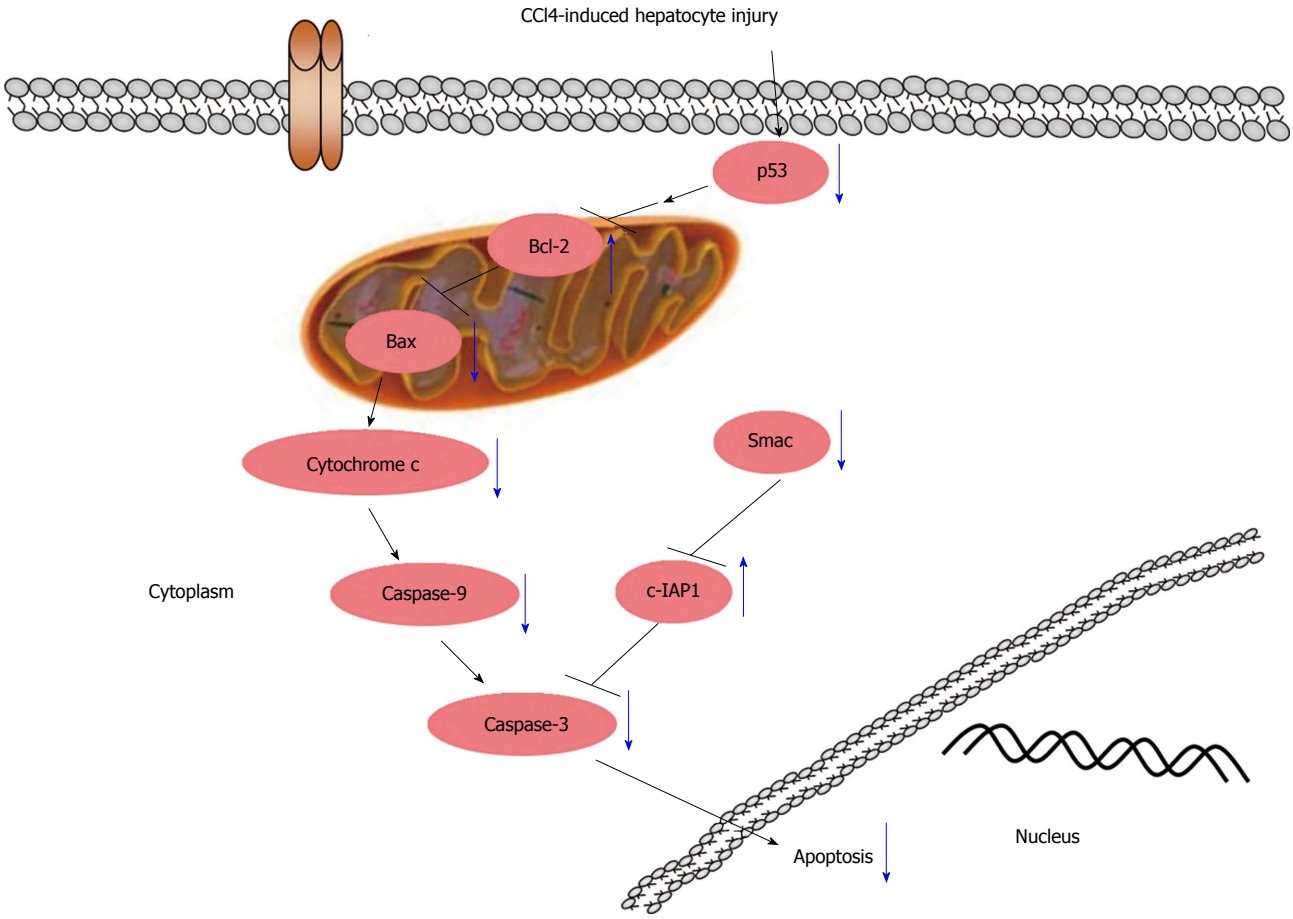

Figure 5 Schematic diagram of the effect of glycyrrhizic acid on the interruption of p53 signaling in carbon tetrachloride-induced hepatocyte apoptosis (blue arrows). Glycyrrhizic acid (GA) suppressed the activation of p53, decreased the expression level of Bax and increased the expression level of Bcl-2, which resulted in reduced cytochrome C release from the mitochondria into the cytoplasm, and inactivated caspase-9 and -3; GA also significantly inhibited Smac release from mitochondria into the cytoplasm and elevated the expression level of c-IAP1, resulting in inhibition of caspase-3 activity. Ultimately, GA suppressed the apoptosis of hepatocytes.

and the inhibition of proteins associated with apoptosis in the mitochondria. These findings suggest that GA can attenuate CCl_4-induced hepatocyte apoptosis *via* a p53-mediated mitochondrial pathway and can retard the progression of liver fibrosis induced by CCl_4 in rats.

COMMENTS

Background

Liver fibrosis, induced by various pathological factors, is a common outcome in many chronic liver diseases, and is a serious threat to human health. However, there have been few effective therapies for the treatment of hepatic fibrosis in recent years. The authors investigated whether glycyrrhizic acid (GA) could attenuate hepatocyte apoptosis *via* a p53-mediated mitochondrial pathway and retard the progression of liver fibrosis induced by CCl_4 in rats.

Research frontiers

In this study, the authors found that GA attenuated hepatocyte apoptosis *via a* p53-mediated mitochondrial pathway and retarded the progression of liver fibrosis induced by carbon tetrachloride (CCl_4) in rats, which may be a potential alternative treatment approach in patients with liver injury.

Innovations and breakthroughs

This study sought to investigate the effects of GA on p53-dependent apoptosis in CCl_4-induced hepatic injury. The study data showed that GA protected against CCl_4-induced hepatocyte apoptosis by regulating the Bcl-2 family of proteins, expression of Smac and caspase cleavage.

Applications

This study provides valuable experimental evidence for future anti-liver fibrosis drug studies, and may provide an effective therapy for retarding the process of liver fibrosis.

Terminology

Liver fibrosis, induced by various pathological factors, is a common outcome in many chronic liver diseases, and eventually leads to liver cirrhosis. Apoptosis is gene-controlled and auto-programmed cell death in order to maintain homeostasis. Apoptosis is different from necrosis, as it is an initiative process rather than a passive process and involves gene activation, expression and regulation.

Peer review

This is a good study in which the authors presented experimental evidence that GA exerts anti-apoptotic effects *via* a p53-dependent mitochondrial pathway in CCl_4-induced hepatocyte apoptosis in rats. The results are interesting and suggest that GA could protect against CCl_4-induced hepatocyte apoptosis by regulating Bcl-2 family of proteins, expression of Smac and caspases cleavage.

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doi:10.3748/wjg.v19.i24.3792

World J Gastroenterol 2013 June 28; 19(24): 3792-3801
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Annexin A2 silencing inhibits invasion, migration, and tumorigenic potential of hepatoma cells

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Supported by The Society Development of Nantong, No. HS2012034; the Jiangsu Health Projects, No. BL2012053 and No. K201102; the Priority Academic Program Development of Jiangsu, and the International S and T Cooperation Program of China, No. 2013DFA32150
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Received: March 13, 2013 Revised: April 30, 2013
Accepted: May 18, 2013
Published online: June 28, 2013

Abstract

AIM: To investigate the effects of Annexin A2 (ANXA2) silencing on invasion, migration, and tumorigenic potential of hepatoma cells.

METHODS: Human hepatoma cell lines [HepG2, SMMC-7721, SMMC-7402, and MHCC97-H, a novel human hepatocellular carcinoma (HCC) cell line with high metastasis potential] and a normal hepatocyte cell line (LO2) were used in this study. The protein and mRNA expression levels of ANXA2 were analysed by western blotting and real-time polymerase chain reaction, respectively. The intracellular distribution profile of ANXA2 expression was determined by immunofluorescence and immunohistochemistry. Short hairpin RNA targeting ANXA2 was designed and stably transfected into MHCC97-H cells. Cells were cultured for *in vitro* analyses or subcutaneously injected as xenografts in mice for *in vivo* analyses. Effects of ANXA2 silencing on cell growth were assessed by cell counting kit-8 (CCK-8) assay (*in vitro*) and tumour-growth assay (*in vivo*), on cell cycling was assessed by flow cytometry and propidium iodide staining (*in vitro*), and on invasion and migration potential were assessed by transwell assay and wound-healing assay, respectively (both *in vitro*).

RESULTS: The MHCC97-H cells, which are known to have high metastasis potential, showed the highest level of ANXA2 expression among the four HCC cell types examined; compared to the LO2 cells, the MHCC97-H expression level was 8-times higher. The ANXA2 expression was effectively inhibited (about 80%) by ANXA2-specific small hairpin RNA (shRNA). ANXA2 expression in the MHCC97-H cells was mainly localized to the cellular membrane and cytoplasm, and some localization was detected in the nucleus. Moreover, the proliferation of MHCC97-H cells was obviously suppressed by shRNA-mediated ANXA2 silencing *in vitro*, and the tumour growth inhibition rate was 38.24% *in vivo*. The percentage of MHCC97-H cells in S phase dramatically decreased (to 27.76%) under ANXA2-silenced conditions. Furthermore, ANXA2-silenced MHCC97-H cells showed lower invasiveness (percentage of invading cells decreased to 52.16%) and suppressed migratory capacity (migration distance decreased to 63.49%). It is also worth noting that shRNA-mediated silencing of ANXA2 in the MHCC97-H cells led to abnormal apoptosis.

CONCLUSION: shRNA-mediated silencing of ANXA2

suppresses the invasion, migration, and tumorigenic potential of hepatoma cells, and may represent a useful target of future molecular therapies.

Key words: Annexin A2; Small hairpin RNA; Hepatocellular carcinoma; Invasion; Migration; Tumorigenic potential

Core tip: The overexpression of annexin A2 (ANXA2) is closely related to the high metastasis potential and invasion ability of HCC cells, and ANXA2 deficiency inhibits the invasion, migration, and tumorigenic potential of hepatocellular carcinoma (HCC) cells, which not only provides further insight into the pathogenesis of HCC but also provides a potential predictive biomarker for HCC prognosis and a potential therapeutic target.

Zhang HJ, Yao DF, Yao M, Huang H, Wang L, Yan MJ, Yan XD, Gu X, Wu W, Lu SL. Annexin A2 silencing inhibits invasion, migration, and tumorigenic potential of hepatoma cells. *World J Gastroenterol* 2013; 19(24): 3792-3801 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3792.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3792

INTRODUCTION

Hepatocellular carcinoma (HCC) is currently the 3rd leading cause of cancer-related death, and its worldwide incidence is increasing; epidemiologic studies have revealed a strong association between HCC and chronic liver disease and cirrhosis[1,2]. Surgical therapy with liver transplantation or resection remains the mainstay of curative therapy for patients in the early stage of HCC[3,4]. However, even with radical resection, 60%-70% of patients develop metastasis and experience recurrence within 5 years of surgery[5-7]. Although several clinicopathologic features have been identified as contributing factors to this disease's poor prognosis (*e.g.,* poorly-differentiated phenotype, large-sized tumour, and portal venous invasion), the underlying mechanisms of HCC development remain unclear[8,9]. Gaining a detailed understanding of the molecular processes of HCC will likely identify specific diagnostic and prognostic markers and facilitate development of novel targeted therapeutic strategies. To this end, recent studies have identified annexin A2 (ANXA2) as an important mediator of malignant transformation and development of HCC[10,11].

As one of the best characterized components of the annexin family, ANXA2 is a calcium-dependent phospholipid-binding protein[12,13]. Up-regulation of ANXA2 expression and its phosphorylation at tyrosine 23 (mediated by c-Src) has been observed in clinical samples of human HCC[14]; in addition, the tyrosine 23 phosphorylation-dependent cell-surface localization of ANXA2 was found to be required for the invasive and metastatic properties of pancreatic cancer[15]. Further studies of this ability to promote tumour metastasis have elucidated the molecular process in which ANXA2 induces plasminogen conversion to plasmin, thereby leading to activation of metalloproteinases, degradation of extracellular matrix components, and promotion of neoangiogenesis[10,16-18]. Thus, ANXA2-targeted interference by small hairpin RNA (shRNA) may represent an effective therapeutic strategy to mediate the biological behaviours of hepatoma cells.

In the present study, ANXA2 overexpression was found in MHCC97-H cells, a novel human HCC cell line with high metastasis potential, which was then used to investigate the effects of ANXA2 silencing on cell invasion, migration, and tumorigenic potential of HCC cells.

MATERIALS AND METHODS

Plasmid construction

ANXA2-specific shRNA targeting nucleotides 94-113 downstream of the transcription start site of ANXA2 was synthesized as previously described[16] and used to construct an experimental plasmid (pRNAT-U6.1-shRNA) by inserting into a *BamH* I -*Hind*Ⅲ linearized pRNAT-U6.1/Neo shRNA expression vector (Biomics Biotechnologies Co., Ltd., Nantong, China). A negative control vector (pRNAT-U6.1-negative) was constructed similarly with a shRNA sequence that does not suppress the expression of genes expressed in humans, rats, or mice (Biomics Biotechnologies Co., Ltd.). All inserted sequences were verified by DNA sequencing.

Cell culture and transfection

Human hepatoma cell lines (HepG2, SMMC-7721, and SMMC-7402) and a normal hepatocyte cell line (LO2) were obtained from Biomics Biotechnologies Co., Ltd. The MHCC97-H cell line was obtained from the Liver Cancer Institute of the Affiliated Zhongshan Hospital of Fudan University (Shanghai, China). For all cell lines, maintenance growth was carried out in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc., Frederick, MD, United States) supplemented with 10% foetal bovine serum (FBS) at 37 ℃ in a humidified atmosphere of 5% CO_2/95% air.

For experimentation, the MHCC97-H cells were seeded into 6-well plates, allowed to grow to 90%-95% confluence, and transfected with the plasmids (pRNAT-U6.1-negative or pRNAT-U6.1-shRNA) using PolyJet™ *In Vitro* DNA Transfection Reagent (SignaGen Laboratories, Gaithersburg, MD, United States) according to the manufacturer's instructions. At 48 h after transfection, cells were selected by culturing in the presence of 400 μg/mL of G418 (Life Technologies, Inc.) for 2 wk followed by 200 μg/mL of G418 for an additional 2 wk. Individual G418-resistant monoclonals were obtained by performing a limiting dilution with subsequent proliferation in medium supplemented with 200 μg/mL of G418 to generate the stably transfected experimental (MHCC97-H/

ANXA2-shRNA) and control (MHCC97-H/control-shRNA) cell lines.

RNA isolation and cDNA synthesis

Total RNA was isolated from mouse liver tissue specimens (50 mg) using the TRIzol Reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Integrity of the isolated RNA was qualitatively assessed by 1% agarose gel electrophoresis and quantitatively assessed by ultraviolet spectrophotometry (absorbance at 260 nm, A_{260}; SmartSpec™ Plus; Bio-Rad Life Science Research and Development Co., Ltd., Shanghai, China); purity of the isolated RNA was indicated by an absorbance ratio at $A_{260/280}$ of < 1.8. The total RNA (1 μg) was applied as template for cDNA synthesis using the First-Strand cDNA Synthesis Kit (Fermentas Inc., Burlington, Canada) and following the manufacturer's instructions.

Quantitative real-time polymerase chain reaction

The cDNA samples (4 μg in 4 μL) were subjected to quantitative real-time polymerase chain reaction (qPCR) using an Applied Biosystems StepOne™ Real-Time PCR System (Life Technologies, Inc.) with the manufacturer's recommended protocol. The reaction solution (50 μL) contained 25 μL of 2 × SYBR Premix ExTaq (Takara Biotechnology Co., Ltd., Dalian, China), 2 μL of gene-specific primers, 1 μL of 50 × ROX Reference Dye I , and 18 μL deionized water. The following primers were used: ANXA2: forward, 5'-TGAGCGGGATGCTTTGAAC-3' and reverse, 5'-ATCCTGTCTCTGTGCATTGCTG-3'; β-actin (internal control): forward, 5'-ATTGCCGACAGGATGCAGA-3' and reverse, 5'-GAGTACTTGCGCTCAGGAGGA-3'[16]. Negative control reactions with no template (deionized water) were also included in each run. The optimized PCR conditions were: one cycle at 95 ℃ for 2 min; 40 cycles at 95 ℃ for 10 s, 62 ℃ for 1 min; 1 cycle at 72 ℃ for 2 min. The relative quantitative analysis was performed by comparison of the $2^{-\Delta\Delta Ct}$ values.

Western blotting

Total cell protein was extracted by sonication with 2 × sample buffer (0.25 mol/L Tris-HCl, 10% 2-mercaptoethanol, 4% sodium dodecyl sulphate (SDS), and 10% sucrose). Protein concentration was determined using the Enhanced Bicinchoninic Acid Protein Assay Kit (Beyotime Institute of Biotechnology, Haimen, China). Samples (20 μg) were resolved by 15% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. After blocking with 5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, United States) in Tris-buffered saline (pH 7.5; 100 mmol/L NaCl, 50 mmol/L Tris, and 0.1% Tween-20), the membranes were immunoblotted overnight at 4 ℃ with monoclonal primary antibody rabbit anti-human ANXA2 (1:500 dilution) and monoclonal primary antibody mouse anti-human β-actin (1:500) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States), washed three times, followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:1000). Immunoreactive bands were visualized by chemiluminescence detection (Milipore Corp., Billerica, MA, United States) and analysed by densitometric analysis using the ImageJ software (version 1.30v; National Institutes of Health Research Services Branch, Bethesda, MD, United States). The ANXA2 protein level is expressed as relative ratio, which was calculated as signal intensity (SI) of ANXA2 divided by SI of β-actin[19].

Immunofluorescence assay

Cells (1×10^6) were plated on cover slips and cultured for 24 h in 24-well plates. After three washes with phosphate-buffered saline (PBS), the cells were fixed with 4% paraformaldehyde for 10 min and blocked with 3% BSA in PBS for 30 min at 37 ℃. Then, the samples were incubated overnight at 4 ℃ with the anti-ANXA2 (1:100). After three washes with PBS, the samples were incubated for 1 h at 37 ℃ in the dark with the corresponding Cy3-labeled goat anti-rabbit immunoglobulin G (IgG) secondary antibody (1:500; Beyotime Institute of Biotechnology), washed three times with PBS, stained with 4,6-diamidino-2-phenylindole for 5 min, washed three times with PBS, and sealed with 50% glycerine. The immunostaining analysis was conducted by an multifunction microscope (IX71; Olympus Corp., Tokyo, Japan).

Cell proliferation assay

Cell proliferation was evaluated using the cell counting kit-8 (CCK-8; Beyotime Institute of Biotechnology) and following the manufacturer's instructions. MHCC97-H (untransfected), MHCC97-H/control-shRNA and MHCC97-H/ANXA2-shRNA cells were seeded in 96-well plates (2×10^3 cells/well in 100 μL medium) and cultured for 24 h. Wells with medium alone (no cells) served as blank controls. CCK-8 solution (10 μL/well) was added to the culture medium and incubated for 2 h, after which the A_{450} was measured by a microplate reader (Synergy HT; BioTek Corp., Winooski, VT, United States) at various time points. Experiments were performed in triplicate.

Cell cycle assay

Cell cycle assay was performed using the cell cycle and apoptosis analysis kit (Beyotime Institute of Biotechnology) and following the manufacturer's instructions. MHCC97-H (untransfected), MHCC97-H/control-shRNA and MHCC97-H/ANXA2-shRNA cells were seeded in 6-well plates (1.0×10^6 cells/well in 2 mL medium). After 24 h of culturing, the cells were digested with trypsin enzyme, washed with pre-cooled PBS, and fixed in pre-cooled 70% ethanol for 24 h at 4 ℃. The cells were then stained with propidium iodide and analysed by flow cytometry (FACSCalibur; Becton Dickinson Medical Devices Co Ltd., Shanghai, China) to determine the cell cycle distribution. Experiments were performed in triplicate[20].

Transwell assay

MHCC97-H (untransfected), MHCC97-H/control-shRNA

and MHCC97-H/ANXA2-shRNA cells were plated at 1.0×10^5 cells/well in 0.5 mL of serum-free medium in 24-well matrigel-coated transwell units with polycarbonate filters (8 μm pore size; Costar Inc., Milpitas, CA, United States). The outer chambers were filled with 0.5 mL of medium supplemented with 10% FBS. After 24 h, the cells were fixed in methanol and stained with crystal violet. The top surface of the membrane was gently scrubbed with a cotton bud, and the cells that had invaded through the membrane filters were counted. The invasion inhibition rate (%) was calculated as [(A - B)/A) × 100], where A was the invading cells' percentage for the MHCC97-H group and B was the invading cells' percentage for the MHCC97-H/ANXA2-shRNA group.

In vitro wound-healing assay

The various HCC cell types were cultured in 12-well plates; at subconfluency, the cell monolayer was scratched (wound) with a plastic pipette tip, washed with serum-free medium, and cultured in DMEM medium supplemented with 0.1% FBS. The relative migration distance (%) travelled at various time points (0 and 8 h) was calculated as [(Ax - Bx)/(A_{mock} - B_{mock}) × 100%], where A was the wound width prior to incubation and B was the wound width after incubation[21].

Xenograft tumour-growth assay

The animal protocol was approved by the Ethical Review Committee for Animal Experimentation of Nantong University (China). Specific-pathogen free BALB/C nude mice (6-wk-old and 20 ± 3 g; Super-B and K Laboratory Animal Co., Ltd., Shanghai, China) were randomly assigned to three groups for xenografting of MHCC97-H (untransfected; $n = 4$), MHCC97-H/control-shRNA ($n = 4$), and MHCC97-H/ANXA2-shRNA ($n = 4$) cells. For each group, right flank subcutaneous injection was made with 2×10^7 of the respective HCC cells suspended in 0.2 mL of DMEM medium. Four mice injected with normal saline alone represented the control (non-xenografted) group. Over 21 d of growth, tumour size was routinely measured using callipers and used to calculate the tumour volume by the formula: [(length × width^2)/2]. On post-injection day 21, the animals were sacrificed for liver excision and complete tumour resection. The tumorigenicity inhibition rate (%) was calculated for each HCC-xenografted group as [(tumour $weight_{control}$ - tumour $weight_{shRNA}$)/tumour $weight_{control}$) × 100][22].

Histopathological examination of resected xenografted tumours

Resected tissues were processed for haematoxylin and eosin staining by dehydrating, sectioning (3 μm thick), and mounting on glass slides. For analysis, the sections were rehydrated in distilled water for 2 min, stained with haematoxylin for 5 min, washed with tap water three times for 5 min each, dehydrated with 95% ethanol for 5 s stained with eosin for 2 min, washed with 70% ethanol two times for 5 min each, and air dried.

Immunohistochemical examination of resected xenografted tumours

Resected tissues were processed for were immunohistochemical analysis by formalin fixing, embedding in paraffin, sectioning (3 μm thick), and mounting on glass slides. For analysis, the sections were deparaffinised by soaking in xylene for two times at 10 min each, dehydrated by soaking in an ethanol to distilled water gradient for 5 min at each serial dilution, washed with PBS (pH 7.4) three times, and incubated in endogenous peroxidase blocking solution for 5 min (Immunostain EliVision Kit; Maixin Biotech Inc., Fuzhou, China). The treated sections were subjected to antigen-retrieval by boiling in 0.01 mol/L citrate buffer (pH 6.0) for 10 min (650 W microwave) and blocking of non-specific antibody binding by pretreatment with 0.5% BSA in PBS. After rinsing with PBS, the processed sections were incubated overnight at 4 ℃ with ANXA2 antibody (1:500), washed three times with 0.05% Tween-20 in PBS, and stained with the chromogen 3, 3'-diaminobenzidine tetrahydrochloride. The slide was then rinsed with distilled water, counterstained with haematoxylin, dehydrated, and air-dried. Negative control sections were generated by the same procedure except that the non-specific mouse IgG antibody was used. All samples were evaluated by light microscope by an expert who was blinded to the group and outcome. ANXA2 staining intensity is expressed as an immunoreactive score[23].

Statistical analysis

Results are expressed as mean ± SD. Significance of differences detected between groups was assessed by one-way analysis of variance followed by the least significant difference test or Newman-Keuls test. A P value of < 0.05 was set as the threshold of significance.

RESULTS

ANXA2 is over-expressed in HCC cells

As shown in Figure 1A, the ANXA2 protein level detected in MHCC97-H cells was the highest among the four HCC cell lines and was 8-times higher ($P < 0.05$) than that detected in the normal cell line LO2. Additionally, the level of ANXA2 mRNA expression was significantly higher in the MHCC97-H cells than that in the other HCC cells and the LO2 cells (Table 1; $P < 0.001$). Thus, the MHCC97-H cell line was selected for subsequent study of the effects of shRNA targeting ANXA2 on cell invasion, migration, and tumorigenic potential of hepatoma cells.

ANXA2 expression in MHCC97-H cells is inhibited by shRNA in vitro

The silencing efficiency of ANXA2-specific shRNA in MHCC97-H cells reached approximately 80%. As shown in Table 2 and Figure 1B, the expression levels of ANXA2 mRNA and protein were significantly lower in the MHCC97-H/ANXA2-shRNA cells than in the MHCC97-H/control-shRNA cells and in the MHCC97-H

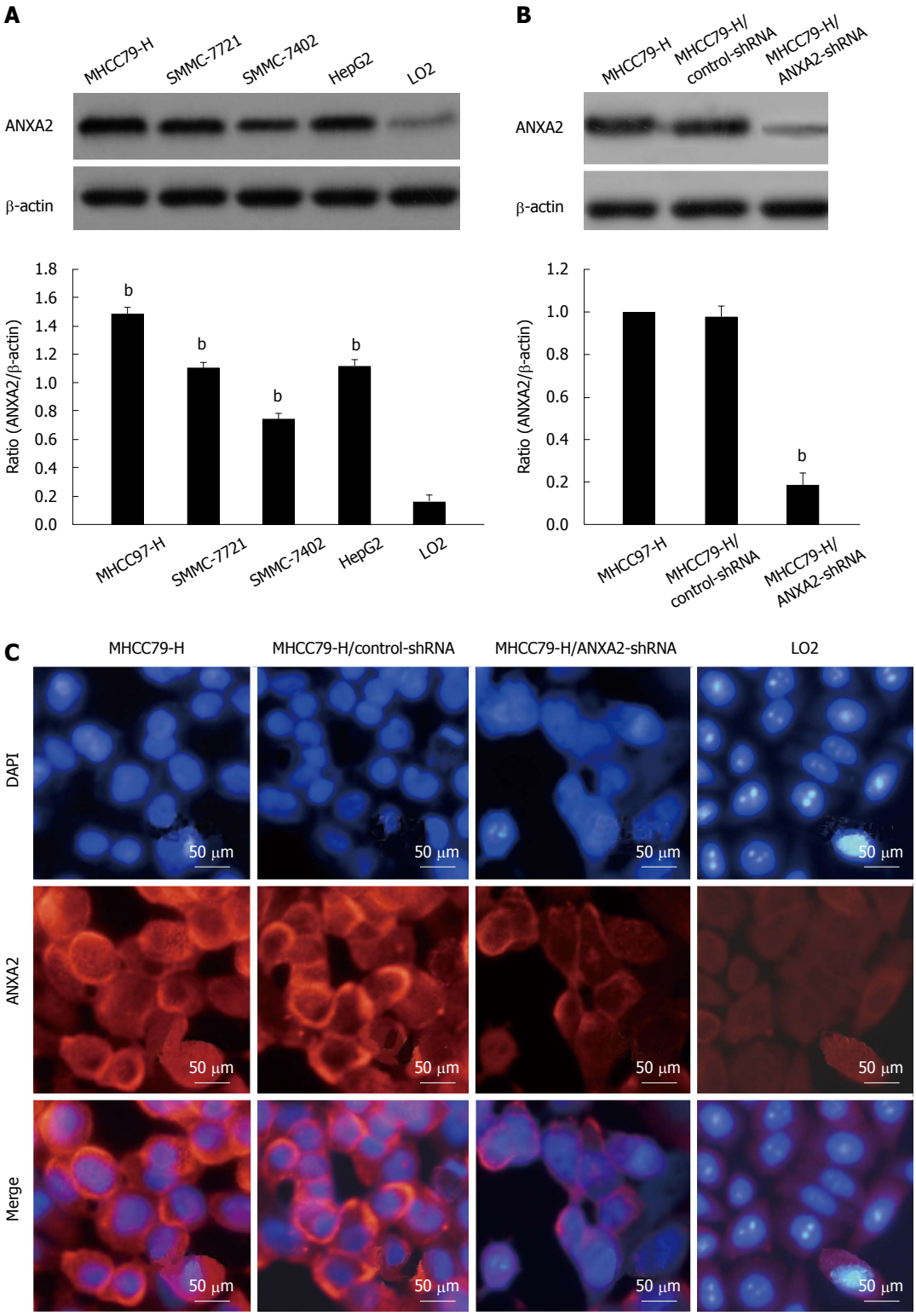

Figure 1 Annexin A2 expression level in hepatoma cells and silencing efficiency of small hairpin RNA in MHCC97-H cells. A: Representative Western blotting images of hepatocellular carcinoma cell lines and the normal hepatic cell line LO2. $^{b}P < 0.01$ *vs* LO2; B: Representative Western blotting images of annexin A2 (ANXA2) silencing upon transfection of small hairpin RNA (shRNA). $^{b}P < 0.01$ *vs* MHCC97-H; C: Representative immunofluorescence images of ANXA2 cellular distribution (× 400). DAPI: 4,6-diamidino-2-phenylindole.

(untransfected) cells. The levels detected in the MHCC97-H/control-shRNA cells and MHCC97-H cells were not significantly different. As shown in Figure 1C, ANXA2 (red fluorescence) expression in the MHCC97-H cells was mainly localized to the cellular membrane and cytoplasm, and some localization was detected in the nucleus. The MHCC97-H/ANXA2-shRNA cells showed obviously lower density of the red immunofluorescent signal

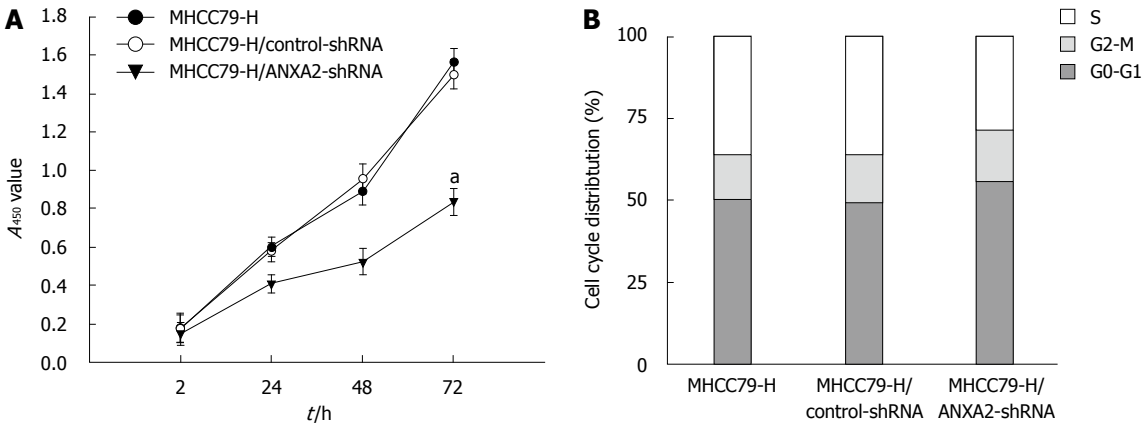

Figure 2 Effect of small hairpin RNA-mediated annexin A2 silencing on proliferation and cell cycling of MHCC97-H cells. A: Cellular proliferation assay. [a]*P* < 0.05 *vs* the A_{450} of MHCC97-H cells at 72 h; B: Cell cycle assay. *P* < 0.05 for percentage of MHCC97-H/annexin A2 (ANXA2)-small hairpin RNA (shRNA) cells in S phase *vs* that of MHCC97-H cells.

Table 1 Expression level of ANXA2 mRNA in hepatocellular carcinoma cells and normal hepatic cells

Group	*n*	Ct_{ANXA2}	$Ct_{\beta\text{-actin}}$	$2^{-\Delta\Delta Ct}$
LO2	5	25.16 ± 0.09	20.86 ± 0.03	1.00
HepG2	5	22.14 ± 0.15	20.66 ± 0.02	7.07 ± 0.35[b]
SMMC-7402	5	22.87 ± 0.15	20.80 ± 0.14	4.68 ± 0.31[b]
SMMC-7721	5	22.21 ± 0.12	20.72 ± 0.10	7.02 ± 0.19[b]
MHCC97-H	5	21.85 ± 0.26	20.78 ± 0.13	9.45 ± 0.53[b]

[b]*P* < 0.001 *vs* the LO2 group.

Table 2 Inhibition of ANXA2 mRNA expression by small hairpin in MHCC97-H cells

Group	*n*	Ct_{ANXA2}	$Ct_{\beta\text{-actin}}$	$2^{-\Delta\Delta Ct}$
MHCC97-H	5	21.84 ± 0.11	20.77 ± 0.16	1.00
MHCC97-H/control-shRNA	5	21.83 ± 0.21	20.72 ± 0.10	0.97 ± 0.04
MHCC97-H/ANXA2-shRNA	5	24.24 ± 0.55	20.80 ± 0.14	0.20 ± 0.05[b]

[b]*P* < 0.001 *vs* the MHCC97-H group. shRNA: Small hairpin RNA.

in the cellular membrane and cytoplasm, as compared to both MHCC97-H and MHCC97-H/control-shRNA cells. Thus, the ANXA2-shRNA was capable of down-regulating ANXA2 expression. Intriguingly, the nuclei of the MHCC97-H/ANXA2-shRNA cells (blue fluorescence) showed signs of early apoptosis (fragmentation with condensed chromatin), as compared to the nuclei of the MHCC97-H cells or the MHCC97-H/control-shRNA cells.

ANXA2 deficiency inhibits proliferation of MHCC97-H cells in vitro

The effects of ANXA2 deficiency on proliferation and cell cycling of MHCC97-H cells are shown in Figure 2. At 72 h post-transfection, the MHCC97-H/ANXA2-shRNA cells showed significantly lower proliferation potential than the MHCC97-H cells or MHCC97-H/control-shRNA cells (*P* < 0.05) (Figure 2A). The proliferation rates of the MHCC97-H cells and the MHCC97-H/control-shRNA cells were not significantly different. The percentage of MHCC97-H/ANXA2-shRNA cells in S phase was significantly lower than that in the MHCC97-H cells (27.76% *vs* 36.14%, *P* < 0.05), whereas the percentages of MHCC97-H/ANXA2-shRNA cells in G_0-G_1 phase and G_2-M phase were significantly higher than those in the MHCC97-H cells (both *P* < 0.05) (Figure 2B). Thus, shRNA targeted suppression of ANXA2 inhibits the growth of MHCC97-H/shRNA cells *in vitro*.

ANXA2 silencing suppresses the invasion and migration potential of MHCC97-H cells in vitro

As shown in Figure 3, the invasive potential of MHCC-97-H/ANXA2-shRNA cells was significantly lower than that of the MHCC97-H cells (52.16% *vs* 86.14%, *P* < 0.05). The invasion inhibition rate in MHCC97-H/ANXA2-shRNA cells reached up to 39.45%. In addition, the migration potential of MHCC97-H/ANXA2-shRNA cells was significantly lower than that of the MHCC97-H cells (63.49% *vs* 100%, *P* < 0.05). The migration inhibition rate in MHCC97-H/ANXA2-shRNA cells reached up to 36.51%. The migration inhibition rates of the MHCC97-H cells and the MHCC97-H/control-shRNA cells were not significantly different.

Down-regulation of ANXA2 inhibits xenograft tumour growth in vivo

As shown in Figure 4A, the tumour volumes of MHCC-97-H/ANXA2-shRNA xenograft group were remarkably smaller than those of the MHCC97-H xenograft group or the MHCC97-H/control-shRNA xenograft group. Unlike the non-xenografted group (Figure 4A), all three xenografted groups showed obvious emaciation, especially the MHCC97-H group and the MHCC97-H/control-shRNA group. As shown in Figure 4B, by day 21 after cell injection the average tumour weight for the MHCC97-H/ANXA2-shRNA group was distinctly lower than that for either the MHCC97-H group or the MHCC97-H/control-shRNA group (both *P* < 0.05). The shRNA-mediated inhibition rate of xenografted tumours reached 38.2%. The

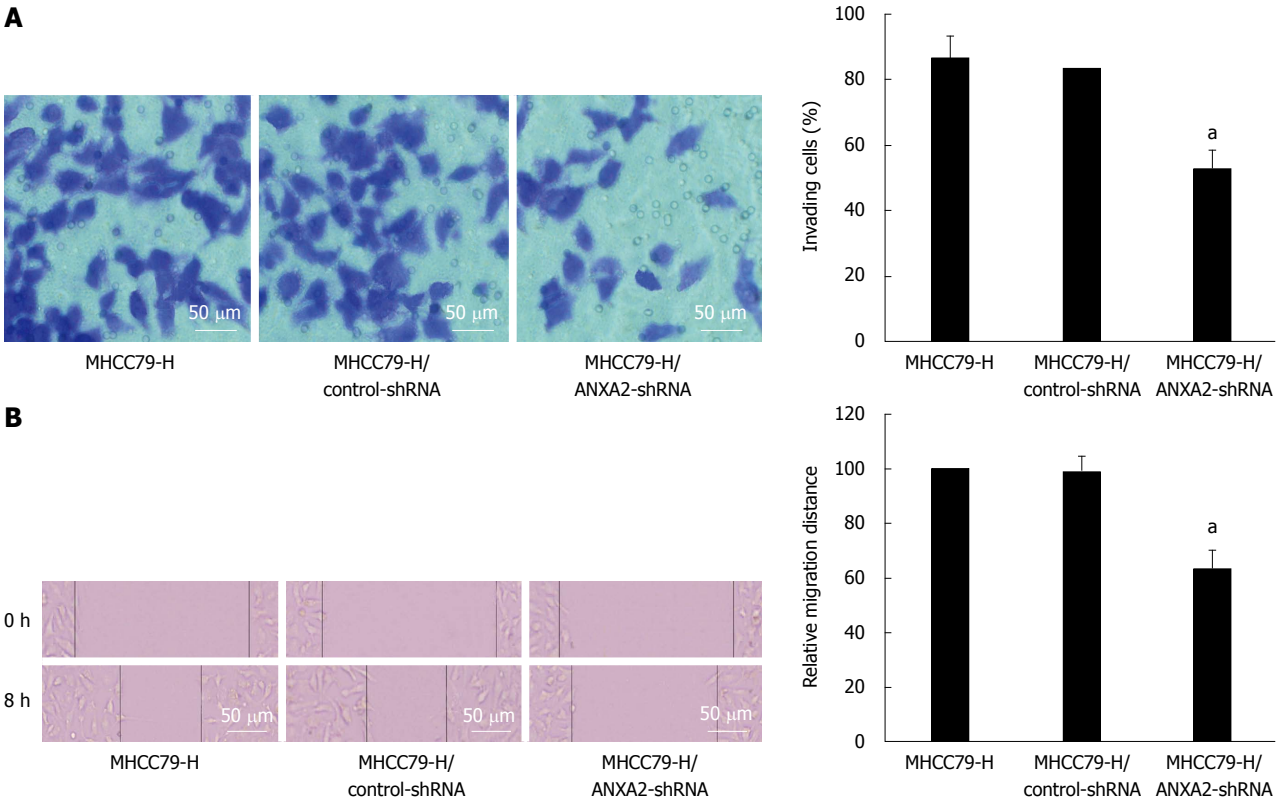

Figure 3 Suppressive effect of small hairpin RNA -mediated annexin A2 silencing on the invasion and migration potential of MHCC97-H cells. A: Representative images of invasive cells (stained with crystal violet) from: the MHCC97-H group; the MHCC97-H/control-small hairpin RNA (shRNA) group; the MHCC97-H/annexin A2 (ANXA2)-small hairpin RNA (shRNA) group; B: Representative images of cell migration. [a]*P* < 0.05 *vs* MHCC97-H cells.

Table 3 Intensity of ANXA2 expression in xenograft tumours

Group	*n*	ANXA2 intensity				*Z*
		-	+	++	+++	
MHCC97-H	4	0	0	0	4	
MHCC97-H/control-shRNA	4	0	0	1	3	1.000
MHCC97-H/ANXA2-shRNA	4	1	3	0	0	2.530[a]

[a]*P* < 0.05 *vs* the MHCC97-H group. shRNA: Small hairpin RNA.

tumour growth curve over 21 d indicated that ANXA2 silencing in MHCC97-H cells reduced their tumorigenic potential *in vivo* (Figure 4C).

As shown in Figure 4D, the morphological characteristics of the xenograft tumours derived from MHCC97-H/ANXA2-shRNA cells were not fundamentally different from the tumours derived from the other cell types. Similar to the *in vitro* observations, ANXA2 expression was mainly localized to the cellular membrane in the MHCC97-H/ANXA2-shRNA tumour tissues and to both the cellular membrane and cytoplasm in the MHCC97-H tumour tissues and the MHCC97-H/control-shRNA tumour tissues. Moreover, the ANXA2 expression level in the xenograft tumours of the MHCC97-H/ANXA2-shRNA group was significantly lower than that in the xenograft tumours of the MHCC97-H group (Table 3). The ANXA2 expression levels in the xenograft tumours of the MHCC97-H group and the MHCC97-H/control-shRNA group were not significantly different.

DISCUSSION

In the present study ANXA2 was found to be up-regulated in HCC cells, particularly those with high metastasis potential and invasion ability. Furthermore, shRNA-mediated silencing of ANXA2 was shown to inhibit the invasion, migration, and tumorigenic potential of hepatoma cells. These results not only provide further insight into the pathogenic mechanisms of HCC, but also suggest ANXA2 as a potential target of future molecular therapies.

Enhanced expression and activation of ANXA2 in cancerous tissues has been reported for many different tumour types, including HCC[14,24,25], which led investigators to consider the potential anti-tumour benefit of inhibiting its gene expression or protein phosphorylation. Knockdown of ANXA2 in glioma cells led to a remarkable decrease in tumour size and suppression of tumour progression and proliferation[26]. Similarly, our results showed that the proliferation ability of MHCC97-H/ANXA2-shRNA cells was significantly suppressed both *in vitro* and *in vivo*. In another previous study of non-small cell lung cancer (NSCLC), ANXA2 knockdown in human adenocarcinoma A_{549} cells led to defective proliferation *in vitro* and reduced tumour growth *in vivo*; additionally, the knockdown cells were arrested at the G2 phase[27]. In the current study, MHCC97-H cells with shRNA-mediated silencing of ANXA2 showed a significantly lower percentage of cells in the S phase than the MHCC97-H cells

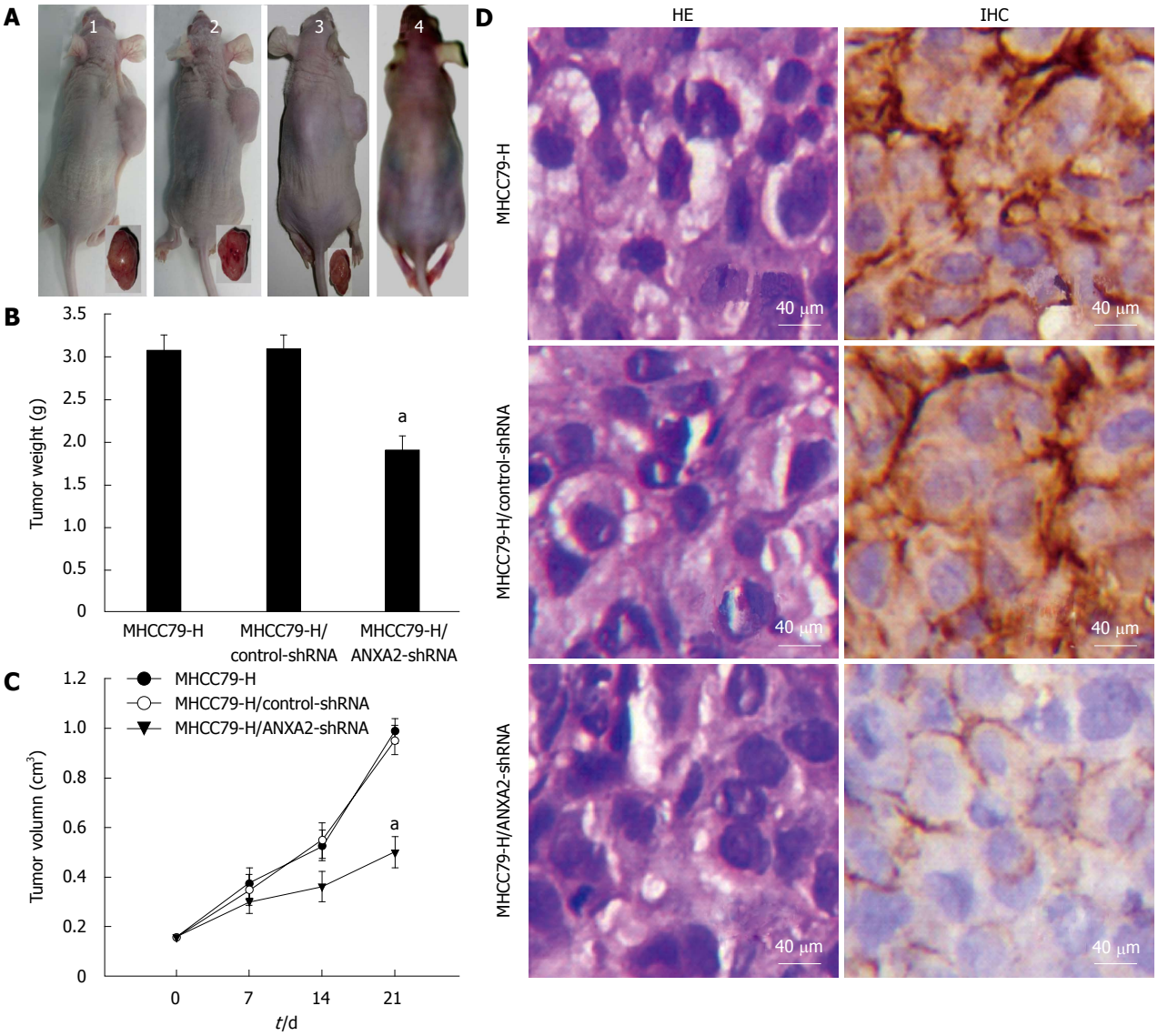

Figure 4 Inhibitive effect of small hairpin RNA-mediated annexin A2 silencing on xenograft tumour growth *in vivo*. A: Representative images of xenografted and control mice and resected tumours. The MHCC97-H (untransfected) group (1); the MHCC97-H/control-small hairpin RNA (shRNA) group (2); the MHCC97-H/annexin A2 (ANXA2)-shRNA group (3); the blank control group (4). Tumorigenic nude mice appeared obviously emaciated, especially the those in the MHCC97-H group and MHCC97-H/control-shRNA group; B: Average tumour weights. $^{a}P < 0.05$ *vs* MHCC97-H group; C: Tumour growth rates. $^{a}P < 0.05$ *vs* MHCC97-H group at post-injection day 21; D: Representative immunohistochemical analysis and hematoxylin and eosin staining results (× 400). The density of ANXA2 staining (brown) in the cytoplasm of MHCC97-H/ANXA2-shRNA cells was obviously lower than that for the MHCC97-H cells or the MHCC97-H/control-shRNA cells. ANXA2 was mainly localized in the membrane of the MHCC97-H/ANXA2-shRNA cells, and localized in both the membrane and cytoplasm of the MHCC97-H cells and the MHCC97-H/control-shRNA cells. The morphological characteristics of subcutaneous xenograft tumours derived from MHCC97-H/ANXA2-shRNA cells were not fundamentally different from the other tumours.

with endogenously enhanced ANXA2. This results from both studies indirectly indicate a promising anti-proliferative effect for ANXA2 silencing.

While previous studies have shown that increased ANXA2 levels are associated with enhanced tumour cell proliferation and HCC development[21,27], our study achieved a relatively low inhibition of tumour growth upon ANXA2 silencing. In the NSCLC study described above, the downstream effects of ANXA2 silencing were explored and a significant effect on p53 expression was discovered[27]. It is possible that ANXA2 may facilitate cell proliferation partly through the regulation of p53 *via* JNK/c-Jun in HCC, since disruption of the p53/miRNA-34 axis has been shown to result in abnormal apoptosis and tumour progression[28]. Furthermore, since ANXA2 is only one of the increased proteins in HCC[1,2], ANXA2 deficiency is expected to only partly, rather than wholly, suppress tumour growth.

Previous molecular studies have determined that ANXA2 promotes invasion and migration of human HCC cells *in vitro via* its interaction with HAb18G/CD147[18,21]. Depletion of HAb18G/CD147 produced a rounded morphology, which is associated with amoeboid movement, while depletion of ANXA2 resulted in an elongated morphology, which is associated with mesenchymal movement. HAb18G/CD147 was also found to promote

cell motility of HCC cells by regulating ANXA2-activated RhoA and Rac1 signalling pathways[18,21]. Plasmin, a downstream factor of ANXA2 activity, is essential for metastatic progression due to its activation of metalloproteinases and degradation of extracellular matrix components[10,29]. In another previous study of human adenomyosis, ANXA2 was shown to function as a key mediator of metastasis and proangiogenesis of endometrial cells[30]. Accumulating evidence has suggested that interactions between ANXA2 and its binding partners contribute to the tumour microenvironment, and together act to enhance metastasis[10]. Our observations of ANXA2 silencing suppressing the invasion and migration potential of hepatoma cells suggest that the invasion and migration potential of HCC cells are correlated with ANXA2 expression level. Furthermore, our finding that ANXA2 silencing is sufficient to inhibit invasion of HCC cells support the notion that ANXA2 is a potential therapeutic target for treating tumour development (through angiogenesis) and progression (through metastasis)[31,32].

The effects of ANXA2 on p53 may modulate apoptotic processes, which are critically associated with cell survival and may also have effects on proliferation[33]. Additionally, ANXA2 has been characterized as one of the factor H ligands binding to apoptotic cells, and has been shown to promote cell apoptosis in systemic lupus erythematosus *via* this mechanism[34]. In the current study, obvious apoptosis was observed in MHCC97-H/ANXA2-shRNA, suggesting that ANXA2-shRNA could suppress the proliferation and invasion ability of hepatoma cells with high metastasis potential, possibly by promoting cell apoptosis. In a previous study of the expression characteristics and diagnostic value of ANXA2 in HCC, we discovered an intermediate state of ANXA2 level in HCC adjacent tissue[35]. In the current study, we defined the distribution profile of ANXA2 expression in HCC cells, with the primary localization occurring in the cell membrane and cytoplasm and some localization occurring in the nucleus.

In conclusion, the results presented herein suggest that ANXA2 is up-regulated in HCC cells with high metastasis potential and invasion ability, and demonstrated that shRNA-mediated silencing of ANXA2 inhibits the invasion, migration, and tumorigenic potential of HCC cells. Collectively, these data reveal a link between the level of ANXA2 expression and HCC metastasis, as well as suggest the potential utility of ANXA2 as a predictive biomarker for HCC prognosis and as a therapeutic target of molecular-based strategies. It, therefore, will be important to carry out further investigations to identify additional signalling modulators and to delineate pivotal regulatory mechanisms involving ANXA2 and HCC metastasis.

COMMENTS

Background

Cell invasion and tumour progression remain two of the major obstacles that must be overcome before hepatocellular carcinoma (HCC) is finally conquered. Annexin A2 (ANXA2) is a 36-kDa calcium-dependent, phospholipid-binding protein expressed on various cell types. In addition, ANXA2 expression is up-regulated in various tumour types, where it plays multiple roles in tumorigenesis and development.

Research frontiers

Up-regulated ANXA2 expression has been detected in clinical samples of HCC, and ANXA2 phosphorylation has been demonstrated as essential for invasion and metastasis of pancreatic cancer. Moreover, the mechanism by which ANXA2 promotes tumour metastasis has been defined as induction of the conversion of plasminogen to plasmin. However, the effect of small hairpin RNA targeting ANXA2 on biological behaviours of hepatoma cells has not yet been reported. In the present study, ANXA2 overexpression was found in MHCC97-H cells, a novel HCC cell line with high metastasis potential, which was then used to investigate the effects of ANXA2 silencing on cell invasion, migration, and tumorigenic potential of HCC.

Innovations and breakthroughs

This study provides the first reported evidence of endogenous ANXA2 up-regulation in HCC cells with high metastasis potential and invasion ability, and of ANXA2 deficiency inhibiting the invasion, migration, and tumorigenic potential of HCC cells. Collectively, these data not only reveal a link between the level of ANXA2 expression and HCC metastasis, but also indicate the potential utility of this factor as a predictive biomarker for HCC prognosis and as a potential therapeutic target.

Applications

The results provide further insight into the pathogenesis of HCC. These data provide foundational knowledge for further expansion in future studies to identify the additional signalling modulators involved in this pathogenic mechanism. Moreover, the newly identified tumorigenic roles of AXNA2 suggest its utility as a target of anti-metastatic and anti-proliferative therapies.

Peer review

The paper is good design and illustration, deserved to be published.

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P- Reviewers Mazzanti R, Xu RY **S- Editor** Zhai HH
L- Editor A **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3802

World J Gastroenterol 2013 June 28; 19(24): 3802-3809
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Role of activin A in carbon tetrachloride-induced acute liver injury

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Supported by The Natural Science Foundation of China, Grants No. 30801005, No. 81273199 and No. 81270513; and the Project of Science and Development of Jilin Province (to Liu ZH)
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Received: February 16, 2013 Revised: April 12, 2013
Accepted: May 8, 2013
Published online: June 28, 2013

Abstract

AIM: To investigate the expression and role of activin A in a mouse model of acute chemical liver injury.

METHODS: Acute liver injury in C57BL/6 male mice was induced by intraperitoneal injection with carbon tetrachloride (CCl_4) (0.5 mL/kg, body weight) dissolved in olive oil (1:19 v/v). Mice were sacrificed 1, 3, 5 and 7 d after the treatment. The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum were examined and pathological changes of liver observed by hematoxylin and eosin staining to evaluate the liver injury. Activin A protein levels in serum and hepatic tissue homogenate of mice were detected by enzyme-linked immunosorbent assay, and the expression pattern of activin A protein in livers of mice was examined by immunohistochemistry. Activin type ⅡA receptor (ActRⅡA) and Smad3 expressions in the liver were analyzed by real-time quantitative reverse transcription-polymerase chain reaction. In order to further investigate the role of activin A, we also utilized activin A blocking experiment by anti-activin A antibody (500 μg/kg, body weight) injection into mouse tail vein.

RESULTS: In CCl_4-treated mice, serum ALT and AST levels were significantly increased, compared with that in control mice ($P < 0.01$). Furthermore, the serious necrosis was observed around hepatic portal areas in CCl_4-treated mice. Simultaneously, activin A levels in serum and hepatic tissue homogenate of mice treated with CCl_4 for 1, 3 and 5 d increased significantly, compared with that in control mice ($P < 0.01$). Activin A protein expression in hepatocytes not within the necrotic area was also upregulated in mice following CCl_4 treatment. Not only activin A, but also ActRⅡA and activin signaling molecule Smad3 mRNA expressions in injury liver induced by CCl_4 were significantly higher than that in control liver. In addition, levels of serum ALT and AST in CCl_4-treated mice were significantly decreased by injection of anti-activin A antibody to block endogenous activin A action, compared with that in CCl_4-treated mice by injection of immunoglobulin G instead of anti-activin A antibody ($P < 0.01$), and the severity of liver injury was also reduced remarkably.

CONCLUSION: These data show that activin A is involved in CCl_4-induced acute liver injury. Blocking activin A actions may be a therapeutic approach for acute liver injury.

Key words: Liver injury; Carbon tetrachloride; Activin A; Immunohistochemistry

Core tip: The objective of this study was to investigate the expression and role of activin A in acute liver injury. A carbon tetrachloride (CCl_4)-induced acute liver injury mouse model was used. Liver injury effects were examined by measuring alanine aminotransferase and aspartate aminotransferase levels in serum and liver pathological changes. Activin A protein expression levels were detected by enzyme-linked immunosorbent assay and immunohistochemistry. Activin type ⅡA receptor and Smad3 expressions in liver were analyzed by real-time quantitative reverse transcription-polymerase chain reaction. We found that activin A is involved in CCl_4-induced acute liver injury, suggesting activin A could be a potential therapeutic option for acute liver injury diseases.

Wang DH, Wang YN, Ge JY, Liu HY, Zhang HJ, Qi Y, Liu ZH, Cui XL. Role of activin A in carbon tetrachloride-induced acute liver injury. *World J Gastroenterol* 2013; 19(24): 3802-3809 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3802.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3802

INTRODUCTION

Activin A, a member of the transforming growth factor-β (TGF-β) superfamily, has a wide range of biological roles[1-3]. It is distributed widely in various tissues and produced by numerous cells including macrophages, T-helper 2 cells and hepatocytes[4-6]. It plays important roles in regulation of pituitary hormone release, neuron survival, hematopoiesis and the early development of embryos[7-10]. The expression of activin A is closely related to liver diseases, and abnormal expression of activin A and its signal proteins are found during the development of virus hepatitis, hepatic fibrosis, liver cancer and other diseases[11-14]. Activin A affects the function of hepatocytes by inhibiting DNA synthesis while stimulating the synthesis of the extracellular matrix of hepatic stellate cells, which can result in the hepatic fibrosis[2,11,12].

The liver is an important metabolic organ in the body, and can be injured by various factors, which include viral infection, trauma, and chemical reagents. Carbon tetrachloride (CCl_4)-induced liver injury is a classic model of chemical liver injury in mice[15]. Activin A expression increased significantly in CCl_4-induced chronic liver injury in mice, marking it as an important factor in liver fibrosis development[16]. However, it is still unclear whether activin A is involved in the process of CCl_4-induced acute chemical liver injury.

In the present study, the expression and role of activin A were investigated in CCl_4-induced acute chemical liver injury in mice. Further, the role of activin A in the process of acute liver injury was confirmed by *in vivo* blockade of activin A action.

MATERIALS AND METHODS

Reagents

CCl_4 was purchased from Beijing Chemical Factory (batch number 20050106). Olive oil was obtained from Beijing KeLipei Tsui olive oil Development Centers. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay kit was provided by NJBI (Nanjing, China). Anti-activin A antibody was obtained from Sigma Company. Trizol reagent was provided by Invitrogen Corporation. SYBR fluorescence quantitative reverse transcription-polymerase chain reaction (PCR) kit was purchased from Takara Company.

Animals

C57BL/6 male mice were provided by Animal Center of Jilin University (Changchun, China). All animal experiments were performed following an institutionally approved protocol in accordance with the Jilin University Guide for the care and use of laboratory animals.

Preparation of CCl_4-induced acute liver injury mouse model

C57BL/6 mice were randomly divided into the olive oil control group and the CCl_4 group (n = 24). In the control group, mice were treated with olive oil (10 mL/kg) by intraperitoneal injection; in the CCl_4 group, mice were injected intraperitoneally with CCl_4 (0.5 mL/kg) + olive oil (9.5 mL/kg) (1:19 v/v). Mice were executed 1, 3, 5 and 7 d after the treatment, serum was collected for the determination of transaminases and activin A levels, and hepatic tissues were obtained for pathological examination and immunohistochemical staining.

Determination of serum transaminases ALT and AST

The serum transaminases ALT and AST levels were detected by assay kit according to the manufacturer's protocol (NJBI, Nanjing, China). Briefly, 25 μL AST or ALT substrates and 5 μL serum were added into one well of polystyrene microtiter plates at 37 ℃ for 30 min. And then 25 μL of 2,4-dinitrophenylhydrazine was added in to all wells at 37 ℃ for 30 min. Finally, 250 μL of 0.4 mol/L sodium hydroxide was added to stop the reactions at room temperature for 15 min, and the absorbance at 510 nm in each well was measured with an enzyme-linked immunosorbent assay (ELISA) reader (BioRad Laboratories, Hercules, CA, United States). The levels of AST or ALT are expressed in U/L.

Pathological examination

The right lobe of each mouse liver was collected, fixed with 40 g/L paraformaldehyde for 24 h, embedded in paraffin, and sliced into a thickness of 3-4 μm. The sections were deparaffinized and pathological liver change were examined by hematoxylin and eosin (HE) staining.

Detection of activin A levels

To prepare the mouse hepatic tissue homogenate, 50 mg mouse liver was added to 1 mL lysis buffer (1% Triton

Table 1 Primer sequences for polymerase chain reaction

Genes	Primers	Sequences	Product size (bp)
GAPDH	Sense	5′-GATTGTTGCCATCAACGACC-3′	371
	Antisense	5′-GTGCAGGATGCATTGCTGAC-3′	
Activin A	Sense	5′-GAGAGGAGTGAACTGTTGCT-3′	514
	Antisense	5′-ATGACTGTTGAGTGGAAGG-3′	
ActR Ⅱ A	Sense	5′-ATTGGCCAGCATCCATCTCTTG-3′	296
	Antisense	5′-TGCCACCATCATAGACTAGATTC-3′	
	Antisense	5′-ACTCTGTGGCTCAATTCCTGCT-3′	
Smad3	Sense	5′-CGGTCAAGAGCCTGGTCAAGA-3′	241
	Antisense	5′-TTGAAGGCGAACTCACACAGC-3′	

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ActR Ⅱ A: Activin type Ⅱ A receptor.

X-100, pH 7.5, 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid, 2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium fluoride, 4 μg/mL leupeptin, 1 μg/mL aprotinin). The lysate was centrifuged for 30 min at 12000 rpm at 4 ℃ and the supernatant was harvested. The levels of activin A in serum and hepatic tissue homogenates of mice were determined by ELISA according to the manufacturer's protocol (R and D).

Real-time quantitative reverse transcription-PCR

Total hepatic tissue RNA was extracted using the Trizol reagent according to the manufacturer's protocol (Invitrogen), and then activin βA, Activin type Ⅱ A receptor (ActR Ⅱ A) and Smad3 mRNA expressions were examined by SYBR real-time-PCR kit using a ABI PRISM 7700 sequence detection system (Perkin-Elmer Applied Biosystems) in a two-stage, single-tube reaction. The following reaction conditions were used: stage one, 95 ℃, 10 s for 1 cycle; stage two, 95 ℃, 5 s and 60 ℃, 31 s for 40 cycles, collecting fluorescence in this phase; stage three, 95 ℃, 15 s, 60 ℃ 1 min, 95 ℃ 15 s for 1 cycle. Primers were synthesized by BECL (Shanghai, China), and the sequences are shown in Table 1. The reverse transcription (RT)-PCR products were quantitatively analyzed according to a standard cDNA calibration curve[17].

Immunohistochemical staining

The right lobe of the liver was fixed by 40 g/L paraformaldehyde for 24 h, embedded in paraffin, and then sliced into a thickness of 3-4 μm. The sections were deparaffinized, and 3% hydrogen peroxide (H_2O_2)-methanol was used to block endogenous peroxidase at room temperature for 30 min. Two percent of bovine serum albumin in 0.01 mol/L phosphate-buffered saline (PBS) was used to block nonspecific reactivity by a preincubation of section for 30 min. The sections were then incubated in anti-activin A antibody (1:400) at 4 ℃ overnight. The sections were washed with PBS, bound antibodies were detected with SP1 kit (ZSJQ, Beijing, China) and immunoreactive products were visualized in 0.05% diaminobenzidine and 0.03% H_2O_2. The sections were counterstained with hematoxylin, dehydrated, cleared, counted and observed under an Olympus microscope (BX51). In control staining, the sections were incubated with normal mouse immunoglobulin G (IgG) instead of anti-activin A antibody[18].

Blocking experiment

C57BL/6 mice adaptive feeding for 7 d were randomly divided into four groups (n = 12). The IgG control group (IgG Cont), in which each mouse was injected intraperitoneally with olive oil (10 mL/kg), 2 h later, injected by tail vein with normal IgG (500 μg/kg); the antibody control group (Anti-A Cont), in which each mouse was injected intraperitoneally with olive oil (10 mL/kg), 2 h later, injected by tail vein with anti-activin A antibody (500 μg/kg); the CCl_4 group (IgG + CCl_4), in which each mouse was injected intraperitoneally with CCl_4 (0.5 mL/kg) + olive oil (9.5 mL/kg), 2 h later, injected by tail vein with IgG (500 μg/kg); the antibody plus CCl_4 group (Anti-A + CCl_4), in which each mouse was injected intraperitoneally with CCl_4 (0.5 mL/kg) + olive oil (9.5 mL/kg), 2 h later, injected by tail vein with anti-activin A antibody (500 μg/kg). Sera and livers of these mice were collected 3 d after CCl_4 injection for determination of serum ALT and AST levels and pathological examination of livers.

Statistical analysis

The statistical software SPSS 10.0 was used to analyze all data. $P < 0.05$ was considered statistically significant.

RESULTS

The establishment of the acute liver injury mouse model

Change of serum transaminases ALT and AST levels is an important indicator of liver injury. Our results revealed that serum ALT and AST levels were significantly elevated on days 1, 3, 5 and 7 after the administration of CCl_4, compared with that in control group, $P < 0.01$ (Figure 1A). Furthermore, lobular architecture in the control mouse liver was clear and the hepatic cells arranged in neat rows by HE staining. In mouse liver 1 d following CCl_4 treatment, there were round necrotic lesions around the hepatic lobule portal area, and 3 d after CCl_4 treatment, there was inflammatory cell infiltration in the hepatic lobule portal area (Figure 1B).

Increase of activin A levels in mice treated with CCl_4

Activin A levels in serum and hepatic homogenates of experimental mice were determined by ELISA. The results showed that activin A levels increased significantly in serum and hepatic homogenate from CCl_4-induced acute liver injury mice for 1, 3 and 5 d, compared with that in control group, $P < 0.01$ (Figure 2). These levels peaked on days 1 and 3, and returned to normal levels by day 7.

Elevation of activin A protein expression in liver of mice treated with CCl_4

To explore the reason for increased activin A levels in the serum of CCl_4-treated experimental mice and the

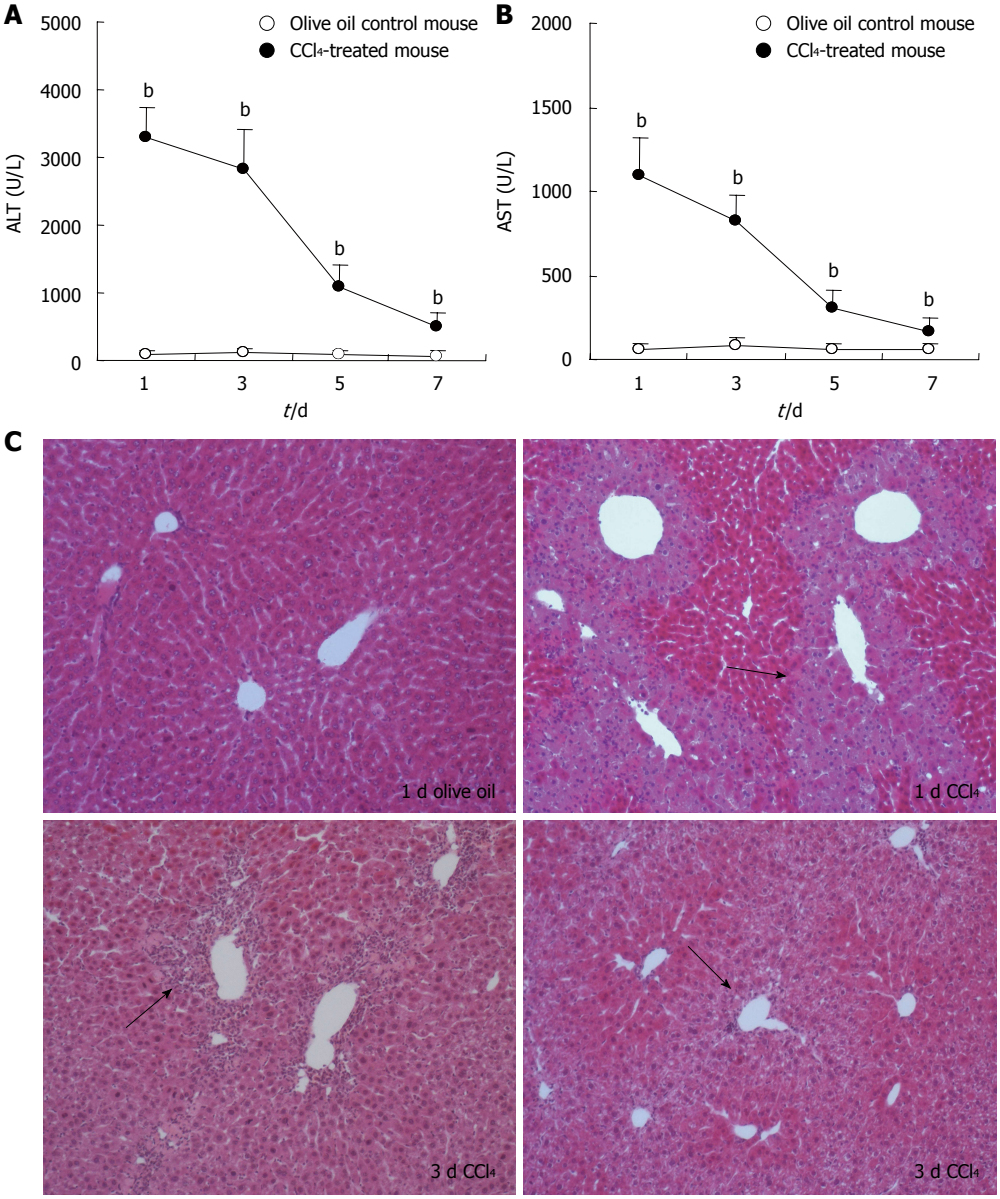

Figure 1 Examination of serum alanine aminotransferase and aspartate aminotransferase levels and pathological changes of liver in carbon tetrachloride-treated mice. A: Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were detected by enzyme-linked immunosorbent assay in olive oil control mouse and carbon tetrachloride (CCl_4)-treated mouse. $^bP < 0.01$ *vs* control; B: Pathological change of liver was analyzed by hematoxylin and eosin staining. Arrows represent lesion area (magnification, × 100).

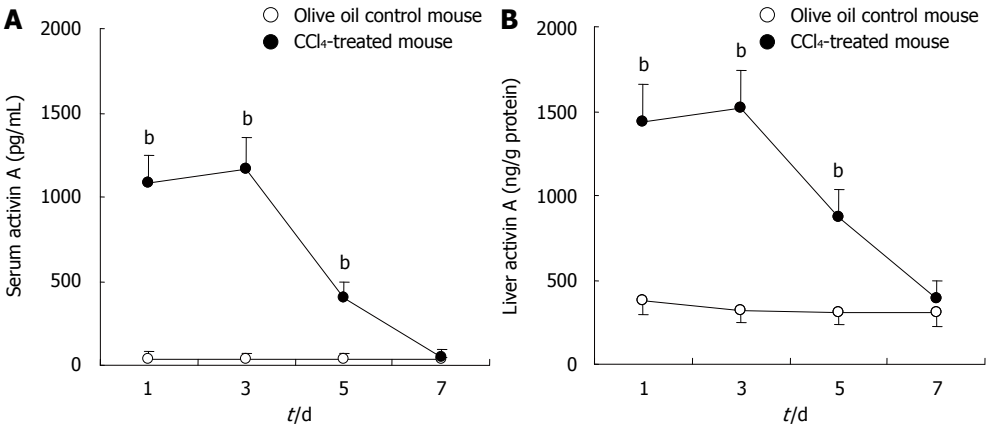

Figure 2 Detection of levels of activin A in serum and hepatic homogenates of mouse treated with carbon tetrachloride by enzyme-linked immunosorbent assay. CCl_4: Carbon tetrachloride. $^bP < 0.01$ *vs* control.

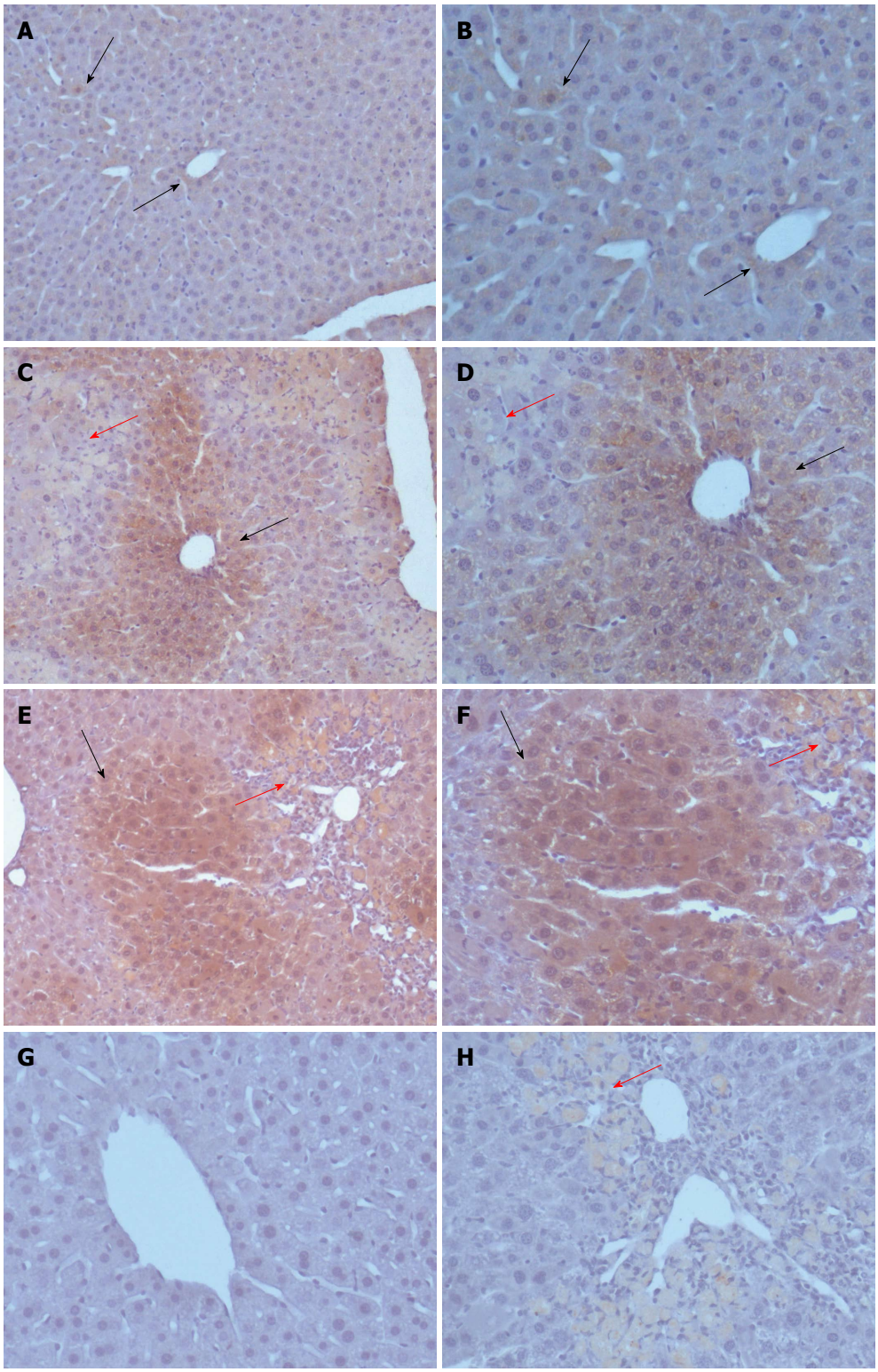

Figure 3 Expression of activin A protein in liver of mouse assessed by immunohistochemical staining. A, B: Activin A expression was examined by using anti-activin A antibody in the same liver tissues on day 1 after olive oil treatment; C, D: Activin A expression was examined by using anti-activin A antibody in the same liver tissues on day 1 after carbon tetrachloride (CCl_4) treatment; E, F: Activin A expression was examined by using anti-activin A antibody in the same liver tissues on day 3 after CCl_4 treatment; G, H: The procedural background control staining was represented by using normal mouse immunoglobulin G instead of anti-activin A antibody in livers of olive oil-treated and CCl_4-treated mice. Red arrows represent lesion area and black arrows represent positive staining for activin A. A, C, E: Magnification × 100; B, D, F, G, H: Magnification × 200.

expression pattern of activin A in acute liver injury, the expression of activin A protein was examined by immunohistochemical staining. The results revealed that activin A protein expression was detectable in the livers of olive oil-treated mice and in injured livers from CCl_4-treated mice. In addition, activin A protein expression in hepatocytes with non-necrotic area was up-regulated on days 1 and 3 after CCl_4 treatment (Figure 3).

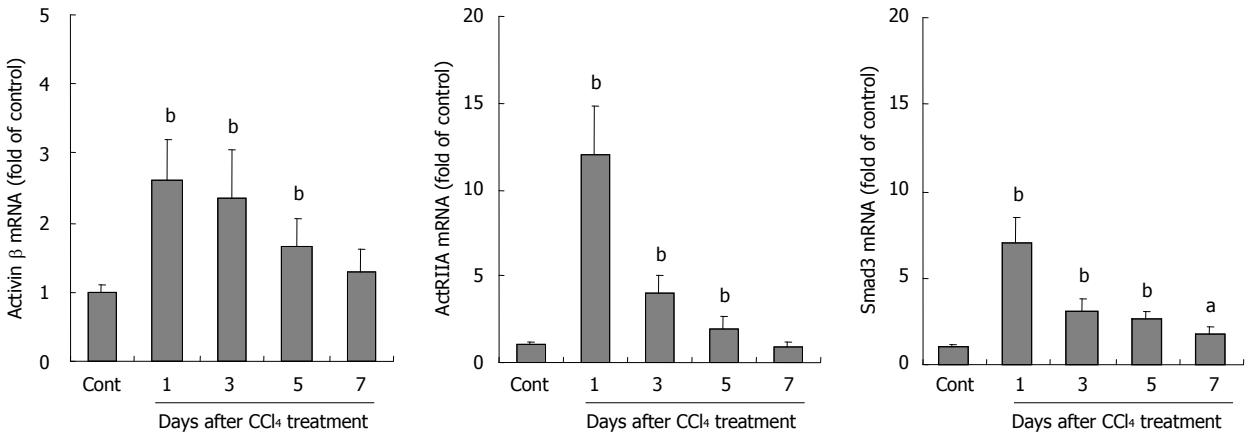

Figure 4 Assay of mRNA expressions of activin βA and activin signal molecules in liver of mouse by real-time quantitative reverse transcription-polymerase chain reaction. The mRNA levels in olive oil control group (Cont) were adjusted to 100%. All values (mean ± SD) were expressed as % of that in control. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

Up-regulation of activin βA, ActRIIA and Smad3 mRNA expressions in liver of mice treated with CCl4

Activin A is a dimeric glycoprotein formed by two βA subunits. To further investigate activin and its signal molecule expression in liver injury, activin βA, ActR Ⅱ A and Smad3 mRNA expressions were examined by real-time quantitative RT-PCR. The results showed that not only activin βA mRNA, but also ActR Ⅱ A and intracellular signal protein Smad3 mRNA expressions were significantly increased in livers from CCl_4-treated mice when compared with that in control group, $P < 0.01$ (Figure 4).

Protective effect of anti-activin A antibody on CCl4-treated mouse liver

In order to further confirm that activin A was involved in acute liver injury, pathological changes of livers in CCl_4-treated mice were examined after injection of an anti-activin A antibody to block endogenous activin action. The results revealed that serum ALT and AST levels in mice treated with anti-activin A + CCl_4 were significantly lower than that in IgG + CCl_4 control mice (Figure 5A, $P < 0.01$). Furthermore, the results showed that the necrotic severity around the hepatic lobule portal area in mice treated with anti-activin A + CCl_4 was remarkably reduced compared with that in IgG + CCl_4 control mice (Figure 5B).

DISCUSSION

Biologic activity of activin

Activin A as a multifunctional factor plays an important role in acute phase immune response, and its expression levels are increased in several inflammatory diseases such as septicemia, inflammatory bowel disease, and rheumatoid arthritis[19-22]. Recent studies have reported that activin A is closely related to the liver diseases hepatitis and hepatic fibrosis[3,14,16]. Activin receptors belong to serine/threonine kinase receptors of the TGF-β superfamily, are divided into ActR Ⅰ and ActR Ⅱ. Activin binds directly to the type Ⅱ receptor on the cell membrane, and then recruits the type Ⅰ receptor[23,24]. Further, the activated type Ⅰ receptor propagates the signal through a cascade reaction elicited by downstream Smad proteins into the nucleus to activate target genes[25,26].

Damage effects of CCl4 on the liver

CCl_4 is a chemical reagent that induces mouse liver injury, a classic chemical model for studying liver injury. The principle of injury is that in the function of cytochrome P450, CCl_4 generates chloromethyl free radicals (-CCl_3), which enable the peroxidation of the membrane lipids on the liver cell membrane or subcellular structure causing the rise of membrane permeability that results in the release of large number of ALT in the cytoplasm into blood. Another possibility is covalent binding of -CCl_3 and hepatic microsomal lipids and proteins that damage the integrity of the structure and function of hepatic cell membranes[27-29]. Additionally, various factors can further aggravate CCl_4-induced liver injury in mice, such as the increase of activin A expression in chronic liver injury[16].

Role of activin in liver injury

Abnormal expression of activin A is related to a variety of liver diseases, and activin A can directly induce hepatocyte apoptosis and inhibit hepatocyte proliferation and DNA synthesis[2,12,30]. To investigate whether activin A is involved in acute chemical liver injury, C57BL/6 mice were injected with CCl_4 through peritoneal cavity to establish acute liver injury model. The results showed that serum ALT and AST increased, and serious necrosis of hepatic tissue was observed by HE staining in CCl_4-treated mice. Simultaneously, activin A levels increased significantly in serum and hepatic tissue homogenate of mice treated with CCl_4. By immunohistochemical staining, the elevated expression of activin A protein was also observed in the injured livers of CCl_4-treated mice. Since CCl_4 induced necrotic death of hepatocytes in large area, which necrotic hepatocytes could not release cytokines, but the decomposition product of necrotic tissues as inflammatory stimulator could induce hepatocytes activation to secrete activin A. Thus, activin A protein was expressed in hepatocytes around the necrotic area and the

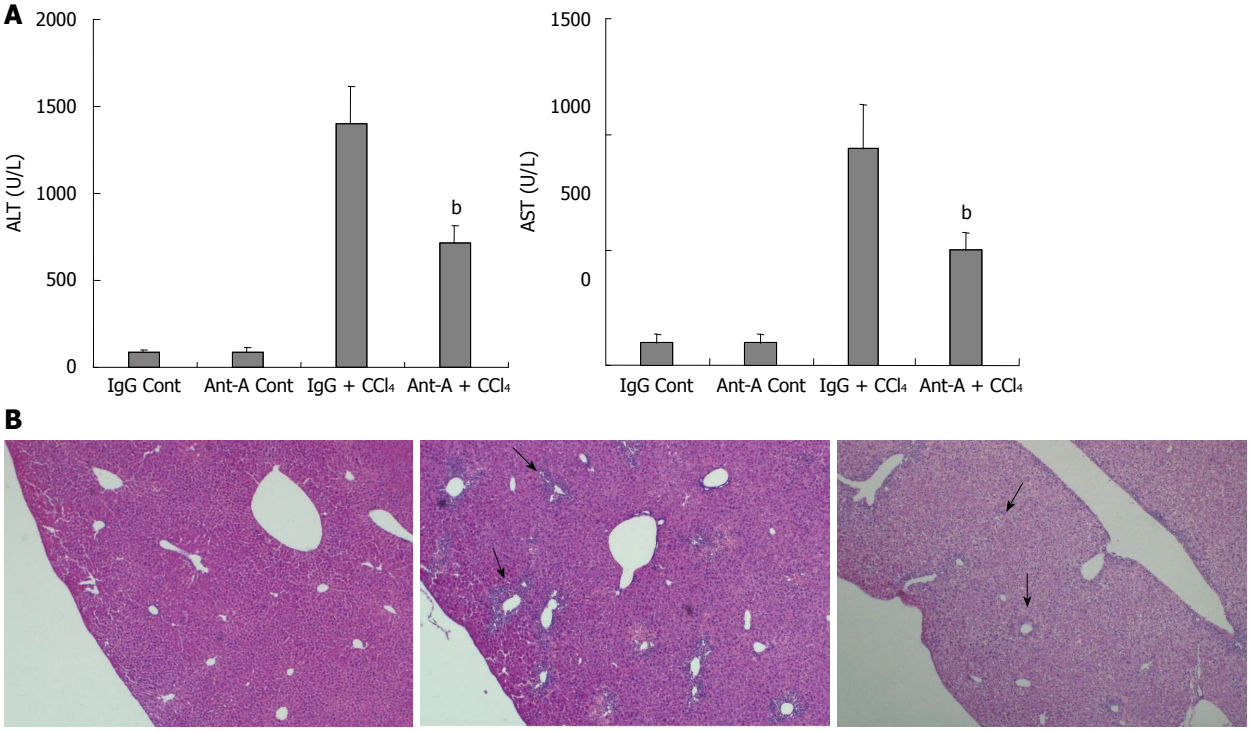

Figure 5 Effects of anti-activin A antibody *in vivo* on serum alanine aminotransferase and aspartate aminotransferase levels and pathological change of liver in carbon tetrachloride-treated mouse. A: Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were detected in mouse 3 d after carbon tetrachloride (CCl_4) treatment. Immunoglobulin G (IgG) + Cont, IgG control group; Anti-A + Cont, anti-activin A control group; IgG + CCl_4, IgG plus CCl_4 group; Anti-A + CCl_4, anti-activin A plus CCl4 group. $^bP < 0.01$ *vs* IgG + CCl_4 group; B: Pathological change of liver in mouse 3 d after CCl_4 treatment was analyzed by hematoxylin and eosin staining. Arrows represent lesion area (magnification × 40).

activated hepatocytes have lager clear nuclei. In addition, the real-time quantitative RT-PCR results revealed that in CCl_4-treated mice, activin A, ActR Ⅱ A and intracellular activin signal protein Smad3 mRNA expressions significantly increased. In order to further confirm the role of activin A in CCl_4-induced liver injury, pathological changes of livers in CCl_4-treated mice were examined after injection with anti-activin A antibody to block endogenous activin action. These results revealed that activin A might participate in the process of CCl_4-induced liver injury *via* inhibiting proliferation and DNA synthesis of hepatocytes to aggravate the CCl_4-induced liver injury, and blockade of activin A actions by anti-activin A antibody could significantly reduce the levels of serum transaminases and the necrosis severity of hepatic tissue in CCl_4-treated mice.

In summary, these data suggested that the activin A-ActR Ⅱ A-Smad signal transduction cascade was induced in CCl_4-treated mice. Further, activin A was involved in the process of CCl_4-induced acute chemical liver injury of mice in an autocrine/paracrine manner. Activin A may be a potential therapeutic target for acute liver injury disease.

COMMENTS

Background

Activin A is an important mediator of liver cells, not only in proliferation of hepatocytes, but also in activation of hepatic stellate cells, secretion of liver extracellular matrix, as well as the formation and development of liver injury. Carbon tetrachloride (CCl_4)-induced liver injury is a classical model of chemical liver injury in mice. Previous studies reported that the expression of activin A increased significantly in CCl_4-induced chronic liver injury in mice, and it was an important factor leading to liver fibrosis. The effect of activin A on the process of CCl_4-induced acute chemical liver injury has not been reported.

Research frontiers

Previous studies have demonstrated the effect of activin A on CCl_4-induced chronic liver injury in mice, however, the effect and mechanism of activin A on CCl_4-induced acute liver injury remains undefined.

Innovations and breakthroughs

New therapies that aim to block biological role of activin A in liver injury diseases are being actively explored and evaluated. Authors believe this study to be the first to explore the effect of activin A on acute liver injury.

Applications

The findings that block the biological action of activin A may be clinically relevant to potential liver injury therapeutics.

Terminology

Activin is a multifunctional factor of transforming growth factor-β superfamily. There are three types of activins formed by homo- or hetero-dimerization of two inhibin subunits (βA and βB), activin A (βAβA), activin B (βBβB) and activin AB (βAβB). Activin A is an important mediator in CCl_4-induced acute liver injury in mice, and blockade of biological action of activin A may be a potential therapeutics for acute liver injury diseases.

Peer review

This is a good descriptive study in which authors analyze the role of activin A in carbon tetrachloride-induced acute liver injury in mouse. The results are interesting and suggest that activin A is involved in the process of CCl_4-induced acute liver injury in an autocrine/paracrine manner. Further, the authors show that an antibody-mediated blockade of activin A biological action may provide insights into potential therapeutics.

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P- Reviewers Ding WX, Ohkohchi N, Shimizu Y, Tarantino G
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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3810

World J Gastroenterol 2013 June 28; 19(24): 3810-3818
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Quality of life following laparoscopic Nissen fundoplication: Assessing short-term and long-term outcomes

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Supported by EVO-funding of the Central Hospital of Central Finland
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Received: January 3, 2013 Revised: March 31, 2013
Accepted: April 9, 2013
Published online: June 28, 2013

Abstract

AIM: To investigate the quality of life following laparoscopic Nissen fundoplication by assessing short-term and long-term outcomes.

METHODS: From 1992 to 2005, 249 patients underwent laparoscopic Nissen fundoplication. Short-term outcome data including symptom response, side effects of surgery, endoscopy, and patient's perception of overall success were collected prospectively. Long-term outcomes were investigated retrospectively in patients with a median follow-up of 10 years by assessment of reflux symptoms, side effects of surgery, durability of antireflux surgery, need for additional treatment, patient's perception of success, and quality of life. Antireflux surgery was considered a failure based on the following criteria: moderate to severe heartburn or regurgitation; moderate to severe dysphagia reported in combination with heartburn or regurgitation; regular proton pump inhibitor medication use; endoscopic evidence of erosive esophagitis Savary-Miller grade 1-4; pathological 24-h pH monitoring; or necessity to undergo an additional surgery. The main outcome measures were short- and long-term cure rates and quality of life, with patient satisfaction as a secondary outcome measure.

RESULTS: Conversion from laparoscopy to open surgery was necessary in 2.4% of patients. Mortality was zero and the 30-d morbidity was 7.6% (95%CI: 4.7%-11.7%). The median postoperative hospital stay was 2 d [interquartile range (IQR) 2-3 d]. Two hundred and forty-seven patients were interviewed for short-term analysis following endoscopy. Gastroesophageal reflux disease was cured in 98.4% (95%CI: 95.9%-99.6%) of patients three months after surgery. New-onset dysphagia was encountered postoperatively in 13 patients (6.7%); 95% reported that the outcome was better after antireflux surgery than with preoperative medical treatment. One hundred and thirty-nine patients with a median follow-up of 10.2 years (IQR 7.2-11.6 years) were available for a long-term evaluation. Cumulative long-term cure rates were 87.7% (81.0%-92.2%) at 5 years and 72.9% (64.0%-79.9%) at 10 years. Gastrointestinal symptom rating scores and RAND-36 quality of life scores of patients with treatment success were similar to those of the general population but significantly lower in those with failed antireflux surgery. Of the patients available for long-term follow-up, 83% rated their operation a success.

CONCLUSION: For the long-term, our results indicate decreasing effectiveness of laparoscopic antireflux

surgery, although most of the patients seem to have an overall quality of life similar to that of the general population.

Key words: Laparoscopy; Nissen fundoplication; Long-term outcome; Antireflux; Gastrointestinal symptom rating scores; RAND-36

Core tip: Current evidence suggests that laparoscopic fundoplication is more effective than medical therapy for the short- and medium-term treatment of gastroesophageal reflux disease. This study examined short-term and long-term outcomes after laparoscopic Nissen fundoplication. Short-term outcomes were assessed by symptom response, side effects of surgery, endoscopy and the patient's perception of overall success. Long-term outcomes were examined by addressing multiple domains affected by the operation including reflux symptoms, side effects of surgery, durability of antireflux surgery, selective objective testing, need for additional medical or surgical treatment, the patient's perception of overall success, and long-term quality of life.

Kellokumpu I, Voutilainen M, Haglund C, Färkkilä M, Roberts PJ, Kautiainen H. Quality of life following laparoscopic Nissen fundoplication: Assessing short-term and long-term outcomes. *World J Gastroenterol* 2013; 19(24): 3810-3818 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3810.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3810

INTRODUCTION

Medical treatment with proton pump inhibitors (PPIs) heals esophagitis and suppresses heartburn in over 90% of patients[1-3]. Despite the fact that effective maintenance therapies do exist, patients often require lifelong medication, and compliance with long-term maintenance therapy is sometimes difficult to achieve[1-3]. Furthermore, concerns exist regarding long-term safety of PPI medication[4]. Current evidence suggests that laparoscopic fundoplication is more effective than medical therapy for the short- and medium-term treatment of gastroesophageal reflux disease[5-10]. In contrast to medical therapy that inhibits only acid reflux, antireflux surgery is designed to prevent reflux of all gastric content. This is achieved by creating a mechanical antireflux valve that increases lower esophageal sphincter pressure and markedly reduces the rate of spontaneous transient relaxations accompanied by reflux[11,12]. Side effects of Nissen fundoplication such as dysphagia, increased bloating and flatulence, and inability to belch or vomit may limit the success of antireflux surgery[12,13]. Patients may also need revision surgery to improve symptom control, or revert to the use of long-term medical therapy following recurrent symptoms.

Optimal management of chronic gastroesophageal reflux disease is uncertain as treatment options include either modern medical therapy using proton pump inhibitors or antireflux surgery[5]. In addition, long-term quality of life studies are limited. This study examined short-term and long-term outcomes after laparoscopic Nissen fundoplication. Short-term outcomes were assessed by symptom response, side effects of surgery, endoscopy and the patient's perception of overall success. Long-term outcomes were examined by addressing multiple domains affected by the operation including reflux symptoms, side effects of surgery, durability of antireflux surgery, selective objective testing, need for additional medical or surgical treatment, the patient's perception of overall success, and long-term quality of life.

MATERIALS AND METHODS

Study population

From 1992 to 2005, a total of 249 patients underwent laparoscopic Nissen fundoplication for gastroesophageal reflux disease at the fourth Department of Surgery, Helsinki University Central Hospital (120 operations done by three surgeons: Kellokumpu I, Haglund C and Roberts PJ from 1992 to 1997) and at the Central Hospital of Central Finland (129 operations done by Kellokumpu I from 1998 to 2005). Indications for antireflux surgery were residual symptoms while on medical therapy or endoscopic esophagitis after at least three months of intensive acid suppression therapy, or both; dependence on continuous medication and expenses; and pathological 24-h esophageal pH monitoring in symptomatic patients without preoperative endoscopic signs of erosive esophagitis. Diagnosis of gastroesophageal reflux disease was based on upper gastrointestinal endoscopy on 24-h ambulatory pH measurement according to standard criteria as established by Johnson *et al*[14], and on esophageal manometry to evaluate the lower esophageal resting pressure and pre-existing motility disorders[15]. Antisecretory medication was halted 7 d before assessment of esophageal function. Preoperative and short-term outcome data were entered in a prospective database. Long-term outcome data were collected retrospectively.

Surgical technique

Laparoscopic floppy Nissen fundoplication was performed by the standard 5-trocar technique as described[16]. Every patient had prophylactic anticoagulation and compressive elastic stockings. The fundus was routinely mobilized by ligating and dividing the short gastric vessels (Kellokumpu I and Haglund C)[16]. The Rossetti-Hell modification[17] with mobilization of the fundus posteriorly to the upper pole of the spleen without division of short gastric vessels was preferred by Roberts PJ. A short (20 mm) fundic wrap was constructed around the distal esophagus with 2-0 non-absorbable sutures. Nasogastric tubes (18 F) and bougies (36 F) allowed calibration of the fundic wrap and closure of the hiatus. A posterior hiatoplasty was performed with 2-0 non-absorbable stitches

when the esophageal hiatus was enlarged. The left side of the wrap was anchored to the cardia with an additional 1-2 sutures. A nasogastric tube was left in place until the following morning when intake of liquids was started. Solid intake was usually started on the second or third postoperative day. All patients received dietary instructions before their discharge.

Evaluation of short-term outcomes

Preoperative and short-term symptom assessment three months after surgery was based on a standardized questionnaire and interview about heartburn, regurgitation, and dysphagia according to the DeMeester-Johnson reflux scale[18]. Regurgitation was graded as: none = 0, mild = 1 (occasional after straining, large meal, or lying down), moderate = 2 (predictable with position change, straining, or lying down), severe = 3 (history of aspiration). Heartburn grades were none = 0, mild = 1 (recognizable symptom, no prior history of medical treatment), moderate = 2 (primary reason for medical visit or medical problem), severe = 3 (constant, marked disability in activities of daily living). Dysphagia grades were none = 0, mild = 1 (occasional with coarse foods), moderate = 2 (require liquids to clear), severe = 3 (history of meat impaction necessitating medical attention)[18]. In addition, the frequency of heartburn, regurgitation and dysphagia was rated as absent = 0, occasional (less than once every two weeks) as 1, frequent symptoms (more than once every two weeks but less than daily) as 2, and daily symptoms (at least one attack/day to constant symptoms) as 3[19].

Side effects of surgery such as disturbed belching ability, bloating (defined as abdominal swelling), and flatulence were analyzed with the patients assessing their degree of disturbance by any postoperative decrease (belching) or increase (flatus, bloating) on a scale where 0 = no change, 1 = mild change, 2 = moderate change, and 3 = severe change.

Upper gastrointestinal endoscopy was routinely performed preoperatively and at three months after surgery. Endoscopic classification of esophagitis was based on the modified Savary-Miller 5-grade classification[20]. The state and localization of the fundic wrap and the presence of paraesophageal hernia were checked postoperatively.

Overall short-term success of the operation was assessed by the patient's subjective perception of the outcome after surgery compared to that with medical therapy and patient's willingness to undergo surgery again for similar preoperative conditions.

Evaluation of long-term outcome

Long-term outcome was investigated in a subgroup of patients with a median follow-up of 10 years and was based on the same self-completed, standardized questionnaire about heartburn, regurgitation, and dysphagia[18,19], and side effects of surgery as in the short-term. Patients also reported time to symptom recurrence and the use of PPI medication as well as the date of any re-fundoplication. Finally, long-term outcome of surgery was assessed by a self-rated 7-point Likert scale describing outcome as good as expected (0), mildly (+1), moderately (+2) or markedly (+3) better or worse as -1, -2, -3 than expected and by willingness to undergo surgery again under similar preoperative conditions. During the long-term follow-up, objective testing including upper gastrointestinal endoscopy, barium examination, and esophageal function studies was not routinely performed.

Long-term quality of life assessment was done by the self-completed Gastrointestinal Symptom Rating Scale (GSRS)[21,22], and overall quality of life by RAND-36 (SF-36)[23,24]. The GSRS is a disease-specific instrument comprising 15 items, each rated on a 7-point Likert scale from no discomfort to very severe discomfort, and further divided into five dimensions: abdominal pain syndrome (abdominal pain, hunger pain and nausea), reflux syndrome (heartburn and acid regurgitation), diarrhea syndrome (diarrhea, loose stools and urgent need for defecation), indigestion syndrome (borborygmus, abdominal distension, eructation and increased flatus), and constipation syndrome (constipation, hard stools and feeling of incomplete evacuation). The higher the GSRS score, the more the patient suffers from gastrointestinal symptoms. The Finnish RAND-36[23] covers eight areas of health status, including physical functioning, role limitation due to physical health problems, bodily pain, general health, energy, social functioning, role limitation due to personal emotional problems, and emotional well-being. The raw responses were recoded according to the original version of the RAND-36, each item being scored on a 0-to-100 scale with higher scores indicating better quality of life[23,24]. Questionnaires were sent to the patients in May 2006 and returned by November 2006. Incomplete answers were completed by telephone interview.

Definition of failed antireflux surgery

Consistent with Lundell *et al*[25], laparoscopic Nissen fundoplication was considered failed if at least one of the following criteria was present: persistence or recurrence of moderate to severe heartburn or regurgitation[18] occurring more than once every two weeks (grade 2) or daily (grade 3)[19] or both; moderate to severe dysphagia[18] reported in combination with heartburn or regurgitation or both; the use of daily or weekly PPI medication; endoscopic evidence of erosive esophagitis Savary-Miller grade 1-4; pathological 24-h pH monitoring; and necessity to undergo redo surgery.

Ethics

The Central Hospital of Central Finland ethics committee approved this study (K-Sshp Dnro 47/2005). The causes of death were obtained from the National Cause of Death Registry.

Statistical analysis

The data are presented as mean ± SD, as median with interquartile range (IQR), or as counts with percentages. The 95%CIs are given for the most important outcomes.

Table 1 Baseline clinical characteristics *n* (%)

Follow-up	Study population (*n* = 249)	Long-term (*n* = 139)
Age, yr (mean ± SD)	51.2 ± 11.2	51.5 ± 10.6
Sex, male/female	131/118	76/62
ASA classification		
I	130 (52.0)	85 (61.2)
II	96 (38.4)	40 (28.8)
III	23 (9.2)	14 (10.1)
Duration of GERD in years, median (IQR)	6 (5.0-10.0)	6 (4.0-10.0)
Preoperative medication		
PPI	222 (89.2)	120 (86.3)
H2-antagonist and/or prokinetic	27 (10.8)	19 (13.7)
Duration of medical treatment, mo, median (IQR)	18 (8.0-36.0)	18 (6.0-36.0)
Preoperative *Helicobacter pylori* eradication	36 (14.6)	20 (14.4)
Preoperative symptom severity grade 1/2/3 as %[1]		
Heartburn	0/49.8/48.6	0/55.4/42.4
Regurgitation	5.6/87.6/4.4	5.0/89.2/4.3
Dysphagia	12.0/9.2/0	12.9/11.5/0
pH < 4 of total time, median (IQR) as %[2]	12.0 (8.0-18.7)	11.5 (7.4-19.1)
DeMeester score, median (IQR)	42.5 (28.4-71.3)	41.0 (26.2-69.3)
Lower esophageal sphincter pressure in mmHg[3], median (IQR)	11.0 (8.0-16.0)	11.0 (7.5-17.0)
Preoperative grading of esophagitis (Savary-Miller)		
None	68 (27.3)	36 (25.9)
Grade 1 (single erosive lesion, one longitudinal fold)	22 (8.8)	16 (11.5)
Grade 2 (multiple erosive lesions, more than one fold)	98 (39.4)	57 (41.0)
Grade 3 (circumferential lesions)	13 (5.2)	3 (2.2)
Grade 4 (chronic ulcer or stricture ± Gr. 1-3)[4]	5 (2.0)	3 (2.2)
Grade 5 (Barrett's esophagus ± Gr. 1-3)	43 (17.3)[5]	24 (17.2)[6]

[1]DeMeester *et al*[18]; [2]24-h pH monitoring in 232 patients; [3]Esophageal manometry in 228 patients; [4]Grade 4 (4 strictures, 1 ulcer); [5]Barrett's esophagus only 18 (7.2%) and associated with Gr. 1-2 erosive changes in 24 patients (9.6%) and grade 3 in 1 (0.4%); [6]Barrett's esophagus only 10 (7.2%) and associated with Gr. 1-2 erosive changes in 14 patients (10.1%). GERD: Gastroesophageal reflux disease; PPI: Proton pump inhibitor; IQR: Interquartile range.

Groups were compared using the Mann-Whitney *U* test or the χ^2 test. The statistical significance between groups in HRQL measures was evaluated by bootstrap-type analysis of co-variance because of the violation of distribution assumptions. Repeated measures for dichotomous outcomes were analyzed by Cochran's *Q* test and the marginal homogeneity test. Time-to-failure analysis was based on the product limit estimate (Kaplan-Meier) of the cumulative "survival" function. The Finnish general population values[23] for the eight Rand-36 domains were weighted to match the gender- and age-distribution of the study population. Statistical analyses were performed with Stata statistical software, release 12.1 (StataCorp, College Station, TX, United States).

RESULTS

Baseline clinical characteristics of the study population are shown in Table 1. Esophageal dilatations up to 18 mm in diameter were performed preoperatively in three of the four patients having esophageal stricture. Frequent or daily heartburn in 89.9% and regurgitation in 87.2% of the patients were the main presenting symptoms. DeMeester-Johnson severity grades are shown in Table 1, and operative data and surgical outcome in Table 2. While major intraoperative complications occurred in three patients, no deaths occurred. A distal esophageal perforation caused by diathermy scissors in one patient and by an inadvertent push of the calibration tube in another patient with esophageal stricture were suture-repaired and covered by the fundic wrap. A small fundic perforation caused by Harmonic scissors-induced thermal injury occurred postoperatively in one patient and necessitated a laparotomy and suture repair. Overall, 30-d morbidity was 7.6% (95%CI: 4.7-11.7) (Table 2). Pleural empyema in one patient with esophageal injury necessitated a thoracotomy and decortication. Comparing the two treatment centers and time periods, 30-d morbidity was similar (10.0% *vs* 5.4%, $P = 0.17$), but operation time [median 135 min (IQR 116.3-180 min) *vs* 75 min (IQR 65-90 min), $P < 0.001$] and postoperative hospital stay [median 3 d (IQR 2-4 d) *vs* 2 d (IQR 1-2 d), $P < 0.001$] were shorter during the latter period.

Short-term outcomes

Two of the 249 operated patients could not be contacted 3 mo after surgery, leaving 247 patients for short-term analysis. Based on symptom control and endoscopically-verified healing of erosive esophagitis, gastroesophageal reflux disease was cured in 243 of the 247 patients [98.4% (95%CI: 95.9%-99.6%)]. Only four patients reported short-term failures. Two had persisting reflux symptoms and erosive esophagitis (grade 2) with partly disrupted plication. One had erosive esophagitis (grade 1) and too distally placed and partly disrupted plication. Another one who had an apparently normal fundoplication had persistence of erosive esophagitis (grade 1). Three patients underwent a reoperation, and one was treated with

Table 2 Operative data and short-term surgical outcome *n* (%)

Variables	*n* = 249
Type of surgery	
Floppy Nissen fundoplication	213 (85.5)
Rosetti-Hell fundoplication	36 (14.5)
Hiatoplasty	235 (94.4)
Conversion to open surgery[1]	7 (2.8)
Operation time, min, median (IQR)	90 (70.3-135.0)
Mortality (30 d)	0 (0.0)
Intraoperative complications	3 (1.2)
Esophageal perforation	2 (0.8)
Burn injury of the fundus	1 (0.4)
Postoperative morbidity (30 d)[2]	19 (7.6)
General	12 (4.8)
Atelectasis + fever	5 (2.0)
Pneumonia	7 (2.8)
Surgical	9 (3.6)
Pleural empyema	1 (0.4)
Fundic perforation (burn injury)	1 (0.4)
Port-site hernia and bowel obstruction	1 (0.4)
Port-site bleeding	2 (0.8)
Excessive pain at port-site	1 (0.4)
Gastric dilatation	1 (0.4)
Wound infection	1 (0.4)
Urinary retention	1 (0.4)
Reoperation rate[3]	5 (2.0)
Relaparotomy	4 (1.6)
Thoracotomy and decortication	1 (0.4)
Postoperative hospital stay, d, median (IQR)	2 (2-3)

[1]Reasons for conversion: intraoperative esophageal perforation (n = 1), technical difficulties in dissection (n = 4), obesity (n = 1), problem with CO_2-insufflation (n = 1); [2]Figures in the column include some patients with more than one complication; [3]Reoperations: bowel obstruction (n = 1), fundic perforation (n = 1), port-site bleeding (n = 2), thoracotomy and decortication for pleural empyema (n = 1). IQR: Interquartile range.

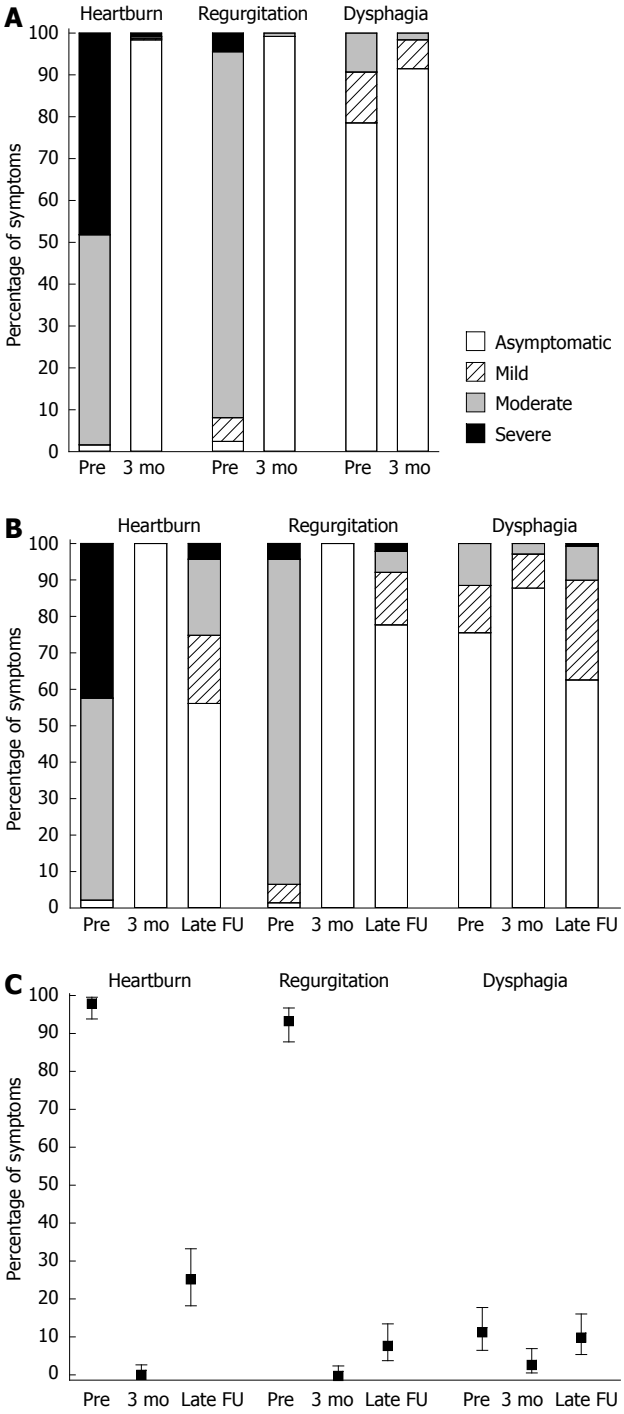

Figure 1 Change in point prevalence and severity of typical reflux symptoms. A: Patients were assessed for short-term symptoms (n = 247); B: Patients were assessed for long-term symptoms (n = 139); C: Measurement of the changes in long-term moderate to severe symptoms (n = 139). Differences between pre- and post-operative symptoms were significant for heartburn and regurgitation (P < 0.001), late dysphagia (P = 0.016). FU: Follow-up.

PPI-medication. None of the patients used PPI-medication at three months post-surgery. Barrett's esophagus without erosive changes was observed in 45 patients 3 mo after surgery and 2 patients had strictures, of which 1 was dilated. No cases of paraesophageal hernia were found. The short-term failure rate was similar between the two centers and time periods (1.7% *vs* 1.6%, P = 0.94). Two gastric ulcers and one case of acute gastritis were encountered in three patients with ulcer-like dyspepsia within 3-5 mo after surgery. All had biopsy-verified *Helicobacter pylori* infection and were treated by eradication therapy and followed-up by endoscopy and biopsies.

The patients observed typical reflux symptoms, especially heartburn and regurgitation, changed markedly after surgery. While 4 patients were asymptomatic for heartburn before and after surgery, 239 patients with reporting heartburn preoperatively [98.4% (95%CI: 95.8%-99.5%)] were completely symptom free (Figure 1A). Regurgitation was ameliorated in 239 [99.2% (97.0%-99.9%)] of the 241 patients, while 6 were asymptomatic before and after surgery (Figure 1A). Transitory new-onset dysphagia or worsening of preoperative dysphagia within 3 mo after surgery was reported by 176 patients (71.3%), but overall, dysphagia was significantly reduced from baseline. Dysphagia was alleviated in 45 [84.9% (72.4%-92.3%)] of the 53 patients having preoperative dysphagia. New onset dysphagia (grade 1 in all) was reported by 13 of the 194 patients (6.7%) without preoperative dysphagia.

A decreased ability to belch was reported by 164 [66.4% (60.1%-72.3%)], increased flatulence by 183 [74.1% (68.2%-79.4%)] and increased bloating by 61 [24.7% (19.4%-72.3%)] of the 247 patients. The ability to vomit could not be evaluated at three months.

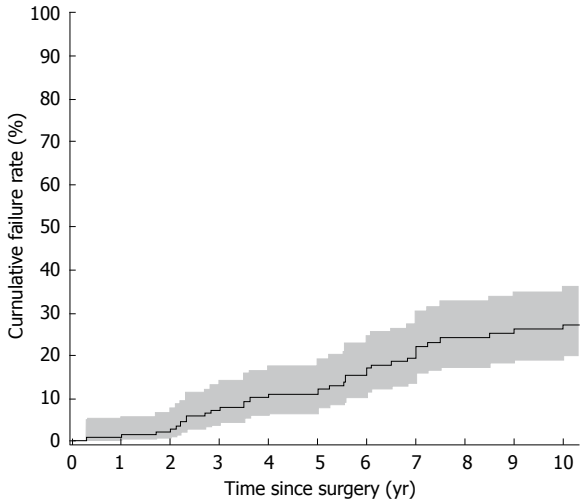

Figure 2 Cumulative failure rates for laparoscopic Nissen fundoplication. Surgical failure is shown as a function of time after median follow-up of 10 years (n = 139) (gray part shows 95%CI).

At their first clinical visit, 235 [95.1% (95%CI: 92.7%-98.0%)] patients reported better outcome after antireflux surgery than with preoperative medical treatment and were willing to have the procedure repeated in situations similar to preoperative ones.

Long-term outcomes

A cohort of the first 180 consecutive patients operated on between 1992 and 2002 was selected to obtain a median follow-up time of 10 years until November 2006. Of these, 14 had died of the following non-gastroesophageal reflux disease (GERD), related causes: myocardial infarctions (n = 3), intoxications (n = 2), cerebral infarction, pulmonary embolism, aortic rupture, gastric cancer, pneumonia, necrotizing fasciitis, chemotherapy-related sepsis, cerebral trauma and intracranial hemorrhage, and severe myasthenia gravis. In addition, 1 patient had severe dementia, 1 had undergone esophageal resection for Barrett's esophagus-related adenocarcinoma, 3 returned incomplete questionnaires, and 22 patients could not be contacted, leaving 139 available for evaluation of late outcomes [median follow-up time 10.2 years (IQR 7.2-11.6 years), response rate 85%]. During the long-term follow-up, 24-h pH monitoring and endoscopy was done in 32 patients; 6 showed endoscopically verified disrupted fundoplication and pH < 4 median 25.1% (IQR 13.0%-42.8%) of total time, and 26 had intact fundoplication and pH median 0.6% (IQR 0.1%-2.4%).

The crude long-term failure rate was 24.5% (34 of 139 patients): 6 patients (4.3%) underwent reoperation for symptomatic and objective recurrence caused by defective plication, 16 (11.5%) had heartburn, regurgitation, dysphagia and PPI medication on a daily/weekly basis, 11 patients (7.9%) not using PPI medication reported frequent/daily symptoms, and 1 was asymptomatic while on medication (Figure 1B and C). The cumulative 5-year failure rate was 12.3% (95%CI: 7.8%-36.0%) and the 10-year failure rate 27.1 % (20.1%-36.0%) (Figure 2), and cure rates 87.7% (81.0%-92.2%) and 72.9% (64.0%-79.9%), respectively. One patient underwent a reoperation for paraesophageal hernia and one for late trocar-site hernia.

Decreased belching ability in the long-term was reported by 61.2% (52.5%-69.3%), increased rectal flatulence by 91.4% (85.4%-95.5%) and bloating by 71.9% (63.7%-79.2%). Total inability to vomit was reported by 43 patients (30.9%).

The mean GSRS score describing overall discomfort related to gastrointestinal symptoms was 1.9 (95%CI: 1.8-2.0) in patients cured by antireflux surgery and 2.8 (2.4-3.1) in those with failed antireflux surgery ($P < 0.001$). Mean scores for all GSRS dimensions in comparison to a healthy control population are shown in Figure 3A. The failure of antireflux surgery and post-fundoplication side effects were reflected in the reflux syndrome score and indigestion syndrome score. Mean values of RAND-36 QoL scores were similar to the scores of the Finnish age- and gender-matched reference population for patients without treatment failure but were significantly impaired for those with failed antireflux surgery (Figure 3B).

Long-term outcomes were subjectively graded as good as expected (0) by 18.0%, moderately better (+2) by 14.4%, markedly better (+3) by 50.4%, and worse (-1) 5.8%, moderately worse (-2) 8.6% and markedly worse (-3) by 2.9%. At the time of this survey, 118 patients [84.9% (95%CI: 77.8%-90.4%)] were willing to undergo surgery again under similar preoperative conditions, whereas 21 patients (15.1%) chose medical treatment as a better initial treatment option.

DISCUSSION

Laparoscopic Nissen fundoplication has become the method of choice in antireflux surgery providing good short-term outcome in over 90% of patients, an associated morbidity rate of less than 10%, and only a 5% incidence of new-onset dysphagia[5-10,12,13]. Here, we show that laparoscopic Nissen fundoplication effectively abolished heartburn and regurgitation, alleviated most preoperative dysphagia, and cured erosive esophagitis in most patients in the short term. This was achieved with low morbidity, a short hospital stay, a low incidence of new-onset dysphagia, and good patient satisfaction, with approximately 95% of the patients reporting a better outcome after surgery compared with non-surgical medical therapy. Mechanical side effects of total fundoplication may lead to a functional obstruction in the gastroesophageal junction and to an inability to vent air from the stomach[12]. As a result, we observed common postoperative side effects including inability to belch, abdominal bloating, and increased flatulence. However, these mild side effects did not seem to limit the success of antireflux surgery.

Long-term outcomes after laparoscopic antireflux surgery in this study were examined in multiple domains including symptom response, side effects of surgery, durability of the antireflux surgery, patient's subjective

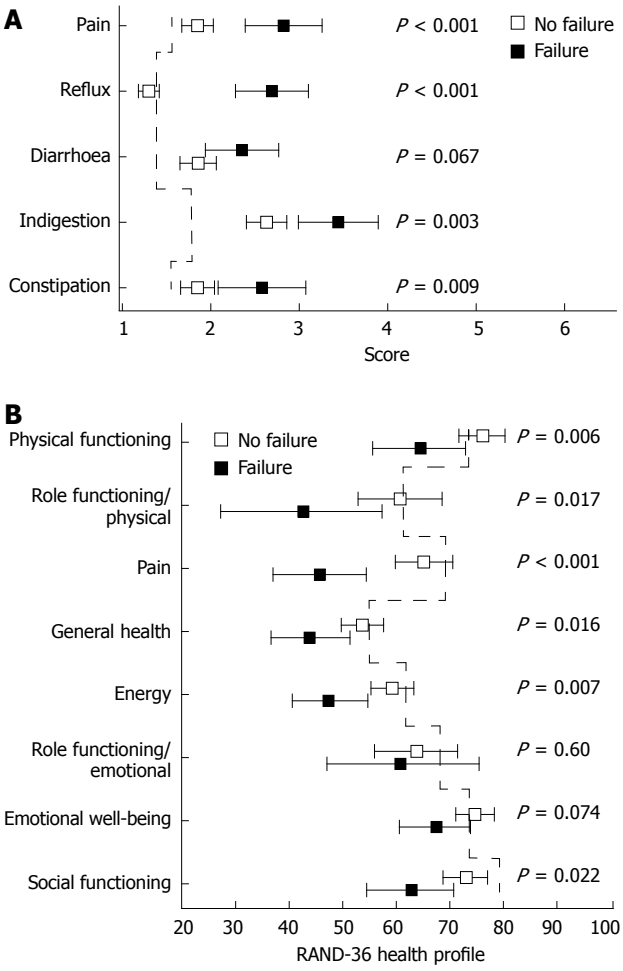

Figure 3 Comparing successful *vs* failed laparoscopic Nissen fundoplication by rating common gastrointestinal symptoms. A: Gastrointestinal symptom rating scores (GSRS) according to treatment success or failure (dotted line = healthy controls[22]); B: RAND-36 scores according to treatment success or failure (dotted line = age-matched and sex-matched general population[23]). *P* values were age- and sex-adjusted for treatment success *vs* failure.

perception of the overall success of the operation, and quality of life. In addition, treatment failure was defined according to composite criteria in contrast to previous studies[26-30] expressing the clinical outcome as the point prevalence of reflux-associated symptoms and patient satisfaction. However, our 5-year (88%) and 10-year (73%) cure rates compare well to previous studies reporting long-term control of reflux in 74%-90% of patients[26-31]. Our results also compare well to the nationwide long-term outcome after laparoscopic antireflux surgery in Sweden demonstrating a treatment failure in 25%-29% of the patients[32]. Only one randomized study comparing medical and surgical therapies for GERD and including patients with good response to PPI therapy, has reported similar remission rates at five years (85% *vs* 92%) between laparoscopic antireflux surgery and modern antisecretory medication[9]. Similar outcomes after laparoscopic Nissen fundoplication were obtained in our study despite the inclusion of patients reporting residual symptoms while on medical antisecretory treatment.

Patient well-being or quality of life are among the most important outcome measures in the field of functional surgery. The GSRS is a useful patient-related scale for evaluating the outcome of treatment for GERD[21]. In our study, the GSRS reflected well the long-term success of antireflux surgery as well as the side effects of antireflux surgery; it differentiated patients experiencing treatment failure from those being cured and from healthy controls[22]. Certain side effects, including increased bloating and rectal flatulence, manifest as indigestion syndrome and seem to be inevitable for most patients following fundoplication.

Overall quality of life was evaluated with RAND-36 (SF-36) because it is well validated in Finnish[23]. Consistent with previous studies[26,30] our results revealed that the quality of life of patients with long-term treatment success is similar to that of an age- and sex-matched general population. On the other hand, failed antireflux surgery and symptom recurrence significantly worsened quality of life in most dimensions.

Patients were also given a chance to evaluate the result of their surgery in the long-term. Here, 83% of the patients rated their surgical result to be as good as or better than expected, while only 17% felt that their surgical result was only fair or poor. Similarly, 118 patients (85%) were willing to undergo surgery again under similar preoperative conditions.

A major challenge with this study and other similar studies was its retrospective nature and the lack of systematic long-term assessment by endoscopy and ambulatory 24-h pH monitoring[31]. The significance of the use of antireflux medication in the long term is also debatable without objective measurements and may not be a reliable marker of surgical failure. On the other hand, we used standardized symptom questionnaires, composite criteria, and quality of life assessment to define treatment failures. Furthermore, it is the symptom response experienced by patients that ultimately determines the success or failure of antireflux surgery and quality of life, not the change in objective measurements. It is also well known that the outcome after antireflux surgery is dependent on surgeon's experience and quality of the surgery. Satisfactory short-term outcomes indicate, however, that the quality of surgery has been as good in this study as elsewhere. The long-term outcome was investigated in 139 of the first 180 consecutive patients selected for the 10-year follow-up. The compliance in this study compares favorably with that in earlier studies with 85% of the eligible patients returning the questionnaires for long-term evaluation.

In the short-term, laparoscopic antireflux surgery effectively alleviated symptoms of gastroesophageal reflux disease and cured erosive esophagitis. Postoperative adverse effects were usually mild and patient satisfaction was good. For the long-term, our results indicate decreasing effectiveness of laparoscopic antireflux surgery, although most of the patients seem to have an overall quality of life similar to that of the general population.

COMMENTS

Background

Optimal management of chronic gastroesophageal reflux disease (GERD) is uncertain because treatment options can include either modern medical therapy that uses proton pump inhibitors or antireflux surgery.

Research frontiers

Only one randomized study comparing medical and surgical therapies for GERD, has reported similar remission rates at 5 years. Data on long-term (10-year) cures and quality of life after antireflux surgery *vs* medical therapy are limited.

Innovations and breakthroughs

To assess long-term outcomes, the authors examined several domains affected by the operation and defined treatment failures based on composite criteria and quality of life assessment. The results show that the excellent short-term results after laparoscopic Nissen fundoplication deteriorate over time. Quality of life analysis with the Gastrointestinal Symptom Rating Scale reflected well the side effects of antireflux surgery and differentiated patients having treatment failure from those being cured and from healthy controls. Overall quality of life (RAND-36) of patients with long-term treatment success was similar to that of an age- and sex-matched general population. Failed antireflux surgery and symptom recurrence significantly worsened the quality of life in most dimensions.

Applications

By defining treatment outcomes with composite criteria and understanding how quality of life is affected by failed antireflux surgery, this study represents a potential new strategy for assessment of the long-term outcome after antireflux surgery.

Peer review

The authors examined the short- and long-term outcome and quality of life after laparoscopic fundoplication. By addressing several domains affected by the operation and using composite criteria to define treatment failure, they show the excellent short-term results after laparoscopic Nissen fundoplication deteriorate over time. They also show failed antireflux surgery is associated with reduced quality of life. Use of composite criteria and quality of life assessment to define treatment failure represents a potential new strategy for the assessment of long-term outcomes following antireflux surgery.

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P- Reviewers Clark J, Detry O **S- Editor** Wen LL **L- Editor** A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3819

World J Gastroenterol 2013 June 28; 19(24): 3819-3823
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Is alcoholic pancreatitis associated with enteroviral infection?

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Received: April 24, 2012 Revised: September 29, 2012
Accepted: October 30, 2012
Published online: June 28, 2013

Abstract

AIM: To investigate whether enteroviral infection might trigger acute pancreatitis in patients made susceptible due to high alcohol consumption.

METHODS: Patients with alcohol-induced acute pancreatitis were analyzed for signs of simultaneous or preceding enteroviral infection. We studied the serum samples of 40 patients hospitalized for alcohol-induced acute pancreatitis and 40 controls recruited from an alcohol detoxification center. Reverse transcription-polymerase chain reaction (RT-PCR) was used to detect enterovirus RNA and diagnose acute viremia. Immunoglobulin G (IgG), immunoglobulin A (IgA) and immunoglobulin M (IgM) enteroviral antibodies were measured using enzyme immunoassay to detect subacute and previous infections. The samples were considered positive when the antibody titers were ⩾ 15 IU. Furthermore, using RT-PCR, we studied pancreatic biopsy samples obtained during surgery from nine patients with chronic pancreatitis, one patient with acute pancreatitis and ten control patients with pancreatic carcinoma for evidence of persisting enteroviral RNA in the pancreatic tissue.

RESULTS: No enterovirus RNA indicating acute viremia was detected by RT-PCR in the serum samples of any patient or control. A high incidence of positive antibody titers was observed in both study groups: IgM antibodies had positive titers in 5/40 (13%) *vs* 4/40 (10%), $P = 0.723$; IgG in 15/40 (38%) *vs* 19/40 (48%), $P = 0.366$; and IgA in 25/40 (63%) *vs* 33/40 (83%), $P = 0.045$, patients and controls, respectively. Ten (25%) patients had severe pancreatitis and two (5%) required treatment in intensive care. The median length of hospitalization was 7 d (range: 3-47 d). The severity of acute pancreatitis or the length of hospitalization was not associated with enteroviral IgM, IgG or IgA antibodies. Five pancreatic biopsy samples tested positive with RT-PCR, three (8%) in the control group and two (5%) in the patient group ($P = 0.64$).

CONCLUSION: The rate of enteroviral infection is not increased in patients with alcohol-induced acute pancreatitis when compared to alcoholics with similar high alcohol use.

Key words: Pancreatitis; Alcoholic; Pancreatitis; Acute necrotizing; Enterovirus

Khan J, Nordback I, Seppänen H, Lappalainen-Lehto R, Järvinen S, Oikarinen S, Tauriainen S, Räty S, Hyöty H, Sand J. Is alcoholic pancreatitis associated with enteroviral infection? *World J Gastroenterol* 2013; 19(24): 3819-3823 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3819.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3819

INTRODUCTION

Although heavy alcohol consumption is known to be associated with the development of acute pancreatitis, surprisingly little is known of the actual mechanism behind this association. Furthermore, only a small proportion of heavy drinkers ever develop acute pancreatitis even during long-term follow up[1]. Excessive alcohol consumption has been reported to cause 9%-70% of all cases of acute pancreatitis[2-7]; being predominant in some countries (*e.g.*, United States, Hungary and Finland), whereas gallstones are predominant in many other countries such as China, Greece and Italy. While alcohol remains a clear risk factor for acute pancreatitis, a multitude of other factors that may be genetic or environmental could be involved in triggering or modulation of the disease.

One previously suggested co-factor possibly associated with the induction of acute alcohol-associated pancreatitis is enteroviral infection. Human enteroviruses typically cause mild respiratory or gastrointestinal infections, but are also associated with myocarditis and aseptic meningitis. Over 100 enterovirus serotypes have been identified, including the polio virus. Other enteroviruses are classified as *coxsackie A* and *B* viruses, *enteric cytopathogenic human orphan* viruses or as numbered serotypes (*e.g.*, *enterovirus 70*). The evidence suggesting an association between enteroviruses and acute pancreatitis is mostly derived from case reports[8-12] and historical serological studies[13,14]. Evidence of enterovirus infection in the pancreatic beta cells has been reported by several authors[15-17]. More recently, Ozsvár *et al*[18] reported a significant rise in *coxsackie B* virus antibody titers in acute and chronic pancreatitis patients. Recent animal studies further support a possible connection between enteroviral infection and pancreatitis[19-22]. Jerrells *et al*[23] reported that mice on an alcohol diet and infected with a strain of *coxsackie B* virus developed more severe pancreatitis than control mice, and that even typically avirulent strains produced severe pancreatitis in these mice. Clemens *et al*[24] showed that the pancreas of mice on an alcohol diet had impaired regeneration potential compared to control mice which may be associated with the severity of acute pancreatitis and the development of chronic pancreatitis. These studies suggest that enteroviruses may play a triggering role in at least a portion of human alcoholic pancreatitis.

To the best of our knowledge, there are no studies addressing the association between enteroviral infection and alcohol-associated acute pancreatitis in humans, where the alcohol intake of the non-pancreatitis controls has been comparable. The aim of this study was to ascertain whether patients suffering from alcohol-associated acute pancreatitis show evidence of simultaneous or preceding enteroviral infection in greater numbers than control subjects with similar recent alcohol consumption, but no previous or current pancreatitis. In addition, we analyzed pancreatic biopsy samples obtained from chronic pancreatitis patients and control patients during surgery to evaluate whether chronic pancreatitis specimens showed signs of persistent enteroviral genome in the pancreas.

MATERIALS AND METHODS

This study was a retrospective analysis of previously collected serum samples from a prospective study[25]. The study patients were recruited between January 2001 and November 2005. The samples for the first group, 40 patients hospitalized due to their first alcohol-associated acute pancreatitis, were collected during the first days of hospitalization. The samples for the control group, 40 alcoholics recruited from an alcohol detoxification center, were collected during their stay in the center. The patients were diagnosed with acute pancreatitis when they met the following criteria: acute epigastric pain that led to hospitalization, clinical signs consistent with acute pancreatitis together with serum amylase activity of at least three times the upper normal range, elevated serum inflammation markers (C-reactive protein concentration and leukocyte count), and/or the findings of acute pancreatitis on imaging. Alcohol was considered the probable etiology when the patient reported high alcohol intake during the alcohol use disorders identification test (AUDIT) or in a thorough interview of the patient or the family and other etiologies were excluded by laboratory testing and imaging[26]. Heavy alcohol consumption was similarly identified in the control subjects. Previously diagnosed pancreatitis or any acute illness were exclusion criteria when recruiting the control subjects.

The length of hospitalization, the development of complications and the need for and duration of treatment in the intensive care unit in alcohol-associated acute pancreatitis patients were recorded together with basic information such as body mass index (BMI), age and gender. Acute pancreatitis was considered severe when it met the Atlanta criteria[27]. The AUDIT questionnaire, amount of alcohol consumption (g/wk) preceding hospitalization and amount of smoking were elicited by a person specialized in addiction problems. The control group was matched according to age and reported amount of alcohol consumption. Thirty-two (80%) of the patients were male with a median age of 47 years (range: 18-73 years) and median BMI of 26 kg/m^2 (range: 19-34 kg/m^2). In the control group, 25 (63%) patients were male with a median age of 46 years (range: 22-66 years) and median BMI of 26 kg/m^2 (range: 16-34 kg/m^2). The median AUDIT scores were 22 (range: 5-38) in the patient group and 29 (range: 15-36) in the control group.

The biopsy samples were collected from 20 patients who underwent pancreatic surgery: one with alcohol-associated acute pancreatitis, nine with chronic pancreatitis and ten with pancreatic carcinoma. The development of chronic pancreatitis was alcohol associated in five and idiopathic in four patients. None of the patients with pancreatic carcinoma had a history of acute pancreatitis or excessive alcohol consumption. They were operated on between December 2001 and March 2006. The biopsy

samples were analyzed for the presence of enteroviral RNA using a highly sensitive reverse transcription-polymerase chain reaction (RT-PCR) method which amplifies a sequence common to all known enterovirus serotypes. The details of this method have been described earlier[28]. Frozen tissue samples were disrupted and homogenized using the TissueRuptor homogenizator (Qiagen, Hilden, Germany). RNA was extracted from the homogenized sample using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions.

The serum samples were stored at -70 ℃ during the interval between their acquisition and analysis. Evidence of enteroviral infection was analyzed by detecting immunoglobulin G (IgG), immunoglobulin A (IgA) and immunoglobulin M (IgM) class antibodies by enzyme immunoassay and by detecting enteroviral-RNA using the RT-PCR method described above. IgM class enterovirus antibodies were measured against a mixture of three enterovirus antigens (coxsackie virus B3, coxsackie virus A16 and echovirus 11) using a capture enzyme immunoassay as previously described[29]. IgG and IgA class antibodies were measured against a synthetic enterovirus peptide antigen (sequence KEVPALTAVETGAT-C derived from an immunodominant region of capsid protein VP1, which is a common epitope for several enteroviruses) as described earlier[30-32]. The samples were considered positive when the antibody titers were ⩾ 15 EIU.

Statistical analysis

Statistical testing was performed with SPSS statistical software using Pearson's correlation, χ^2 test, Mann-Whitney *U*-test and Fisher's Exact test. *P* values ⩽ 0.05 were considered statistically significant. This study was performed according to the Helsinki Declaration and was approved by the Ethics Committee of Tampere University Hospital.

RESULTS

Ten (25%) patients had severe pancreatitis according to the Atlanta criteria. Of these, six patients had necrotizing pancreatitis, one developed infected necrosis and three developed pseudocysts. Two patients required treatment in intensive care. The median length of hospitalization was 7 d (range: 3-47 d).

No enterovirus RNA was detected by RT-PCR in any patient or control subject. IgM antibodies had positive titers in 5/40 (13%) *vs* 4/40 (10%), *P* = 0.723; IgG in 15/40 (38%) *vs* 19/40 (48%), *P* = 0.366; and IgA in 25/40 (63%) *vs* 33/40 (83%), *P* = 0.045, patients and controls, respectively. The severity of acute pancreatitis or the length of hospitalization was not associated with enteroviral IgM, IgA or IgG antibodies.

Three pancreatic biopsy samples from patients with pancreatic carcinoma and two biopsy samples from patients with chronic pancreatitis tested positive for enteroviral RNA. The etiology of chronic pancreatitis was alcohol consumption in both patients. The tissue specimen from the patient with alcohol-induced acute pancreatitis was negative for enteroviral RNA.

DISCUSSION

In this study, we ascertained whether patients hospitalized for their first alcohol-induced acute pancreatitis had evidence of simultaneous or preceding enteroviral infection. In animal studies, enterovirus infection has been found to cause pancreatitis and, furthermore, simultaneous consumption of alcohol has been found to exacerbate the pancreatic insult. We hypothesized that enteroviral infection might be the triggering factor in at least some of the patients with their first alcohol-induced acute pancreatitis.

All the samples analyzed in this study were stored frozen. To the best of our knowledge, no studies have been reported on the possible adverse effects of prolonged storage and thawing of samples of enteroviral antibodies or on RT-PCR sensitivity. In general, repeated freezing and thawing may slightly alter the results observed, but the cycles generally do not affect samples to any clinically significant extent[33-35].

No evidence of acute viremia was found in any of the patients. Positive IgM antibodies reflect subacute disease and 13% of our patients tested positive, with a similar rate in the control group. We also report a relatively high number of patients with positive IgA and IgG antibody titers. However, this was also the case in the control group. IgG antibodies remain elevated long after the infection, while IgA antibodies usually disappear within a few months. Therefore, we suspect that this finding reflects the fact that our patients and controls were of lower socio-economic background with a tendency to acquire such infections at an increased rate when compared to the general population. An association between lower socio-economic status and increased enteroviral infection rate has previously been reported[36,37]. Thus, our findings do not suggest a role for enteroviral infection in the pathogenesis of alcohol-induced acute pancreatitis in humans, at least to a clinically significant extent. In fact, IgG and IgA class enterovirus antibodies tended to be at lower levels in the pancreatitis group, which may reflect the general immunosuppression associated with this disease.

A surprisingly high percentage of pancreatic tissue samples obtained during surgery, from patients operated on either for chronic pancreatitis or carcinoma of the pancreas, tested positive for enteroviral RNA in RT-PCR. In an earlier study, Lászik *et al*[38] studied pancreatic tissue specimens obtained during surgery for acute pancreatitis using *in situ* hybridization and reported no evidence of enteroviral infection in any of the samples. In the present study, we did not investigate whether enteroviral genome was present in the acini or the islets of Langerhans in the pancreas. Recent studies suggest a role for enteroviral infection in the genesis of type 1 diabetes[39], and, furthermore, direct beta cell involvement[15-17]. It is therefore possible, although not certain, that the high percentage

of enteroviral genome observed in the tissue samples in our study also came from the islets of Langerhans in this patient material.

In conclusion, we report no evidence of an increased rate of enteroviral infection in patients hospitalized for their first alcohol-induced acute pancreatitis when compared to alcoholics with similarly heavy alcohol consumption, but with no history or signs of acute or chronic pancreatitis. The rate of positive results in pancreatic tissue samples was clearly higher in our study than reported elsewhere, although the sample size was small.

COMMENTS

Background

Although heavy alcohol consumption is known to be associated with the development of acute pancreatitis, surprisingly little is known of the actual mechanism behind this association. Furthermore, only a small proportion of heavy drinkers ever develop acute pancreatitis even during long-term follow up.

Research frontiers

One previously suggested co-factor possibly associated with the induction of acute alcohol-associated pancreatitis is enteroviral infection. Human enteroviruses typically cause mild respiratory or gastrointestinal infections, but are also associated with myocarditis and aseptic meningitis.

Innovations and breakthroughs

There are no studies addressing the association between enteroviral infection and alcohol-associated acute pancreatitis in humans, where the alcohol intake of the non-pancreatitis controls has been comparable.

Applications

The aim of this study was to ascertain whether patients suffering from alcohol-associated acute pancreatitis show evidence of simultaneous or preceding enteroviral infection in greater numbers than control subjects with similar recent alcohol consumption, but no previous or current pancreatitis.

Peer review

This study partially answered a question in etiology of acute pancreatitis. It was well designed retrospective study.

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P- Reviewers Muniraj T, Sezgin O **S- Editor** Gou SX
L- Editor Webster JR **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3824

World J Gastroenterol 2013 June 28; 19(24): 3824-3830
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Characteristics of allergic colitis in breast-fed infants in the absence of cow's milk allergy

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Supported by OTKA-K105530, -K81117 and ETT-028-02; the János Bolyai Research Grant, to Veres G; and the János Bolyai Research Scholarship of the Hungarian Academy of Sciences
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Received: October 4, 2012 Revised: February 5, 2013
Accepted: March 23, 2013
Published online: June 28, 2013

Abstract

AIM: To investigate the characteristics of mucosal lesions and their relation to laboratory data and long-term follow up in breast-fed infants with allergic colitis.

METHODS: In this study 31 breast-fed infants were prospectively evaluated (mean age, 17.4 wk) whose rectal bleeding had not ceased after a maternal elimination diet for cow's milk. Thirty-four age-matched and breast-fed infants (mean age, 16.9 wk) with no rectal bleeding were enrolled for laboratory testing as controls. Laboratory findings, colonoscopic and histological characteristics were prospectively evaluated in infants with rectal bleeding. Long-term follow-up with different nutritional regimes (L-amino-acid based formula or breastfeeding) was also included.

RESULTS: Iron deficiency, peripheral eosinophilia and thrombocytosis were significantly higher in patients with allergic colitis in comparison to controls (8.4 ± 3.2 μmol/L *vs* 13.7 ± 4.7 μmol/L, $P < 0.001$; 0.67 ± 0.49 G/L *vs* 0.33 ± 0.17 G/L, $P < 0.001$; 474 ± 123 G/L *vs* 376 ± 89 G/L, $P < 0.001$, respectively). At colonoscopy, lymphonodular hyperplasia or aphthous ulceration were present in 83% of patients. Twenty-two patients were given L-amino acid-based formula and 8 continued the previous feeding. Time to cessation of rectal bleeding was shorter in the special formula feeding group (mean, 1.4 wk; range, 0.5-3 wk) when compared with the breast-feeding group (mean, 5.3 wk; range, 2-9 wk). Nevertheless, none of the patients exhibited rectal bleeding at the 3-mo visit irrespective of the type of feeding. Peripheral eosinophilia and cessation of rectal bleeding after administration of elemental formula correlated with a higher density of mucosal eosinophils.

CONCLUSION: Infant hematochezia, after cow's milk allergy exclusion, is generally a benign and probably self-limiting disorder despite marked mucosal abnormality. Formula feeding results in shorter time to cessation of rectal bleeding; however, breast-feeding should not be discouraged in long-lasting hematochezia.

Key words: Rectal bleeding; Breast-feeding; Allergic colitis; Colonoscopy; Amino-acid formula

Core tip: Rectal bleeding is a common problem in otherwise healthy breast-fed infants; our primary aim was to find characteristic lesions at colonoscopy and determine the cessation of rectal bleeding when administering different nutritional regimes (L-amino-acid based formula or breast-feeding). Our secondary aim was to find correlations between laboratory data, severity of mucosal lesions and cessation of rectal bleeding in allergic colitis infants with no cow's milk allergy.

Molnár K, Pintér P, Győrffy H, Cseh Á, Müller KE, Arató A, Veres G. Characteristics of allergic colitis in breast-fed infants in the absence of cow's milk allergy. *World J Gastroenterol* 2013; 19(24): 3824-3830 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3824.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3824

INTRODUCTION

Rectal bleeding is a common problem in otherwise healthy breast-fed infants[1]. The differential diagnosis of this condition includes anal fissures, infectious colitis, congenital bleeding disorders, inflammatory bowel disease (IBD) and, most frequently, allergic colitis (AC)[2]. Currently there is no reliable diagnostic test available for AC, and the diagnosis is often made presumptively in healthy infants with rectal bleeding who show no anal fissures or infectious colitis[3]. AC is believed to be a hypersensitive gastrointestinal disorder to allergens present in breast milk, and formula is regarded as a form of food allergy in infancy[4]. As a resulting first-line therapeutic intervention, cow's milk (CM) is generally eliminated from the maternal diet[5].

Recent guidelines state that the elimination diet for the mother should be continued for a minimum of at least 2 wk, and up to 4 wk in cases of AC[6]. According to the guidelines, if an infant has no cessation of rectal bleeding after 4 wk of maternal CM-free diet then the patient has no CM allergy. It is of interest that the majority of infants with rectal bleeding, despite the general belief, have no CM allergy as an underlying cause of hematochezia. This phenomenon was supported by recent studies showing that even in breast-fed infants with rectal bleeding, CM allergy is more uncommon than previously believed[7]. Only 18% of 40 infants with rectal bleeding had proven CM allergy, none had positive specific immunoglobulin E (IgE) to cow's milk, egg or wheat on admission, and only 5% had a positive skin-prick test to cow's milk[8]. Nevertheless, an atopy patch test was useful to identify sensitization for cow's milk (50%), soy (28%), egg (21%), rice (14%) and wheat (7%) in 14 AC infants with multiple food allergy[9].

Lymphonodular hyperplasia (LNH), patchy granularity and aphthous lesions were found at colonoscopy in patients with AC; however, most patients showed no abnormal mucosal lesions[5,7,8]. These studies included patients with short-term rectal bleeding which may not have shown the development of characteristic lesions at colonoscopy. Therefore, in our study, there was a minimum 4-wk timeline between onset of rectal bleeding and the procedure of colonoscopy. We hypothesized that 4 wk would be enough to find a typical mucosal abnormality in otherwise healthy breast-fed infants with rectal bleeding not caused by CM allergy.

Taken together, our primary aim was to find characteristic lesions at colonoscopy and determine the cessation of rectal bleeding when administering different nutritional regimes (L-amino-acid based formula or breast-feeding). Our secondary aim was to find correlations between laboratory data, severity of mucosal lesions and cessation of rectal bleeding in AC infants with no CM allergy.

It should be noted that there is no previous, prospective study in AC patients with no CM allergy.

MATERIALS AND METHODS

Patients

During a 4-year period (January 2006-February 2010) at the First Department of Pediatrics, Semmelweis University, breast-fed infants were invited to join the present study. Inclusion criteria were age less than 6 mo, exclusive breast-feeding, normal stool cultures (*Salmonella, Shigella, E. coli, Yersinia, Campylobacter*), and no cessation of rectal bleeding after introduction of a maternal elimination diet for CM for a minimum of 4 wk. All subjects were evaluated by history and physical examination for signs of fissure, infection and allergic diseases. Rectal bleeding as bloody spots or streaks with or without mucus was confirmed macroscopically before the colonoscopy. A complete blood count with differential, C-reactive protein, prothrombin time, activated partial thromboplastin time, serum albumin, serum IgE, and specific IgE to common food antigens including CM, egg, wheat, rye, soy, fish, nuts, peanuts, sesame, almond, tomato, banana, celery, carrots, apple, peach, lemon, and orange were obtained. Thirty-four age-matched and breast-fed infants (mean age, 16.9 wk; range, 4-25 wk; girls, 15) with no rectal bleeding (apnea, 10; gastro-esophageal reflux, 12; minor trauma, 7; minor surgical intervention, 5) were enrolled as controls for the laboratory testing. Characteristics of patients and controls are summarized in Table 1.

The Institutional Ethical Committee approved the study; written parental informed consent was obtained.

Evaluation of colonoscopy

To characterize allergic colitis and exclude other rare forms of rectal bleeding in infancy such as angiodysplasia, hemangioma, polyp, IBD or blue rubber bleb syndrome, colonoscopy with multiple biopsies was performed using a flexible pediatric gastroscope with narrow band imaging technique included (Olympus GIF-Q180, Olympus Hungary KFT). Ambulatory colonoscopy was done by the same gastroenterologist (Veres G) with the assistance of a trained endoscopy nurse. The procedure was done under general anesthesia. The colon was gently cleansed by a single water enema (5 mL/kg) before the procedure.

Evaluation of histology

Using standard biopsy forceps, 4 biopsies were taken from the sigmoid colon (about 10 cm from the anal verge) targeted to areas with gross endoscopic findings. Biopsy samples were fixed in 4% buffered formalin for 24 h then embedded into paraffin. Hematoxylin eosin (HE) stain was performed on 3-4 μm thin slides. The biopsy was ex-

Table 1 Clinical characteristics and parameters related to atopy in infants with allergic colitis and control subjects *n* (%)

Characteristic	AC (*n* = 30)	Controls (*n* = 34)
Gender (F:M)	19:11	19:15
Age at presentation (wk, mean)	17.2	16.9
Family history of atopy	17 (57)	13 (38)
Atopic dermatitis	8 (27)	7 (21)
Duration of hematochezia (wk, mean)	8.6	-
Increased level of IgE (> 5 U/mL)	3	Not done
Positivity to specific IgE to food antigens	0	Not done

AC: Allergic colitis; M: Male; F: Female; IgE: Immunoglobulin E.

amined for routine histology by a pathologist (Győrffy H). Eosinophils were counted in 10 high-power fields (HPF) and the average number of cells was recorded. Although there are no standard accepted criteria for the diagnosis of AC, several studies have demonstrated eosinophilic infiltration (≥ 6/HPF) in the lamina propria of the left colon or rectum[10-12]. Based on previous studies[11,13], patients with AC were subdivided according to the number of eosinophils on biopsy specimens. AC1 consisted of patients with eosinophils between 6 and 19/HPF, and AC2 with eosinophils ≥ 20/HPF. Using these criteria, AC2 patients fulfill the diagnostic criteria for eosinophilic colitis.

Treatment and follow-up

All subjects were followed up for 6 mo to assess outcome, including visits after one week following colonoscopy, after 3 mo, and after 6 mo. Elemental L-amino acid formula (Neocate; SHS Int., Liverpool, United Kingdom) was offered to the parents to treat their children. Assessment of rectal bleeding during the follow-up period was qualitative and assessed by parental self-report. In addition, if macroscopic bleeding eased, parents were asked to provide feces to exclude occult bleeding (HSV10; Diagnosticum Zrt, Budapest, Hungary). Because macroscopic bleeding was the main inclusion criteria, the cessation of macroscopic bleeding was considered to be the endpoint.

Up to 1 year of age, patients received AA formula or continued breast-feeding. At the age of 1 year, CM was introduced to both groups with no macroscopic bleeding in the follow-up. After 4 wk of the challenge, parents were asked to provide feces to exclude occult bleeding (HSV10; Diagnosticum Zrt, Budapest, Hungary). In addition to CM, parents were asked to avoid eggs, wheat, soya, nuts and fish ("six food elimination diet") up to one year of age. After CM introduction, other foods were introduced gradually (wheat, soya, eggs, fish and nuts).

At the age of 6 mo, introduction of supplemental feeding (except the 6 foods listed above) was recommended irrespective of whether the patient was on the formula or the breast-feeding arm. Subjects with worsening of symptoms or having a severe form of colitis underwent repeat colonoscopy with biopsy. Six month timepoint was chosen for this procedure to allow enough time for healing in the latter group.

Statistical analysis

Our data followed normal distribution, therefore parametric statistical tests were implicated and data were expressed as means with standard deviation. For comparison of datasets, unpaired *t* test and analysis of variance (ANOVA) with Bonferroni's post hoc tests were used. For analyses of contributing effects, factorial ANOVA, logistic regression and Pearson's correlation were used. The level of significance was 5% ($P < 0.05$) and Bonferroni's adjustment for multiple comparisons was introduced where needed. Statistical analysis was performed with Statistica 8 (Statsoft, Tulsa, OK, United States).

RESULTS

Clinical characteristics of infants with rectal bleeding

Thirty-one healthy, breast-fed infants (mean age, 17.4 wk; range, 5-26 wk; 12 girls) were enrolled. The mean (range) duration of bloody stools before colonoscopy was 8.7 (4-16) wk (Table 1). All mothers strictly followed a CM-free diet with the help of a trained dietician. In addition to CM-free diet, 7 mothers had an elimination diet for egg and 4 had an exclusion diet for egg, fish, wheat, nuts and soy. In all patients, rectal bleeding was the main symptom that prompted the request for gastroenterological evaluation. In addition to bloody stools, watery (42%) or mucous stools (68%) were common. None of the patients had fissures at rectal examination. Stool frequency averaged 4.2 (range, 1-7) bowel movements per day. Three patients had a classic history of infantile colic. One patient had hemangioma on the thorax with no involvement of the liver. One patient had transient hypothyroidism. The others had no previous hospitalization or chronic disease. Growth was reported as normal in all patients whose weight and height charts were reviewed for an objective assessment of their growth pattern.

During follow-up, one subject had failure to thrive and was subsequently diagnosed with infantile Crohn's disease. She was 24 wk old at study entry with a history of rectal bleeding for 12 wk. Her data were excluded from the study (final study group consisted of 30 infants).

History of atopy, analysis of IgE-mediated hypersensitivity

Eight patients (27%) had atopic dermatitis which is comparable with the percentage of control subjects (21%). Seventeen patients (57%) had a positive family history of atopy among first-degree relatives which was higher than in controls (38%). Only 3 patients had an elevated level of serum IgE (> 5 kU/L). Specific IgE to the most common food antigens was determined in 25 of 31 patients (81%) with negative results (Table 1).

Laboratory investigation

The laboratory abnormalities are presented in Table 2. The mean iron level was significantly decreased in patients (8.43 ± 3.2 μmol/L) in comparison to controls (13.7 ± 4.7 μmol/L, $P < 0.001$). We found a markedly increased thrombocyte count in patients (474 ± 123 G/L)

Table 2 Laboratory data on admission in infants with allergic colitis, allergic colitis 1, allergic colitis 2 and control subjects (mean ± SD)

Characteristic	AC (n = 30)	AC1 (n = 19)	AC2 (n = 11)	Controls (n = 34)
Hemoglobin (g/L)	115 ± 13	114 ± 12	115 ± 14	113 ± 10
Iron (μmol/L)	8.4 ± 3.2[b]	8.9 ± 2.9[b]	7.6 ± 4.0[b]	13.7 ± 4.7
Thrombocytes (G/L)	474 ± 123[b]	458 ± 126[b]	497 ± 122[b]	376 ± 89
Eosinophils (G/L)	0.67 ± 0.49[b]	0.51 ± 0.45	0.91 ± 0.47[a]	0.33 ± 0.17

[a]$P < 0.05$ *vs* allergic colitis (AC), [b]$P < 0.01$ *vs* controls. Allergic colitis 1 (AC1) consisted of patients with eosinophils between 6 and 19/ high power field (HPF), and allergic colitis 2 (AC2) with eosinophils ⩾ 20 eosinophils/HPF.

Table 3 Frequency of laboratory abnormalities in infants with allergic colitis on admission and at 6-mo visit n (%)

Characteristic	On admission	6-mo visit	ND
Thrombocytosis (> 450 × 10^9/L)	16 (60)	5	2
Iron deficiency (< 9 μmol/L)	15 (50)	4	2
Eosinophilia (> 5% of leukocytes)	12 (40)	5	1

The number of patients who had previous abnormal parameters and no control analysis depicted in the last column. ND: Not done.

when compared with controls (376 ± 89 G/L, $P < 0.001$). Similarly, patients exhibited increased peripheral eosinophilia (6.7 ± 4.9 G/L) when compared with controls (3.3 ± 1.7 G/L, $P < 0.001$). On the other hand, 60% of patients had thrombocytosis, 50% showed low iron level, and 40% demonstrated eosinophilia (Table 3). There was no difference concerning hemoglobin level between patients and controls.

Patients were subdivided into two groups (AC1 and AC2: eosinophilic colitis) according to the number of eosinophils determined by histology on biopsies (see above). Iron level, hemoglobin and thrombocytes were comparable in AC1 and AC2. However, peripheral eosinophils were significantly elevated in AC2 (9.1 ± 4.7 G/L) when compared with AC1 (5.1 ± 4.5 G/L, $P < 0.01$).

Only one patient had marked anemia which required blood transfusion. This patient was the only one who had low albumin level (< 35 g/L). Her other characteristics (including macroscopic and microscopic mucosal abnormalities) were not different from those of the other subjects. Laboratory data in patients who were subsequently diagnosed with Crohn's disease showed iron deficiency (4 mol/L) without anemia (113 g/L), and thrombocytosis (524 × 10^9/L).

Characterization of colonoscopy

At colonoscopy, the cecum was reached in 23 (74%) patients. In all other subjects the colonic mucosa was visualized from the anus to the splenic flexure, two of them to the hepatic flexure. Three patients had normal visual colonoscopy findings. Twenty-seven (90%) showed abnormal mucosal lesions such as LNH, aphthous ulceration, and marked erythema (2 patients). LNH or aphthous ulceration was present in 25/30 patients (83%)

Table 4 Frequency of colonoscopic abnormalities and number of eosinophils on histology/high power field of infants with allergic colitis 1 and allergic colitis 2 n (%)

Characteristic	AC1 (n = 19)	AC2 (n = 11)
LNH	15 (79)	7 (64)
Aphthous ulceration	8 (42)	6 (55)
LNH and aphthous ulceration	6 (32)	5 (45)
Marked erythema	1 (5)	1 (9)
No. of eosinophils/HPF (mean)	12.2	29.5

LNH: Lymphonodular hyperplasia; AC1: Allergic colitis 1; AC2: Allergic colitis 2; HPF: High power field.

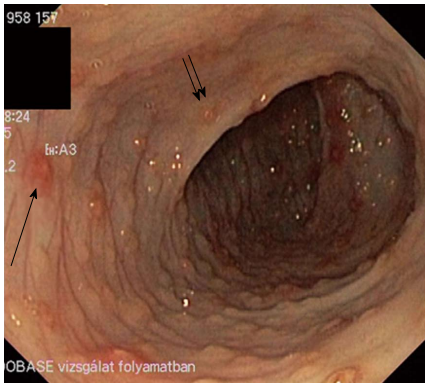

Figure 1 Endoscopic findings of allergic colitis in a breastfed infant. Lymphonodular hyperplasia with circumscribed erythema (arrow) and tiny bleeding spot (double arrow) in the descending colon in a breast-fed infant with allergic colitis.

(Figure 1). LNH was found in 73% of patients (22/30) and aphthous ulceration in 47% of the patients (14/30). Subdividing the patients with regard to the number of eosinophils on biopsies, LNH was seen in 15 patients with AC1 (79%) and in 7 patients with AC2 (64%). Aphthous ulceration was visualized in 8 patients with AC1 (42%) and in 6 patients with AC2 (55%). More patients with AC2 (45%) depicted both phenomenon, which were present in 32% of subjects with AC1. However, the discrepancies failed to reach significance (Table 4).

LNH and aphthous ulceration without any specific signs for IBD were present in the patient with Crohn's disease at her first colonoscopy. She was the only patient with no cessation of rectal bleeding at the 3-mo visit. Deep ulceration in the transverse colon and non-caseating granulomas on histology established the diagnosis of Crohn's disease at her second colonoscopy (after 6-mo visit). Abnormal findings at colonoscopy are depicted in Table 4. There were no adverse events recorded under general anesthesia and colonoscopy.

Characterization of histology

Light microscopy revealed normal mucosal architecture in all patients studied, including the first biopsy of the patient with Crohn's disease which showed normal mucosal architecture with an elevated number of eosinophils (29/HPF). All patients had more than 6 eosinophils/HPF on histol-

Table 5 Baseline characteristics and cessation of rectal bleeding in patients with amino acid-based formula feeding and breast-feeding [mean (range)]

Characteristic	Amino-acid based formula feeding	Breast-feeding
No. of patients	22	8
Age at presentation (wk)	16.0 (5-26)	20.6 (16-25)[a]
Rectal bleeding at baseline (wk)	7.9 (4-14)	10.5 (6-16)
Cessation of rectal bleeding (wk)	1.4 (0.5-3)	
Rectal bleeding at 3-mo visit (*n*)	0	0

[a]$P < 0.05$ *vs* formula feeding.

ogy (Table 4). All three patients with normal macroscopic findings at colonoscopy showed an elevated eosinophil count on biopsies (6, 20 and 39 cells/HPF, respectively). Nineteen patients had an eosinophil count between 6 and 19/HPF (AC1) and 11 patients had more than 20/HPF (AC2). Mean number of eosinophils was 12.2/HPF in AC1 and 29.5/HPF in AC2 (eosinophilic colitis).

Follow-up and clinical outcome, laboratory data

Based on marked mucosal abnormalities and the long-lasting rectal bleeding, we decided to treat patients with special formula. At the first visit one week after colonoscopy, elemental L-amino acid formula was offered to the parents to treat their children. Parents refused the formula feeding in 8 cases (5 in AC1 and 3 in AC2) and continued the previous feeding type. Twenty-two patients received the special formula (14 in AC1 and 8 in AC2). There were no significant differences concerning baseline characteristics between the 2 groups.

The duration of bleeding was significantly shorter in the special formula feeding group (mean, 1.4 wk; range, 0.5-3 wk) when compared with the breast-feeding group (mean, 5.3 wk; range, 2-9 wk). Nevertheless, none of the patients exhibited gastrointestinal complaints or visible rectal bleeding at the 3-mo visit irrespective of the type of feeding (Table 5). Three patients with serum iron level below 5 μmol/L were treated with iron-containing syrup (Maltofer; dosage, 2 drops/kg) for 6 wk. At 6-mo visit, control blood count and iron level were analyzed in patients who had abnormal levels on admission.

Follow-up and colonoscopy, histology

Due to the cessation of bleeding, only four parents agreed for a control colonoscopy to be performed. At baseline all of them had LNH and aphthous ulceration. One patient was in AC1 (13 eosinophils/HPF) and 3 patients were in AC2 (eosinophils > 30/HPF). At control colonoscopy none of the patients had aphthous ulceration; only scattered LNH without bleeding spots were visible. The number of eosinophils normalized in AC1 patients (5/HPF) and markedly decreased in AC2 patients (8-17/HPF).

Correlation analysis and risk factors

Correlation analysis revealed that the time to stop the bleeding was negatively correlated with the administration of elemental formula ($P < 0.001$, $r = -0.762$). In addition, the number of eosinophils on biopsy specimens at baseline correlated negatively with elemental formula concerning cessation of rectal bleeding ($P = 0.025$, $r = -0.667$). There was no correlation between abnormal serum laboratory data (thrombocytes, eosinophils, iron) and the ending of rectal bleeding. In patients with AC1 and AC2, we found similar correlations between elemental diet and the time to stop the bleeding.

DISCUSSION

The elimination of CM from the diet of the lactating mother is a commonly used and recommended practice[10-12] for infants with rectal bleeding. However, recent studies have suggested that there are significant proportions of infants with rectal bleeding with no CM allergy[8,9,13]. Based on these results we focused on AC patients with no cessation of rectal bleeding after a minimum of 4 wk of maternal CM-free diet. Four weeks is a long enough time-span to exclude rare forms of surgical diseases, to cure an undetected viral gastrointestinal disorder or for elimination of CM protein from patients[6,8,14].

In our study, frequency of atopic dermatitis, measurement of serum IgE, and specific IgE to different foods were not significant factors in the diagnostic procedure, as has been suggested by previous studies[14,15]. On the other hand, a recent Italian study suggested a delayed-type immunogenic mechanism in this process, hence the atopy patch test was useful in the diagnostic procedure in 14 AC infants with multiple gastrointestinal food allergy[9].

In our study, iron deficiency, peripheral eosinophilia and thrombocytosis were significantly higher in AC patients in comparison to controls. Surprisingly, no previous study has analyzed iron levels in patients with AC. Peripheral eosinophilia and thrombocytosis were reported previously[7,8,15]. To determine the risk factors for the clinical outcome, none of the 3 abnormal laboratory findings correlated with the cessation of rectal bleeding.

Theoretically, colonoscopy would have detected other rare causes of rectal bleeding such as polyps, hemangioma, blue rubber bleb syndrome and angiodysplasia. However, all but 3 patients showed mucosal abnormalities such as LNH, aphthous ulceration and marked focal erythema. As a bleeding source, LNH with circumscribed erythema or/and central pit-like bleeding spots (Figure 1) or aphthous ulceration were present in 25 patients with AC (83%). This is in contrast to previous studies, which reported a much lower percentage of characteristic abnormalities at colonoscopy. In a prospective evaluation of 34 infants with rectal bleeding, only 10 (29%) showed LNH including three of them with normal histology[5]. Xanthakos *et al*[7] followed 22 infants younger than 6 mo of age with hematochezia. Only 12 patients showed gross endoscopic abnormalities at colonoscopy including diffuse erythema, friability, LNH and aphthous ulceration. In addition, a recent study followed 40 infants with visible rectal bleeding where 41% of patients had normal mucosa, 33% had aphthous ulceration, and 51% depicted focal erythema with no report of LNH[8]. One of the ex-

planations for the high rate of LNH and aphthous ulceration found in our study is the long-lasting rectal bleeding (minimum 4 wk). LNH has been described as a sign of food allergy in children with CM allergy[16-18]. In our patients with no CM allergy, LNH was also a characteristic abnormality at colonoscopy. We do not think that LNH is a specific sign of food allergy or AC, just an indicator of a focal inflammation beneath the epithelium. However, LNH with circumscribed erythema or/and central pit-like bleeding spots and aphthous ulceration may have a role in the diagnostic procedure of AC.

The number of eosinophils on biopsies in patients with AC is a question of debate[19]. Previous studies[20,21] have suggested, as a diagnostic criteria for AC, eosinophilic infiltration in the lamina propria of ≥ 6/HPF, whereas Machida *et al*[5] suggested for this entity of ≥ 20/HPF. All patients in the present study had ≥ 6 eosinophils/HPF in the lamina propria. To find a possible difference of laboratory, colonoscopy and clinical outcome, patients were subdivided to moderate (AC1) or high (AC2) mucosal density of eosinophils. It should be noted that AC2 patients fulfill the present criteria for eosinophilic colitis (≥ 20 eosinophils/HPF)[22]. Peripheral eosinophilia and cessation of rectal bleeding after administration of elemental formula correlated with the higher number of eosinophils on biopsies. In conclusion, these data suggest that both groups represent similar entities, and a cut-off > 20 eosinophils/HPF for eosinophilic colitis may be artificial.

Due to the marked mucosal abnormality and the long-lasting rectal bleeding, elemental formula was given to the patients. It was refused by 8 cases who continued their previous feeding. Elemental diet shortened the time to cessation of hematochezia; nevertheless, it should be emphasized that none of the patients exhibited gastrointestinal complaints or rectal bleeding at the 3-mo visit irrespective of the type of feeding. This result suggests that the underlying cause could be food-related other than CM, hence AA formula shortened the resolutions of symptoms. Probably, our study population may have multiple food allergy; at least, we cannot exclude this because the nursing mothers conducted only limited types of food elimination diet (beside CM).

The beneficial effect of amino acid-based formula was reported previously[23], suggesting an allergy-related mechanism even in AC patients with no CM allergy.

On the other hand, we cannot exclude the possibility that our patients had no food allergy at all. This phenomenon is similar to infantile eczema[24] or eosinophilic esophagitis in infancy, where considerable proportions of patients have no food allergy but an allergic-immunologic component does exist[25]. Exclusive amino acid formula-based dietary trials resulted in more than 90% remission in children with eosinophilic esophagitis. Similarly to current guidelines in AC, empiric elimination diets of avoidance of foods commonly known to cause hypersensitivity reactions resulted in 50%-74% disease remission in eosinophilic esophagitis[26]. Taken together, previous studies and the beneficial effect of AA formula in the present study suggest that our study population probably has multiple food allergy. One of the most interesting points of data in our study is that patients were able to tolerate a non-restricted diet after a period of elimination diet (AA-based formula) or after a more prolonged persistence of symptoms on breast-feeding. Acquired tolerance may explain this finding, which is supported by our previous study where depleted FOXP3 regulatory T cells normalized after feeding of AA formula[27].

Nevertheless, the only patient who had not ceased the rectal bleeding was subsequently diagnosed with infantile Crohn's disease and she was excluded from the study. As reported previously[7,28,29], at baseline, none of her laboratory, colonoscopy or histology parameters were different from the patients with AC.

In conclusion, rectal bleeding in infancy, even after exclusion of CM, is generally benign and is probably a self-limiting disorder, despite the marked mucosal abnormality. Iron deficiency, peripheral eosinophilia and thrombocytosis are characteristic findings in these patients. At colonoscopy, LNH and aphthous ulceration may be characteristic signs. Administration of amino acid formula shortened the timeline of the rectal bleeding in patients with AC; however, breast-feeding should not be discouraged in this population with long-lasting hematochezia.

COMMENTS

Background

Allergic inflammation of the large intestine is believed to be a hypersensitive gastrointestinal disorder to allergens present in breast milk, and formula is regarded as a form of food allergy in infancy. As a resulting first line therapeutic intervention, cow's milk is generally eliminated from the maternal diet; however, significant numbers of infants with rectal bleeding, despite the general belief, have no cow's milk allergy as an underlying cause of hematochezia. Currently there is no reliable diagnostic test available for allergic colitis, and the diagnosis is often made presumptively in healthy breast-fed infants with rectal bleeding who show no anal abnormalities or infectious colitis.

Research frontiers

A research group in Hungary investigated the characteristics of endoscopic findings in colonic mucosal lesions and their relation to laboratory data in breast-fed infants with allergic colitis. Long-term follow-up with different nutritional regimes (L-amino acid-based formula or breast-feeding) was also included. According to the literature there is no previous, prospective study in allergic colitis patients with no cow's milk allergy.

Innovations and breakthroughs

In this study, 31 breast-fed infants were prospectively evaluated whose rectal bleeding had not ceased after a maternal elimination diet for cow's milk allergy. As controls, 34 age-matched and breast-fed infants without rectal bleeding were enrolled for the laboratory testing. In the laboratory findings, iron deficiency and elevation of peripheral eosinophilic cell and platelet counts were significantly higher in patients with allergic colitis in comparison to controls. At colonoscopy, increments of lymphoid tissue appearing as a lump (called lymphonodular hyperplasia) or aphthous ulceration were present in 83% of patients. Twenty-two patients were given L-amino acid-based formula and 8 continued the previous feeding. Time to cessation of rectal bleeding was shorter in the special formula feeding group when compared with the breast-feeding group. Nevertheless, none of the patients exhibited rectal bleeding at the 3-mo visit irrespective of the type of feeding. The elevated peripheral eosinophilic cell count and cessation of rectal bleeding after administration of elemental formula correlated with the higher density of mucosal eosinophilic cells in the colon.

Applications

Administration of amino acid formula shortened the timeline of rectal bleeding in patients with allergic colitis; however, breast-feeding should not be discouraged in this population with long-lasting rectal bleeding. These data suggest that the significance of real food allergy in breast-fed infants with allergic colitis

should be revised in the future. This phenomenon is similar to infantile eczema or eosinophilic esophagitis in infancy where considerable proportions of patients had no food allergy but an allergic-immunologic component does exist. It may indicate that the "food allergy" theory is currently much less applicable, and probably a significant number of nursing mothers continue an unnecessary restriction diet worldwide.

Terminology

Allergic colitis is explained as an allergic condition of the gastrointestinal tract in breast-fed infants that results from an immune response to the allergens present in breast milk.

Peer review

This is a very interesting article from a teaching institution in Hungary. It is well written and conclusions are valid.

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P- Reviewer Camilleri-Brennan J **S- Editor** Wen LL
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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3831

World J Gastroenterol 2013 June 28; 19(24): 3831-3840
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Active treatments are a rational approach for hepatocellular carcinoma in elderly patients

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Received: January 4, 2013 Revised: February 10, 2013
Accepted: April 28, 2013
Published online: June 28, 2013

Abstract

AIM: To determine whether an active intervention is beneficial for the survival of elderly patients with hepatocellular carcinoma (HCC).

METHODS: The survival of 740 patients who received various treatments for HCC between 1983 and 2011 was compared among different age groups using Cox regression analysis. Therapeutic options were principally selected according to the clinical practice guidelines for HCC from the Japanese Society of Hepatology. The treatment most likely to achieve regional control capability was chosen, as far as possible, in the following order: resection, radiofrequency ablation, percutaneous ethanol injection, transcatheter arterial chemoembolization, transarterial oily chemoembolization, hepatic arterial infusion chemotherapy, systemic chemotherapy including molecular targeting, or best supportive care. Each treatment was used alone, or in combination, with a clinical goal of striking the best balance between functional hepatic reserve and the volume of the targeted area, irrespective of their age. The percent survival to life expectancy was calculated based on a Japanese national population survey.

RESULTS: The median ages of the subjects during each 5-year period from 1986 were 61, 64, 67, 68 and 71 years and increased significantly with time ($P < 0.0001$). The Child-Pugh score was comparable among younger (59 years of age or younger), middle-aged (60-79 years of age), and older (80 years of age or older) groups ($P = 0.34$), whereas the tumor-node-metastasis stage tended to be more advanced in the younger group ($P = 0.060$). Advanced disease was significantly more frequent in the younger group compared with the middle-aged group ($P = 0.010$), whereas there was no difference between the middle-aged and elderly groups ($P = 0.75$). The median survival times were 2593, 2011, 1643, 1278 and 1195 d for 49 years of age or younger, 50-59 years of age, 60-69 years of age, 70-79 years of age, or 80 years of age or older age groups, respectively, whereas the median percent survival to life expectancy were 13.9%, 21.9%, 24.7%, 25.7% and 37.6% for each group, respectively. The impact of age on actual survival time was significant ($P = 0.020$) with a hazard ratio of 1.021, suggesting that a 10-year-older patient has a 1.23-fold higher risk for death, and the overall survival was the worst in the oldest group. On the other hand, when the survival benefit was evaluated on the basis of percent survival to life expectancy, age was again found to be a significant explanatory factor ($P = 0.022$); however, the oldest group showed the best survival among the five different age groups. The youngest group revealed the worst outcomes in this analysis, and the hazard ratio of the oldest against the youngest was 0.35 for death. The survival trends did not differ substantially between the survival time and percent survival to

life expectancy, when survival was compared overall or among various therapeutic interventions.

CONCLUSION: These results suggest that a therapeutic approach for HCC should not be restricted due to patient age.

Key words: Hepatocellular carcinoma; Population aging; Survival; Life expectancy; Active intervention

Core tip: Progressive population aging worldwide demands consensus development for decision making to treat elderly patients. A simple comparison of survival days is confounded by aging; therefore, age compensation is mandatory to evaluate survival benefits among different age groups. In this study, age difference was compensated by life expectancy in a hepatocellular carcinoma cohort. The authors suggested that age itself might not be a critical determinant for the selection of a therapeutic option. This study emphasizes the importance of clarifying risk determinants specific for elderly patients with respect to individual aspects and medical economy.

Suda T, Nagashima A, Takahashi S, Kanefuji T, Kamimura K, Tamura Y, Takamura M, Igarashi M, Kawai H, Yamagiwa S, Nomoto M, Aoyagi Y. Active treatments are a rational approach for hepatocellular carcinoma in elderly patients. *World J Gastroenterol* 2013; 19(24): 3831-3840 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3831.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3831

INTRODUCTION

The Japanese population is aging more rapidly than that of any other nation in the world. In 1990, approximately one in eight people in Japan were aged 65 years or older[1]. This level was already the highest in Asia, although somewhat lower than in most developed European countries. Since then, the population of Japan has aged because of an increasing life expectancy and a falling birth rate[2]. As a result, as many as one in four of the Japanese population will be at least 65 years old by the year 2025[3], which is a much higher ratio than that predicted for any other country.

There is much controversy concerning medical interventions for elderly patients. Zhang *et al*[4] reported that an interventional scheme should not be changed on the basis of the age of patients facing the treatment of myocardial infarction, whereas Teo *et al*[5] reported that the addition of percutaneous coronary intervention to optimal medical therapy did not improve the clinical outcomes in patients 65 years of age or older. An active treatment is recommended for elderly patients suffering from subarachnoid hemorrhage[6,7]. However, a conservative treatment was reported to be superior for elderly patients in the management of traumatic dental axis fracture[8]. Although some benefit of active treatment for hepatocellular carcinoma (HCC) in elderly patients has been suggested, the debate still continues as to how fast patients suffering from HCC are actually getting older and whether the survival benefit offered by active interventions is comparable between the young and the elderly.

In this report, aging trends among patients actively treated for HCC were evaluated over 25 years from 1986 to 2011 at a single institution in Japan. To compare the survival benefits between the relatively young and the elderly, survival was compared after adjusting the absolute survival time or life expectancy. Finally, a case presentation of an elderly patient who was successfully managed is used to illustrate the risks and benefits of active interventions for HCC in elderly patients.

MATERIALS AND METHODS

Patients

Clinicopathological data were retrospectively analyzed for 918 patients who were admitted between 1983 and 2011 for the first time for management of HCC in our hospital. The subjects' basic characteristics are shown in Table 1. To compare the ages of patients during each 5-year interval of the overall study period, 840 patients were selected who were admitted between 1986 and 2010. Only 740 patients who had already died or who had been followed for longer than a year in our hospital were included in survival analyses using actual survival time. Among these 740 cases, 504 could be allocated a life expectancy because these data are available for each age and gender since 1996 in Japan. To compare survival among different age groups, patients were classified into five groups according to their ages; 49 years of age or younger (-49), 50-59 years of age (50s), 60-69 years of age (60s), 70-79 years of age (70 s) and 80 years of age or older (80+).

Hepatic nodules were radiographically diagnosed as HCC when they fulfilled at least one of the following criteria based on dynamic computed tomography (CT)/magnetic resonance imaging and/or CT during hepatic arteriography/CT during arterial portography: (1) the typical hemodynamics of classical HCC, with a substantial arterial phase enhancement followed by a washout with a corona-like peripheral enhancement in an equilibrium phase; or (2) with similar characteristics as coexisting nodules that had already been diagnosed as HCC. Otherwise, a histological diagnosis was made.

Therapeutic options were principally selected according to the clinical practice guidelines for HCC from the Japanese Society of Hepatology, 2009[9]. The treatment most likely to achieve regional control capability was chosen, as far as possible, in the following order: resection, radiofrequency ablation (RFA), microwave coagulation (MWC), percutaneous ethanol injection (PEI), transcatheter arterial chemoembolization (TACE), transarterial oily chemoembolization (TOCE), hepatic arterial infusion chemotherapy (HAIC), systemic chemotherapy including molecular targeting or best supportive care. Each treatment was used alone or in combination, such as RFA af-

Table 1 Basic characteristics

Age (yr)	67.0 (60.0-73.0)[1]
Gender (male/female)	647/271
HBsAg (-/+/ND)	582/196/140
anti-HCV (-/+/ND)	278/534/106
AIH/PBC/alcohol/NASH/BCS	10/6/85/16/2
Child-Pugh (A/B/C/ND)	704/172/36/6
Size (mm)	28.0 (19.0-45.0)[1]
Stage (Ⅰ/Ⅱ/Ⅲ/Ⅳ)[2]	163/306/287/162
Therapy (Loco/IVR/Cx/others/BSC)	459/332/78/11/38

[1]Median and interquartile range; [2]General rules for the clinical and pathological study of primary liver cancer from Liver Cancer Study Group of Japan. ND: Not determined; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; NASH: Nonalcoholic steatohepatitis; BCS: Budd-Chiari syndrome; Loco: Logo-regional treatments, including resection, radiofrequency ablation, microwave coagulation and percutaneous ethanol injection; IVR: Interventional radiology including transcatheter arterial chemoembolization, and transarterial oily chemoembolization; Cx: Chemotherapy including hepatic arterial infusion chemotherapy, systemic chemotherapy and molecular targeting therapy; Others: Other therapies including stereotactic body radiation, proton beam and liver transplantation; BSC: Best supportive care; HBsAg: Hepatitis B surface antigen; HCV: Hepatitis C virus.

ter TACE or TACE following HAIC, with a clinical goal of striking the best balance between functional hepatic reserve and the volume of the targeted area. Stereotactic radiotherapy was considered when loco-regional treatments were indicated but not applicable, whereas liver transplantation was selected by an exclusive decision process. Treatments were classified into four groups: (1) loco-regional including resection, RFA, MWC and PEI; (2) interventional radiology (IVR) including TACE and TOCE; (3) chemotherapy (Cx) including HAIC and systemic chemotherapy; and (4) other, including stereotactic radiotherapy, proton beam and liver transplantation, which were applied to only 11 patients in total. If pleural treatments were added as an adjunct, the case was classified into a group according to the applied treatment with the highest regional control capability.

Measuring hepatitis B surface antigen (HBsAg), anti-hepatitis C virus (HCV), anti-mitochondrial, anti-M2 and anti-nuclear antibodies serologically defined background liver diseases. A habitual daily alcohol intake of more than 60 g was considered alcohol abuse. Nonalcoholic steatohepatitis was diagnosed on the basis of histological findings, whereas Budd-Chiari syndrome was diagnosed angiographically. Patients who were negative for all of the above criteria were considered not definitive for a background liver disease. The institutional review board of our institution, which did not require informed consent for a retrospective study using medical records or imaging examinations, approved the present study, which conformed to the ethical guidelines of the 2008 Declaration of Helsinki.

Serum biochemistry and histological examination

HBsAg and anti-HCV antibodies were detected by a chemiluminescence immunoassay using the ARCHITECT HBsAg QT and ARCHITECT HCV kits (Abbott Japan Co. Ltd., Chiba, Japan), respectively. Serum anti-mitochondrial and anti-M2 antibodies were quantified using the commercial kits AMA FluoroAID-1 and Mesacup mitochondria M2 (MBL Co. Ltd., Nagoya, Japan), respectively. Total and *Lens culinaris* agglutinin A-reactive α-fetoprotein (AFP) serum concentrations were quantified with a liquid-phase binding assay system (LiBASys; Wako Pure Chemical Indus-tries Ltd., Osaka, Japan). L3 was calculated as a percentage of *Lens culinaris* agglutinin A-reactive species against total AFP. Serum des-γ-carboxy prothrombin (DCP) was measured using an electrochemiluminescence immunoassay (Wako Pure Chemical Industries Ltd, Osaka, Japan). Other blood biochemistries were routinely measured in the clinical laboratories of our hospital.

Two expert histologists independently rendered histological diagnoses based on microscopic observations of tissues stained with hematoxylin and eosin, silver, iron, periodic acid-Schiff, periodic acid-Schiff with diastase digestion, and azan. When there was any discordance between the two histologists, the specimen was reviewed to reach a consensus diagnosis.

Life expectancy and percent life expectancy

The Japanese life expectancy per year for each gender at a specific age is available for 1996 onwards and was downloaded from the Ministry of Health, Labour and Welfare[1]. The life expectancy for our cohort was plotted in three dimensions using O-Chart Standard software (ONO SOKKI Co., Ltd., Yokohama, Japan). The survival time-for each case was divided by the life expectancy to obtain the percent life expectancy (%LE).

Statistical analysis

Patient ages were compared using the Kruskal-Wallis test, and Dunn's multiple comparison tests were used to compare the different periods in 5-year intervals. The influence of multiple factors on survival was evaluated using Cox regression analysis. The comparisons of categorical data were performed with the Fisher's exact test or the χ^2 test among three or two different age groups, respectively. Overall survival was demonstrated by calculating Kaplan-Meier survival fractions. All analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, United States), except for the multivariate analysis, which was performed using PASW statics 17.0 (SPSS Inc., Chicago, United States). A two-tailed *P* value less than 0.05 was considered statistically significant after Bonferroni correction.

RESULTS

Patients receiving active treatments for HCC are continuously aging in Japan

The median ages for each 5-year interval steadily increased from 61 (interquartile range: 55-66) years of age (from 1986 to 1990) to 71 (63-76) years of age (from 2006 to 2010), as shown in Figure 1A. The median age was sig-

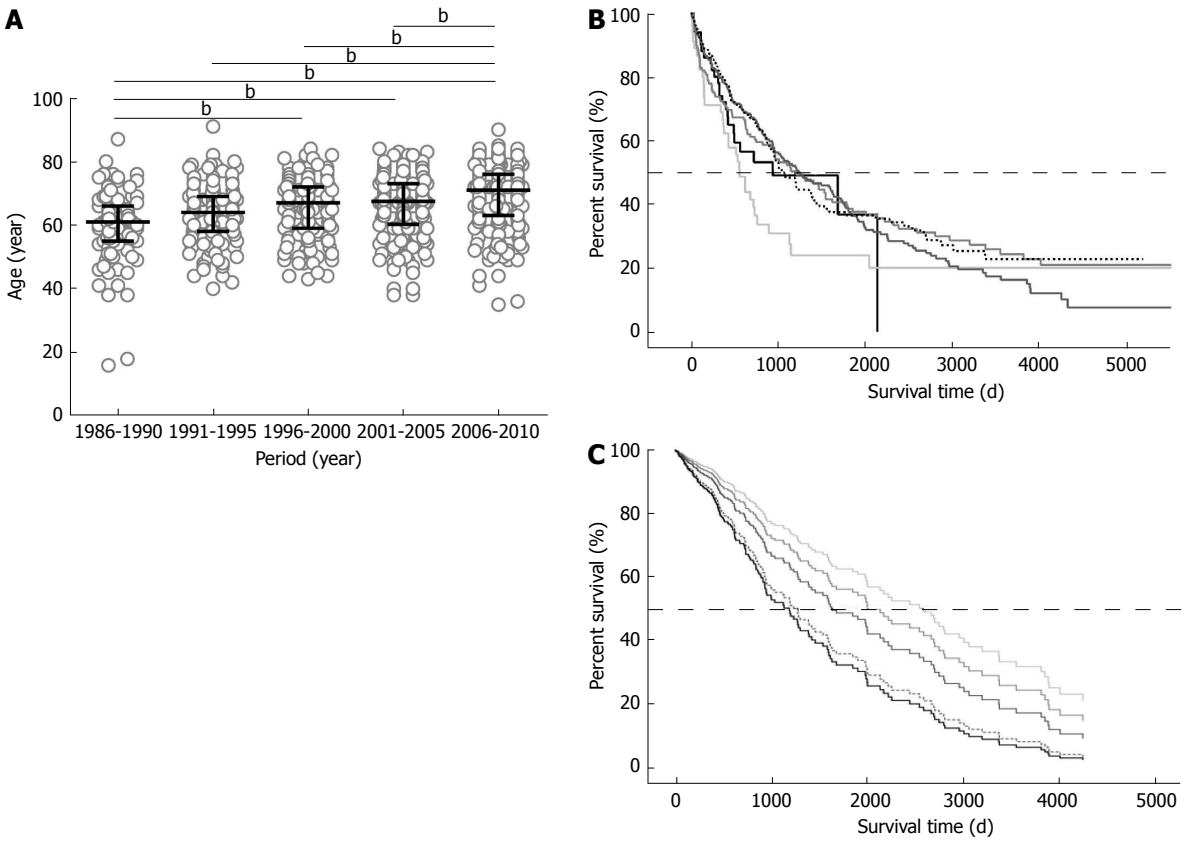

Figure 1 Age distribution in different periods and the survival of patients with hepatocellular carcinoma. A: The age of patients who were admitted for the management of hepatocellular carcinoma was plotted for each 5-year interval since 1986: the median ages were significantly different among the different periods ($P < 0.0001$); B: The overall survival of 740 patients in five age groups who have already died or have been followed for longer than 1 year was calculated on the basis of Kaplan-Meier survival fractions: the median survival time of all cases was 1094 d; C: Overall survival was compared among the different age groups after compensation for background characteristics using a Cox proportional hazard model and was significantly different among age groups ($P = 0.020$). The solid black and dotted lines are the survival curves of the 80 years of age or older and 70-79 years of age groups, respectively. The other lines are 60-69 years of age, 50-59 years of age and 49 years of age or younger groups, indicated in colors ranging from dark to pale. $^{b}P < 0.01$. The horizontal bars (A) indicate the median and interquartile range. The dotted horizontal lines (B and C) indicate a position of 50% survival.

nificantly different among the periods ($P < 0.0001$), and the median age of these patients increased by 10 years in the last 20 years. The patients admitted from 2006 to 2010 were significantly older than the patients who were hospitalized during any other periods ($P < 0.001$ *vs* 1986-1990, 1991-1995, 1996-2000; and $P < 0.01$ *vs* 2001-2005).

Aging is a significant factor affecting overall survival time in HCC

The median survival time for 740 patients who were deceased or were followed longer than 1 year was calculated from the Kaplan-Meier survival fractions as 1094 d. When the patients' ages were categorized into the five groups to test the dependence of survival on age, there was no significant survival difference among the groups ($P = 0.41$), and the least median survival time was 553 d in the -49 group, as shown in Figure 1B. For the Cox regression analysis, 379 cases were included because they were not missing values for the 10 explanatory variables: (1) age (years); (2) gender (male/female): (3) HBsAg (-/+); (4) anti-HCV (-/+);(5) AFP ($\log_{10}$); (6) L3 (%); (7) DCP ($\log_{10}$); (8) Child-Pugh class (A/B/C); (9) HCC stage (Ⅰ / Ⅱ / Ⅲ / Ⅳ); and (10) therapy (loco-regional/IVR/Cx/other). Among these 10 variables, age, AFP, DCP, HBsAg, anti-HCV, Child-Pugh class, HCC stage and therapy were determined to be significant factors that influenced survival time (Table 2). The impact of age on survival time was found to be significant ($P = 0.020$), with a hazard ratio of 1.021, suggesting that a 10-year-older patient has a 1.23-fold higher risk of death. When the survival differences among the five age groups were estimated with the Cox proportional hazards model on the basis of the above 10 explanatory factors, overall survival was poorer with age and was the worst in the 80+ group, as shown in Figure 1C. The risk of death in the 80+ group was 2.41-times higher when compared with that of the -49 group.

Fractional life expectancy is an indicator of survival benefit adjusted for age

It may be reasonable to assume that older patients will have shorter survival times irrespective of effective treatments, preserved functional hepatic reserve, or other factors, simply because of their shorter residual length of life. To compare the survival from the point of aging, survival was normalized by life expectancy. A ratio

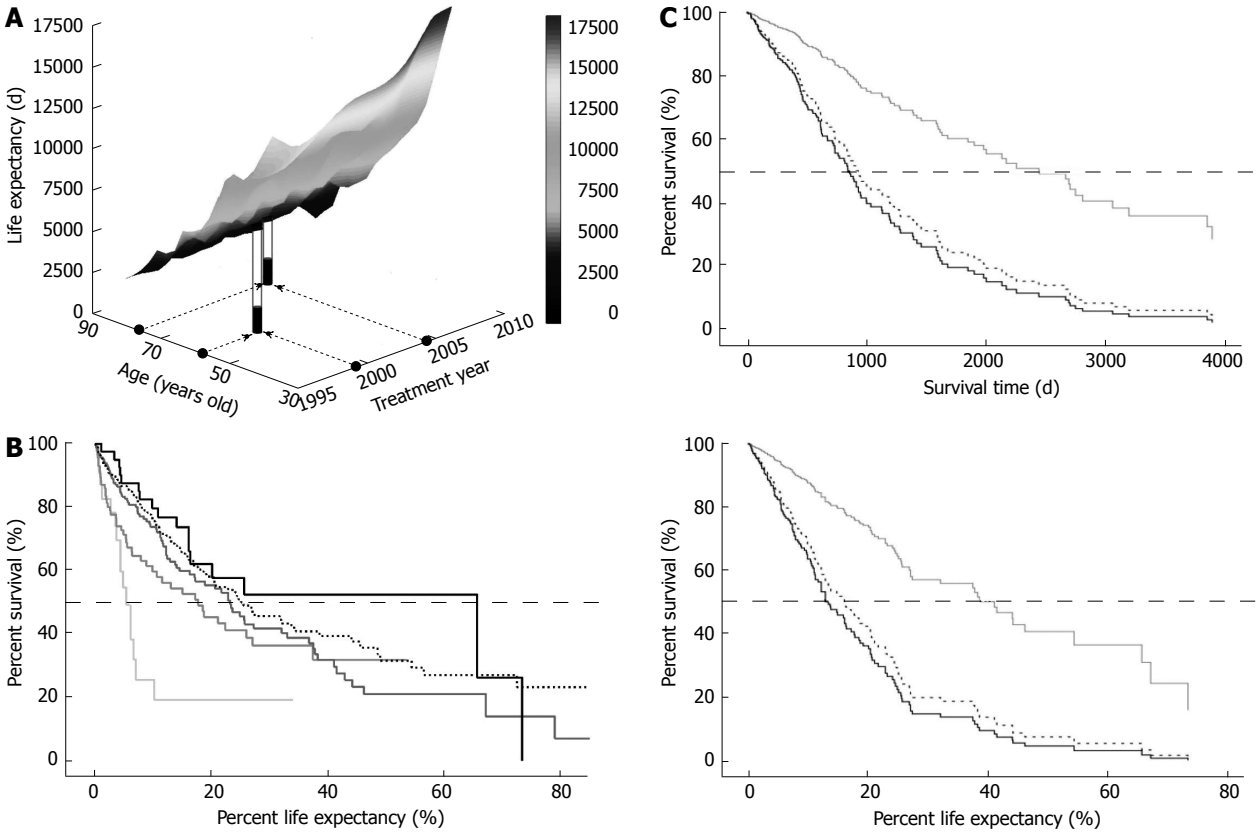

Figure 2 Life expectancy and percent life expectancy of patients with hepatocellular carcinoma. A: Life expectancy (LE) for each case was plotted in a three-dimensional space. The percent LE (%LE) was defined as the ratio between survival time and LE and is shown for representative cases. The LE of a male at 59 years of age in the year 1999 is 7928 d, whereas the LE is 3760 d for a 77-year-old male in the year 2004 (white piles). Both patients survived for 1779 d, as indicated by the black piles, with %LE values of 22.4% and 48.6%, respectively; B: A survival proportion was expressed in %LE in the five different age groups, and the median %LE of all 504 cases was 22.9%. The solid black and dotted lines are the survival curves of 80 years of age or older and 70-79 years of age group, respectively. The other lines represent 60-69 years of age, 50-59 years of age and 49 years of age or younger groups, in colors ranging from dark to pale, respectively; C: In a cohort of 328 patients for whom LE is available, the survival among patients receiving loco-regional, interventional radiology (IVR), or chemotherapy (Cx) treatments was evaluated on the basis of absolute time (upper panel) or %LE (lower panel). The solid black and dotted lines are survival curves for Cx and IVR, respectively, and the gray line represents the loco-regional group. The dotted horizontal lines indicate a position of 50% survival.

of survival days to the expected residual life length is defined as the %LE. Life expectancy data for each age and gender are available from 1996 onward in Japan; therefore, life expectancy was plotted for the 504 cases in our cohort (Figure 2A). Overall survival based on %LE revealed a median survival percentage of 22.9%. When the survival based on %LE was compared among the five different age groups, the median survival was significantly different among the groups (Figure 2B, $P < 0.0001$), ranging from 5.4% in the -49 group to the best rate of 65.7% in the 80+ group.

Among 504 patients, 174 cases were excluded from further analyses because a therapeutic intervention was never performed or because one or more of the 10 explanatory candidate factors was not measured. Among the remaining 330 cases, only two patients received therapies that were categorized in "other". Finally, 328 cases were subjected to Cox regression analysis to investigate the survival differences associated with different therapeutic modalities. As shown in Figure 2C, the survival curves were very similar to the evaluations based on survival time (upper) and %LE (lower). Both analyses showed that loco-regional therapies far surpassed IVR and Cx in terms of survival benefit.

When the relationship between survival time and %LE was evaluated in each case, however, it became clear that the two survival indicators were not consistent. For example, the same survivals of 1779 d for males at 59 and 77 years of age in 1999 and 2004 gave rise to very different %LE values of 22.4% and 48.6%, respectively, as indicated in Figure 2A. In another example, a 69-year-old female in 2002 and a male of the same age in 1999 survived 2693 and 1980 d, respectively. Because their life expectancies were 7125 and 5168 d, respectively, the shorter absolute survival value for the male surpassed the female's longer survival in terms of %LE at 37.8% and 38.3%, respectively. Taken together, %LE is a potential alternative for evaluating survival benefit in HCC patients among different age groups.

Elderly patients survive for the highest percentage of their life expectancy after receiving active treatments for HCC

Although the overall survival trends and survival benefits

Table 2 Cox regression analysis for survival time

Variable	Significance	HR	95%CI for HR	
			Lower	Upper
Age	0.020	1.021	1.003	1.040
Gender	0.652	1.079	0.775	1.503
HBsAg	0.029	1.598	1.051	2.432
anti-HCV	0.036	1.503	1.027	2.198
AFP	0.000	1.314	1.135	1.521
L3	0.288	1.004	0.997	1.012
DCP	0.005	1.216	1.061	1.395
Child-Pugh class				
A	0.000			
B	0.000	2.307	1.562	3.408
C	0.001	3.373	1.617	7.035
Tumor stage[1]				
Ⅰ	0.000			
Ⅱ	0.148	1.504	0.865	2.616
Ⅲ	0.051	1.751	0.997	3.074
Ⅳ	0.000	5.715	2.985	10.939
Therapy category				
Loco-regional	0.000			
IVR	0.000	2.567	1.800	3.661
Chemotherapy	0.000	2.861	1.675	4.889
Others	0.000	6.151	2.505	15.107

[1]General rules for the clinical and pathological study of primary liver cancer from Liver Cancer Study Group of Japan. HR: Hazard ratio; AFP: α-fetoprotein; L3: Percentage of fucosylated fraction in AFP; DCP: Des-γ-carboxy prothrombin; Loco-regional: Therapies including resection, radiofrequency ablation, microwave coagulation and percutaneous ethanol injection; IVR: Interventional radiology including transcatheter arterial chemoembolization and transarterial oily chemoembolization; Chemotherapy: Therapies including hepatic arterial infusion chemotherapy, systemic chemotherapy and molecular targeting therapy; Others: Other therapies including stereotactic body radiation, proton beam and liver transplantation; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen.

Table 3 Cox regression analysis for percent life expectancy

Variable	Significance	HR	95%CI for HR	
			Lower	Upper
Gender	0.072	0.717	0.499	1.030
HBsAg	0.043	1.709	1.018	2.869
anti-HCV	0.043	1.597	1.015	2.511
AFP	0.000	1.348	1.145	1.587
L3	0.421	1.004	0.995	1.012
DCP	0.015	1.243	1.043	1.482
Child-Pugh class				
A	0.001			
B	0.001	2.144	1.393	3.300
C	0.084	2.375	0.891	6.332
Tumor stage[1]				
Ⅰ	0.000			
Ⅱ	0.554	1.216	0.637	2.320
Ⅲ	0.180	1.554	0.816	2.960
Ⅳ	0.000	3.879	1.871	8.045
Therapy category				
Loco-regional	0.000			
IVR	0.000	2.755	1.816	4.179
Chemotherapy	0.000	3.365	1.884	6.010
Others	0.246	3.359	0.435	25.971
Age group				
49 years/younger	0.240			
50s	0.242	0.598	0.253	1.415
60s	0.107	0.496	0.211	1.164
70s	0.047	0.436	0.192	0.990
80 years/older	0.041	0.348	0.126	0.958

[1]General rules for the clinical and pathological study of primary liver cancer from Liver Cancer Study Group of Japan; HR: Hazard ratio; AFP:α-fetoprotein; L3: Percentage of fucosylated fraction in AFP; DCP: Des-γ-carboxy prothrombin; Loco-regional: Therapies including resection, radiofrequency ablation, microwave coagulation and percutaneous ethanol injection; IVR: Interventional radiology including transcatheter arterial chemoembolization, and transarterial oily chemoembolization; Chemotherapy: Therapies including hepatic arterial infusion chemotherapy, systemic chemotherapy and molecular targeting therapy; Others: Other therapies including stereotactic body radiation, proton beam and liver transplantation; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen.

of different therapies were consistent between the analyses using dependent variables of survival time and %LE, the two analyses indicated substantially different survival benefits when the survival was compared among the different age groups. A comparison based on survival time in 330 cases revealed that the 80+ group had the worst survival (Figure 3A upper panel), consistent with a similar finding in the initial cohort of 760 patients (Figure 1C). However, when the survival benefit was evaluated on the basis of %LE, age was again found to be a significant explanatory factor (P = 0.022), but the 80+ group showed the best survival among the five different age groups, as shown in the lower panel of Figure 3A. Intriguingly, the -49 group revealed the worst outcomes in this analysis. The hazard ratio of the 80+ group against the -49 group was 0.35 for death (Table 3). The other significant explanatory factors for %LE were HBsAg and anti-HCV, AFP, DCP, Child-Pugh score, tumor-node-metastasis (TNM) stage, and therapeutic options, and the maximal hazard ratios for each variable were 1.71, 1.60, 1.35, 1.24, 2.14, 3.88 and 3.37, respectively, suggesting that age is one of the most powerful determinants of %LE.

Tumors were more advanced in the young

Functional hepatic reserve and anatomical tumor extent were compared among three groups: a younger group of -49 and 50s, a middle-aged group consisting of 60s and 70s, and an elderly group of 80+. As shown in the upper panel of Figure 3B, the functional hepatic reserve, as assessed by the Child-Pugh class, did not differ among the three groups (P = 0.34), while the TNM stage tended to be more advanced in the younger group (Figure 3B middle panel, P = 0.060). Advanced disease was significantly more frequent in the younger group as compared to the middle-aged group (P = 0.010), whereas there was no difference between the middle-aged and elderly groups (P = 0.75). A similar trend was observed for HBsAg positivity (P < 0.0001). In the younger group, HBsAg was positive in 45.7% of patients, while it was positive in only 14.8% and 6.7% of patients in the middle-aged and elderly groups, respectively, which led to a significant difference between the younger and middle-aged groups (P < 0.0001), but no significant difference between the middle-aged and elderly groups (P = 0.23).

Case

This case is an example of an 85-year-old Japanese male

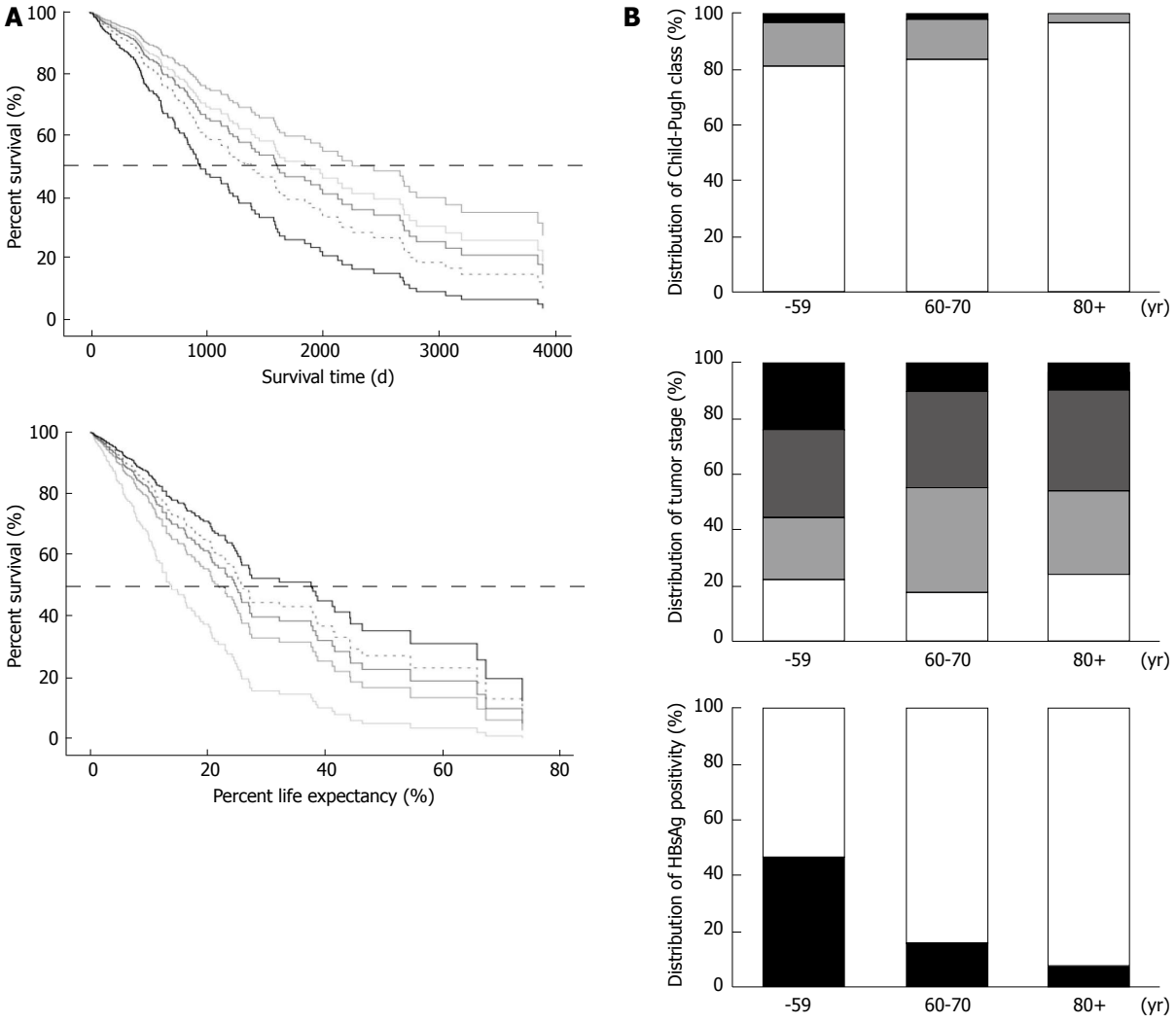

Figure 3 Differences in survival and background characteristics by age groups. A: In a cohort of 330 patients for whom life expectancy (LE) data are available, the survival of five different age groups was evaluated on the basis of absolute time (upper panel) or percent LE (lower panel). The solid black and dotted lines are the survival curves of the 80 years of age or older and 70-79 years of age groups, respectively. The other lines are 60-69 years of age, 50-59 years of age and 49 years of age or younger groups, indicated in colors ranging from dark to pale. The oldest group showed the worst survival in days but the best in percent LE; significantly better than that of the youngest group ($P = 0.041$); B: The distributions of Child-Pugh class (upper), tumor stage (middle), and hepatitis B surface antigen (HBsAg) positivity (lower) among three age groups: 59 years of age or younger, 60-79 years of age, and 80 years of age or older. For the hepatic reserve, the white, grey and black columns indicate Child-Pugh A, B and C classes, respectively, whereas tumor stages from Ⅰ to Ⅳ are represented in order from white to black. The black column reveals that HBsAg was positive in the lower graph. There was no significant difference in terms of functional hepatic reserve among the three groups, although anatomical tumor extent and frequency of positive reaction for HBsAg were significantly higher in the youngest group as compared with the middle-aged group ($P = 0.010$ and $P < 0.0001$, respectively). The dotted horizontal lines indicate a position of 50% survival.

who was admitted to our hospital on April 2009 for the treatment of an HCC that was approximately 20 mm in diameter and located in segment 6 (Figure 4A). He suffered from HCV infection and diabetes mellitus, for which he had used insulin by injection for more than a decade, and he had one prior hepatic resection for HCC. His life expectancy upon admission was 6.27 years. RFA was completed after TACE through the right intercostal space under ultrasound guidance. Twenty-five months after the ablation, however, a follow-up CT revealed that the HCC had spread to wide areas of segments 6 and 7 with portal vein tumor thrombus that extended up to the right main trunk (Figure 4B). Relying on the Child A-class preserved hepatic functional reserve, HAIC was our recommendation at this stage, delivered through a catheter that was implemented and connected to a port under the skin. After receiving written informed consent from the patient, 125 mg of 5-fluorouracil and 5 mg of cis-diamminedichloroplatinum were infused over 23 h and 60 min, respectively, through the common hepatic artery, and repeated for 5 consecutive days. After 2 d of no drug administration, the same schedule was performed for the following 2 wk. The 5-d HAIC was then repeated every 2-3 mo. The tumor and portal vein tumor thrombi gradually disappeared to reveal an enormous tumor reduction by July 2012 (Figure 4C). A new lesion appeared in segment 5 and gradually enlarged to 15 mm in diameter; therefore, RFA was performed again on August 2012 (Figure 4D). In September 2012, the patient turned 89 years old and has survived for 42 mo (55.8% of LE)

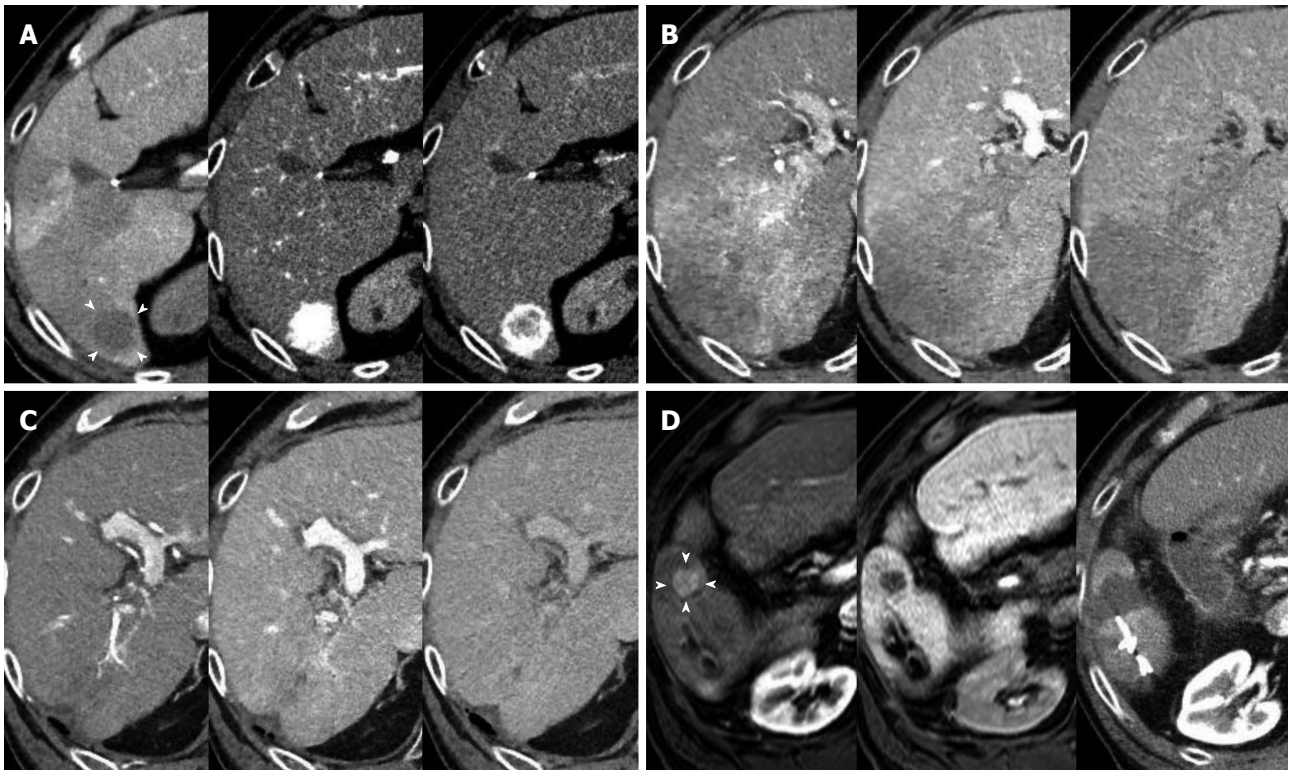

Figure 4 Representative follow-up images of successfully treated hepatocellular carcinoma in an elderly patient. A: A classical hepatocellular carcinoma (HCC) was detected in segment 6 of the liver on April 12, 2009 as demonstrated by (1) a lower intensity up on computed tomography (CT) during arterial portography, indicated by arrowheads (left); (2) vigorous staining during the arterial phase of the CT during hepatic arteriography (middle); and (3) washout with a corona-like peripheral enhancement during the equilibrium phase of the CT during hepatic arteriography (right); B: A dynamic CT 25 mo after the initial radiofrequency ablation revealing recurrent HCCs, which had spread to large areas of segments 6 and 7 and extended to the main trunk of the right portal vein. The images were obtained during arterial, portal and equilibrium phases of the dynamic CT study, shown in order from left to right; C: After hepatic arterial infusion chemotherapy *via* a catheter using 5-fluorouracil and cis-diamminedichloroplatinum for 15 mo, an enormous tumor reduction, including the portal vein tumor thrombus, was achieved. The images were obtained during arterial, portal, and equilibrium phases of the dynamic CT study, shown in order from left to right; D: A new 10-mm lesion appeared in segment 6 during hepatic arterial infusion chemotherapy treatment, and a second radiofrequency ablation (RFA) was applied 41 mo after the initial RFA. Magnetic resonance imaging study using a contrast medium of gadolinium ethoxybenzyl diethylene-triamine-pentaacetic-acid showing the arterial supply (left, arrowheads) and a defect in the hepatobiliary phase (middle) of the study. An arterial phase image of the dynamic CT (right) obtained one day after RFA revealing that the ablated area of lower intensity included the target.

since the initial RFA. He is in good shape with a PS of 0 and without severe complaints.

DISCUSSION

In this study, we introduced a new indicator, the %LE, to evaluate whether active treatments for HCC are beneficial for patients over 80 years of age. Considering that morbidity, mortality and other health-related outcomes are generally compared in populations after adjusting for age structures, the age should also be normalized when survival is compared among different age groups. Based on the assumption of a community downscaling to an individual, the life expectancy adjustment among individuals should correspond to age adjustment among communities. As shown in Figure 1B and C, there is a large difference in survival curves between the Kaplan-Meier fractions and the Cox hazards after compensation using 10 explanatory factors. The worst survival of the -49 group in the Kaplan-Meier analysis was the best survival in the Cox regression. In contrast, when %LE was used, the order of survival was consistent between the Kaplan-Meier and Cox regression analyses (Figures 2B and 3A lower panel). The explanatory factors for survival time and %LE in the Cox regression analyses were consistent, and the analyses using survival time or %LE revealed similar survival curves among the different therapeutic approaches (Figure 2C); therefore, it is suggested that the factor that explains the large difference between the Kaplan-Meier and Cox regression analyses using survival time is age. Therefore, it is assumed that the impact of age on survival irrespective of liver pathophysiology can be normalized using %LE instead of survival time. The worst survival of the -49 group in the %LE analysis concurs with the common clinical experience in Japan of higher HBsAg positivity in the younger generation of HCC, leading to the onset of HCC at advanced stages[10]. Taken together, these data suggest that the %LE can be a useful factor to compare survival benefit, especially between cohorts of patients with large differences in age.

It is controversial whether active intervention is beneficial to elderly patients, and inconsistent recommendations have been reported for various diseases, including HCC. Studies that demonstrate an adverse influence of

aging on survival in HCC were all reported more than 20 years ago[11-14], whereas recent reports emphasized the benefit of active treatments in the elderly[15-18], suggesting improvements of the medical and social environments over the past decades. Unfortunately, however, all recent reports discussed survival benefits in the elderly principally based on Kaplan-Meier survival fractions. As shown in Figure 1B, a simple comparison of survival time does not reveal survival differences among various age groups. However, one should not conclude that age is not a significant factor for survival on the basis of Kaplan-Meier analysis, because survival varies widely among the different age groups in the Cox regression analysis (Figure 1C). Although our conclusion is consistent with previous reports that a therapeutic scheme should not be changed because of a patient's age, this study explains the rationale of active intervention for HCC in the elderly more theoretically. However, our conclusions are based on the study from a single institution and a limited number of patients. To establish the best approach for the elderly patients, a multicenter study should be conducted using a larger cohort.

Generally, our institution applies the same process to decide a treatment strategy irrespective of the patient's age, and this approach led to the best survival in the elderly in terms of %LE, as shown in this study. We also presented the case of a patient over 80 years of age, for whom active interventions such as RFA, TACE or HAIC were safely applied and provided survival benefit. Although it is important to thoroughly assess the risks and to obtain written informed consent, because aging is associated with a progressive deterioration of organ function that reduces the functional reserve to recover from stress/complications[19], our data suggested that age itself should not be a reason to change a treatment strategy. The number of elderly patients in one institution is generally limited, as is the case in this study; therefore, it is difficult to clarify risk determinants that are specific for elderly patients. Future trials should include efforts to enroll the elderly and to clarify factors specific for them that determine outcomes, not only for physical function, but also for quality of life and independence. Facing the world's highest proportion of elderly people, Japanese society should play a primary role in the application of guidelines for the elderly subset by achieving maximal benefits while minimizing risks.

COMMENTS

Background

Increasing life expectancy and a falling birth rate have lead to population aging worldwide, especially in developed countries. As a result, the proportion of elderly patients is steadily rising in many diseases, including hepatocellular carcinoma (HCC). There is much controversy concerning medical interventions for elderly patients; therefore, it is important to clarify a treatment strategy specific for the elderly in terms of both survival benefit and medical resources.

Research frontiers

Aging itself has a significant impact on survival days; therefore, a simple comparison of survival days among different age groups may not be suitable for evaluating survival benefit with regard to aging. Another approach is required to compensate for the effect of aging on survival.

Innovations and breakthroughs

To avoid the confounding effect between survival days and aging, the research team led by Takeshi Suda from Division of Gastroenterology and Hepatology, Niigata University introduced percent life expectancy instead of survival days as a novel indicator for the evaluation of survival benefit. Using percent life expectancy, the authors finally concluded that a therapeutic approach for HCC should not be restricted because of patient age.

Applications

Facing progressive population aging, medical society should play a primary role in the application of guidelines for the elderly subset by achieving maximal benefits while minimizing risks. In future trials with larger cohorts, percent life expectancy should play an important role in evaluating survival benefits associated with aging.

Terminology

Computed tomography (CT) during hepatic arteriography and CT during arterial portography are one of the best ways to evaluate perfusion characteristics of a nodular lesion in the liver, and provide the diagnostic rationale for HCC. Radiofrequency ablation therapy and percutaneous ethanol injection are puncture-based locoregional approaches for HCC. Both are usually applied percutaneously under ultrasound guidance, and utilize radiofrequency waves and anhydrous ethanol, respectively, to degenerate cancer cells. Transcatheter arterial chemoembolization and transarterial oily chemoembolization are interventional radiology including the embolization of hepatic arteries feeding HCC. These techniques employ gelatin and oil for embolization, respectively, and gelatin achieves a prolonged obstruction. The tumor markers of α-fetoprotein, L3, and des-γ-carboxy prothrombin are commonly measured as useful independent indicators for the biological malignant potential of HCC.

Peer review

Facing the world highest elderly ratio in Japan, it is necessary to clarify whether a medical decision process that is applied for the general population provides similar benefits for elderly patients in terms of survival. For this purpose, the authors introduced a new indicator, percent life expectancy, to normalize aging effects, and evaluated the survival benefit among different age groups. Many developed countries, such as United States and China, are also facing population aging; therefore, it is important to clarify risk determinants specific for the elderly in terms of individual aspects and medical economy. This manuscript will have a significant impact and the hepatology community will recognize the importance of the gerontological aspect.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3841

World J Gastroenterol 2013 June 28; 19(24): 3841-3846
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Anus-preserving rectectomy *via* telescopic colorectal mucosal anastomosis for low rectal cancer: Experience from a Chinese cohort

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Supported by Grants from the National Natural Science Foundation of China, No. 81041025 and 81000189

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Received: May 14, 2013 Revised: May 24, 2013
Accepted: June 1, 2013
Published online: June 28, 2013

Abstract

AIM: To investigate the safety and efficacy of anus-preserving rectectomy *via* telescopic colorectal mucosal anastomosis (TCMA) for low rectal cancer.

METHODS: From August 1993 to October 2012, 420 patients including 253 males and 167 females with low rectal cancer underwent transabdominal and transanal anterior resection, followed by TCMA. The distance between the anus and inferior margin of the tumor ranged from 5 to 7 cm, and was 5 cm in 6 patients, 6 cm in 127, and 7 cm in 287 patients. Tumor-node-metastasis staging showed that 136 patients had stage Ⅰ, 252 had stage Ⅱ and 32 had stage Ⅲ. Fifty-six patients with T3 or over received preoperative neoadjuvant chemoradiotherapy.

RESULTS: The postoperative follow-up rate was 91.9% (386/420) with a median time of 6.4 years. All 420 patients underwent radical resection. No postoperative death occurred. Postoperative complications included anastomotic leakage in 13 (3.1%) patients and anastomotic stenosis in 7 (1.6%). The local recurrence rate after surgery was 6.2%, the hepatic metastasis rate was 13.2% and the pulmonary metastasis rate was 2.3%. The 5-year survival rate was 74.0% and the disease-free survival rate was 71.0%. Kirwan classification showed that continence was good in 94.4% of patients with stage I when scored 12 mo after resection.

CONCLUSION: TCMA for patients with low rectal cancer leads to better quality of life and satisfactory defecation function, and lowers anastomotic leakage occurrence, and might be one of the safe operative procedures in anus-preserving rectectomy.

Key words: Low rectal cancer; Rectectomy; Telescopic colorectal mucosal anastomosis; Reconstruction; Abdominoperineal resection

Core tip: Li *et al* developed the telescopic colorectal mucosal anastomosis technique based on the experiences and lessons from several sphincter-preserving operations under preconditions of low rectal resection, which improved the anastomotic stoma and alleviated tension. With this modified technique used over the past 20 years, the incidence of anastomotic leakage was significantly decreased and the long-term outcome was satisfactory with good anal function and a lower rate of incontinence.

Li SY, Chen G, Bai X, Zuo FY, Chen G, Du JF, Wei XJ, Cui W. Anus-preserving rectectomy *via* telescopic colorectal mucosal anastomosis for low rectal cancer: Experience from a Chinese cohort. *World J Gastroenterol* 2013; 19(24): 3841-3846 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3841.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3841

INTRODUCTION

Abdominoperineal resection (APR) is thought to be the gold standard in the treatment of low rectal cancer less than 5 cm from the anal verge[1,2]. However, over the last 50 years, surgeons realized that permanent colostomy led to inconvenience in terms of the social life of patients and mental health issues[3,4]. Dixon developed the operative procedure of anterior resection, in which end-end anastomosis of the sigmoid colon and rectum was performed after radical resection of low rectal cancer. However, it was difficult to perform this operation in overweight patients with a narrow pelvis[5]. Parks *et al*[6] proposed a colon-anal anastomosis, which was modified by Bacon's operation with the preservation of both internal and external anal sphincters. Due to satisfactory clinical results, this type of operation was popular in European countries, however, a temporary diverting stoma was routinely required to ensure healing of the anastomotic stoma[7]. Heald *et al*[8] reported total mesorectal excision (TME) for the first time, involving resection of the mesorectum more than 5 cm from the distal margins of the tumor, which reduced the local recurrence rate, and improved the survival rate of patients with rectal cancer[9,10]. However, potential ischemia of the distal bowel during such surgery could lead to an increased rate of anastomotic leakage.

Li *et al*[11] developed the telescopic colorectal mucosal anastomosis (TCMA) based on the experiences and lessons from several sphincter-preserving operations under preconditions of low rectal resection, which improved the anastomotic stoma and alleviated tension. With this modified technique, the incidence of anastomotic leakage was significantly decreased and the long-term outcome was satisfactory with good anal function and a lower rate of incontinence[11-13]. In this study, we summarized the influential factors for high-incidence anastomotic leakage after sphincter-preserving surgery in radical rectal resection (8.1%-18.0%), including anastomotic skills, blood supply and tension of the anastomotic stoma[6,8,14-16].

MATERIALS AND METHODS

Patients

From August 1993 to August 2012, we treated 1510 patients with rectal cancer surgically at the Department of General Surgery, General Hospital of Beijing Military Command, China. Of these patients, 576 (38.1%) underwent Miles' procedure and 420 (27.8%) underwent TCMA for rectal carcinoma less than 7 cm from the anal verge. There were 253 male and 167 female patients, with an average age of 55.7 years (range: 21-91 years). The distance between the lower margin of the tumor and the anal verge varied from 5 to 7 cm, and was 7 cm in 287 patients, 6 cm in 127, and 5 cm in 6 patients. The distance was measured with a rigid sigmoidoscope. All 420 patients were examined by rectal touch, colonoscopy, barium enema, magnetic resonance imaging (MRI) and endorectal ultrasonography. Primary malignant rectal neoplasms were confirmed by biopsy, and preoperative tumor-node-metastasis (TNM) staging of the patients was also carried out.

Table 1 Comparison of pathological stages of 56 patients who underwent preoperative neoadjuvant therapy (T stage)

Preoperative stage	Cases	Postoperative stage (*n*)
T3	50	T0 (4), T1 (24), T2 (22)
T4	6	T0 (0), T1 (0), T2 (4), T3 (2)
Total	56	T0 (4), T1 (24), T2 (26), T3 (2)

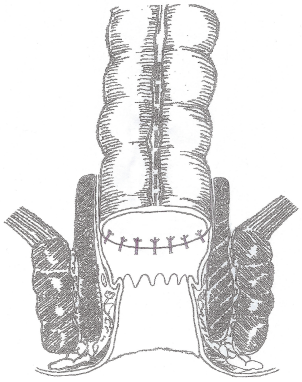

Figure 1 Schematic layout of the anus-preserving procedure *via* trans-abdominal radical anterior resection and trans-anal telescopic colorectal mucosal anastomosis.

Preoperative TNM staging in the 420 patients was as follows: stage Ⅰ, n = 136; stage Ⅱ, n = 252 and stage Ⅲ, n = 32. Of these patients, 56 above T3 received neoadjuvant chemoradiotherapy. They were given capecitabine 1500 mg twice a day orally for 1 mo. Radiotherapy was added during the chemotherapy cycle (45-50 Gy in 25-28 fractions to the pelvis). The tumor staging (assessed by MRI) after 6-8 wk of neoadjuvant therapy was as follows: T0, n = 4; T1, n = 24; T2, n = 26; T3, n = 2 (Table 1).

Surgical procedures

Surgical procedures were performed according to the TME principles and the methods previously described by Li *et al*[11]. The schematic layout of the anus-preserving procedure *via* TCMA is shown in Figure 1. Under general anesthesia and continuous epidural anesthesia, the patient was placed in the lithotomy position. The procedures routinely involved high ligation of the inferior mesenteric artery and dissection to the levator ani under direct vision. The rectum was mobilized to the pelvic floor as low as possible to facilitate the perianal approach. If the lower edge of the tumor was reached, a clamp was applied below the tumor to close the rectum when possible.

Following the abdominal approach, sufficient relaxation of the anal sphincter was achieved by finger expansion under continuous epidural anesthesia. Wide exposure of the operative field above the dentate line was achieved using the "5-stitches-suspension" method (Figure 2A). To prevent bleeding, 2-3 mL of saline adrenaline solution (1:10000) was injected into the anal canal 1.0 cm

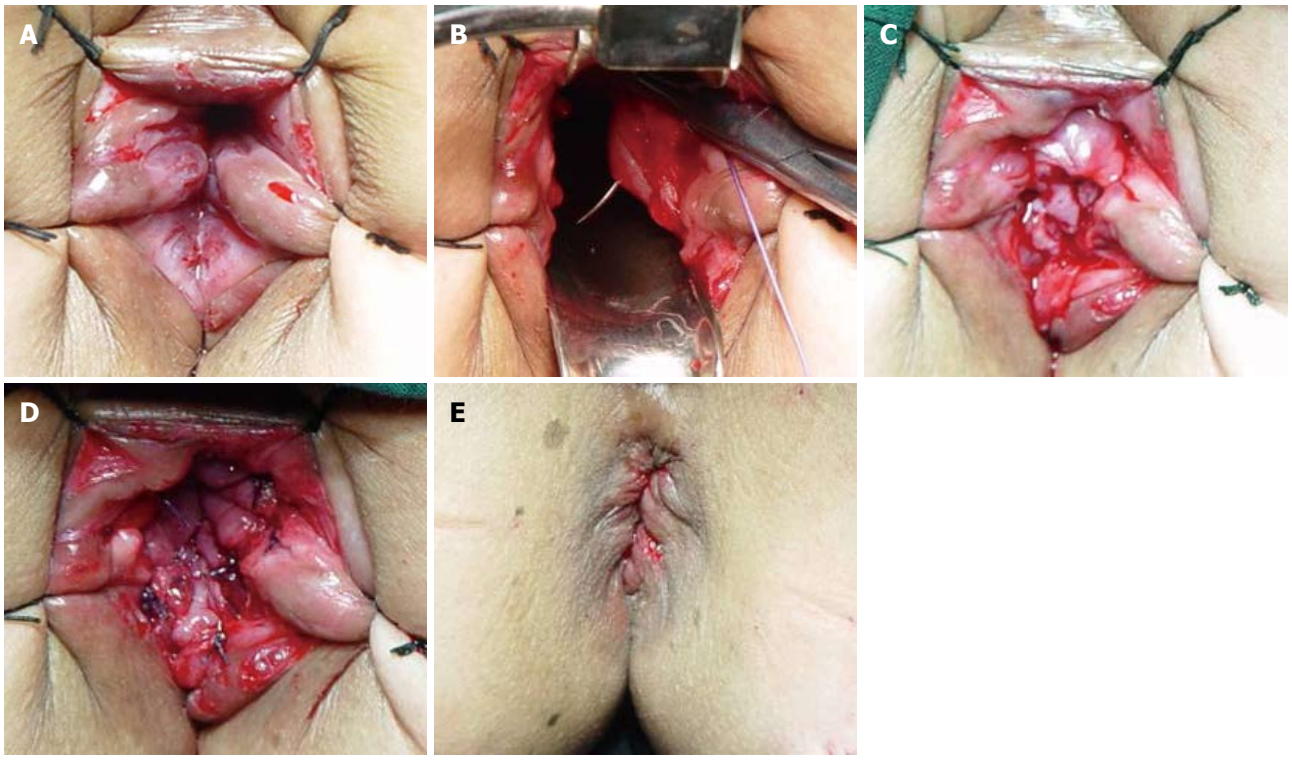

Figure 2 Surgical procedures. A: The "5-stitches-suspension" method; B: Dissection was performed by mobilizing the rectum through the mucosal plane to 2-4 cm above the dentate line; C: Telescopic colorectal mucosal anastomosis (TCMA) of the sero-muscular layer and muscular sheath was performed at 2.0 cm above the dentate line; D: Four interrupted absorbable sutures in the distal end of the colon and the residual of the rectal mucosa were also placed at the 12, 3, 6 and 9 o'clock positions, followed by 4-8 additional sutures; E: After TCMA, the 5-suspension-stitches were removed, and the anastomotic stoma was repositioned.

above the dentate line, which resulted in swelling of the mucosa. A circumferential incision of the mucosa was made at 1.5-2.0 cm above the dentate line. Dissection was performed by mobilizing the rectum through the mucosal plane to approximately 2-4 cm (Figure 2B), and then the distal margin of the rectum was clamped and cut, with preservation of the entire muscular sheath of the rectum. Later, the distal end of the colon was pulled through the anus, and TCMA of the sero-muscular layer and muscular sheath was performed at 2.0 cm above the dentate line (Figure 2C). Four interrupted absorbable sutures were placed at the 12, 3, 6 and 9 o'clock positions in the lithotomy position, respectively, for fixation and relaxation. Similarly, 4 interrupted absorbable sutures in the distal end of the colon and the residual rectal mucosa were also placed at the 12, 3, 6 and 9 o'clock positions, followed by 4-8 additional sutures (Figure 2D). To remove the dermal sutures and reposition the anastomotic stoma back in the anal canal (Figure 2E), a pelvic drainage tube was placed before closure of the abdominal wall, and was removed 4-5 d after surgery.

Postoperative adjuvant radiation and chemotherapy

Patients with greater than T2 stage received 7-12 cycles of postoperative systemic chemotherapy with the mFOLFOX4 protocol (oxaliplatin, 5-fluorouracil and calcium folinate). Eighty-eight patients with T4 stage and 23 patients with positive circumferential margins after resection were given postoperative pelvis radiotherapy at a total dose of 10-20 Gy before adjuvant chemotherapy.

Complications and follow-up

Patients were seen within one month following resection for monitoring postoperative complications, such as bleeding, pelvic abscess and anastomotic leakage. Follow-up was performed every 3 mo for 2 years, every 6 mo for 3 years and then every 1 year thereafter. All patients underwent digital examination, laboratory studies (stool analysis including occult blood, serum carcinoembryonic antigen levels) and imaging examination (abdominal ultrasound, chest X-ray, and pelvic computed tomography/MRI). Colonoscopy was performed every 6 mo for 5 years after surgery. Anastomotic stenosis was confirmed by colonoscopy under direct vision within 1 year after operation. Local recurrence was defined as the first clinical, radiologic and/or pathologic evidence of tumor of the same histologic type within the pelvis 2-3 years after surgery. Distant recurrence was defined as clinical, radiologic, and/or pathologic evidence of systemic disease outside the pelvis, at sites including liver and lungs 5 years after surgery. Death of patients was recognized as the end of follow-up. All the clinical data were collected from the follow-up records at different time-points.

Anal function evaluation

A questionnaire on anal function was completed after surgery according to Kirwan staging criteria[17].

Statistical analysis

Overall survival and disease-free survival were calculated by the Kaplan-Meier method. Statistical analysis was per-

Table 2 Patient characteristics

Characteristics	Data
Patients (*n*)	420
Age (yr)	55.7 (range: 21.0-91.0)
Gender	
Male	253
Female	167
Distance of tumor from anal verge (cm)	
7	287
6	127
5	6
Preoperative tumor stage	
Ⅰ	136
Ⅱ	252
Ⅲ	32
Postoperative tumor stage	
Ⅰ	142
Ⅱ	250 (Ⅱa: 177, Ⅱb: 61, Ⅱc: 12)
Ⅲ	28 (Ⅲa: 13, Ⅲb: 9, Ⅲc: 6)
Differentiation of tumors	
Well differentiated	148
Moderately differentiated	249
Poorly differentiated	16
Adenomatous canceration	7
Temporary diverting stoma	
Yes	0
No	420
Surgical time (min)	130 (range: 110-190)
Intraoperative blood loss (mL)	360 (range: 150-1200)
Hospital stay (d)	13 (range: 7-31)

formed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, United States).

RESULTS

The postoperative follow-up rate in this series was 91.9% (386/420), with a median time of 6.4 years. All 420 patients underwent radical resection. The distance between the distal margins of the tumor ranged from 2 to 5 cm (mean 3.2 cm). Negative distal margins were confirmed pathologically in all 420 cases, while positive circumferential margins were observed in 23 cases (5.4%). A pathological diagnosis was made in 148 patients with well differentiated adenocarcinoma, in 249 patients with moderately differentiated adenocarcinoma, in 16 patients with poorly differentiated adenocarcinoma, and in 7 patients with adenomatous canceration.

According to the TNM staging principles of the 2010 National Comprehensive Cancer Network guidelines, postoperative pathological staging showed: 142 patients with stage Ⅰ, 250 patients with stage Ⅱ (Ⅱa: 177 patients, Ⅱb: 61 patients, and Ⅱc: 12 patients) and 28 patients with stage Ⅲ (Ⅲa: 13 patients, Ⅲb: 9 patients, and Ⅲc: 6 patients) (Table 2).

Mortality and morbidity

No postoperative death occurred in this series. Anastomotic leakage occurred in 13 patients (3.1%), of whom 7 received conservative therapy (total parenteral nutrition and continuous drainage), and 5 underwent transverse colostomy (stoma apothesis after 3 mo). Anastomotic stenosis occurred in 7 patients (1.6%), these patients recovered with continuous expansion of the anus for 1-3 mo. The postoperative local recurrence rate in the series was 6.2% (26 cases), recurrence was seen 2-3 years after surgery. The rate of metastasis to the liver and the lung was 13.2% (51 cases) and 2.3% (9 cases), respectively. The postoperative 5-year survival rate was 74% and the 5-year disease-free survival rate was 71% (Figure 3).

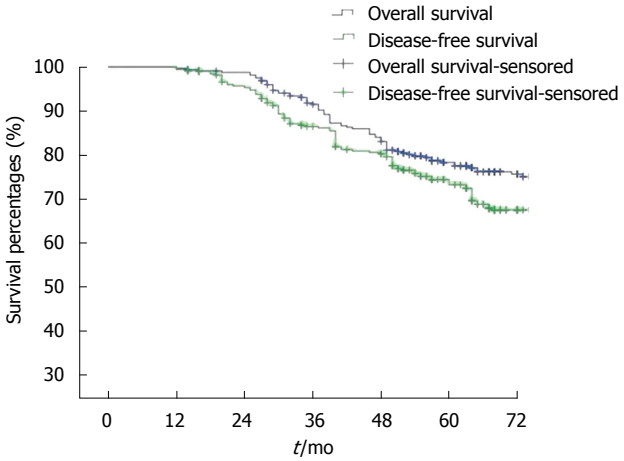

Figure 3 Kaplan-Meier method were used to calculate the 5-year survival rate and the 5-year disease-free survival rate.

Functional results

Enteral nutrition was administered to patients during the early postoperative period. These patients had poor continence, with approximately 6-9 bowel movements per day, which could be controlled to 3-6 times per day following oral intake of compound diphenoxylate 2 pills three times per day. Two to 4 mo after surgery, patients had better continence and recovered anal function 12 mo after surgery. Kirwan staging[17] was stage Ⅰ in 369 patients (94.4%), stage Ⅱ in 20 patients (5.1%), and stage Ⅲ in 2 patients (0.5%).

DISCUSSION

Assessment of therapeutic results

Despite the improved clinical results of anterior resection *via* all types of anus-preserving procedures for treating low rectal cancer, several issues remain controversial such as the incidence of anastomotic leakage, the local recurrence rate and anal function outcome[11,18,19]. In 1993, Li *et al* developed the telescopic anastomosis technique for treating low rectal cancer, focusing on relaxation sutures, while strengthening the anastomotic stoma. The use of this method with wide exposure and a refined surgical technique effectively controlled anastomotic leakage. In our series, the rate of anastomotic leakage was 3.1%, which was significantly lower than the rate of 8.0%-18.0% reported elsewhere. Clinical data indicated that TCMA was a reliable, safe and superior surgical procedure[6,8,14].

The mechanisms of defecation involve both sphincter contraction reflection[20] and complete physiological reflection of the rectal mucosa[21]. In anus-preserving

procedures, low anastomosis may impair the regions of these reflections. Thus, the intraoperative prevention of impairment in such regions could contribute to better anal function after surgery. In TCMA, colorectal anastomosis should be performed on the rectum plane 1.5-2.0 cm above the dentate line, with the preservation of anal sphincter function and enough residual rectum to protect complete physiological nervous reflection of defecation, which is completely different from that of anus-preserving procedures with resection of the internal sphincter[22-24]. Obviously, without impairment of the internal sphincter and normal anal construction, patients could have a recovery of 97.6%-99.5% Kirwan stages I and Ⅱ defecation function 6-12 mo after operation, with improved quality of life.

Long-term clinical outcome depends on radical resection of the tumor and sufficient dissection of lymph nodes according to the TME principles[25], which help to obtain both negative distal and circumferential margins for lowering local recurrence after surgery[26,27]. In our series, patients with T3-4 staging received preoperative neoadjuvant radiochemotherapy to downgrade tumor staging and to facilitate radical resection and anus-preserving procedures. Patients with stage Ⅱ or above underwent postoperative systemic chemotherapy, and those with T4 staging or positive circumferential margins confirmed by pathological examination received postoperative pelvic radiotherapy. The data showed that the patients who received comprehensive treatment exhibited a local recurrence rate of 6.2% and a 5-year survival rate of 74%, which were not significantly different from those of Miles. With 20 years of use in clinical practice, TCMA has been proved to be a safe and feasible treatment for low rectal cancer and one of the effective standard anus-preserving procedures.

Surgical skills and notes

Wide exposure of the operative field by the "5-stitches-suspension" method (Figure 3) and adequate muscle relaxation during anesthesia play a dominant role in TCMA[11]. Perianal anastomosis could be facilitated after satisfactory relaxation of the anal sphincter, and the injury of perianal architectures could be avoided and anal function restored after surgery[11,13].

Perianal dissection through the rectal mucosa plane requires prevention of bleeding by the circumferential injection of saline adrenaline solution (1:10000) 1.0 cm above the dentate line and adequate exposure of the internal sphincteric plane to achieve complete excision of the distal mucosa, while avoiding injury of the internal sphincter[11,13]. In brief, a circumferential incision of the mucosa and internal anal sphincter is made at 1.5-2.0 cm above the dentate line. The dissection continues upward between the mucosa and the superficial layer of the internal sphincter for the resection of 2-4 cm of distal rectal mucosa (Figure 2B).

Relaxation and strengthening prevent anastomotic leakage, using a 4-stitches relaxation suture of the colonal sero-muscular layer and residual rectal muscular sheath[11,13] (Figure 2C). More importantly, penetrating the whole layer of the bowel wall and/or the posterior wall of the vagina is avoided to prevent the occurrence of intestinal and vaginal fistula.

TCMA involves telescopic anastomosis between the whole layer of the colon and residual mucous and the submucous layer of the rectum. The distal margin of the colon should be modified by the resection of adipose tissue with well preserved blood supply to facilitate healing of the anastomotic stoma. Absorbable interrupted sutures are placed at the 6 and 12 o'clock positions, then at the 3 and 9 o'clock positions, followed by the addition of 4-8 sutures to avoid postoperative stenosis (Figure 2D).

After TCMA, the 5-suspension-stitches are removed, and the anastomotic stoma is repositioned. Vaseline gauze can be used for support and is removed 48-72 h after surgery (Figure 2E).

Anus-preserving operations should be performed under the conditions mentioned above in addition to sufficient mobilization of the rectum.

In conclusion, anus-preserving procedures *via* trans-abdominal radical anterior resection and trans-anal TCMA to treat patients with low rectal cancer can achieve satisfactory recovery of anal function with a decreased incidence of anastomotic leakage and a moderate local recurrence rate. In comparison with APR, this modified treatment can improve patient quality of life. TCMA might be one of the standard surgical options in treating low rectal cancer.

COMMENTS

Background

For many years, abdominoperineal resection (APR) was the treatment of choice for most patients with rectal cancer. Recent advances in surgical technique and other treatment modalities have led to a marked increase in the rate of sphincter-sparing operations, with a concomitant decrease in APR as permanent colostomy leads to inconvenience in terms of the social life of patients and mental health issues. In 1993, Li *et al* developed the telescopic colorectal mucosal anastomosis (TCMA) for treating low rectal cancer, focusing on relaxation sutures, while strengthening the anastomotic stoma. This modified technique effectively controlled anastomotic leakage.

Research frontiers

With the modified surgical techniques used over the past 20 years in this study, the incidence of anastomotic leakage was decreased significantly and the long-term outcome was satisfactory with good anal function and a lower rate of incontinence. Anus-preserving procedures *via* trans-abdominal radical anterior resection and trans-anal TCMA in treating patients with low rectal cancer could achieve a satisfactory recovery of anal function with a decreased incidence of anastomotic leakage and a moderate local recurrence rate. In comparison with APR, TCMA can greatly improve the quality of life of the patients, and could be one of the standard surgical options in treating low rectal cancer.

Innovations and breakthroughs

Despite the improved clinical results of anterior resection in treating low rectal cancer by all kinds of anus-preserving procedures, several issues remain controversial such as the incidence of anastomotic leakage, the local recurrence rate and anal functional outcome. The telescopic anastomosis technique was developed to treat low rectal cancer while strengthening the anastomotic stoma. Using this method with wide exposure and refined surgical technique, the anastomotic leakage could be controlled effectively. In this series, the rate of anastomotic leakage was significantly lower than the reported rate elsewhere. Clinical data indicated that TCMA is a reliable, safe and superior surgical procedure for low rectal cancer.

Applications

TCMA could be one of the standard surgical options in treating low rectal cancer. The transabdominal and transanal anterior resection for low rectal cancer *via* colorectal mucosal anastomosis leads to a better life quality with satisfac-

tory defecation function, while lowering the occurrence of anastomotic leakage. Telescopic anastomosis is one of the safe operative procedures in anus-preserving rectectomy for patients with low rectal cancer.

Terminology

APR is considered to be a gold standard for the treatment of low rectal cancer less than 5 cm from the anal verge. It completely removes the distal colon, rectum, and anal sphincter complex using both anterior abdominal and perineal incisions, resulting in a permanent colostomy. The technique of TCMA was developed by Li *et al* for anus-preserving rectectomy in patients with low rectal cancer, which improved the anastomotic stoma and alleviated tension. With this modified technique, the incidence of anastomotic leakage was significantly decreased and the long-term outcome was satisfactory with good anal function and a lower rate of incontinence.

Peer review

This study is innovative and is of interest for surgical community. Methods used are innovative and advanced. Detailed description is provided to allow other investigators to reproduce or validate authors' findings. Results provide sufficient evidence to draw firm scientific conclusions. Sample size and statistical data, especially graphic data, are adequate for a clinical study. However, some revisions should be made on the presentation and evaluation of the results.

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Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3847

World J Gastroenterol 2013 June 28; 19(24): 3847-3853
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Mitochondrial ATP 6 and 8 polymorphisms in irritable bowel syndrome with diarrhea

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Received: February 6, 2013 Revised: March 30, 2013
Accepted: April 9, 2013
Published online: June 28, 2013

Abstract

AIM: To investigate mitochondrial ATP 6 and 8 polymorphisms in the colon and ileum of patients with irritable bowel syndrome with diarrhea (IBS-D).

METHODS: Twenty-eight patients fulfilling the Rome Ⅲ criteria for IBS-D and 28 healthy subjects were investigated. All study participants underwent screening colonoscopy and mucosal biopsies were obtained from the colon and/or terminal ileum. Genomic DNA was extracted from specimens based on standard protocols. Mitochondrial ATP (MT-ATP) 6 and 8 genes in specimens were polymerase chain reaction amplified and sequenced. Sequencing data were analyzed *via* Variant Reporter™ Software and compared with the reference sequence from Genbank (accession No. NC_012920) to indicate possible polymorphisms. The protocol was registered at www.clinicaltrials.gov as NCT01028898.

RESULTS: Twenty-five polymorphic sites of MT-ATP 6 and 8 genes were detected and 12 of them were missense mutations. A median of two polymorphic sites in MT-ATP genes was found in colon specimens of controls while a median of three polymorphic sites was noted in patients with IBS-D (Mann-Whitney test, $P = 0.012$). The variants of the colon and ileum specimens from the same subjects were identical in all but one case. Symptom duration in IBS was not found to be a significant factor associated with the mtDNA polymorphism (Spearman correlation, $P = 0.592$). The mitochondrial DNA change at 8860 was present in all cases of both groups. The frequency of the 8701 polymorphism was found to be the second most frequent; however, no statistical difference was noted between the groups (χ^2 test, $P = 0.584$).

CONCLUSION: Patients with IBS-D have a higher incidence of MT-ATP 6 and 8 polymorphisms than healthy subjects, implying that the mtDNA polymorphism may play a role in IBS-D.

Key words: Irritable bowel syndrome; Diarrhea; Mitochondrial ATP 6 gene; Mitochondrial ATP 8 gene; Polymorphism

Core tip: Mitochondrial DNA, the only source of extranuclear genome, was assessed by Camilleri's group in a study of patients with functional gastrointestinal disorders in 2009 which was the first study exploring the possible role of mitochondrial DNA in irritable bowel

syndrome (IBS). Up till now, few research efforts have focused on this topic and there is little knowledge about it. Present study revealed that mitochondrial ATP 6 and 8 genes were more frequently mutated in IBS with diarrhea than in healthy individuals. We believe this preliminary finding could help promote future research on mitochondrial DNA in IBS.

Wang WF, Li X, Guo MZ, Chen JD, Yang YS, Peng LH, Wang YH, Zhang CY, Li HH. Mitochondrial ATP 6 and 8 polymorphisms in irritable bowel syndrome with diarrhea. *World J Gastroenterol* 2013; 19(24): 3847-3853 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3847.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3847

INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most prevalent functional gastrointestinal disorders and affects 10%-20% of the population[1]. To date, the pathogenesis of IBS remains unclear.

Family[2,3] and twin[4] studies suggested that genetic changes may predispose individuals to IBS, although reported data are controversial[5]. Furthermore, some candidate nuclear genes are associated with IBS (serotonin transporter and receptor genes and inflammatory markers)[6]. Recently, mitochondrial DNA (mtDNA), the only source of extranuclear genes, was assessed by Camilleri *et al*[7] in a study of patients with functional gastrointestinal disorders. This initial study on the role of haplogroup H, 16519C>T and 3010G>A (located in the D-loop and the 16S rRNA gene) of mitochondrial DNA (mtDNA) indicated that the mitochondrial haplogroup and variations may be associated with gastrointestinal motor and sensory function in gastrointestinal disorders such as IBS. The human mitochondrial genome encodes 13 genes[8] for ATP subunits 6 and 8, and other polypeptides of respiratory complexes crucial for ATP production in mitochondria. ATP synthesis requires ATP synthase, of which subunits 6 and 8 are mtDNA encoded. Based upon this knowledge, we hypothesized that mitochondrial ATP (MT-ATP) 6 and 8 may be involved in the pathogenesis of IBS. In this study, we aimed to investigate the mitochondrial ATP (MT-ATP) 6 and 8 polymorphisms in the colon and ileum of IBS with diarrhea (IBS-D).

MATERIALS AND METHODS

Subjects

Twenty-eigh patients who satisfied Rome Ⅲ criteria[1] for IBS-D and 28 healthy controls were enrolled at the Chinese PLA General Hospital between June 2009 and May 2011. Inclusion criteria for IBS with diarrhea were: (1) male and female individuals aged 18-60 years; (2) recurrent abdominal pain or discomfort associated with a change in stool frequency or form over a 3-mo period; (3) loose or watery stool more than 25% of the time, and hard stool less than 25% of the time; (4) symptom onset at least 6 mo prior to diagnosis; and (5) pain or discomfort at least 2 d a week during the screening period. Controls were healthy subjects undergoing routine health evaluation, with normal bowel movements (*i.e.*, fewer than 3/d and more than 3/wk) and no gastrointestinal complaints in the previous year. Patients were excluded in both IBS-D and control groups if they: (1) were pregnant or lactating; (2) were found to have abnormal colonoscopic findings; (3) had symptoms of a significant clinical illness; (4) had previous gastrointestinal surgery; and (5) had diabetes or other diseases affecting gastrointestinal function. This study was approved by the Ethics Committee of Chinese PLA General Hospital and written informed consent was obtained from each subject. The protocol was registered at www.clinicaltrials.gov as NCT01028898. This study was conducted in compliance with the principles of the Declaration of Helsinki.

Endoscopy procedure

All study participants underwent screening colonoscopy with a CF-H260AI or CF-Q260AI colonoscope (Olympus, Tokyo, Japan); terminal ileum intubation was attempted when applicable. During withdrawal of the scope, the colonic mucosa was carefully visualized and biopsies of the sigmoid colon were obtained (FB-55U-1 biopsy forceps; Olympus, Tokyo, Japan). Terminal ileal biopsies were obtained from 10 patients with IBS-D.

All colonoscopies were performed at the Chinese PLA General Hospital and samples were stored in covered storage tubes at -20 ℃ before analysis.

Polymerase chain reaction and mtDNA sequencing

The MT-ATP 6 and 8 genes were polymerase chain reaction amplified and sequenced according to the mitoSEQr™ protocol (Applied Biosystems, Foster City, CA, United States).

Polymerase chain reaction reaction: Genomic DNA was extracted from specimens based on standard protocols[9]. Thermocycling conditions with the AB 9700 (Applied Biosystems, Foster City, CA, United States) were as follows: Heat activation at 96 ℃ for 5 min, followed by 40 cycles at 94 ℃ for 30 s, 60 ℃ for 45 s, 72 ℃ for 45 s; final extension at 72 ℃ for 10 min. Polymerase chain reaction (PCR) reaction clean-up was performed by adding 2 μL of ExoSAP-IT® (USB Corporation), incubating at 37 ℃ for 30 min and heat inactivation at 80 ℃ for 15 min.

Sequencing reaction and electrophoresis: A forward and reverse sequencing reaction mix was prepared from ready-to-use resequencing sets (Applied Biosystems, Foster City, CA, United States). The sequencing master mixes contained the M13 forward and reverse primers. Cycling conditions were: heat activation at 96 ℃ for 1 min, followed by 25 cycles at 96 ℃ for 10 s, 50 ℃ for 5 s, and 60 ℃ for 4 min. Sequencing reaction clean-up was performed. PCR products were electrophoresed using the

Table 1 Demographic data and clinical profile of the participants

	Control (n = 28)	IBS (n = 28)	P value
Age (yr)	43.6 ± 1.9	41.6 ± 2.2	> 0.05[1]
Gender (male:female)	22:6	18:10	> 0.05[2]
Disease duration (yr)	N/A	3.6 ± 0.6	N/A

[1]P values were obtained using t test (t = -0.682, df = 54, P = 0.498); [2]Pearson's χ^2 test (χ^2 = 1.400, df = 1, P = 0.237) between the control and patients with irritable bowel syndrome (IBS). Values expressed as mean ± SE. N/A: Not available.

AB 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, United States).

Analysis of the sequences of MT-ATP 6 and 8 genes: Sequencing data were analyzed *via* Variant Reporter™ Software version 1.0 (Applied Biosystems, Foster City, CA, United States) and compared with the reference sequence from Genbank (accession No. NC_012920) to indicate possible polymorphisms. The corresponding changes of amino acid with missense polymorphisms were excerpted from MITOMAP (www.mitomap.org). For polymorphism not recorded in MITOMAP, HmtDB (http:// www.hmtdb.uniba.it: 8080/ hmdb/) was applied.

Statistical analysis

The χ^2 and Student's t test were used to analyze demographic data between control and IBS-D patients, wherever applicable. Since MT-ATP 6 and 8 genes overlap in the mtDNA genome, these two genes were considered as a single gene during analysis. The non-parametric Mann-Whitney U test was performed to analyze polymorphism frequency between groups. The Spearman rank correlation coefficient was used to determine the association between the presence of the mtDNA polymorphism and IBS-D duration. A P value of less than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS software (Chicago, IL, United States).

RESULTS

Characteristics of study participants

The characteristics of study participants are summarized in Table 1. No significant difference was found in age or gender between patients with IBS-D and controls.

mtDNA polymorphisms in colon and terminal ileum

The nuclear positions and base changes of the mtDNA polymorphism, as compared with the Genbank mtDNA sequence (accession No. NC_012920) are listed in Table 2. Twenty-five polymorphic sites in MT-ATP 6 and 8 genes were observed, of which 48% (12/25) were missense. No deletions or insertions were detected.

The median 2 polymorphic sites (range 1-3) in the MT-ATP 6 and 8 genes were found in control colon specimens (Table 3) while the median 3 polymorphic sites (range 1-4) were found in IBS-D patients (Table 4). Similarly, 1-4 polymorphic sites in MT-ATP 6 and 8 genes from the terminal ileum specimens were found in IBS-D patients (Table 5). When comparing with mtDNA polymorphisms in the colon and terminal ileum, all were identical except in one case (IBS22).

Table 2 Summary of mitochondrial DNA mutations in the ATP 6 and 8 genes

Nucleotide position	Base change (N>T)	mtDNA region	Amino acid change
8392	G>A	ATP8	syn
8394	C>T	ATP8	Missense
8414	C>T	ATP8	Missense
8459	A>G	ATP8	Missense
8473	T>C	ATP8	syn
8563	A>G	ATP8 and ATP6	ATP6: missense; ATP8: syn
8584	G>A	ATP6	Missense
8684	C>T	ATP6	Missense
8697	G>A	ATP6	syn
8701	A>G	ATP6	Missense
8705	T>C	ATP6	Missense
8727	C>T	ATP6	syn
8772	T>C	ATP6	syn
8784	A>G	ATP6	syn
8793	T>C	ATP6	syn
8794	C>T	ATP6	Missense
8829	C>T	ATP6	syn
8856	G>A	ATP6	syn
8860	A>G	ATP6	Missense
8943	C>T	ATP6	syn
8994	G>A	ATP6	syn
9053	G>A	ATP6	Missense
9123	G>A	ATP6	syn
9128	T>C	ATP6	Missense
9180	A>G	ATP6	syn

mtDNA: Mitochondrial DNA; syn: Synonymous; N: Sequence obtained from National Center for Biotechnology Information genbank; T: Mutated sequence in the specimens; A: Adenine; T: Thymine; G: Guanine; C: Cytosine.

Association between clinical profiles and mtDNA polymorphisms

Polymorphisms in the MT-ATP 6 and 8 genes were increased in IBS patients as compared with controls (non-parametric Mann-Whitney test, U = 245, P = 0.012; Figure 1). In addition, the difference in percentage of missense polymorphisms between groups was statistically significant (non-parametric Mann-Whitney test, U = 265, P = 0.016). No correlation was found between the presence of mtDNA polymorphisms and disease duration in IBS-D patients (Spearman rho = 0.106, P = 0.592).

The mtDNA change at 8860 was present in all cases. The frequency of the 8701 polymorphism was 64% (18/28) in the IBS group and 57% (16/28) in controls, not statistically significant (Pearson χ^2 = 0.299, df = 1, P = 0.584). Several IBS-D patients (*e.g.*, IBS13, IBS14 and IBS18) had the same combination of polymorphisms but no similarities in clinical profile (age, gender, duration of disease) were observed.

DISCUSSION

We found that polymorphisms in mitochondrial ATP 6

Table 3 Mitochondrial DNA changes in the colon from controls

Case	Nucleotide position															
	8392	8394	8414	8473	8563	8584	8701	8705	8727	8794	8860	8994	9053	9180	Missense	Sum
1								+			+				2	2
2							+				+			+	2	3
3											+		+		2	2
4							+				+				2	2
5											+				1	1
6							+				+				2	2
7							+				+		+		3	3
8											+				1	1
9					+					+	+				3	3
10											+				1	1
11							+				+				2	2
12		+					+				+				3	3
13							+				+				2	2
14						+	+				+				3	3
15							+				+				2	2
16						+			+		+				2	3
17							+				+				2	2
18						+	+				+				3	3
19				+			+				+				2	3
20											+		+		2	2
21						+	+				+				3	3
22	+						+				+				2	3
23											+				1	1
24											+	+			1	2
25											+				1	1
26							+				+			+	2	3
27			+				+				+				3	3
28											+				1	1

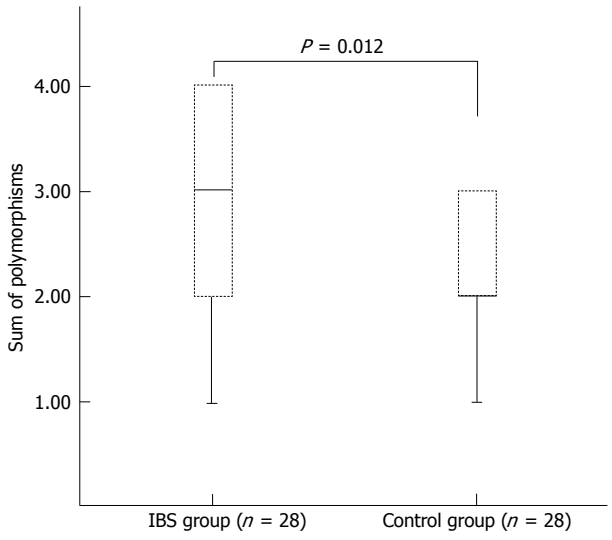

Figure 1 Box plots showing the interquartile range (box) and median (solid line). An increase of mitochondrial DNA polymorphisms in irritable bowel syndrome (IBS) group was observed ($P = 0.012$).

and 8 genes were more frequent in IBS with diarrhea, than in healthy individuals. Nearly half of the mutations studied were non-synonymous, the meaning of which from a pathophysiological point of view has not been previously clarified.

Blood samples were used typically when analyzing mtDNA. However, previous studies reported that mtDNA changes in the blood were not always the same as those from organ tissues[10,11]. IBS is anatomically attributed to the lower gastrointestinal tract[12]; thus we have chosen colonic and ileal tissues rather than blood. We found that there was almost no difference in mtDNA variants between colon and ileum specimens from the same individual with IBS, except for one case. Both the small and large intestines, harboring the same mtDNA mutation, indicate that a similar pathogenesis may exist in both locations.

mtDNA is highly polymorphic[13,14], and may occur across the entire mtDNA genome, with the MT-ATP gene as one of the hot spots. A high frequency of mtDNA polymorphisms has been implicated in a variety of diseases[15,16], including mitochondrial diseases, degenerative diseases, aging, tumors and so on. Thus far, studies investigating the association between mtDNA and IBS are scarce. Camilleri's group reported that the mtDNA polymorphism in functional gastrointestinal disorders may be associated with digestive functions, such as satiation, gastric emptying, and pain[7]. Moreover, the mtDNA haplotype might affect the risk of developing IBS. Human mtDNA haplogroups are defined by a particular single nucleotide polymorphism (SNP). Haplotype H (7028C genotype) is predominant in the European population[17,18]; hence, Caucasians are often divided into Haplotype H and non-Haplotype H groups. Camilleri studied the difference between these groups, and found that constipation-predominant and mixed IBS were more likely to be associated with Haplotype H. Most Chinese people are Haplotype A, D and G, but not Haplotype

Table 4 Mitochondrial DNA changes in the colon from irritable bowel syndrome group

Case	Nucleotide position																				
	8392	8414	8459	8473	8563	8584	8684	8697	8701	8772	8784	8793	8794	8829	8856	8860	8943	9123	9128	9180	Missense	Sum
IBS1																+					1	1
IBS2		+							+							+					3	3
IBS3								+								+					1	2
IBS4																+					1	1
IBS5					+								+			+					3	3
IBS6		+							+							+					3	3
IBS7						+	+		+							+					4	4
IBS8										+						+					1	2
IBS9	+															+					1	2
IBS10						+					+			+		+					2	4
IBS11									+							+				+	2	3
IBS12									+							+	+				2	3
IBS13		+		+					+							+					3	4
IBS14		+		+					+							+					3	4
IBS15									+			+			+	+					2	4
IBS16									+			+			+	+					2	4
IBS17									+							+					2	2
IBS18		+		+					+							+					3	4
IBS19			+										+			+					3	3
IBS20									+							+				+	2	3
IBS21									+							+					2	2
IBS22									+							+					2	2
IBS23				+		+			+							+					3	4
IBS24						+	+		+							+					4	4
IBS25									+							+					2	2
IBS26																+		+			1	2
IBS27																+			+		2	2
IBS28									+							+			+	+	3	4

IBS: Irritable bowel syndrome.

Table 5 Mitochondrial DNA changes in terminal ileum from irritable bowel syndrome group

Case	Nucleotide position											
	8414	8459	8473	8563	8584	8684	8697	8701	8772	8794	8860	Sum
IBS1											+	1
IBS2	+							+			+	3
IBS3							+				+	2
IBS5				+						+	+	3
IBS7					+	+		+			+	4
IBS17								+			+	2
IBS18	+		+					+			+	4
IBS19		+								+	+	3
IBS21								+			+	2
IBS22									+		+	2

IBS: Irritable bowel syndrome.

H[19], which is why we did not investigate the haplotype association with IBS in our study.

More than 3000 mtDNA polymorphisms are reported in various mtDNA databases[20], and are often categorized as neutral or pathogenic. The role of pathogenic mtDNA mutations in mitochondrial diseases has been established[21,22], while many polymorphisms in the mtDNA genome present in aging, tumorigenesis and other disease states are considered to be of little pathogenic significance[22,23]. Although efforts have been made to discover the underlying mechanism of these "neutral" polymorphisms, we are far from having a complete understanding of it. Recently, accumulation of mtDNA polymorphisms was proposed to underlie the pathogenesis of these diseases[24-26]. In our study, the polymorphisms detected were neutral, and cumulative polymorphisms in MT-ATP genes were higher in the IBS group. Accumulation of mtDNA polymorphism may play a role in IBS pathogenesis, however, data are limited on functional studies of neutral polymorphisms. In one study, neutral polymorphisms in the mtDNA control region were found to play a role in individual differences such as exercise tolerance[27]. Another study showed that 8701 and 10398 polymorphisms were associated with changes

in the mitochondrial matrix pH and intracellular calcium dynamics in the cell line[28], which may help understand the functional significance of some neutral mtDNA polymorphisms. We also identified the 8701 polymorphism, but found no statistical difference in this polymorphism between IBS-D and controls. How these neutral polymorphisms are involved in functional diseases such as IBS-D remains unknown and further research is needed.

This study has several strengths and limitations. A major strength of this study lies in its direct analysis of small intestinal and colon samples, which are considered the areas of IBS symptomatology. As mentioned previously, blood mtDNA represents the status of circulating mitochondria, not specifically that of the bowel. However, due to the nature of intestinal biopsy, it is difficult to recruit a large number of subjects to participate. The relatively small number of study subjects limits our conclusions. Also, there is another concern that no functional studies focusing on these polymorphisms were involved, limiting the interpretation of the results. Further studies with larger sample sizes from multiple centers are required.

In conclusion, we found that cumulative polymorphisms in the mitochondrial ATP genes are associated with IBS-D. While it remains unknown how these polymorphisms are associated with ATP synthase function and cellular energy production in the intestine, our study has important implications for future research on the mtDNA genome in IBS.

ACKNOWLEDGMENTS

We would like to thank Dr. Qing-Sen Liu, Dr. Wen Li and Dr. En-Qiang Linghu for their support. We are also grateful to Drs. Yuan-Zi Yu, and Min Min for their technical assistance. This work was presented in abstract form at UEG Week 2012 in Amsterdam.

COMMENTS

Background

Irritable bowel syndrome (IBS) is one of the most prevalent functional gastrointestinal disorders and affects 10%-20% of the population. To date, the pathogenesis of IBS remains unclear. Family and twin studies suggested that genetic changes may predispose individuals to IBS.

Research frontiers

A previous study indicated that the mitochondrial (MT) haplogroup and variations may be associated with gastrointestinal motor and sensory function in gastrointestinal disorders such as irritable bowel syndrome. Up till now, few research efforts have focused on this topic and little knowledge is available about it.

Innovations and breakthroughs

The authors sequenced the coding region of mitochondrial DNA and found that polymorphisms in MT-ATP 6 and 8 genes were more frequent in IBS with diarrhea, than in healthy individuals.

Applications

This study results implied that the MT-DNA polymorphism may play a role in irritable bowel syndrome with diarrhea, but further studies are needed.

Terminology

MT-DNA: Human MT DNA is approximately 16.6 kbp long, which is inherited solely from the mother and encodes 13 genes for ATP subunits 6 and 8, and other polypeptides of respiratory complexes crucial for ATP production in mitochondria; IBS: According to the Rome Ⅲ criteria, irritable bowel syndrome is defined as a functional bowel disorder in which abdominal pain or discomfort is associated with defecation or a change in bowel habit, and with features of disordered defecation.

Peer review

In the short paper, the authors analyzed the polymorphisms of MT-ATP 6 and 8 genes in the colon and ileum of IBS with diarrhea (IBS-D). Based on clinical samples, they concluded that patients with IBS-D have a higher incidence of MT-ATP 6 and 8 polymorphisms, compared with healthy controls. This finding is interesting and will be helpful for clinicians to emphasize the importance of the mtDNA polymorphism of IBS-D patients.

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P- Reviewers Clave P, Igor SL, Ren XF **S- Editor** Song XX
L- Editor Ma JY **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3854

World J Gastroenterol 2013 June 28; 19(24): 3854-3860
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Laparoscopic splenectomy for treatment of splenic marginal zone lymphoma

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Received: January 10, 2013 Revised: April 28, 2013
Accepted: May 17, 2013
Published online: June 28, 2013

Abstract

AIM: To investigate the short-term and long-term efficacy and safety of laparoscopic splenectomy (LS) for treatment of splenic marginal zone lymphoma (SMZL).

METHODS: A total of 18 continuous patients who were diagnosed with SMZL and underwent LS in our department from 2008 to 2012 were reviewed. The perioperative variables and long-term follow-up were evaluated. To evaluate the efficacy and safety of this procedure better, we also included 34 patients with liver cirrhosis who underwent LS, 49 patients with immune thrombocytopenia (ITP) who underwent LS, and 20 patients with SMZL who underwent open splenectomy (OS). The results observed in the different groups were compared.

RESULTS: No differences were found in the sex and Child-Pugh class of the patients in SMZL-LS, SMZL-OS, ITP, and liver cirrhosis groups. The splenic length of the patients in the SMZL-LS group was similar to that in the SMZL-OS and liver cirrhosis groups but significantly longer than in the ITP group. The SMZL-LS group had a significantly longer operating time compared with the SMZL-OS, ITP, and liver cirrhosis groups, and the SMZL-LS group exhibited significantly less blood loss compared with the SMZL-OS group. No difference was found in the length of the postoperative hospital stay between the SMZL-LS, SMZL-OS, ITP, and liver cirrhosis-LS groups. After surgery, 6 (33.3%) SMZL-LS patients suffered slight complications. During mean follow-up periods of 13.6 and 12.8 mo, one patient from the SMZL-LS group and two from the SMZL-OS group died as a result of metastasis after surgery. None of the ITP and liver cirrhosis patients died.

CONCLUSION: LS should be considered a feasible and safe procedure for treatment of SMZL in an effort to improve the treatment options and survival of patients.

Key words: Splenic marginal zone lymphoma; Laparoscopic splenectomy; Open splenectomy; Liver cirrhosis; Immune thrombocytopenia

Core tip: Laparoscopic splenectomy (LS) achieves excellent results for treatment of benign hematological diseases. The role of LS in treatment of splenic marginal zone lymphoma (SMZL) is difficult to define due to the associated splenomegaly, which may influence long-term outcomes. We investigated the perioperative variables and long-term follow-up of 18 SMZL patients who underwent LS and compared them with SMZL patients who underwent open splenectomy, immune thrombocytopenia patients who underwent LS, and liver cirrhosis patients who underwent LS. LS should be considered an appropriate treatment strategy for SMZL

patients in an effort to improve the treatment options and survival of these patients.

Wu Z, Zhou J, Wang X, Li YB, Niu T, Peng B. Laparoscopic splenectomy for treatment of splenic marginal zone lymphoma. *World J Gastroenterol* 2013; 19(24): 3854-3860 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3854.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3854

INTRODUCTION

Due to their low incidence rate, it is difficult and often ambiguous to determine the appropriate strategy for the treatment/management of splenic masses, which are considered uncommon diseases[1]. The most common splenic malignancy is lymphoma[1]. Splenic marginal zone lymphoma (SMZL) with or without villous lymphocytes is a disorder that was recently recognized as a distinct pathological entity in the World Health Organization classification[2]. This disease mainly affects elderly and middle-aged patients with a median age of 65 years[3]. At diagnosis, SMZL presents as an indolent and disseminated disease that is originally recognized after histopathological examination of surgically removed spleens as SMZL itself, or by means of morphological and immunophenotypic characterization of circulating neoplastic lymphocytes as splenic lymphoma with villous lymphocytes[4-6]. Cytopenia and lymphocytosis are frequently observed[7]. To date, there is no definitive standard treatment for SMZL. Approximately 2/3 of the patients are asymptomatic at diagnosis, and as many as one third of the patients will never require therapy. The diagnosis of this disease in patients who do not undergo splenectomy involves the morphological and immunophenotypic analysis of the peripheral blood and bone marrow[8].

When splenectomy is indicated, laparoscopic splenectomy (LS) is the favored approach for treatment of benign hematological disorders. The role of LS in the treatment of a variety of hematological diseases, such as immune thrombocytopenia (ITP) and thrombotic thrombocytopenia, for which all other medical therapies have been exhausted has been elaborately documented[8]. The technical success, minimal morbidity, reduced disability, and high patient acceptance have resulted in the classification of LS as the gold standard for treatment of ITP[9,10]. Although splenomegaly was once considered a contraindication for laparoscopy, an increasing number of studies have proven the efficacy and safety of LS in both the short-term and the long-term treatment of splenomegaly and hypersplenism[11,12].

The role of LS in patients with hematological malignancies remains ambiguous due to the skepticism regarding the use of minimally invasive techniques for the management of malignant or potentially malignant splenic diseases[12]. However, the increased incidence of patients with non-Hodgkin's lymphoma, particularly elderly patients, and the relative increase in the number of splenectomies performed in the treatment of hematological malignancies makes this issue particularly germane[13]. To date, there are only a few case studies that have analyzed the use of LS in the treatment of SMZL[1,4,8]. The present study aimed to reveal whether the surgical outcomes of LS are beneficial, safe, and/or secure for the treatment of SMZL to determine whether this procedure should be considered a standard protocol in the management of SMZL. To achieve the most meaningful comparison between patients with similar disease mechanisms, we analyzed 20 patients with SMZL that underwent open splenectomy (OS), 49 with ITP, and 34 with splenomegaly due to liver cirrhosis and portal hypertension who were treated with LS.

MATERIALS AND METHODS

Patients

Our retrospective comparative study was designed to determine the efficacy and surgical outcomes of SMZL patients who underwent LS (SMZL-LS group) and to compare the outcomes with those observed in SMZL patients who underwent OS (SMZL-OS group) and in ITP and liver cirrhosis patients who underwent LS in West China Hospital at Sichuan University in 2008-2012. We include our published report on the use of LS in the management of ITP and liver cirrhosis and compared these results with the outcomes obtained for LS in the management of SMZL.

The chief diagnostic indicator of SMZL was histological confirmation. The diagnosis of ITP was based on bone marrow aspirate that documented a sufficient number of megakaryocytes. All of the patients with liver cirrhosis underwent LS and subsequent liver biopsy. All of the patients were characterized by the principal indicators for splenectomy: diagnostic and therapeutic. The major anticipated therapeutic benefits were the relief of the local symptoms of splenomegaly and the correction of cytopenia.

The patients included in this study underwent a detailed demographic, clinical, and biochemical assessment. The hematological response and liver function were assessed before and 7 d after surgery using peripheral blood count (leukocytes, hemoglobin, and platelets) and total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and albumin assays. At the time of preoperative evaluation for splenectomy, all of the patients underwent a color Doppler ultrasonography (US) scan and computed tomography (CT) to calculate the length of the spleen and to determine the presence of any portal or splenic vein thrombosis (PSVT). Seven days after the operation, all of the patients underwent careful screening for thrombosis. The patients who showed evidence of splenic vein thrombosis by US underwent CT to confirm the extent of thrombosis.

Operative technique of LS

The operative techniques of OS, LS, and hand-assisted

Table 1 Demographic characteristics

Variable	SMZL		Liver cirrhosis	ITP	*P* value[1]	*P* value[2]	*P* value[3]
	LS	OS					
Cases	18	22	34	49			
Age, yr	56.4 ± 10.5	52.0 ± 10.8	47.7 ± 12.2	36.2 ± 15.9	0.191	0.013	0.000
Sex (M/F)	8/10	10/12	16/18	10/39	0.949	0.857	0.049
Child-Pugh class					0.336	0.522	0.282
A	16 (88.9)	17 (77.3)	27 (79.4)	47 (95.9)			
B	2 (11.1)	5 (22.7)	5 (14.7)	2 (4.1)			
C	0	0	2 (5.9)	0 (0)			
Comorbidity							
ITP	1	2					
SLE	1	0					
Pulmonary effusion	1	2					
Herpes zoster	1	1					

Data are presented as mean ± SD or *n* (%). [1]SMZL-LS *vs* SMZL-OS groups; [2]SMZL-LS *vs* liver cirrhosis group; [3]SMZL-LS *vs* ITP groups. SMZL: Splenic marginal zone lymphoma; OS: Open splenectomy; LS: Laparoscopic splenectomy; ITP: Immune thrombocytopenia; M: Male; F: Female; SLE: Systemic lupus erythematosus.

laparoscopic splenectomy (HALS) have been described previously by our group[11,12]. In addition, we removed and biopsied a 1 cm × 1 cm piece of hepatic tissue from the left lobe of the liver of patients with liver cirrhosis.

Follow-up

The mean follow-up time of SMZL patients who underwent LS was 13.6 mo. US and CT studies were performed at 1-mo intervals for 6 mo and at 3-mo intervals thereafter to determine whether the patients relapsed or developed PSVT. Upon the detection of PSVT by CT, we initiated anticoagulation therapy, which consisted of heparin (10000 U/d, intravenously), followed by warfarin. The dose of warfarin was adjusted to achieve an international normalized ratio (INR) of 1.5-2.0. Warfarin was administered until the disappearance of PSVT was confirmed by CT. All of the tests and examinations were repeated depending on the clinical condition of the patient.

Statistical analysis

The continuous variables are expressed as the mean ± SD. The statistical analyses were performed using the SPSS for Windows version 16.0 (SPSS, Chicago, IL, United States). The differences between the variables were compared using Student's *t* test and the χ^2 test. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Demographic characteristics

No differences were found between the demographic characteristics of the SMZL-LS and SMZL-OS groups. The SMZL-LS patients were significantly older than the liver cirrhosis and ITP patients. In addition, women tend to suffer from ITP, and thus there were significant sex differences between the ITP group and both the SMZL-LS and the liver cirrhosis groups (Table 1). There were no differences in the Child-Pugh class between the SMZL-LS group and the SMZL-OS and the liver cirrhosis groups, whereas the ITP patients had normal liver function. The comorbidity of the SMZL patients in both groups is shown in Table 1.

Perioperative outcomes

No patients in the ITP group exhibited conversion, but one patient from the liver cirrhosis group underwent conversion due to bleeding during the operation. In addition, one SMZL-LS patient underwent conversion because the harmonic was unable to stop the bleeding from the VASA during the operation. The SMZL-LS group had a significantly longer operation time compared with the SMZL-OS, ITP and liver cirrhosis groups. The EBL of the SMZL-OS group exhibited the most established blood loss, whereas the estimated blood loss (EBL) of the SMZL-LS and liver cirrhosis groups was not significantly different from that of the ITP group (Table 2). The SMZL-OS group exhibited a higher transfusion rate compared with the SMZL-LS group, whereas the transfusion rates of the other three types of patients were not significantly different. The spleen length of the SMZL-LS group was similar to that of the SMZL-OS and liver cirrhosis groups and longer than that of the ITP patients. The spleens of SMZL and liver cirrhosis patients usually exhibit splenomegaly or massive splenomegaly. The operation methods for the treatment of SMZL were LS ($n = 8$) or HALS ($n = 10$), whereas LS was used for the treatment of patients with liver cirrhosis and ITP.

Postoperative results

No difference was found in the length of postoperative hospital stay between the SMZL-LS, liver cirrhosis and ITP groups, whereas the SMZL-OS group experienced a significantly longer stay (Table 3). Six SMZL-LS, 10 SMZL-OS, 5 liver cirrhosis, and three ITP patients suffered complications. Patients with pulmonary effusion, pancreatic leakage, and abdominal cavity effusion were all cured through conservative treatment, such as somatostatin and drainage. One liver cirrhosis patient experienced

Table 2 Comparison of intraoperative details

Variable	SMZL		Liver cirrhosis	ITP	*P* value[1]	*P* value[2]	*P* value[3]
	LS	OS					
Conversion	1	-	1	0			
Operation time (min)	238.4 ± 37.9	185.9 ± 54.9	210.1 ± 48.5	163.9 ± 67.2	0.001	0.037	0.000
EBL	171.9 ± 228.4	310.0 ± 192.0	150.0 ± 146.1	65.7 ± 114.0	0.045	0.675	0.014
Transfusion	4/18 (22.2)	9/13 (69.0)	3/34 (8.8)	8/49 (16.3)		0.178	0.577
Spleen length (cm)	22.8 ± 5.5	23.7 ± 5.6	23.9 ± 3.9	12.1 ± 3.2	0.624	0.408	0.000
Additional operation							
Liver biopsy	0	0	34	0			
Lymph node biopsy	3	5		0			
LC	1	-	2	5			
Operation Method							
LS	8	-	34	49			
HALS	10	-	0	0			

Data are presented as mean ± SD or *n* (%). [1]SMZL-LS *vs* SMZL-OS groups; [2]SMZL-LS *vs* liver cirrhosis groups; [3]SMZL-LS *vs* ITP groups. SMZL: Splenic marginal zone lymphoma; OS: Open splenectomy; LS: Laparoscopic splenectomy; ITP: Immune thrombocytopenia; HALS: Hand-assisted laparoscopic splenectomy; EBL: Estimated blood loss; LC: Laparoscopic cholecystectomy.

Table 3 Comparison of postoperative details

Variable	SMZL		Liver cirrhosis	ITP	*P* value[1]	*P* value[2]	*P* value[3]
	LS	OS					
PHS (d)	8.17 ± 3.7	10.8 ± 4.2	7.5 ± 2.0	7.6 ± 2.1	0.044	0.378	0.389
Complication							
Pulmonary effusion	3	5	1	0			
Pancreatic leakage	1	2	1	1			
Abdominal cavity effusion	1	1	0	0			
Postoperative bleeding	0	0	1	2			
Portal/splenic vein thrombosis	1	2	2	0			
Total	6 (33.3)	10 (45)	5 (14.7)	3 (6.1)			

Data are presented as mean ± SD or *n* (%). [1]SMZL-LS *vs* SMZL-OS groups; [2]SMZL-LS *vs* liver cirrhosis groups; [3]SMZL-LS *vs* ITP groups. SMZL: Splenic marginal zone lymphoma; OS: Open splenectomy; LS: Laparoscopic splenectomy; ITP: Immune thrombocytopenia; PHS: Postoperative hospital stay.

postoperative bleeding. As a result, an emergency laparotomy and blood transfusion were performed, and the patient was discharged 14 d after LS. Two ITP patients suffered postoperative bleeding and received blood transfusion and conservative medical treatment. Both of these patients recovered 10 d after surgery. Patients were diagnosed with portal splenic vein thrombosis by postoperative dynamic CT. These patients received anticoagulation therapy consisting of heparin (10000 U/d *iv*) followed by warfarin. The dose of warfarin was adjusted to achieve an INR of 2. The administration of warfarin was continued every 3 mo until thrombosis disappeared.

After surgery and during follow-up, almost no significant differences in the hematological parameters and liver function outcomes were observed between the SMZL-LS and SMZL-OS groups. Total bilirubin of the liver cirrhosis group was much higher than that of the SMZL-LS and ITP groups because liver cirrhosis usually causes liver damage. The same result was observed in the analysis of the ALT and AST of the three groups of patients. The SMZL-LS and liver cirrhosis patients had a low white blood cell count (WBC) compared with the ITP patients ($P = 0.000$), and the WBCs of the SMZL-LS and liver cirrhosis groups were the same. The platelet counts of the three types of patients were all different. The platelet count of the SMZL-LS group was higher than that of the liver cirrhosis group ($P = 0.000$), and the platelet count of the liver cirrhosis group was higher than that of the ITP group ($P = 0.000$). Postoperative comparison revealed that the liver cirrhosis patients had a higher level of total bilirubin and albumin than the SMZL patients. The ALT and AST levels in these patients were equal. The WBC of the ITP group was higher than that of the lymphoma and liver cirrhosis patients, but the WBC of the lymphoma and liver cirrhosis patients did not differ significantly. The platelet count of the three types of patients exhibited no significant differences (Table 4).

Follow-up outcomes

The SMZL-LS and SMZL-OS groups had a mean follow-up of 13.6 and 12.8 mo, respectively. At these times, none of the patients became septic or experienced wound complications following LS. One SMZL-LS and 2 SMZL-OS patients died as a result of metastasis following surgery. The other 17 patients experienced disease-free survival. None of the patients in the ITP and liver cirrhosis groups died.

Table 4 Comparison of the preoperative and postoperative hematological parameters and liver function variables

Variable	SMZL		Liver cirrhosis	ITP	*P* value[1]	*P* value[2]	*P* value[3]
	LS	OS					
Preoperation							
TBIL (mmol/L)	15.7 ± 8.5	23.3 ± 11.2	28.3 ± 17.2	13.3 ± 6.5	0.023	0.005	0.231
ALT (U/L)	25.1 ± 17.6	30.6 ± 11.1	54.7 ± 44.0	36.2 ± 33.6	0.237	0.009	0.187
AST (U/L)	25.7 ± 16.9	33.9 ± 16.9	59.3 ± 40.1	25.9 ± 21.9	0.133	0.001	0.956
Albumin (g/L)	36.8 ± 6.6	35.1 ± 8.4	37.6 ± 5.7	40.7 ± 5.3	0.478	0.653	0.015
HGB (g/L)	102.7 ± 26.1	106.3 ± 30.3	112.2 ± 22.5	123.7 ± 23.1	0.687	0.175	0.002
WBC ($\times 10^9$/L)	4.2 ± 3.3	4.0 ± 3.2	3.2 ± 2.6	11.3 ± 6.7	0.867	0.255	0.000
PLT ($\times 10^9$/L)	65.8 ± 35.6	56.1 ± 30.5	38.1 ± 15.7	20.6 ± 20.2	0.359	0.000	0.000
Postoperation							
TBIL (mmol/L)	11.9 ± 6.7	16.2 ± 7.8	19.4 ± 11.3		0.078	0.014	
ALT (U/L)	23.4 ± 12.9	28.1 ± 13.9	32.0 ± 25.9		0.279	0.192	
AST (U/L)	27.4 ± 17.7	29.5 ± 14.7	28.6 ± 9.7		0.682	0.753	
Albumin (g/L)	31.2 ± 5.5	33.7 ± 3.9	34.5 ± 3.9		0.114	0.017	
HGB (g/L)	101.1 ± 15.1	99.2 ± 15.1	138.6 ± 172.1	117.3 ± 20.4	0.699	0.362	0.003
WBC ($\times 10^9$/L)	9.9 ± 6.7	11.3 ± 6.5	7.9 ± 1.8	14.4 ± 4.9	0.481	0.122	0.003
PLT ($\times 10^9$/L)	298.8 ± 304.1	318.1 ± 211.1	237.2 ± 165.0	287.8 ± 140.1	0.814	0.346	0.840

Data are presented as mean ± SD. [1]SMZL-LS *vs* SMZL-OS groups; [2]SMZL-LS *vs* liver cirrhosis groups; [3]SMZL-LS *vs* ITP groups. HGB: hemoglobin; PLT: platelet count; TBIL: Total bilirubin; WBC: White blood cell count; SMZL: Splenic marginal zone lymphoma; OS: Open splenectomy; LS: Laparoscopic splenectomy; ITP: Immune thrombocytopenia; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

DISCUSSION

SMZL is globally deemed a low-grade lymphoma with an indolent clinical course. Numerous cases exhibit a protracted straightforward progression, an excellent response to splenectomy or chemotherapy treatment, and sometimes an unmodified clinical picture in the absence of any treatment. The 5-year survival rate ranges from 65% to 78%[14,15]. Retrospective studies have shown that patients who underwent splenectomy exhibited a significantly improved survival rate compared with those patients who underwent chemotherapy[14]. Splenectomy is the generally preferred treatment for SMZL. Although this process is not preventive, splenectomy offers superior swift relief of symptoms and often completely modifies any affiliated cytopenia. Additionally, this surgical procedure offers excellent disease management, which usually makes it possible for individuals to avoid systemic therapy[14]. Although the advantages of LS, such as shorter hospital stay, less scarring, earlier return to activity, and less inflammatory responses[16], have been documented previously, the residual tumor and tumor recurrence should be taken into account in the consideration of LS as an appropriate procedure for the treatment of a potentially malignant lesion.

Extensive experience with LS at many centers has led to its use in the treatment of a wide variety of benign hematological diseases. Furthermore, our previous results demonstrated that LS is an efficient and safe strategy for the treatment of hypersplenism secondary to liver cirrhosis[11]. Our current data suggest that the results of LS for treatment of SMZL are comparable with the results for treatment of ITP and liver cirrhosis, which confirms the safety of this procedure for these diseases. Although the SMZL group included a significantly older patient population compared with the ITP group and exhibited a spleen length comparable to that of the liver cirrhosis patients, the SMZL patients underwent successful operations with low morbidity and no mortality. The significantly longer operating time and the significantly higher blood loss in the SMZL patients compared with the ITP and liver cirrhosis groups were expected but did not correlate with adverse outcomes[9].

The ability to achieve a satisfactory outcome in this difficult patient group is probably related to the technical expertise of the surgeon[9]. It has been shown that splenic size is an independent predictor of postoperative complications[14]. Yano *et al*[17] reported their experience with HALS for the treatment of splenic tumors in 10 patients. They have recommended the HALS approach because it allows easier mobilization of the spleen (particularly with splenomegaly) and easier resection of the adjacent organs or tissue if necessary. However, Makrin *et al*[18] concluded that most splenic tumors can be treated using a completely laparoscopic approach. This total laparoscopic approach may be unsuitable when the tumor is associated with massive splenomegaly; in these cases HALS may be considered. In our study, eight patients underwent total LS, whereas 10 patients underwent HALS. We performed LS on patients with splenic length > 20 cm. To ensure sufficient space throughout the surgical procedure, additional movements of the spleen were required, which escalated the blood loss and the chance of perisplenic organ injury. In contrast, the majority of our patients with splenomegaly underwent LS effectively[19]. In this particular analysis, we attempted to appraise the intraoperative and postoperative consequences with respect to substantial splenomegaly, utilizing LS and HALS for the treatment of SMZL. Of the 81 patients studied by Thieblemont *et al*[20], 44 exhibited spleen lymphoma and anemia, and 13 of these had Coombs-positive hemolytic anemia. Of our 18 patients, 38.9% exhibited Coombs-

positive hemolytic anemia. A study of 309 patients revealed the 50% of the patients remained anemic[21]. However, our comparative study is unique because it analyzed the effectiveness of LS in the treatment of an assortment of diseases, particularly SMZL. Our outcomes demonstrate that, regardless of the numerous strategies for the treatment of SMZL, LS might prove advantageous for a number of reasons, including its significantly shorter hospital stay and low postoperative stress; these findings have been confirmed by several other investigators. Splenectomy frequently contributes to somatic compensation of patients, which results in local relapse in the spleen, prevents continuing dissemination of the primary tumor site, and mostly corrects cytopenia, thereby creating better conditions for chemotherapy[22]. One of the patients enrolled in our study died as a result of metastasis several weeks after surgery; the patient's death was therefore unrelated to our treatment approach.

The sex of the different groups differed significantly, mainly because of the characteristics and epidemiology of SMZL and ITP. The splenic size was an important indicator of the conversion rate, the operation time, and the blood loss. The SMZL and the liver cirrhosis patients had significantly longer spleens. The operation time of the SMZL group was significantly longer than that of the liver cirrhosis and ITP groups, which implies that surgery for lymphoma is more difficult than for liver cirrhosis and ITP. We found that the spleen of the lymphoma patients usually adhered to the greater omentum or intestine. It therefore requires a longer time to separate these tissues and organs. LS is the gold standard for the treatment of ITP. Compared with LS for the treatment of ITP, LS for liver cirrhosis may be more difficult because the blood vessels are thick and varicose. The EBL of the SMZL and the liver cirrhosis groups was higher than that of the ITP group, whereas there was no significant different between the EBL of the SMZL group and that of the liver cirrhosis group. This finding indicates that LS exhibits similar outcomes in the treatment of both types of patients.

Fine-needle aspiration (FNA) was used in the diagnosis of the splenic mass with a high positive rate of approximately 80%-88.9%[23,24]. Previous studies reported a low morbidity rate and no biopsy-site seeding of the tumor. However, the incidentally discovered lesions comprised the minority of the lesions (20%-27%)[1]. Furthermore, this technique may be associated with bleeding complications and the risk of tumor dissemination[21]. Tessier *et al*[1] demonstrated that FNA biopsy is unnecessary unless the patient cannot tolerate splenectomy, that is, in the setting of a solitary splenic mass with no history of malignancy. Based on the results of Tessier *et al*[1], the SMZL patients in our study did not undergo FNA.

In conclusion, we evaluated the safety and efficacy of LS for the treatment of SMZL and compared these results with the outcome of LS for treatment of ITP and liver cirrhosis, and from the use of OS for the treatment of SMZL. Our findings show that LS is usually safe and effective for the treatment of SZML. Although the SMZL patients who underwent LS required a significantly longer operation time than those with ITP and liver cirrhosis, no significant differences were observed in the transfusion requirements, postoperative complications, or length of postoperative hospital stay. LS might be a favored procedure for the treatment of SMZL. However, further research is required to determine more definitely its effectiveness in the treatment of SMZL. Furthermore, the role of HALS as a first-choice approach or an alternative approach for the treatment of massive splenomegaly needs to be investigated.

COMMENTS

Background

Laparoscopic splenectomy (LS) is the favored operative approach for the treatment of benign hematological disorders that require splenectomy. Although splenomegaly was once considered a contraindication for laparoscopy, an increasing number of studies have proven the efficacy and safety of the use of LS for both the short-term and long-term treatment of splenomegaly. However, the role of LS in the treatment of patients with hematological malignancies remains ambiguous due to skepticism regarding the use of minimally invasive techniques for the treatment of malignant or potentially malignant splenic diseases.

Research frontiers

To date, there is no definitive standard for the treatment of splenic marginal zone lymphoma (SMZL). Approximately two-thirds of patients are asymptomatic at the time of diagnosis, and as many as one-third of the patients will never require therapy. However, the incidence of patients with SMZL is increasing, especially in the elderly population. The use of LS for the treatment of hematological malignancy has gradually improved. In this study, the authors demonstrated that LS might be a feasible and safe treatment option for SMZL.

Innovations and breakthroughs

To date, there are only a few case studies that have analyzed the use of LS for the treatment of SMZL. In addition, only a few studies have compared LS and open splenectomy (OS) for the treatment of SMZL. Furthermore, no study has shown differences in the perioperative and long-term outcomes between SMZL, immune thrombocytopenia (ITP), and splenomegaly. This study demonstrated that LS is a feasible and safe procedure for the treatment of SMZL.

Applications

To achieve the most meaningful comparison between patients with similar disease mechanisms, the authors included patients with SMZL who underwent OS, patients with ITP, and patients with splenomegaly due to liver cirrhosis and portal hypertension who were treated with LS. The study revealed that LS is safe for the treatment of SMZL and should be considered in its management.

Terminology

SMZL with or without villous lymphocytes is a disorder that was recently recognized as a distinct pathological entity in the World Health Organization classification. SMZL was originally recognized either after histopathological examination of surgically removed spleens as SMZL itself, or by means of morphological and immunophenotypic characterization of circulating neoplastic lymphocytes as splenic lymphoma with villous lymphocytes.

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This was an interesting study in which the authors analyzed the perioperative and long-term variables in the use of LS for the treatment of lymphoma. This study shows that the morbidity associated with treatment of SMZL is no more than expected compared with the outcomes obtained for LS treatment of other diseases. The results are instructive and suggest that LS is a feasible and safe procedure for the treatment of SMZL.

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P- Reviewer Rizzieri DA **S- Editor** Huang XZ
L- Editor Kerr C **E- Editor** Ma S

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3861

World J Gastroenterol 2013 June 28; 19(24): 3861-3865
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Relationship between hepatocellular carcinoma and hepatitis B virus genotype with spontaneous YMDD mutations

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Received: February 6, 2013 Revised: April 12, 2013
Accepted: May 7, 2013
Published online: June 28, 2013

Abstract

AIM: To investigate the relationship between hepatitis B virus (HBV) genotype with spontaneous YMDD mutations and hepatocellular carcinoma (HCC) in HBV-related cirrhosis.

METHODS: We investigated 264 liver cirrhosis patients who were not treated with antiviral drugs, including 81 patients with HCC. YMDD mutations were detected by fluorescent hybridization bioprobe polymerase chain reaction (PCR) and melting curve assay using the Diagnosis Kit for HBV YMDD Mutation. Serum HBV genotypes were detected by real-time PCR using genotype-specific TaqMan probes. Statistical analysis was performed according to data type using the *t* test, χ^2 test and unconditional logistic regression analysis.

RESULTS: In the HCC group, genotype C strains, spontaneous YMDD mutations, and genotype C strains with YMDD mutations were detected in 33 (40.74%), 13 (16.05%) and 11 (13.58%) patients, respectively. In the liver cirrhosis (LC) group, HBV genotype C strains, spontaneous YMDD mutations, and genotype C strains with YMDD mutations were detected in 33 (18.03%), 7 (3.83%) and 2 (1.09%) patients, respectively. The differences in genotype C strains, spontaneous YMDD mutations, and genotype C strains with YMDD mutations between the two groups were statistically significant (χ^2 = 15.441, *P* = 0.000; χ^2 = 11.983, *P* = 0.001; *P* = 0.000). In the HCC and LC groups, there were seven patients infected by genotype B strains with YMDD mutations and 13 by genotype C strains with YMDD mutations. Further research revealed that HCC occurred in 2 patients infected by genotype B strains with YMDD mutations and 11 infected by genotype C strains with YMDD mutations. The difference was statistically significant (*P* = 0.000). Unconditional logistic regression analysis revealed that patients infected by genotype C strains with spontaneous YMDD mutations had a 7.775-fold higher risk for the development of HBV-related HCC than patients infected by other type HBV strains (*P* = 0.013, 95%CI: 1.540-39.264).

CONCLUSION: Genotype C strains with spontaneous YMDD mutations are an independent risk factor for HCC in LC patients and are important for early warning of HCC.

Key words: Hepatitis B virus; Liver cirrhosis; Primary hepatocellular carcinoma; Hepatitis B virus genotype; YMDD mutation

Core tip: YMDD mutation is a research hotspot globally. Until recently, most research about YMDD mutation focused on the occurrence of lamivudine-related YMDD mutation and its impact on antiviral treatment. In our research, 264 hepatitis B virus (HBV)-related liver cirrhosis patients not treated with antiviral drugs, including 81 with primary hepatocellular carcinoma (HCC), were investigated for the association between infection by different HBV genotype strains with spontaneous

YMDD mutations and occurrence of primary HCC in cirrhosis patients. Infection by genotype C strains with spontaneous YMDD mutations is an independent risk factor for the development of HCC in cirrhosis patients.

Yang JH, Zhang H, Chen XB, Chen G, Wang X. Relationship between hepatocellular carcinoma and hepatitis B virus genotype with spontaneous YMDD mutations. *World J Gastroenterol* 2013; 19(24): 3861-3865 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3861.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3861

INTRODUCTION

According to the estimation of the World Health Organization, about 350 million people worldwide are chronically infected by hepatitis B virus (HBV)[1-3]. HBV infection is endemic in China. The seropositive rate of hepatitis B surface antigen is 7.18% and about 93 million people are chronically infected by HBV[4-6]. Chronic infection by HBV is the major cause of hepatocellular carcinoma (HCC)[7,8], and in addition to HCC, leads to a series of HBV-related liver diseases, including asymptomatic carrier, chronic hepatitis, and liver cirrhosis (LC)[9-11].

HBV replicates actively in host liver cells and the reverse transcriptase domain of HBV polymerases lacks a proofreading function. Thus, the mutation rate of HBV is relatively high. YMDD motif is a highly conserved sequence in domain C of HBV reverse transcriptase. YMDD mutation, also known as M204V/I mutation, is the substitution of methionine by valine or isoleucine and designated as YVDD or YIDD variant[12,13]. Until recently, most research about YMDD mutations has focused on the occurrence of lamivudine-related YMDD mutations and their effect on antiviral treatment[14-16]. During recent years, spontaneous YMDD mutations have been detected in patients with chronic HBV infection not previously treated with antiviral drugs. The relationship between spontaneous YMDD mutation and HBV-related HCC has rarely been reported. Different HBV genotype strains are formatted by accumulation of point mutations in the viral genome. Previous research has revealed that infection by genotype C strains is associated with HCC. However, the relationship between infection by different HBV genotype strains with spontaneous YMDD mutations and the occurrence of HCC in HBV-related LC patients has not been reported before.

In order to investigate the association between infection by different genotype strains with spontaneous YMDD mutation and the occurrence of HBV-related HCC, we investigated 264 cirrhosis patients not previously treated with antiviral drugs, including 81 with HCC.

MATERIALS AND METHODS

Patients

To ensure that HBV genotype and YMDD mutations could be detected by our kit, 264 HBV-related LC patients with serum HBV DNA load > 5×10^3 copies/mL, diagnosed and treated at the Department of Infectious Diseases in our hospital from May 2010 to August 2012, were selected for further research. According to the criteria "Chinese Standard for the Diagnoses and Treatment of Primary Hepatocellular Cancer in 2011" and "Prevention and Treatment Standard of Chinese Viral Hepatitis in 2000", 81 LC patients with HCC and 183 without HCC were selected and assigned to the HCC group and LC group, respectively. In the HCC group, there were 65 male patients (80.25%) and 16 female patients (19.75%). Their ages ranged from 31 to 78 years, with a mean of 53.86 ± 11.05 years. In the LC group, 129 patients (70.49%) were male and 54 patients (29.51%) were female. Their ages ranged from 22 to 79 years, with a mean of 52.66 ± 11.42 years. None of the patients had been treated previously with antiviral drugs and were not affected by other liver injury factors, such as co-infection with hepatitis A virus, hepatitis C virus, hepatitis D virus or hepatitis E virus, alcoholic hepatitis, autoimmune hepatitis, and fatty liver.

Sample collection

Fasting venous blood was collected from these patients. The serum was separated immediately and stored at -70 ℃.

Detection methods

Serum HBV DNA was quantified by real-time polymerase chain reaction (PCR) (Qiagen, Shenzhen, Guangdong Province, China). YMDD mutant types were determined by fluorescence hybridization bioprobe PCR and melting curve assay with the use of the care HBV mutation PCR assay (Qiagen, Shenzhen, China) and distinguished by melting temperature value. HBV genotype was detected by real-time PCR using genotype-specific TaqMan probe (Fuxing, Shanghai, China). Serum HBV markers were tested by enzyme-linked immunosorbent assay (Huamei, Shanghai, China).

Statistical analysis

Statistical analysis including Student's t test, χ^2 test and unconditional logistic regression analysis were performed using SPSS 17.0 software. A difference with $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Patient characteristics are shown in Table 1. Age, sex distribution, hepatitis B e antigen (HBeAg)-positive rate, and serum HBV load did not differ significantly between the HCC and LC groups. Thirteen and seven spontaneous YMDD mutations were detected in the HCC and LC groups, respectively. In the HCC group, 33 (40.74%) patients were infected by genotype C strains and 47 (58.02%) were infected by genotype B strains. In the LC group, 33 (18.03%) patients were infected by genotype C strains

Table 1 Patient characteristics in the hepatocellular carcinoma and liver cirrhosis groups

	HCC group	LC group	t or χ^2 value	P value
No. of patients	81	183		
Age (yr; mean ± SD)	53.86 ± 11.05	52.66 ± 11.42	-0.797	0.426
Sex (male)	65	129	2.742	0.098
HBeAg positive	18	37	0.137	0.712
Serum HBV DNA loads (log10 copies/mL)	5.43 ± 1.16	5.25 ± 1.28	-1.077	0.283
Spontaneous YMDD mutation	13	7	11.983	0.001
Genotype C virus	33	33	15.441	0.000
Genotype B virus	47	146	13.518	0.000
Co-infected by genotype B and C viruses	1	4	-	-

HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus.

and 146 (79.78%) were infected by genotype B strains. The ratio of patients infected by genotype C virus and spontaneous YMDD mutation rate in the HCC group was higher than in the LC group, and these differences were significant.

Ratio of patients infected by genotype B or C strains with spontaneous YMDD mutation

There were 2 (1.09%) and 11 (13.58%) patients infected by genotype C strains with YMDD mutation in the LC (n = 183) and HCC (n = 81) groups, respectively. The ratio of patients infected by genotype C strains with spontaneous YMDD mutation was higher in the HCC than in the LC group, and the difference was significant (P = 0.000). The constituent ratio difference of patients infected by genotype B strains with spontaneous YMDD mutations between the HCC and LC groups was not significant [2 (2.47%) *vs* 5 (2.73%), P = 1.000].

HCC in LC patients infected by genotype B or C strains with spontaneous YMDD mutation

Thirteen patients were infected by genotype C strains with YMDD mutations; 11 in the HCC group and two in the LC group. Seven patients were infected by genotype B strains with YMDD mutations; two in the HCC group and five in the LC group. The occurrence of HCC was significantly higher in patients infected by genotype C strains with YMDD mutation than in patients infected by genotype B strains with YMDD mutation (P = 0.022).

Identification of risk factors for HBV-related HCC

Unconditional logistic regression analysis was performed with the exclusion criterion $P \geqslant 0.05$. Sex, HBV genotype, and genotype C strains with spontaneous YMDD mutation were included in the regression model. As shown in Table 2, the occurrence of HCC in the male patients was 2.114 times higher than in the female patients. Patients infected by genotype C virus were 2.469 times more susceptible to HCC than those infected by genotype B virus. Risk of HCC in patients infected by genotype C strains with spontaneous YMDD mutation was 7.775-fold higher than in other patients. Other factors, including age, HBeAg status, serum HBV DNA load, spontaneous YMDD mutation, and genotype B strains with YMDD mutation, were excluded from the regression model.

DISCUSSION

Prior research has suggested that lamivudine is the major cause of YMDD mutation in HBV P-ORF. However, the mechanism remains unclear. Further research has revealed that strains with YMDD mutation also exist in patients with chronic HBV infection not previously treated with lamivudine[17-20]. Hosaka *et al*[21] have suggested that lamivudine-related YMDD mutation is an independent risk factor for HCC. Our results showed that spontaneous YMDD mutations were detected in LC and HCC patients, and spontaneous YMDD mutation rate in HCC patients was significantly higher than in LC patients. This suggests that spontaneous YMDD mutations are associated with the occurrence of HCC. Unlike the study of Hosaka *et al*[21], our unconditional logistic regression analysis showed that spontaneous YMDD mutation was not an independent risk factor for HBV-related HCC. One possible reason for this difference is that we studied the effect of spontaneous YMDD mutations, and the carcinogenicity of HBV strains with spontaneous YMDD mutations and lamivudine-related YMDD mutations may be different. This needs to be studied further.

Previous research has revealed that HBV-related LC is a leading cause of HCC[22-24]. Infection with genotype C strains can induce continuous gangrenous inflammation in the host liver and increase the risk of LC and HCC[25]. Our results showed that the infection rate of genotype C strains in the HCC group was higher than in the LC group. This suggests that infection by genotype C strains is associated with HCC. Consistent with previous reports, our unconditional logistic regression showed that the risk of HCC in patients infected by genotype C strains was 2.469 times higher than in those infected by genotype B strains, and infection by genotype C strains was an independent risk factor for HCC. This may have been caused by different genotype strains having different biological properties, pathogenicity and carcinogenicity.

Our research revealed that the ratio of patients infected by genotype C strains with spontaneous YMDD mutation was higher in the HCC than LC group. Unconditional logistic regression analysis showed that patients infected by genotype C strains with spontaneous YMDD mutations were 7.775-fold more susceptible to HCC than patients infected by genotype B strains with or without spontaneous YMDD mutations and genotype C strains

Table 2 Multivariate regression analysis for occurrence of hepatitis B virus-related hepatocellular carcinoma

Related factors	*B*	SE	Wald	*P* value	Exp (B)	95%CI
Sex (male)	0.749	0.353	4.500	0.034	2.114	1.059-4.224
HBV genotype C strain	0.904	0.335	7.257	0.007	2.469	1.279-4.764
Spontaneous YMDD mutation in genotype C strain	2.051	0.826	6.161	0.013	7.775	1.540-39.264

HBV: Hepatitis B virus.

without spontaneous YMDD mutations. This suggests that infection by genotype C strains with spontaneous YMDD mutations is an independent risk factor for HCC. Spontaneous YMDD mutation and formation of different genotype strains are the result of mutation in the HBV genome. Mutations in the reverse transcriptase domain and different HBV genotypes may result in changes in amino acid sequence and protein configuration in HBV polymerase. These may change HBV biological properties, influence the process of HBV-related diseases, and increase HBV carcinogenicity. Previous research has shown that mutated HBV may be easier to integrate into host hepatocytes. The integration may lead to chromosome mutation in host cells and increase host chromosomal instability. As a result, chromosome repeat, inversion, deletion and translocation can be detected in many HCC cells. Virus integration may activate many proto-oncogenes and cause mutations in anti-oncogenes. The activation of proto-oncogenes and repression of anti-oncogenes causes loss of control of cell proliferation and differentiation and results in the formation of cell clusters with accelerated division and malignant transformation[26,27].

To the best of our knowledge, this is the first study to show that infection by genotype C strains with spontaneous YMDD mutation is an independent risk factor for the development of HCC in patients with HBV-related LC. Our results pave the way for exploring the molecular biological mechanism of HCC and have important clinical value for early warning of HBV-related HCC.

COMMENTS

Background

YMDD motif is a highly conserved sequence in the C zone of the reverse-transcriptase domain of hepatitis B virus (HBV) polymerase. The YMDD motif is the binding site for lamivudine to interfere with the replication of HBV. YMDD mutation, also known as M204V/I mutation, is the substitution of methionine by valine or isoleucine and designated as YVDD or YIDD variant. YMDD mutation, caused by amino acid substitution, leads to a change in protein configuration, abolishes its binding affinity with lamivudine, and weakens the antiviral activity of lamivudine. The emergence of YMDD mutation can induce a series of symptoms including elevation of serum alanine aminotransferase level, positive conversion of serum HBV DNA, and lamivudine resistance. Resistance to lamivudine increases in parallel with the duration of treatment and affects clinical application of the drug. These findings have made YMDD mutation a research hotspot globally.

Research frontiers

In order to investigate the association between infection by different genotype strains with spontaneous YMDD mutation and occurrence of HBV-related hepatocellular carcinoma (HCC), 264 cirrhosis patients not previously treated with antiviral drugs, including 81 with HCC, were included in our research.

Innovations and breakthroughs

Until recently, most research about YMDD mutations have focused on the occurrence of lamivudine-related mutations and their effect on antiviral treatment. The relationship between spontaneous YMDD mutation and HBV-related HCC and the association between infection by different HBV genotype strains with spontaneous YMDD mutations and the occurrence of HCC in HBV-related liver cirrhosis patients have rarely been reported.

Applications

It is believed that our study is the first to identify infection by genotype C strains with spontaneous YMDD mutation as an independent risk factor for the development of HCC in HBV-related liver cirrhosis. These results pave the way for exploring the molecular biological mechanism of HCC and have important clinical value for early warning of HBV-related HCC.

Terminology

Spontaneous YMDD mutations: Tyrosine (Y)-methionine (M)-aspartic acid (D)-aspartic acid (D) (YMDD) mutation occurs in the absence of known mutagens, such as antiviral drugs. YMDD motif mutations can naturally occur in chronic HBV patients without antiviral treatment.

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This is a case control study. The case and control group should at least have similar characteristics in age, HBV viral load, and hepatitis status (HBV carrier, chronic hepatitis B, liver cirrhosis), otherwise the statistical analysis would be biased.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3866

World J Gastroenterol 2013 June 28; 19(24): 3866-3871
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

META-ANALYSIS

Clinical prognostic factors for disabling Crohn's disease: A systematic review and meta-analysis

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Supported by Centre for Research in Health Informatics Systems and Technologies (CINTESIS)
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Received: December 1, 2012 Revised: December 20, 2012
Accepted: February 7, 2013
Published online: June 28, 2013

Abstract

AIM: To identify demographic and clinical factors associated with disabling Crohn's disease (CD).

METHODS: A systematic review and meta-analysis of observational studies, focusing on the factors that can predict the prognosis of different outcomes of CD was undertaken. PubMed, ISI Web of Knowledge and Scopus were searched to identify studies investigating the above mentioned factors in adult patients with CD. Studies were eligible for inclusion if they describe prognostic factors in CD, with inclusion and exclusion criteria defined as follows. Studies with adult patients and CD, written in English and studying association between clinical factors and at least one prognosis outcome were included. Meta-analysis of effects was undertaken for the disabling disease outcome, using odds ratio (OR) to assess the effect of the different factors in the outcome. The statistical method used was Mantel-Haenszel for fixed effects. The 16-item quality assessment tool (QATSDD) was used to assess the quality of the studies (range: 0-42).

RESULTS: Of the 913 papers initially selected, sixty studies were reviewed and three were included in the systematic review and meta-analysis. The global QATSDD scores of papers were 18, 21 and 22. Of a total of 1961 patients enrolled, 1332 (78%) were classified with disabling disease five years after diagnosis. In two studies, age at diagnosis was a factor associated with disabling disease five years after diagnosis. Individuals under 40 years old had a higher risk of developing disabling disease. In two studies, patients who were treated with corticosteroids on the first flare developed disabling disease five years after diagnosis. Further, perianal disease was found to be relevant in all of the studies at two and five years after diagnosis. Finally, one study showed localization as a factor associated with disabling disease five years after diagnosis, with L3 being a higher risk factor. This meta-analysis showed a significantly higher risk of developing disabling disease at five years after initial diagnosis among patients younger than 40 years of age (OR = 2.47, 95%CI: 1.74-3.51), with initial steroid treatment for first flare (OR = 2.42, 95%CI: 1.87-3.11) and with perianal disease (OR = 2.00, 95%CI: 1.41-2.85).

CONCLUSION: Age at diagnosis, perianal disease, initial use of steroids and localization seem to be independent prognostic factors of disabling disease.

Key words: Crohn's disease; Disabling disease; Prognostic factors; Outcome; Systematic review; Meta-analysis

Dias CC, Rodrigues PP, da Costa-Pereira A, Magro F. Clinical prognostic factors for disabling Crohn's disease: A systematic review and meta-analysis. *World J Gastroenterol* 2013; 19(24): 3866-3871 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3866.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3866

INTRODUCTION

Crohn's disease (CD) occurs in equal proportion in both genders and its incidence has been growing worldwide in the last decades[1]. CD is a disabling disease affecting psychological, familial, and social dimensions of life[2]. Therefore, the need to develop a specific instrument able to evaluate disabilities and identify specific factors as predictors is paramount. This is particularly true since in the last decades the medical treatment options have been dramatically changed. Other strategies are now approaching, namely accelerate step-up and top-down treatment[3]. The top-down strategy is based on the very early use of intensive therapy (immunosuppressants and/or biologics) to maintain a good quality of life from the first flare-up of the disease and prevent any irreversible consequences[3]. Therefore, it is now crucial to identify simple clinical criteria at diagnosis to predict CD outcome. This work aims to systematically review the evidence with respect to predictive clinical prognostic factors for CD.

MATERIALS AND METHODS

A systematic review and meta-analysis of observational studies focusing on the factors that can predict the prognosis of different outcomes of CD was undertaken. The methodology included the definition of eligibility criteria, search strategies, study selection and characteristics, outcome measures, quantitative data synthesis and sensitivity analysis, methodological quality of studies, and statistical data analysis.

Eligibility criteria

Studies that described prognostic factors in CD were eligible for inclusion. The criteria for inclusion were studies with adult patients and CD written in English and studying association between clinical factors and disabling disease. Studies not in English, without available abstract, with genetic or serologic factors, biomarker studies, or those addressing diagnosis or quality of life were excluded.

Search strategy

The main method to search for the eligible articles was a broad literature search using PubMed with the following keywords and MeSH terms: *"crohn disease"[MeSH Terms] OR "crohn"[All Fields] AND predictor [All Fields] OR predictors [All Fields] OR predict [All Fields] OR "prognostic factor" [All Fields] OR "prognostic factors" [All Fields]*. Literature searches were also undertaken in Scopus database and ISI Web of Knowledge using the same search keywords: *crohn disease AND (predictors OR predict OR prognostic factors)*.

Study selection

The studies were screened and selected by two reviewers. First, all titles and abstracts were read and the inclusion and exclusion criteria were applied. Second, the reviewers read the full text of all papers considered for inclusion after abstract selection, again applying the inclusion and exclusion criteria.

Study characteristics

The following properties of each study were recorded: total number of patients, prognostic variables, and percentage of patients with disabling disease.

Outcome measures

The aim of the study is to assess prognostic factors to predict disabling CD.

Methodological quality of included studies

The 16-item quality assessment tool (QATSDD), developed by Higgins *et al*[4], was used to assess the quality of the included studies. This tool includes 16 items, scored between 0 and 3, and can be applied to different types of studies using different approaches. However, two of the items were not evaluated as they only address qualitative studies, hence we only considered a maximum score of 42.

Statistical analysis

Statistical evidence of effects is presented as described in the original studies. Meta-analysis of effects was undertaken for the disabling disease outcome using odds ratio (OR) to assess the effect of the different factors in the outcome. The statistical method used was Mantel-Haenszel for fixed effects. All included estimates are recomputed from original articles descriptions, potentially resulting in slightly different values. All reported *P*-values are 2-sided with a significance level of 5%. Statistical heterogeneity was assessed with the I^2 statistic; values higher than 50% indicate a substantial level of heterogeneity[5]. RevMan v5.1 (The Nordic Cochrane Center, The Cochrane Collaboration, 2011) was used to calculate OR and 95%CI for disabling disease and to derived forest plots showing the results of individual studies and pooled analysis.

RESULTS

Search and study selection

A total of 913 articles were identified using the search

Table 1 Characteristics of the studies

Ref.	Country	Sample size (*n*)	Type of study	Disabling	Follow-up (yr)	Factor	QATSDD
Beaugerie *et al*[6]	France	1123	Retrospective	85.2%	5	Age (under 40 yr) Steroids for 1st flare Perianal disease	22
Loly *et al*[7]	Belgium	361	Retrospective	57.9%	5	Steroids for 1st flare Perianal disease L3	18
Yang *et al*[8]	China	207	Retrospective	71.0%	2	Steroids for 1st flare Perianal disease	21
				80.2%	5	Age (under 40 yr)	

QATSDD: Sixteen-item quality assessment tool.

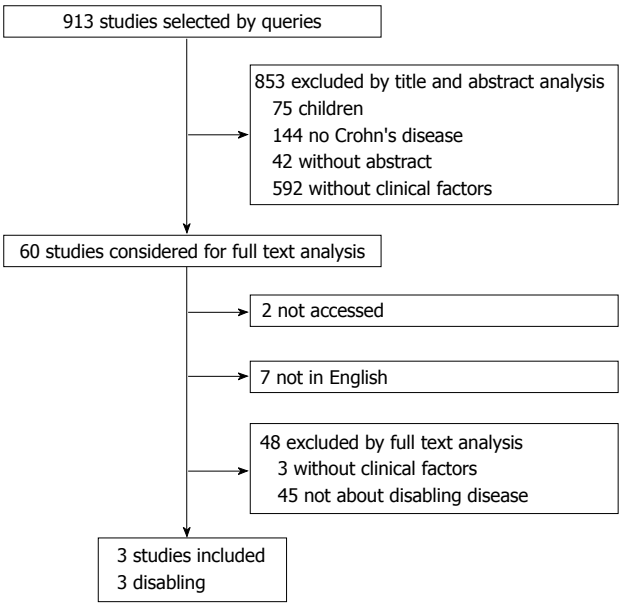

Figure 1 Flowchart of the selection process for this meta-analysis.

strategy. After reading all titles and abstracts, 853 articles were excluded (Figure 1). Sixty studies were reviewed in detail and three articles were included in the study. A new search of the literature focused on the outcome was made in order to find other papers that could have been missed by the generic search. The global QATSDD scores ranged between 18 and 22. The main characteristics of the studies are summarized in Table 1.

Predicting factors of disabling disease

Beaugerie *et al*[6] and Loly *et al*[7] define disabling disease by the presence of at least one of the following criteria: two steroid courses required and/or steroid dependency; further hospitalization after diagnosis for flare up or complications of the disease; chronic symptoms (diarrhea with nocturnal and/or urgent stools, intensive abdominal pain due to intestinal obstruction, fever, fatigue attributable to the disease); joint pain; painful uveitis or pyoderma gangrenous for 12 mo within the five year study; immunosuppressive therapy and intestinal resection or surgical operation for perianal disease. Yang *et al*[8] defined CD as disabling if patients satisfy at least one of the following criteria: require two or more steroids courses and/or steroid dependency; need immunosuppressive therapy; intestinal resection or surgical operation for perianal disease and hospitalization after diagnosis for the treatment of acute exacerbation, or complication of the disease.

According to Beaugerie *et al*[6], 957 of 1123 patients (85.2%) were classified with disabling disease. With a sample of 361 patients, Loly *et al*[7] found 209 patients (57.9%) with disabling disease, while Yang *et al*[8] found 80.2% of 207 patients with disabling disease five years after diagnosis, and 71% of patients already had disabling disease two years after diagnosis.

Different factors were found that could predict disabling disease, namely age at diagnosis, use of steroids, perianal disease, and localization.

Age at diagnosis

Beaugerie *et al*[6] found age at diagnosis as a factor associated with disabling disease. Patients less than 40 years old had a higher risk of developing disabling disease than older patients (OR = 2.1, 95%CI: 1.3-3.6) five years after the diagnosis. Yang *et al*[8] also showed that patients under 40 had a higher risk of developing disabling disease (OR = 3.56, 95%CI: 1.74-7.30).

Results of studies comparing younger patients (under 40) with older patients (over 40) are shown in Figure 2A. A fixed effects model shows that younger patients had a higher risk of disabling disease five years after diagnosis (OR = 2.47, 95%CI: 1.74-3.51). There was no evidence of statistical heterogeneity among the studies (I^2 = 26%).

Steroids for treatment of first flare

Both Beaugerie *et al*[6] and Loly *et al*[7] show patients who had initial requirement of steroids for treating the first flare had a higher risk of developing disabling disease five years after diagnosis when compared to those who did not require treatment (OR = 3.1, 95%CI: 2.2-4.4 and OR = 1.7, 95%CI: 1.02-2.7, respectively). Yang *et al*[8] found similar results two years after diagnosis (OR = 2.142, 95%CI: 1.068-4.298).

Results of these different studies comparing patients with and without steroid requirement treatment are presented in Figure 2B. A fixed effects model shows that patients with steroid treatment had a higher risk of disabling

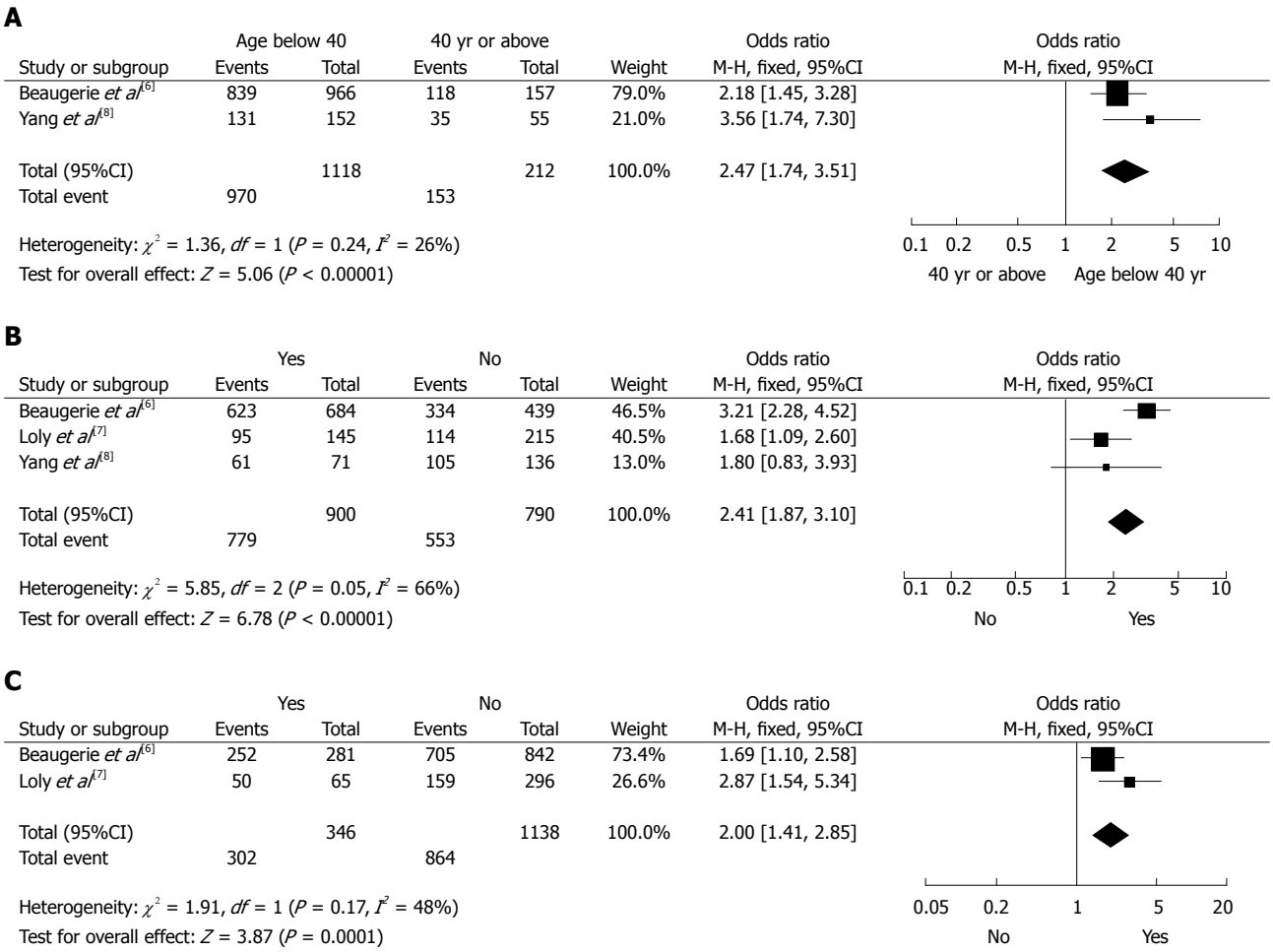

Figure 2 Predictor of disabling disease. A: Age at diagnosis as a predictor of disabling disease; B: The use of steroids for treatment of the first flare as a predictor of disabling disease; C: Perianal disease as a predictor of disabling disease.

disease five years after diagnosis (OR = 2.41, 95%CI: 1.87-3.10). Significant heterogeneity was found among the studies (I^2 = 66%). Nevertheless, all studies found a higher risk of disabling disease for patients on steroids.

Perianal disease

In all three studies, patients with perianal disease had a higher risk of developing disabling disease five years after diagnosis when compared to patients without perianal disease: Beaugerie *et al*[6] (OR = 1.8, 95%CI: 1.2-2.8), Loly *et al*[7] (OR = 2.6, 95%CI: 1.4-5.1), Yang *et al*[8] (two years after diagnosis) (OR = 5.433, 95%CI: 1.585-18.620).

The comparison between patients with and without perianal disease is shown in Figure 2C. A fixed effects model shows the presence of perianal disease as a high risk of disabling disease five years after diagnosis (OR = 2.00, 95%CI: 1.41-3.85). There was no evidence of statistical heterogeneity among the studies (I^2 = 48%).

Localization

One study associated disabling disease to the localization of the disease. In this study, patients with L3 localization had a higher risk of developing disabling disease five years after the diagnosis (OR = 1.74, 95%CI: 1.06-2.8)[7].

DISCUSSION

CD is a chronic disease with no known medical or surgical cure, requiring several appointments and hospitalizations for those afflicted. There are several reasons stressing the importance of prognostic factors: (1) Recent available drugs, namely anti-tumour necrosis factor (TNF), having the potential of inducting mucosal healing and prolonged clinical remission; (2) Mucosal healing has been considered a therapeutic goal; and (3) Early therapeutic interventions are followed by a better outcome. Therefore, it is imperative that therapeutic options are optimized.

The present systematic review and meta-analysis presented some of the factors that could help clinicians identify risk groups for disabling CD. Age, perianal disease, the use of steroids and localization were all associated with disabling disease. Although other markers can help clinicians to predict disease course of CD, namely genetic, serologic and endoscopic findings, we limited this meta-analysis to demographic and clinical characteristics due to feasibility to apply at diagnosis at the bedside.

Three studies address disabling disease and used similar definitions, although in Yang *et al*[8] the presence of chronic symptoms like diarrhea, fever, fatigue, was not

considered. All studies were retrospective and the number of patients in each study ranged from 207 to 1123[6,8]. It was clear that a large number of patients had disabling disease (range: 59%-81%), which gives an indication of the severity of the disease. Moreover, the study with the highest percentage of disabling disease included the least amount of defining characteristics[8]. Our meta-analysis showed that patients under 40 years old and patients with an initial requirement of steroids, or patients with perianal disease had a higher risk of having disabling disease five years following initial diagnosis. These results are in line with the three studies used in the meta-analysis[6-8]. Although the definition of disabling disease in Yang *et al*[8] is slightly different, this study had a lower weight in the final result of the meta-analysis, hence limiting the possible bias. Even though the effect of age at diagnosis was clear in the meta-analysis; further studies are necessary to better assess relative CD risk, including an evaluation of more patients diagnosed after the age threshold. We call into question some of the recent points included in disability definition, namely steroids following the first flare, the need for immunosuppressants, and surgery. The percentage of patients treated with steroids in the first flare (65%) in Beaugerie *et al*[6] was very similar to the percentage of patients who received steroids within in the first year of disease in the North-European population-based study, however this only reflects the step-up strategy, and the population on immunosuppressants was very low[9]. Markowitz *et al*[10] showed children requiring steroids for the treatment of the first flare-up that a very early use of 6-Mercaptopurine was associated with steroid sparing and a more favorable clinical outcome in the 18-mo period following diagnosis. Similar results were observed in those treated with anti-TNF in the first two years of disease[11]. Finally, the role of early surgery in limiting ileal disease with regard to CD prognosis is also debatable. In conclusion, the risk factors analyzed in this meta-analysis should be considered when new scores or approaches are taken concerning risk factors in CD outcome, particularly when more early therapeutic approaches are imminent.

The QATSDD scale, developed by Sirriyeh *et al*[5], allows the comparison of the quality of the included papers even when their designs are different. The included papers consistently presented low quality scores, especially considering the representativeness of the sample and the absence of a critical discussion of strengths and limitations.

The results of this study may need further confirmation due to the small number of reviewed studies and their low quality (maximum QATSDD score of 22 out of 42). Nevertheless, this work presents a step-forward in the definition of clinical predictors for disabling CD, exposing their relevance and impact in disease prognosis.

In summary, this review and meta-analysis showed that age, perianal disease and the use of steroids are associated with disabling disease. The use of these factors in building predictive models for CD prognosis could enhance the initial clinical approach, and therefore improve the clinical outcome of patients with severe disease. However, more elaborate and precise definitions of disabling and severe disease are needed.

COMMENTS

Background

Crohn's disease (CD) occurs in equal proportion in both genders and its incidence has been growing worldwide in the last decades. CD is a disabling disease affecting psychological, familial, and social dimensions of life. Therefore, the need to develop a specific instrument able to evaluate disabilities and identify specific factors as predictors is paramount.

Research frontiers

The top-down strategy is based on the very early use of intensive therapy (immunosuppressants and/or biologics) to maintain a good quality of life from the first flare-up of the disease and prevent any irreversible consequences. Therefore, it is now crucial to identify simple clinical criteria at diagnosis to predict CD outcome.

Innovations and breakthroughs

This work aims to systematically review the evidence with respect to predictive clinical prognostic factors for CD.

Applications

This review and meta-analysis showed that age, perianal disease and the use of steroids are associated with disabling disease. The use of these factors in building predictive models for CD prognosis could enhance the initial clinical approach, and therefore improve the clinical outcome of patients with severe disease. However, more elaborate and precise definitions of disabling and severe disease are needed.

Peer review

It is one of the first work searching the role of CD in disability of the patient. The authors performed an extensive review of multiple manuscript related with the topic. The manuscript is very well prepared and written and can be accepted for publication.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3872

World J Gastroenterol 2013 June 28; 19(24): 3872-3882
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

META-ANALYSIS

Meta-analysis of radiofrequency ablation in combination with transarterial chemoembolization for hepatocellular carcinoma

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Received: December 11, 2012 Revised: March 12, 2013
Accepted: April 3, 2013
Published online: June 28, 2013

Abstract

AIM: To compare radiofrequency ablation (RFA) and transcatheter arterial chemoembolization (TACE) with RFA monotherapy in hepatocellular carcinoma (HCC).

METHODS: We searched PubMed, Medline, Embase and Chinese databases (CBMdisc and Wanfang data) for randomized controlled trails comparing RFA plus TACE and RFA alone for treatment of HCC from January 2000 to December 2012. The overall survival rate, recurrence-free survival rate, tumor progression rate, and safety were analyzed and compared. The analysis was conducted on dichotomous outcomes and the standard meta-analytical techniques were used. Pooled odds ratios (ORs) with 95%CIs were calculated using either the fixed-effects or random-effects model. For each meta-analysis, the χ^2 and I^2 tests were first calculated to assess the heterogeneity of the included trials. For $P < 0.05$ and $I^2 > 50\%$, the assumption of homogeneity was deemed invalid, and the random-effects model was used; otherwise, data were assessed using the fixed-effects model. All statistical analysis was conducted using Review manager (version 4.2.2.) from the Cochrane collaboration.

RESULTS: Eight randomized controlled trials were identified as eligible for inclusion in this analysis and included 598 patients with 306 treated with RFA plus TACE and 292 with RFA alone. Our data analysis indicated that RFA plus TACE was associated a significantly higher overall survival rate ($OR_{1\text{-year}} = 2.96$, 95%CI: 1.84-7.74, $P < 0.001$; $OR_{2\text{-year}} = 3.72$, 95%CI: 1.24-11.16, $P = 0.02$; $OR_{3\text{-year}} = 2.65$, 95%CI: 1.81-3.86, $P < 0.001$) and recurrence-free survival rate ($OR_{3\text{-year}} = 3.00$, 95%CI: 1.75-5.13, $P < 0.001$; $OR_{5\text{-year}} = 2.26$, 95%CI: 1.43-3.57, $P = 0.0004$) *vs* that of RFA alone. The tumor progression rate in patients treated with RFA alone was higher than that of RFA plus TACE (OR = 0.60, 95%CI: 0.42-0.88, $P = 0.008$) and there was no significant difference on major complications between two different kinds of treatment (OR = 1.20, 95%CI: 0.31-4.62, $P = 0.79$). Additionally, the meta-analysis data of subgroups revealed that the survival rate was significantly higher in patients with intermediate- and large-size HCC underwent RFA plus TACE than in those underwent RFA monotherapy; however, there was no significant difference between RFA plus TACE and RFA on survival rate for small HCC.

CONCLUSION: The combination of RFA with TACE has advantages in improving overall survival rate, and provides better prognosis for patients with intermediate- and large-size HCC.

Key words: Radiofrequency ablation; Transcatheter arterial chemoembolization; Hepatocellular carcinoma; Meta-analysis

Core tip: This study aimed to compare the effectiveness and prognosis of combination of transcatheter

arterial chemoembolization (TACE) and radiofrequency ablation (RFA) with that of RFA alone in hepatocellular carcinoma (HCC). To the best of our knowledge, there has been no comprehensive comparison on these two treatments in terms of small-, intermediate- and large-size HCC. Our analysis demonstrated that effectiveness of TACE combined with RFA was better than that of RFA for treatment of intermediate- and large-size HCC. We provide important evidence that TACE-RFA for intermediate- and large-size HCC may be performed more widely in clinical practice.

Ni JY, Liu SS, Xu LF, Sun HL, Chen YT. Meta-analysis of radiofrequency ablation in combination with transarterial chemoembolization for hepatocellular carcinoma. *World J Gastroenterol* 2013; 19(24): 3872-3882 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3872.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3872

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies. HCC ranks fifth for men and eighth for women and accounts for > 660000 new cases annually worldwide[1-3]. Due to poor baseline liver function, over tumor burden, or hepatic vessel invasion of HCC patients, it is barely possible to perform surgical resection. Transcatheter arterial chemoembolization (TACE) as a palliative therapy has become one of the most widely performed treatments for unresectable HCC[4,5]. However, the complete necrosis rate of tumors after TACE have only reached 10%-20%, and the 1-, 3- and 5-year overall survival rates range from 49%-71.9% to 23%-62.5% and 9%-17% in most studies[6-11]. Radiofrequency ablation (RFA) as a thermal *in situ* destruction technique has been proved to be a safe and effective treatment. RFA has been accepted as one of the best treatment options for small HCC[12,13]. However, it is difficult for RFA to achieve complete ablation in the treatment of relatively large HCC. Therefore, novel approaches to treating HCC patients have been extensively pursued and may offer opportunities for longer survival of patients with HCC. In recent years, the combination of interventional therapies has been widely performed for treatment of HCC. One such combined strategy is the combination of RFA and TACE.

Previous studies have reported that combination of RFA and TACE is more effective for induction of a significantly higher complete tumor necrosis rate than RFA monotherapy is, and improves overall survival rate in patients with HCC[14-16]. However, other studies assessing the clinical efficacy of RFA plus TACE and RFA alone for treatment of HCC have reported conflicting outcomes[17-19]. Hence, whether RFA combined with TACE or RFA monotherapy is the better treatment choice for HCC has long been debated. Meta-analysis is a suitable method to resolve this conflict. Several randomized controlled trials have been published in an attempt to answer the above question. A meta-analysis of these trials to analyze and compare comprehensively the clinical efficacy and safety of RFA combined with TACE and RFA monotherapy will provide clinicians with an unbiased opinion and valuable information about the efficacy of these treatment options. Comparison of these two treatments could help stratify the benefits of treatment choices for patients with HCC. Hence, this meta-analysis was designed to compare comprehensively the efficacy and safety of combination of RFA and TACE with RFA monotherapy for treatment of patients with HCC.

MATERIALS AND METHODS

Study selection

A search of the literature was conducted in PubMed, Medline, Embase and Chinese databases (CBMdisc and Wanfang data) from January 2000 to December 2012, using the following MeSH search headings: "hepatocellular carcinoma", "radiofrequency ablation" and "transcatheter arterial chemoembolization". A limit was set on the randomized controlled trials, which was conducted to identify studies comparing the effectiveness and safety of the combination of RFA and TACE with that of RFA monotherapy for HCC. No language restriction was imposed in this search.

Criteria for inclusion and exclusion

To be eligible for the present meta-analysis, studies were required to have an integrated baseline of patients and outcomes: (1) study design: randomized controlled trials on RFA plus TACE *vs* RFA monotherapy in the treatment of HCC; (2) baseline of population: randomization of no fewer than 30 formally diagnosed HCC patients with average age, percentage male, Child-Pugh class, tumor size, and tumor stage; and (3) results: studies were required to have good descriptions of the results for overall survival rate, recurrence-free survival rate, tumor progression rate, and major complications. Abstracts, letters, reviews without original data, expert opinions, editorials, case reports and studies lacking control groups were excluded from the analysis.

Statistical analysis

All analysis was conducted on dichotomous outcomes and the standard meta-analytical techniques were used. Pooled odds ratios (ORs) with 95%CIs were calculated using either the fixed-effects or random-effects model. For each meta-analysis, the χ^2 and I^2 tests were first calculated to assess the heterogeneity of the included trials. $P < 0.05$ and $I^2 > 50\%$ was considered significant. For $P < 0.05$ and $I^2 > 50\%$, the assumption of homogeneity was deemed invalid, and the random-effects model was used; otherwise, data were assessed using the fixed-effects model. The risk of the publication bias of the included trials was assessed using the symmetry of the funnel plot. Statistical analysis was performed using the

Table 1 Baseline characteristics of the trials included in the meta-analysis (mean ± SD)

Ref.	Country	Design	Treatment	No. of patients	Age (yr)	Sex (male/female)	Tumor size (cm)	Child-Pugh class (A/B/C)
Peng *et al*[14]	China	RCT	TACE + RFA	69	57.5 ± 10.0	60/9	≤ 5.01	60/9/0
			RFA	70	55.1 ± 9.5	55/15	-	59/11/0
Cheng *et al*[15]	China	RCT	TACE + RFA	96	≤ 75[1]	NA	3 < TS ≤ 7.5[1]	NA
			RFA	100	-	-	-	NA
Yang *et al*[16]	China	RCT	TACE + RFA	24	59.1 ± 11.4	18/6	6.6 ± 0.6	NA
			RFA	12	61.0 ± 10.4	8/4	5.2 ± 0.4	NA
Shibata *et al*[18]	Japan	RCT	TACE + RFA	46	67.2 ± 8.9	31/15	1.7 ± 0.6	32/14/0
			RFA	43	69.8 ± 8.0	33/10	1.6 ± 0.5	33/10/0
Morimoto *et al*[20]	Japan	RCT	TACE + RFA	19	70 (57-78)	15/4	3.6 ± 0.7	12/7/0
			RFA	18	73 (48-84)	12/6	3.7 ± 0.6	16/2/0
Kang *et al*[22]	China	RCT	TACE + RFA	19	52.2	14/5	6.7 ± 1.1	12/7/0
			RFA	18	50.7	14/4	6.2 ± 1.2	12/6/0
Shen *et al*[23]	China	RCT	TACE + RFA	18	52.7 (20-72)	5/13	5.6 (2.2-15.8)	4/14/0
			RFA	16	56.1 (36-75)	3/13	5.0 (2.3-12.3)	6/10/0
Zhang *et al*[24]	China	RCT	TACE + RFA	15	57.8 (39-72)	12/3	4.6 (2.3-7.1)	NA
			RFA	15	61.8 (38-78)	13/2	4.1 (2.4-6.0)	NA

[1]Total data of relative study. RCT: Randomized controlled trial; NA: Not applicable; TS: Tumor size; RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization.

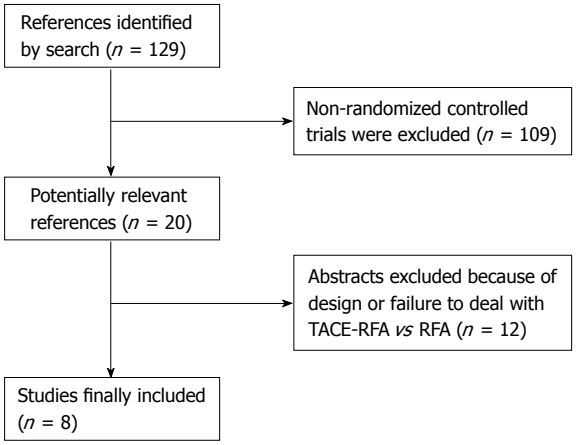


Figure 1 Flow chart of searching strategy for study inclusion. RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization.

software programs Review manager (version 4.2.2.) from the Cochrane collaboration. $P < 0.05$ was considered significant.

RESULTS

Selection of trials

This meta-analysis yielded a total of 129 studies. Based on the inclusion and exclusion criteria, we included eight randomized controlled trials[14,15,18,20-24] (Figure 1). There was a total of 598 patients with 306 treated with RFA plus TACE and 292 treated with RFA alone (Table 1). Among these studies, there were eight, three, six and two studies that reported comparative data for overall survival rate at 1, 2, 3 and 5 years, respectively; five, three and two studies reported comparative data for recurrence-free survival rate at 1, 3 and 5 years, respectively (Tables 2 and 3). In the small HCC (tumor size ≤ 3 cm) subgroup, there were two studies with comparative data on survival rate at 1 and 3 years, respectively. In the intermediate-size HCC (3 cm < tumor size ≤ 5 cm) subgroup, there were four, three and two studies with comparative data on survival rate at 1, 3 and 5 years, respectively. In the large-size HCC (tumor size > 5 cm) subgroup, there were four and three studies with comparative data on survival rate at 1 and 3 years, respectively (Tables 2 and 3). There were six and three studies with comparative data on tumor progression rate and major complications, respectively (Tables 2 and 3). The quality of the included studies was detected using Review manager (version 4.2.2.) programs, and was judged to be high quality (Figure 2A-D).

Meta-analysis results

Heterogeneity assessment: In the analysis of the effects of 1-, 2- and 3-year overall survival rates ($P_{1\text{-year}} = 0.77$, $I^2 = 0\%$; $P_{2\text{-year}} = 0.56$, $I^2 = 0\%$; $P_{3\text{-year}} = 0.33$, $I^2 = 13.1\%$); 1-, 3- and 5-year recurrence-free survival rates ($P_{1\text{-year}} = 0.39$, $I^2 = 3.5\%$; $P_{3\text{-year}} = 0.60$, $I^2 = 0\%$; $P_{5\text{-year}} = 0.37$, $I^2 = 0\%$); tumor progression rates ($P = 0.18$, $I^2 = 34.8\%$); major complications ($P = 0.91$, $I^2 = 0\%$), the meta-analysis data indicated that there was no significant heterogeneity among the studies, thus the fixed-effects model was used to pool the results. However, in the analysis of the effect of 5-year overall survival rates, there was significant heterogeneity among the studies ($P_{5\text{-year}} = 0.03$, $I^2 = 79.3\%$), thus the random-effects model was used to pool the results (Figures 2 and 3).

Overall survival rate: There was a significant difference in 1-, 2- and 3-year overall survival rate between treatment with RFA plus TACE and RFA alone, and the meta-analysis data suggested that RFA plus TACE was associated with a significantly higher 1-, 2- and 3-year overall survival rate than RFA monotherapy was ($OR_{1\text{-year}} = 2.96$, 95%CI: 1.84-7.74, $P < 0.001$; $OR_{2\text{-year}} = 3.72$, 95%CI: 1.24-11.16, $P = 0.02$; $OR_{3\text{-year}} = 2.65$, 95%CI: 1.81-3.86, $P < 0.001$).

Table 2 Prognosis of patients reported in the trials included in the meta-analysis

Ref.	Treatment	No. of patients	Recurrence- free survival rate			Overall survival rate			
			1 yr	3 yr	5 yr	1 yr	2 yr	3 yr	5 yr
Peng *et al*[14]	TACE+RFA	69	80.00%	45.00%	40.00%	94.00%	NA	69.00%	46.00%
	RFA	70	64.00%	18.00%	18.00%	82.00%		47.00%	36.00%
Cheng *et al*[15]	TACE+RFA	96	NA	NA	58.00%	83.00%	NA	55.00%	31.00%
	RFA	100			42.00%	67.00%		32.00%	8.00%
Yang *et al*[16]	TACE+RFA	24	29.00%	NA	NA	68.00%	NA	NA	NA
	RFA	12	34.70%			57.00%			
Shibata *et al*[18]	TACE+RFA	46	71.30%	48.80%	NA	100.00%	100.00%	84.80%	NA
	RFA	43	74.30%	29.70%		100.00%	88.80%	84.50%	
Morimoto *et al*[20]	TACE+RFA	19	67.00%	NA		100.00%	93.00%	93.00%	NA
	RFA	18	56.00%	28.00%		89.00%	89.00%	80.00%	
Kang *et al*[22]	TACE+RFA	19	NA	NA	NA	84.20%	42.10%	36.80%	NA
	RFA	18				66.10%	22.20%	16.70%	
Shen *et al*[23]	TACE+RFA	18	63.90%	50.00%	NA	87.50%	NA	73.30%	NA
	RFA	16	30.00%	18.70%		52.20%		20.40%	
Zhang *et al*[24]	TACE+RFA	15	NA	NA	NA	100.00%	NA	NA	NA
	RFA	15				80.00%			

NA: Not applicable; RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization.

Table 3 Efficacy and major complications of radiofrequency ablation-transcatheter arterial chemoembolization *vs* radiofrequency ablation for treatment of hepatocellular carcinoma

Variables	No. of studies furnishing data	Results		OR (95%CI)	*P* value	I^2
		RFA-TACE	RFA			
Efficacy overall survival rate						
1 yr	8	89.20%	76.00%	2.96 (1.84- 7.47)	< 0.001	0.00%
2 yr	3	85.70%	73.40%	3.72 (1.24-11.16)	0.02	0.00%
3 yr	6	66.70%	45.70%	2.65 (1.81- 3.86)	< 0.001	13.10%
5 yr	2	37.50%	19.40%	2.78 (0.85-9.12)	0.09	79.30%
Recurrence-free survival rate						
1 yr	5	67.60%	60.30%	1.59 (0.99-2.55)	0.05	3.50%
3 yr	3	46.60%	22.40%	3.00 (1.75-5.13)	< 0.001	0.00%
5 yr	2	50.90%	32.30%	2.26 (1.43-3.57)	0.0004	0.00%
Survival rate (TS ⩽ 3 cm)						
1 yr	2	98.90%	91.00%	8.42 (1.01- 70.56)	0.05	NA
3 yr	2	78.10%	71.90%	1.35 (0.67-2.74)	0.4	0.00%
Survival rate (3 cm < TS ⩽ 5 cm)						
1 yr	4	95.30%	84.60%	3.46 (1.29-9.28)	0.01	0.00%
3 yr	3	78.20%	53.90%	3.58 (1.79-7.15)	0.0003	28.60%
5 yr	2	49.30%	15.40%	5.34 (2.42-11.75)	< 0.001	0.00%
Survival rate (TS < 5 cm)						
1 yr	4	75.90%	52.50%	2.91 (1.60-5.29)	0.0004	0.00%
3 yr	3	43.10%	10.30%	6.96 (3.01-16.07)	0.00001	0.00%
Tumor progression rate	6	31.60%	42.80%	0.60 (0.42-0.88)	0.008	34.80%
Major complications	3	3.70%	3.10%	1.20 (0.31-4.62)	0.79	0.00%

NA: Not applicable; TS: Tumor size; RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization.

However, there was no significant difference in overall survival rate between these two treatments for 5-year overall survival rate ($OR_{5\text{-year}}$ = 2.78, 95%CI: 0.85-9.12, P = 0.09) (Figure 2A-D).

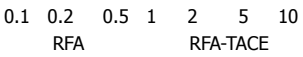
Recurrence-free survival rate: There was a significant difference in 3- and 5-year recurrence-free survival rate between treatment with RFA plus TACE and RFA alone, and the meta-analysis data suggested that RFA plus TACE was associated with a significantly higher 3-and 5-year recurrence-free survival rate than RFA monotherapy was ($OR_{3\text{-year}}$ = 3.00, 95%CI: 1.75-5.13, P < 0.001; $OR_{5\text{-year}}$ = 2.26, 95%CI: 1.43-3.57, P = 0.0004). However, there was no significant difference in recurrence-free survival rate between the two treatments at 1 year ($OR_{1\text{-year}}$ = 1.59, 95%CI: 0.99-2.55, P = 0.05) (Figure 2E-G).

Tumor progression rate: There was a significant difference in tumor progression rate between treatment with RFA plus TACE and RFA alone, and the meta-analysis data suggested that RFA monotherapy was associated with a significantly higher tumor progression rate than RFA plus TACE treatment was (OR = 0.60, 95%CI: 0.42-0.88, P = 0.008) (Figure 3A).

A

Study or sub-category	RFA-TACE	RFA	Weight	OR, fixed, 95%CI	Quality
Zhang *et al*[24]	15/15	12/15	1.86%	8.68 [0.41, 184.28]	A
Shen *et al*[23]	16/18	8/16	4.48%	8.00 [1.37, 46.81]	A
Kang *et al*[22]	16/19	12/18	9.25%	2.67 [0.55, 12.88]	A
Cheng *et al*[15]	80/96	67/100	52.01%	2.46 [1.25, 4.86]	A
Yang *et al*[16]	16/24	7/12	14.79%	1.43 [0.34, 5.95]	A
Shibata *et al*[18]	46/46	43/43		Not estimable	A
Morimoto *et al*[20]	19/19	16/18	2.01%	5.91 [0.26, 132.00]	A
Peng *et al*[14]	65/69	57/70	15.60%	3.71 [1.14, 12.01]	A
Total (95%CI)	306	292	100.00%	2.96 [1.84, 4.74]	

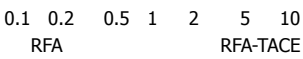
Total events: 273 (RFA-TACE), 222 (RFA)
Test for heterogeneity: χ^2 = 3.32, *df* = 6 (*P* = 0.77), I^2 = 0%
Test for overall effect: *Z* = 4.49 (*P* < 0.00001)



B

Study or sub-category	RFA-TACE	RFA	Weight	OR, fixed, 95%CI	Quality
Kang *et al*[22]	8/19	4/18	64.87%	2.55 [0.61, 10.71]	A
Shibata *et al*[18]	46/46	38/43	11.54%	13.29 [0.71, 247.91]	A
Morimoto *et al*[20]	18/19	16/18	23.59%	2.25 [0.19, 27.22]	A
Total (95%CI)	84	79	100.00%	3.72 [1.24, 11.16]	

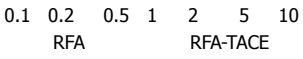
Total events: 273 (RFA-TACE), 222 (RFA)
Test for heterogeneity: χ^2 = 3.32, *df* = 6 (*P* = 0.77), I^2 = 0%
Test for overall effect: *Z* = 4.49 (*P* < 0.00001)



C

Study or sub-category	RFA-TACE	RFA	Weight	OR, fixed, 95%CI	Quality
Shen *et al*[23]	13/18	3/16	2.65%	11.27 [2.22, 57.20]	A
Kang *et al*[22]	7/19	3/18	5.85%	2.92 [0.62, 13.76]	A
Cheng *et al*[15]	53/96	32/100	42.22%	2.62 [1.46, 4.69]	A
Shibata *et al*[18]	39/46	36/43	17.03%	1.08 [0.35, 3.39]	A
Morimoto *et al*[20]	18/19	14/18	2.28%	5.14 [0.52, 51.29]	A
Peng *et al*[14]	48/69	33/70	29.98%	2.56 [1.28, 5.14]	A
Total (95%CI)	267	265	100.00%	2.65 [1.81, 3.86]	

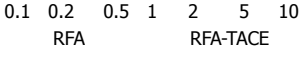
Total events: 178 (RFA-TACE), 121 (RFA)
Test for heterogeneity: χ^2 = 5.75, *df* = 5 (*P* = 0.33), I^2 = 13.1%
Test for overall effect: *Z* = 5.04 (*P* < 0.00001)



D

Study or sub-category	RFA-TACE	RFA	Weight	OR, fixed, 95%CI	Quality
Cheng *et al*[15]	30/96	8/100	47.83%	5.23 [2.25, 12.13]	A
Peng *et al*[14]	32/69	25/70	52.17%	1.56 [0.79, 3.07]	A
Total (95%CI)	165	170	100.00%	2.78 [0.85, 9.12]	

Total events: 62 (RFA-TACE), 33 (RFA)
Test for heterogeneity: χ^2 = 4.83, *df* = 5 (*P* = 0.03), I^2 = 79.3%
Test for overall effect: *Z* = 1.69 (*P* = 0.09)



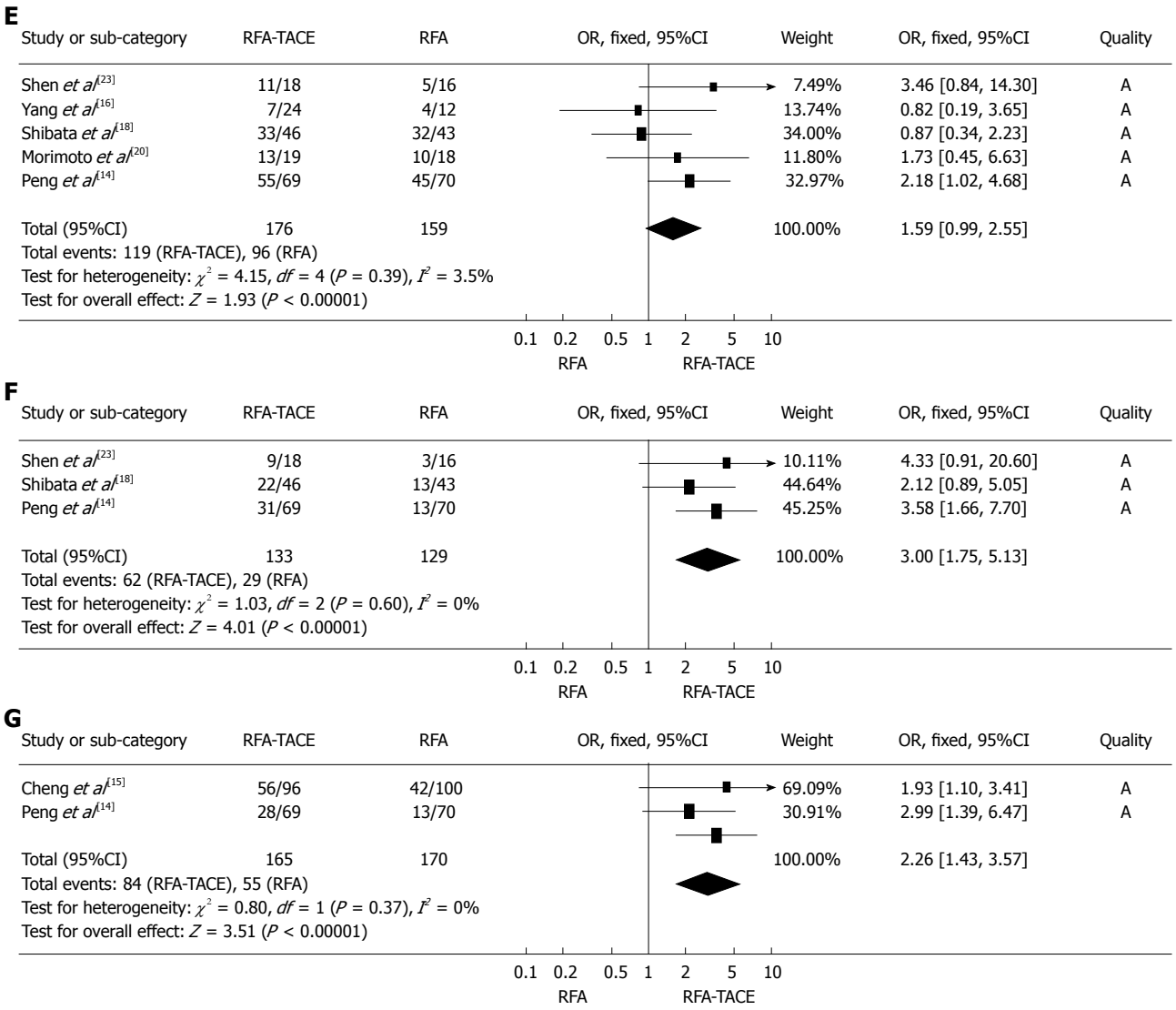


Figure 2 Comparison of combination of radiofrequency ablation and transcatheter arterial chemoembolization with radiofrequency ablation alone for hepatocellular carcinoma in terms of overall survival rates (A-D) and recurrence-free survival rates (E-G). A, E: Meta-analysis of 1-year results; B: Meta-analysis of 2-year results; C, F: Meta-analysis of 3-year results; D, G: Meta-analysis of 5-year results. RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization; OR: Odds ratio.

Safety

Meta-analysis data showed that there was no significant difference in major complications between treatment with RFA plus TACE and RFA monotherapy (OR = 1.20, 95%CI: 0.31-4.62, *P* = 0.79) (Figure 3B).

Comparison of survival rate in small HCCs (tumor size ≤ 3 cm)

The meta-analysis data revealed that there was no significant difference in survival rate between the two treatments at 1 year (OR: 8.42, 95%CI: 1.01-70.56, *P* = 0.05) and 3 years (OR: 1.35, 95%CI: 0.67-2.74, *P* = 0.40) (Figure 4A and B).

Comparison of survival rate in intermediate-size HCCs (3cm < tumor size ≤ 5cm)

There was a significant difference in survival rate between the two treatments at 1, 3 and 5 years, and the meta-analysis data indicated that RFA plus TACE was associated with a significantly higher 1-, 3- and 5-year survival rate than RFA monotherapy was ($OR_{1\text{-year}}$ = 3.46, 95%CI: 1.29-9.28, *P* = 0.01; $OR_{3\text{-year}}$ = 3.58, 95%CI: 1.79-7.15, *P* = 0.0003; $OR_{5\text{-year}}$ = 5.34, 95%CI: 2.42-11.75, *P* < 0.001) (Figure 4C-E).

Comparison of survival rate in large-size HCCs (tumor size < 5 cm)

There was a significant difference in survival rate between the two treatments at 1 and 3 years, and the meta-analysis data revealed that RFA plus TACE was associated with a significantly higher 1- and 3-year survival rate than RFA monotherapy was ($OR_{1\text{-year}}$ = 2.91, 95%CI: 1.60-5.29, *P* = 0.0004; $OR_{3\text{-year}}$ = 6.69, 95%CI: 3.01-16.07, *P* < 0.001) (Figure 4F and G).

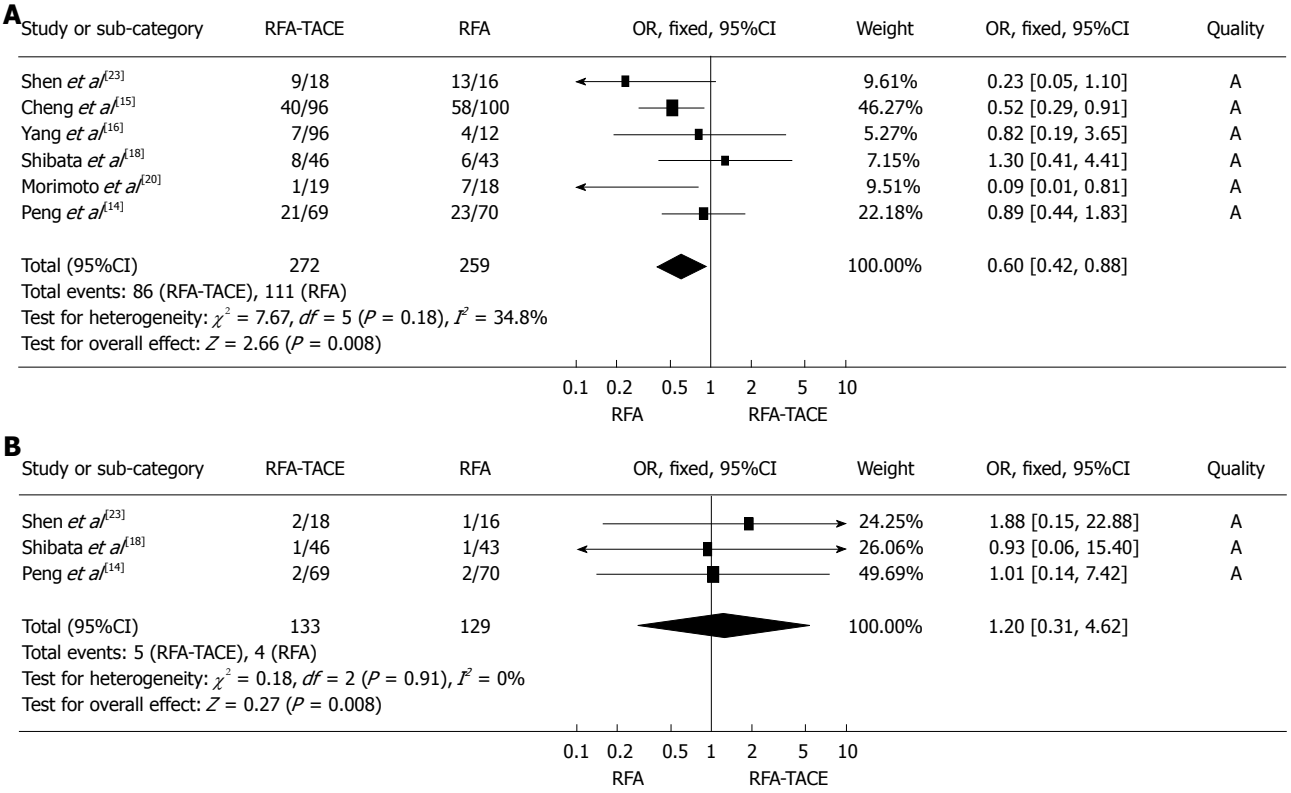


Figure 3 Comparison of combination of radiofrequency ablation and transcatheter arterial chemoembolization with radiofrequency ablation alone for hepatocellular carcinoma in terms of tumor progression rate (A) and major complications (B). RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization; OR: Odds ratio.

Assessment of publication bias

The publication bias in this study was detected using the funnel plot of the meta-analysis results. All of the included studies reported comparative data on 1-year overall survival rate. In the analysis of the effect of 1-year overall survival rate, the symmetry of the funnel plot shape suggested that there was no obvious publication bias in this meta-analysis (Figure 5).

DISCUSSION

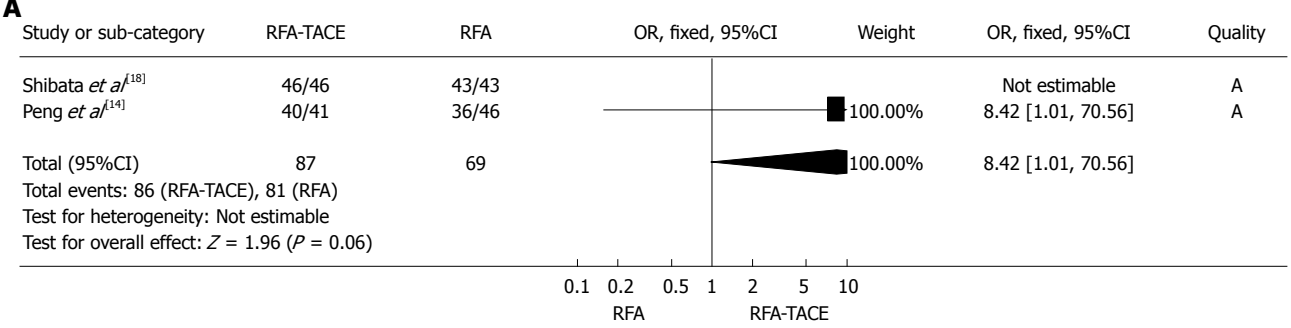
According to our data analysis, we found that the combination of TACE and RFA was associated with higher overall and recurrence-free survival rates than RFA monotherapy was in the treatment of HCC. Additionally, the subgroups analysis indicated that although there were no significant differences between TACE plus RFA and RFA alone in the treatment of small-size HCC, combination of TACE and RFA was associated with a higher survival rate than was RFA monotherapy for patients with intermediate- and large-size HCC. Thus, our analysis suggested that TACE combined with RFA was more effective than RFA monotherapy for treatment for intermediate- and large-size HCC.

Previous studies have reported that RFA combined with TACE is more effective than RFA monotherapy for treatment of HCC[14-16]. However, some other studies have reported conflicting results[17-19]. Meta-analysis is a method that combines data from all eligible studies, and has the advantage of reducing random error, thus obtaining more precise estimates and defining the effect of clinical interventions more precisely[25,26].

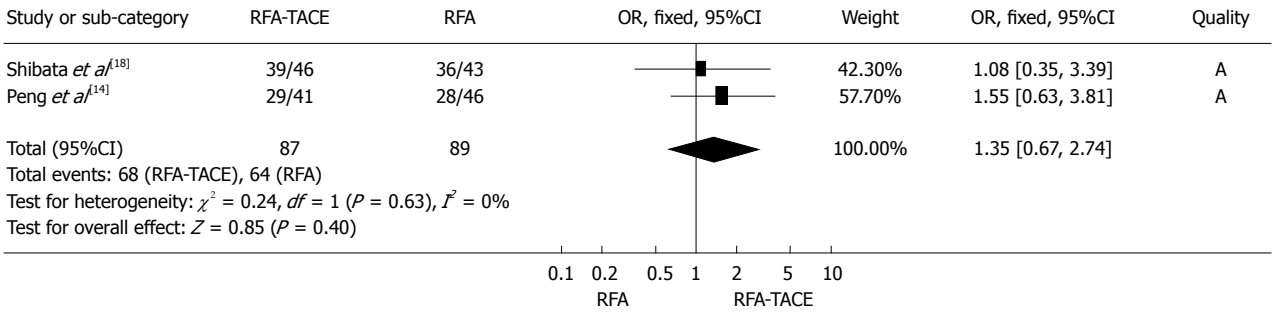
Tumor recurrence and progression are the major risk factors that affect the prognosis of HCC patients. A high rate of intrahepatic recurrence and progression after RFA plus TACE and/or RFA is the main cause of late death of patients with HCC. In the current analysis, the recurrence-free survival and progression rates were compared and analyzed. Our meta-analysis indicated that the 1-, 3- and 5-year recurrence-free survival rate was higher after RFA plus TACE than after RFA alone. However, RFA monotherapy was found to be associated with a higher tumor progression rate than RFA plus TACE was. The residual tumor tissue after RFA was the main cause of tumor recurrence and progression, which may be attributable to insufficient ablation of the primary tumor after RFA monotherapy. RFA combined with TACE has advantages in improving tumor necrosis rate. TACE is expected to reduce the cooling effect of hepatic blood flow in RFA by decreasing hepatic arterial flow, and plays a primary role in inducing tumor destruction[14,20,21]. Additionally, RFA cannot be a suitable treatment for tumors with multiple nodules, and TACE after RFA can effectively induce necrosis of multiple nodules and improve the tumor necrosis rate. This may explain the better results following RFA plus TACE in the treatment of HCC.

As regards the subgroups in our analysis, we found

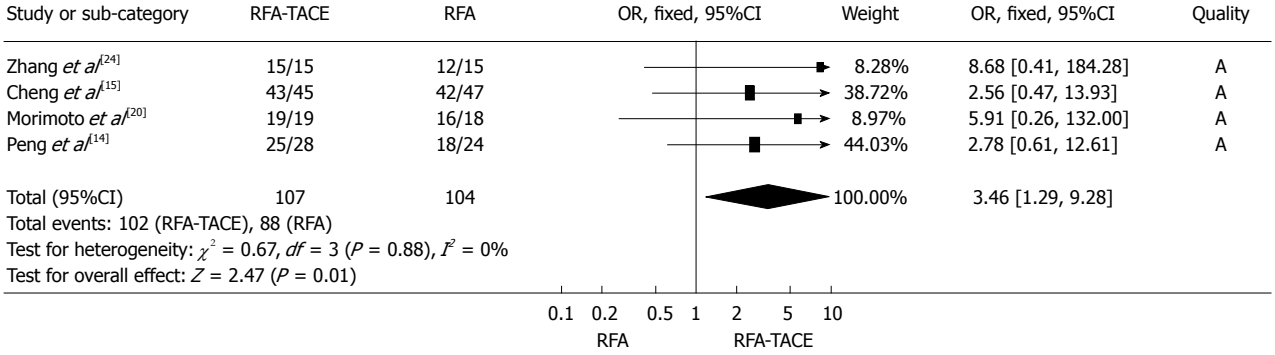
A


Study or sub-category
RFA-TACE
RFA
OR, fixed, 95%CI
Weight
OR, fixed, 95%CI
Quality
Shibata *et al*[18] 46/46 43/43 Not estimable A
Peng *et al*[14] 40/41 36/46 100.00% 8.42 [1.01, 70.56] A
Total (95%CI) 87 69 100.00% 8.42 [1.01, 70.56]
Total events: 86 (RFA-TACE), 81 (RFA)
Test for heterogeneity: Not estimable
Test for overall effect: Z = 1.96 (P = 0.06)
0.1 0.2 0.5 1 2 5 10
RFA
RFA-TACE

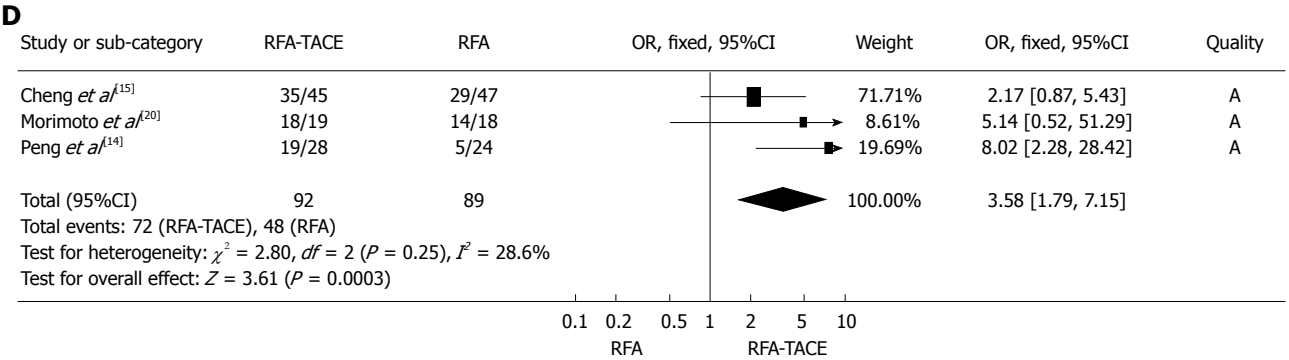
B


Study or sub-category
RFA-TACE
RFA
OR, fixed, 95%CI
Weight
OR, fixed, 95%CI
Quality
Shibata *et al*[18] 39/46 36/43 42.30% 1.08 [0.35, 3.39] A
Peng *et al*[14] 29/41 28/46 57.70% 1.55 [0.63, 3.81] A
Total (95%CI) 87 89 100.00% 1.35 [0.67, 2.74]
Total events: 68 (RFA-TACE), 64 (RFA)
Test for heterogeneity: χ^2 = 0.24, *df* = 1 (*P* = 0.63), I^2 = 0%
Test for overall effect: Z = 0.85 (P = 0.40)
0.1 0.2 0.5 1 2 5 10
RFA
RFA-TACE

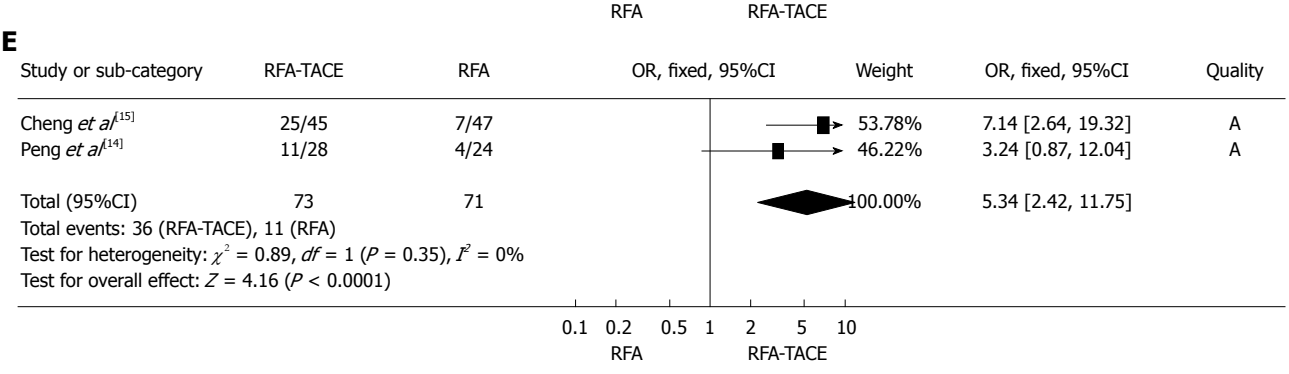
C


Study or sub-category
RFA-TACE
RFA
OR, fixed, 95%CI
Weight
OR, fixed, 95%CI
Quality
Zhang *et al*[24] 15/15 12/15 8.28% 8.68 [0.41, 184.28] A
Cheng *et al*[15] 43/45 42/47 38.72% 2.56 [0.47, 13.93] A
Morimoto *et al*[20] 19/19 16/18 8.97% 5.91 [0.26, 132.00] A
Peng *et al*[14] 25/28 18/24 44.03% 2.78 [0.61, 12.61] A
Total (95%CI) 107 104 100.00% 3.46 [1.29, 9.28]
Total events: 102 (RFA-TACE), 88 (RFA)
Test for heterogeneity: χ^2 = 0.67, *df* = 3 (*P* = 0.88), I^2 = 0%
Test for overall effect: Z = 2.47 (P = 0.01)
0.1 0.2 0.5 1 2 5 10
RFA
RFA-TACE

D


Study or sub-category
RFA-TACE
RFA
OR, fixed, 95%CI
Weight
OR, fixed, 95%CI
Quality
Cheng *et al*[15] 35/45 29/47 71.71% 2.17 [0.87, 5.43] A
Morimoto *et al*[20] 18/19 14/18 8.61% 5.14 [0.52, 51.29] A
Peng *et al*[14] 19/28 5/24 19.69% 8.02 [2.28, 28.42] A
Total (95%CI) 92 89 100.00% 3.58 [1.79, 7.15]
Total events: 72 (RFA-TACE), 48 (RFA)
Test for heterogeneity: χ^2 = 2.80, *df* = 2 (*P* = 0.25), I^2 = 28.6%
Test for overall effect: Z = 3.61 (P = 0.0003)
0.1 0.2 0.5 1 2 5 10
RFA
RFA-TACE

E


Study or sub-category
RFA-TACE
RFA
OR, fixed, 95%CI
Weight
OR, fixed, 95%CI
Quality
Cheng *et al*[15] 25/45 7/47 53.78% 7.14 [2.64, 19.32] A
Peng *et al*[14] 11/28 4/24 46.22% 3.24 [0.87, 12.04] A
Total (95%CI) 73 71 100.00% 5.34 [2.42, 11.75]
Total events: 36 (RFA-TACE), 11 (RFA)
Test for heterogeneity: χ^2 = 0.89, *df* = 1 (*P* = 0.35), I^2 = 0%
Test for overall effect: Z = 4.16 (P < 0.0001)
0.1 0.2 0.5 1 2 5 10
RFA
RFA-TACE

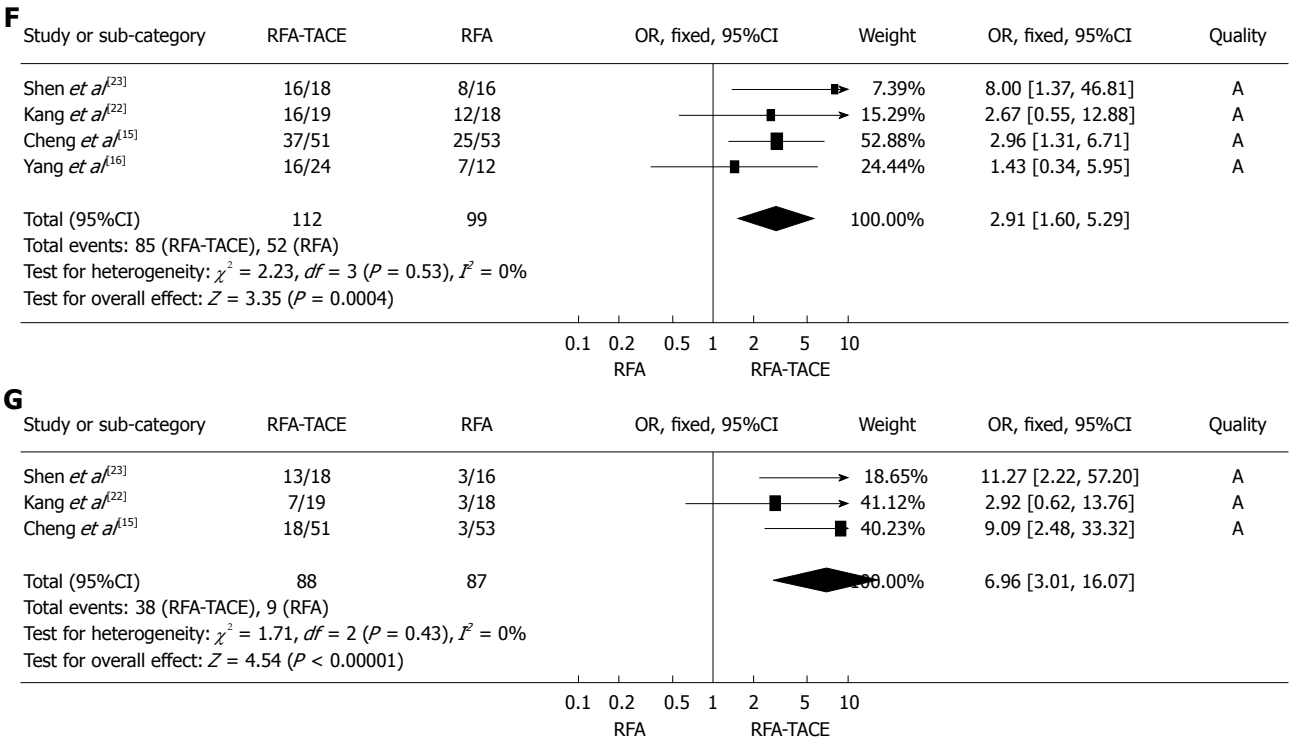


Figure 4 Comparison of combination of radiofrequency ablation and transcatheter arterial chemoembolization with radiofrequency ablation alone for small hepatocellular carcinoma in terms of survival rates. A, B: Tumor size ≤ 3 cm; C-F: 3 cm < tumor size ≤ 5 cm; G, H: Tumor size > 5 cm; A, C, F: Meta-analysis of 1-year results; B, D, G: Meta-analysis of 3-year results; E: Meta-analysis of 5-year results.

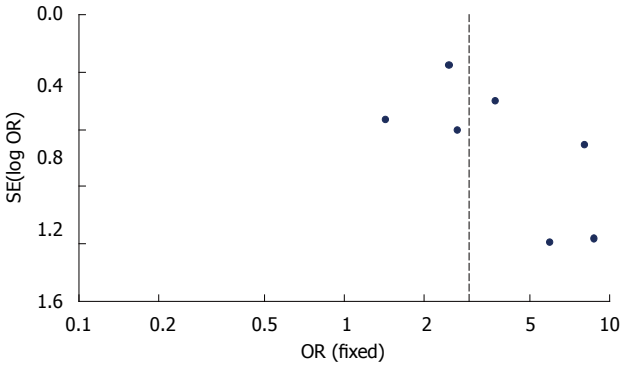


Figure 5 Funnel plot in the analysis of the effect of 1-year overall survival rate. OR: Odds ratio.

that there was no significant difference in survival rate between the two treatments for small HCC. However, there was a significant difference in survival rate between RFA plus TACE and RFA alone for treatment of intermediate- and large-size HCC, and our meta-analysis data showed that RFA plus TACE was associated with a significantly higher survival rate than RFA monotherapy was. RFA as a thermal *in situ* destruction technique was proved to be a safe and effective treatment. It is possible for RFA to achieve complete necrosis for treatment of small HCC. In recent years, RFA has been accepted as one of the best treatment options for small HCC. However, due to the limitation of the ablation area, it is barely possible for RFA to achieve complete necrosis during treatment of a relatively large HCC[27,28]. RFA combined with TACE can resolve this problem effectively. According to the clinical studies and experience, we found that there were some synergistic effects between RFA and TACE in combined treatment: (1) TACE can reduce the cooling effect of hepatic blood flow by decreasing hepatic arterial flow, and improve the effect of percutaneous RFA thermal therapy, which plays a critic role in inducing tumor necrosis[14,20,21]; (2) edematous change in the tumors induced by ischemia and inflammation after TACE is expected to enlarge the area of tumor necrosis during RFA treatment; and (3) RFA combined with TACE can prevent HCC with hepatic artery portal fistula from invading the portal vein and provides a better prognosis for HCC patients[20,29].

The risk of publication bias in this meta-analysis was assessed by the symmetry of the funnel plot[30,31]. All the studies included in the current meta-analysis had comparative data for 1-year overall survival rate. In the analysis of the effect of 1-year overall survival rate, we found that the level of the symmetry of the funnel plot was judged to be high, which indicated that there was no significant publication bias in this meta-analysis. Eight randomized controlled trials were included in this study. The overall quality of the studies was detected using Review Manager from the Cochrane Collaboration and was judged to be high. This suggests that the studies included in the study had strong evidence to support the results of our meta-analysis.

To the best of our knowledge, no meta-analysis has been performed to compare the efficacy and safety of RFA combined with TACE and RFA monotherapy in

terms of overall survival rate, recurrence-free survival rate, tumor progression, and major complications. Our meta-analysis is believed to be the first report to assess comprehensively combination of TACE and RFA compared with RFA alone for treatment of HCC. Additionally, we also analyzed and compared the effectiveness of RFA plus TACE and RFA monotherapy for treatment of small-, intermediate- and large-size HCC. The analysis of these studies is important and useful for precise and objective statistical assessment of the clinical efficacy of RFA plus TACE and RFA alone for treatment of HCC.

Our study had several limitations. First, the heterogeneity of the inclusion criteria (Child-Pugh class, number of tumors, tumor size, tumor stage, and treatment design) might have affected the consistency of the results and caused between-study heterogeneity, which could have affected the overall quality of our study. Second, the etiological factors of HCC such as viral hepatitis, alcoholic liver disease, and autoimmune liver disease, were not considered in the trials. Whether HCC patients with different etiological factors could obtain similar outcomes from treatment with RFA plus TACE needs further research.

In conclusion, this meta-analysis suggests that combination of RFA with TACE is more effective than RFA monotherapy in the treatment of patients with intermediate- and large-size HCC, with significantly higher survival rates achieved with the combined methods. The combination of interventional therapies may be applied more widely in the treatment of HCC.

COMMENTS

Background

Radiofrequency ablation (RFA) and transcatheter arterial chemoembolization (TACE) have been widely accepted as the established non-surgical treatment options for hepatocellular carcinoma (HCC). However, which is the superior treatment choice is still uncertain. Previous studies have suggested that the effectiveness of combination of TACE and RFA is much better than that of RFA monotherapy in HCC. However, other studies assessing the effectiveness of combination of TACE and PRFA compared with RFA alone have reported conflicting results. Hence, it is necessary to design a study to compare comprehensively the effectiveness and safety of TACE plus RFA and RFA monotherapy for treatment of patients with HCC.

Research frontiers

In the current study, a meta-analysis was designed to compare comprehensively the overall survival rates, recurrence-free survival rates, and tumor progression rates for RFA plus TACE with those of RFA monotherapy. Additionally, based on the tumor size, the survival rates of patients with small-, intermediate- and large-size HCC underwent RFA plus TACE compared with those who underwent RFA alone.

Innovations and breakthroughs

The current analysis comprehensively compared the effectiveness and safety of RFA combined with TACE with that of RFA monotherapy in HCC. This analysis indicated that the overall and recurrence-free survival rates of patients treated with RFA plus TACE were significantly higher than those with RFA alone. The authors also found that although there were no significant differences between RFA plus TACE and RFA monotherapy for small HCC, the efficacy of RFA plus TACE was obviously better than that of RFA alone for treatment of intermediate- and large-size HCC.

Applications

The analysis showed that the effectiveness of RFA combined with TACE was much better than that of RFA monotherapy for treatment of intermediate- and large-size HCC. Comparison of these two treatments could provide the theoretical basis for the use of RFA plus TACE for treatment of HCC and help stratify the benefits of treatment choices for patients with HCC.

Terminology

TACE is one of the most widely performed treatments for unresectable HCC, which is a type of interventional radiology. RFA is a thermal *in situ* destruction technique, and it has been proved to be a safe and effective treatment. RFA has been accepted as one of the best treatment options for small HCC.

Peer review

This was an excellent study that addresses an important topic, particularly the part comparing the efficacy and safety of combination of RFA and TACE with RFA monotherapy for treatment of patients with HCC. The report is concise and informative.

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doi:10.3748/wjg.v19.i24.3883

World J Gastroenterol 2013 June 28; 19(24): 3883-3891
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

META-ANALYSIS

Meta-analysis comparison of endoscopic papillary balloon dilatation and endoscopic sphincteropapillotomy

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Author contributions: Zhao HC and Geng XP designed the research; He L, Zhou DC and Zhao HC completed the literature searching; Pan FM, He L and Zhou DC analyzed the data; Zhao HC, He L and Zhou DC wrote the paper.
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Received: January 3, 2013 Revised: March 20, 2013
Accepted: March 28, 2013
Published online: June 28, 2013

Abstract

AIM: To assess endoscopic papillary balloon dilatation (EPBD) and endoscopic sphincteropapillotomy (EST) for common bile duct (CBD) stone removal using a meta-analysis.

METHODS: Randomized controlled trials published from 1990 to 2012 comparing EPBD with EST for CBD stone removal were evaluated. This meta-analysis was performed to estimate short-term and long-term complications of these two treatments. The fixed random effect model or random effect model was established to analysis the data. Results were obtained by analyzing the relative risk, odds ratio, and 95%CI for a given comparison using RevMan 5.1. Statistical significance was defined as $P < 0.05$. Risk of bias was evaluated using a funnel plot.

RESULTS: Of the 1975 patients analyzed, 980 of them were treated with EPBD and 995 were treated with EST. Of the patient population, patients in the EPBD group were younger (OR = -1.16, 95%CI: -1.49 to 0.84, $P < 0.01$). There were no significant differences in gender proportion, average size of stones, number of gallstones, previous cholecystectomy, the incidence of duodenal diverticulum, CBD diameter or the total follow-up time between EST and EPBD groups. Compared with EST, the total stone clearance in the EPBD group decreased (OR = 0.64, 95%CI: 0.42 to 0.96, $P = 0.03$), the use of stone extraction baskets significantly increased (OR = 1.91, 95%CI: 1.41 to 2.59, $P < 0.01$), and the incidence of pancreatitis significantly increased (OR = 2.79, 95%CI: 1.74 to 4.45, $P < 0.0001$). The incidence of bleeding (OR = 0.12, 95%CI: 0.04 to 0.34, $P < 0.01$) and cholecystitis (OR = 0.41, 95%CI: 0.20 to 0.84, $P = 0.02$) significantly decreased. The stone recurrence rate also was significantly reduced in EPBD (OR = 0.48, 95%CI: 0.26 to 0.90, $P = 0.02$). There were no significant differences between the two groups with the incidence of stone removal at first attempt, hours of operation, total short-term complications and infection, perforation, or acute cholangitis.

CONCLUSION: Although the incidence of pancreatitis was higher, the overall stone clearance rate and risk of bleeding was lower with EPBD compared to EST.

Key words: Common bile duct stone; Endoscopic papillary balloon dilatation; Endoscopic sphincteropapillotomy; Meta-analysis

Core tip: A meta-analysis was performed to evaluate the outcomes of endoscopic sphincteropapillotomy (EST) and endoscopic papillary balloon dilatation (EPBD) from previously published reports. Fourteen randomized trials involving 1975 patients were analyzed. Of those, 980 were treated with EPBD and 995 were treated with EST. Differences were observed between the treatments in total stone clearance, short-term complications, and long-term complications. Compared to

EST, the overall stone clearance rate was lower, and the incidence of pancreatitis was higher with EPBD. Thus, EPBD may decrease the incidence of long-term complications and be more suitable for patients who have a high risk of bleeding.

Zhao HC, He L, Zhou DC, Geng XP, Pan FM. Meta-analysis comparison of endoscopic papillary balloon dilatation and endoscopic sphincteropapillotomy. *World J Gastroenterol* 2013; 19(24): 3883-3891 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3883.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3883

INTRODUCTION

Along with the advance of endoscope technology and related instruments, endoscopic sphincteropapillotomy (EST) had become the first choice for common duct stones. But it was associated with some short-term and long-term complications such as pancreatitis, bleeding, cholangitis, perforation of the bile duct, narrow nipple, and sphincter loss of function. Endoscopic papillary balloon dilatation (EPBD), as a replacement for EST, was first reported by Staritz *et al*[1]. Compared with EST, EPBD is a less complicated procedure, and the incidence of short-term complications, such as bleeding and perforation, are decreased, while the function of the Oddi sphincter (OS) is protected. In a multicenter random control study, 117 patients who were definitively diagnosed with bile duct stones were treated by EPBD; after treatment, the incidence of pancreatitis among those patients reached 15.4% and two patients died from post-treatment complications[2]. Thus, a heated dispute still remains in the medical community as to the safety and application of EPBD. In the present study, a meta-analysis was performed to evaluate the curative effect and safety of EPBD relative to EST by systematically reviewing published randomized trials.

MATERIALS AND METHODS

Inclusion criteria

Only randomized controlled trails were considered for review, and no limitations were placed on the article language. In the included published studies, all cases were diagnosed with common duct stones and observational targets included at least one of the following criteria: (1) overall incidence of stone clearance, (2) incidence of a singular stone clearance, (3) incidence of short-term complications, or (4) incidence of long-term complications. EPBD was used as the experimental group while EST was used as the control group.

Search strategy

Studies were identified by searching MEDLINE, EMBASE, Cochrane library, Current Contents and the Science Citation Index in July 2012. Key search terms were "common bile duct stone", "endoscopic retrograde cholangiopancreatography", "endoscopic sphincteropapillotomy", "endoscopic papillary balloon dilatation", "endoscopic balloon dilatation", "EST", "ERCP", "EPBD" and "EBD". Additionally, articles were searched using Google in order to prevent any oversight.

Statistical analysis

Articles were retrieved when they met the inclusion criteria. Two reviewers (He L and Zhou DC) applied the inclusion criteria independently to the retrieved articles as defined in the protocol. Any differences were resolved by discussion. Each included study was critically evaluated for its study quality, based on the criteria used by the Cochrane Collaboration, and level of evidence according to the standards of the Hierarchy of Evidence. One reviewer (He L) extracted information on data extraction sheets and a second reviewer (Zhou DC) checked them.

All data were calculated using RevMan5.1. Relative risks (for dichotomous outcome measures), odds ratio (OR) and weighted mean differences (for continuous outcome measures) with 95%CI were calculated. Statistical significance was defined as $P < 0.05$. If the outcome could not be calculated or the incidence was too small, a qualitative evaluation was given by a detailed explanation. A heterogeneity test was conducted according to the value of I^2, χ^2 and P. If heterogeneity was positive, then a sensitivity analysis was carried out. Any article that showed positive heterogeneity was excluded. Risk of bias was evaluated using a funnel plot.

RESULTS

After the initial screening, 178 relevant articles were considered for review. Of these, 163 articles were not in accordance with the defined inclusion criteria. Among the remaining 15 articles, one was excluded based on positive heterogeneity[3]. A total of 14 articles were included in this meta-analysis with available data from 1975 patients summarized in Table 1[4-16].

Based on results from the analysis of these randomized controlled trials, significant differences were only found in the comparison of mean patient age between the two groups. The average age in the EPBD group was significantly younger than in the EST group (OR = -1.16, 95%CI: -1.49 to -0.84, $P < 0.01$). There were no significant differences in other general characteristics, such as gender ratio (female ratio, OR = 1.10, 95%CI: 0.91 to 1.31, $P = 0.33$; male ratio, OR = 0.91, 95%CI: 0.76 to 1.09, $P = 0.91$), mean diameter of stones (OR = 0.04, 95%CI: -0.22 to 0.30, $P = 0.77$), number of stones (OR = 0.22, 95%CI: -0.11 to 0.46, $P = 0.06$), complicated cholecystolithiasis (OR = 1.13, 95%CI: 0.83 to 1.54, $P = 0.43$), prior-cholecystectomy (OR = 1.03, 95%CI: 0.81 to 1.31, $P = 0.80$), mean diameter of common bile duct (CBD) stones (OR = -0.21, 95%CI: -0.69 to 0.26, $P = 0.38$) or total follow-up time (OR = -0.05, 95%CI: -0.51 to 0.41, $P = 0.83$).

In addition, the analysis showed that there was no

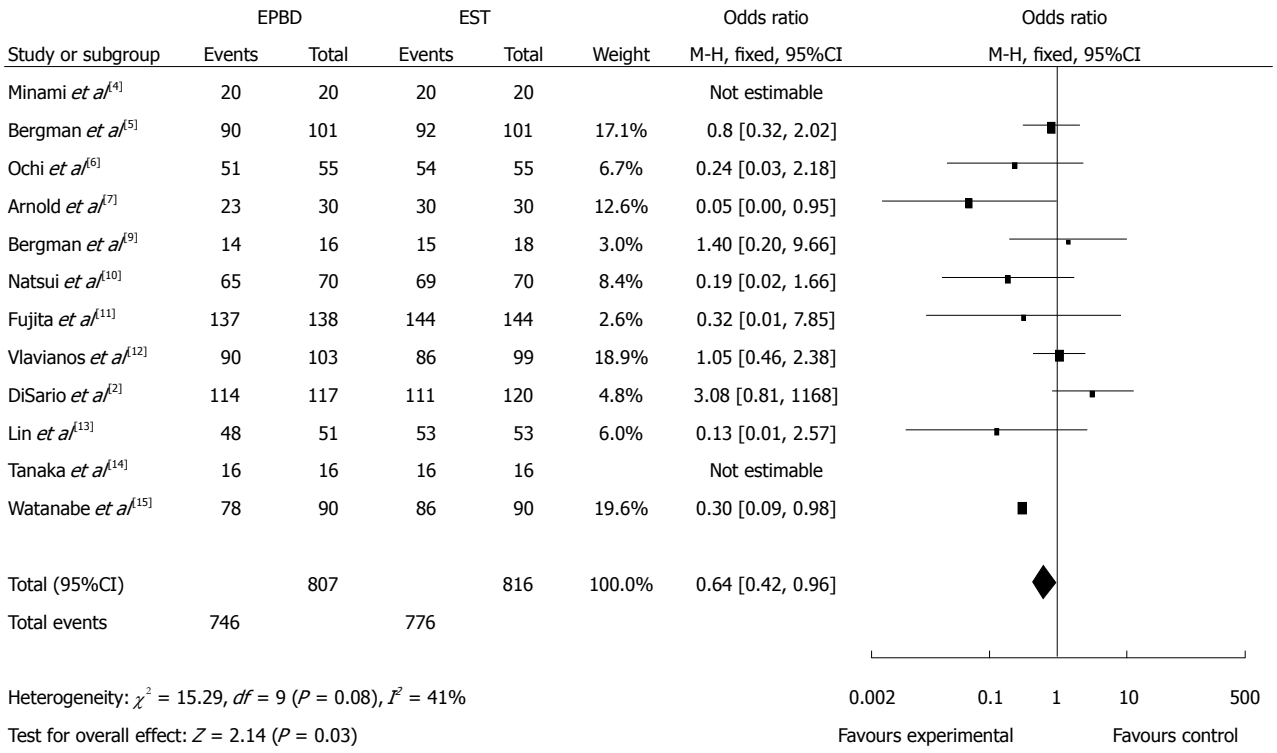


Figure 1 Comparison of total clearance of common bile duct stones between endoscopic papillary balloon dilatation (experimental group) and endoscopic sphincteropapillotomy (control group). EPBD: Endoscopic papillary balloon dilatation; EST: Endoscopic sphincteropapillotomy.

significant difference in the incidence of stone clearance on the first attempt between the survey groups (OR = 0.82, 95%CI: 0.63 to 1.07, $P = 0.15$). Relative to EST, the EPBD procedure was associated with a higher incidence of total clearance of CBD stones (Figure 1; OR = 0.64, 95%CI: 0.42 to 0.96, $P = 0.03$) and with more frequent stone extraction basket use (OR = 1.91, 95%CI: 1.41 to 2.59, $P < 0.01$). There were no significant differences in operation times between the two groups (OR = -0.62, 95%CI: -1.73 to 0.48, $P = 0.27$).

Incidence of short-term complications

Our results show that there was no significant difference in the overall incidence of early complications (OR = 1.33, 95%CI: 0.96 to 1.85, $P = 0.09$). Among the included randomized controlled trials, 11 studies reported the incidence of pancreatitis. Analysis of the data indicated that EPBD increased the incidence of pancreatitis when compared to EST in Western patients (OR = 4.05, 95%CI: 2.06 to 7.94, $P < 0.0001$), but was not significantly different in Asian patients ($P = 0.08$). In a combined analysis of Western and Asian studies, the incidence of pancreatitis was significantly increased in patients who received EPBD treatment (Figure 2A; OR = 2.79, 95%CI: 1.74 to 4.45, $P < 0.0001$).

There were nine studies that reported incidences of bleeding. The analysis showed that the incidence of bleeding in the EPBD group was lower than that of the EST group (Figure 2B; OR = 0.12, 95%CI: 0.04 to 0.34, $P < 0.01$). There was no significant difference in incidence of infection between EPBD and EST (OR = 0.85, 95%CI: 0.46 to 1.56, $P = 0.59$). Likewise, there was no significant difference in the incidence of perforation between EPBD and EST (OR = 0.77, 95%CI: 0.17 to 3.50, $P = 0.73$).

Incidence of long-term complications

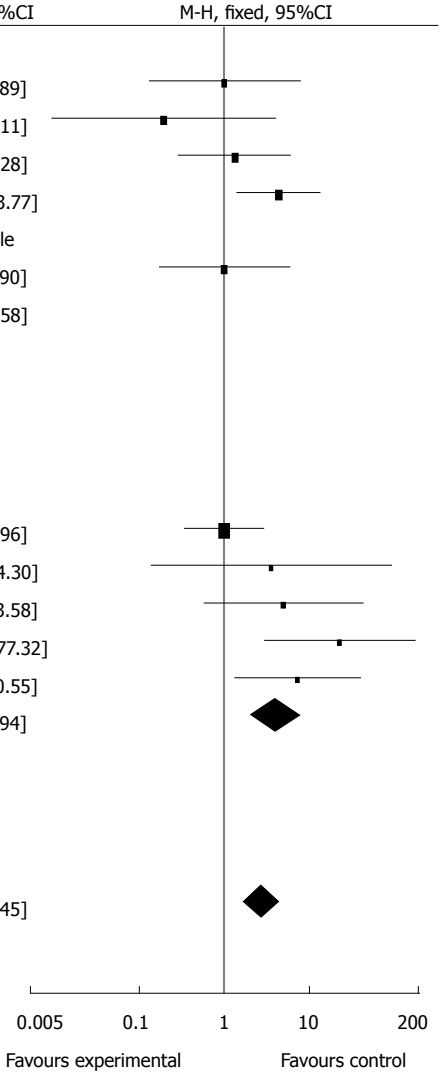
Six of the included studies reported incidences of long-term complications. Overall, the results of our analysis indicated that this incidence was significantly lower if patients were treated by EPBD rather than by EST (Figure 3A; OR = 0.53, 95%CI: 0.36 to 0.77, $P = 0.0008$). Compared to EST, EPBD markedly decreased the incidence of acute cholecystitis (OR = 0.41, 95%CI: 0.20 to 0.84, $P = 0.02$), although there was no significant difference between EPBD and EST in the incidence of acute cholangitis (OR = 0.60, 95%CI: 0.18 to 1.95, $P = 0.39$). Among the included studies, only seven reported recurrence of CBD stones, and our analysis showed that there was no significant difference between EPBD and EST (Figure 3B; OR = 0.66, 95%CI: 0.42 to 1.05, $P = 0.08$). Further analysis of the studies with a follow-up of more than one year indicated that the stone recurrence rate decreased significantly in the EPBD group (Figure 4; OR = 0.48, 95%CI: 0.26 to 0.90, $P = 0.02$).

Study assessment

A funnel plot analysis (Figure 5) was symmetrical on the whole, demonstrating that there was no significant publication bias. In addition, the included studies were of high quality and generally had a low risk of bias for selection, performance, attrition, and reporting as seen in Figure 6.

A

Study or subgroup	EPBD Events	EPBD Total	EST Events	EST Total	Weight	Odds ratio M-H, fixed, 95%CI
3.8.1 Asian						
Minami *et al*[4]	2	20	2	20	7.9%	1.00 [0.13, 7.89]
Ochi *et al*[6]	0	55	2	55	10.9%	0.19 [0.01, 4.11]
Natsui *et al*[10]	4	70	3	70	12.4%	1.35 [0.29, 6.28]
Fujita *et al*[11]	15	133	4	144	15.0%	4.45 [1.44, 13.77]
Lin *et al*[13]	0	51	0	53		Not estimable
Tanaka *et al*[14]	3	16	3	16	10.7%	1.00 [0.17, 5.90]
Subtotal (95%CI)		345		358	56.9%	1.83 [0.93, 3.58]
Total events	24		14			
Heterogeneity: χ^2 = 5.38, *df* = 49 (*P* = 0.25), I^2 = 26%						
Test for overall effect: *Z* = 1.76 (*P* = 0.08)						
3.8.2 Western						
Bergman *et al*[5]	7	101	7	101	28.6%	1.00 [0.34, 2.96]
Bergman *et al*[9]	1	16	0	18	1.9%	3.58 [0.14, 94.30]
Vlavianos *et al*[12]	5	103	1	99	4.3%	5.00 [0.57, 43.58]
DiSario *et al*[2]	18	117	1	129	3.5%	23.27 [3.05, 177.32]
Watanabe *et al*[15]	9	20	2	20	4.8%	7.36 [1.34, 40.55]
Subtotal (95%CI)		357		367	43.1%	4.05 [2.06, 7.94]
Total events	40		11			
Heterogeneity: χ^2 = 9.73, *df* = 4 (*P* = 0.05), I^2 = 59%						
Test for overall effect: *Z* = 4.06 (*P* < 0.0001)						
Total (95%CI)		702		725	100.0%	279 [1.74, 4.45]
Total events	64		25			
Heterogeneity: χ^2 = 15.83, *df* = 9 (*P* = 0.07), I^2 = 43%						
Test for overall effect: *Z* = 4.28 (*P* < 0.0001)						
Test for subgroup differences: χ^2 = 2.67, *df* = 1 (*P* = 0.10), I^2 = 62.5%						



B

Study or subgroup	EPBD Events	EPBD Total	EST Events	EST Total	Weight	Odds ratio M-H, fixed, 95%CI
Minami *et al*[4]	0	20	0	20		Not estimable
Bergman *et al*[5]	0	101	4	101	14.9%	0.11 [0.01, 2.01]
Arnold *et al*[7]	0	30	2	30	8.2%	0.19 [0.01, 4.06]
Bergman *et al*[9]	0	16	3	18	10.7%	0.13 [0.01, 2.81]
Natsui *et al*[10]	0	70	2	70	8.3%	0.19 [0.01, 4.12]
Fujita *et al*[11]	0	138	2	144	8.1%	0.21 [0.01, 4.32]
Lin *et al*[13]	0	51	14	53	44.8%	0.06 [0.01, 0.44]
Tanaka *et al*[14]	0	16	0	16		Not estimable
Watanabe *et al*[15]	0	90	1	90	5.0%	0.33 [0.01, 8.20]
Total (95%CI)		532		816	100.0%	0.12 [0.04, 0.34]
Total events	1		28			
Heterogeneity: χ^2 = 1.22, *df* = 9 (*P* = 0.98), I^2 = 0%						
Test for overall effect: *Z* = 3.97 (*P* < 0.0001)						

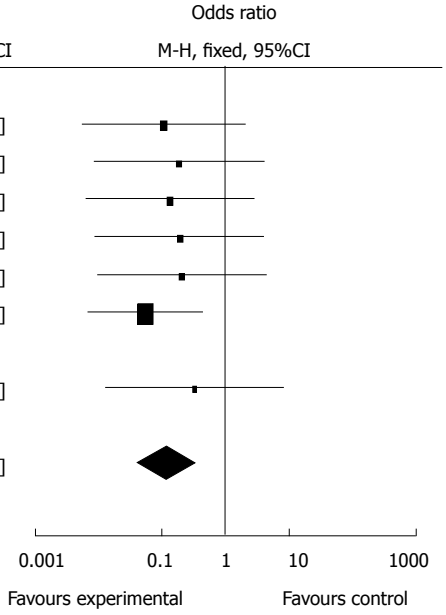
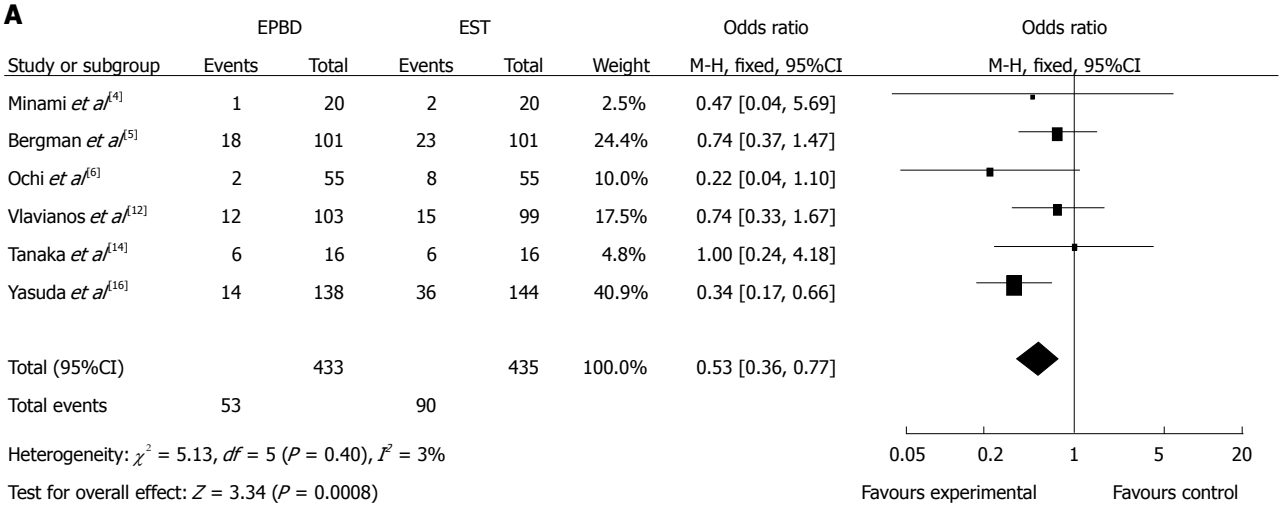


Figure 2 Incidence of pancreatitis (A) and bleeding (B) between endoscopic papillary balloon dilatation and endoscopic sphincteropapillotomy. EPBD: Endoscopic papillary balloon dilatation; EST: Endoscopic sphincteropapillotomy.

A



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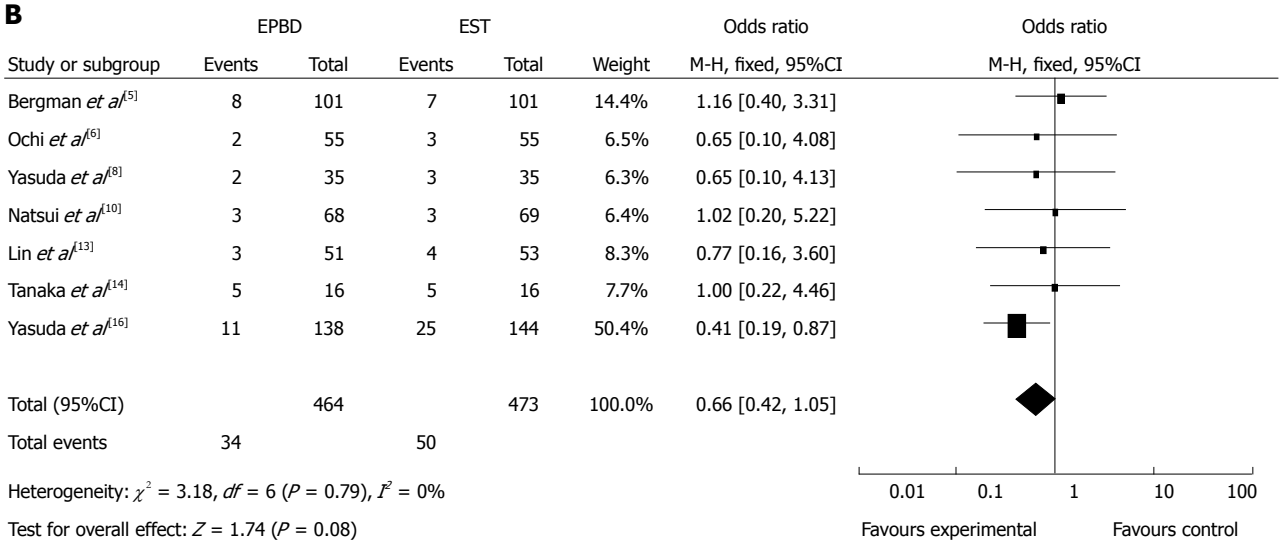

Figure 3 Overall incidence of long-term complications (A) and bile duct stone recurrence rate (B) between endoscopic papillary balloon dilatation and endoscopic sphincteropapillotomy. EPBD: Endoscopic papillary balloon dilatation; EST: Endoscopic sphincteropapillotomy.

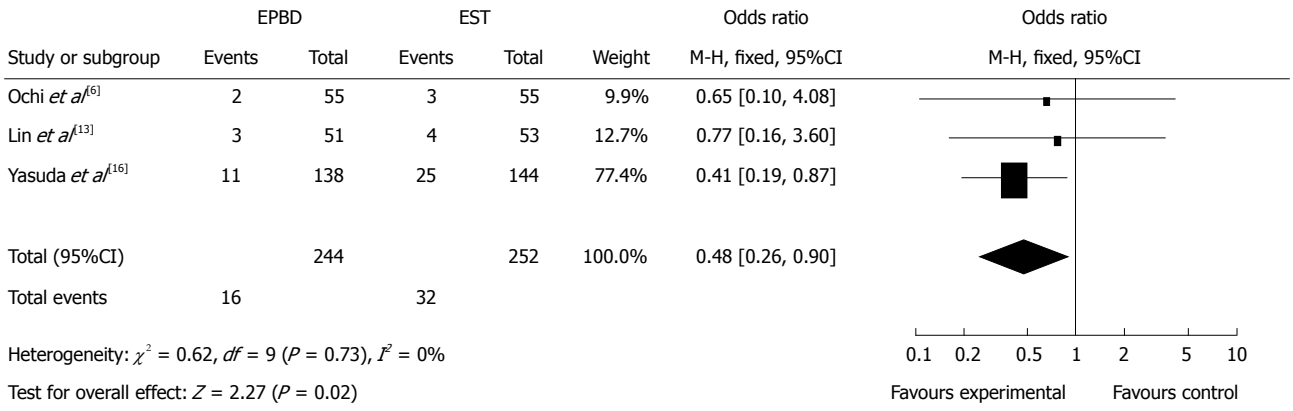

Figure 4 Incidence of bile duct stone recurrence rate between endoscopic papillary balloon dilatation and endoscopic sphincteropapillotomy with a follow-up of more than one year. EPBD: Endoscopic papillary balloon dilatation; EST: Endoscopic sphincteropapillotomy.

However, allocation concealment was only performed in the 5 studies[5,9,12,15,16]. Some outcomes were reported based on the intention to treat using assigned randomization at the beginning of the study[5,9,11,12,15]. In other studies, there was no detail given about randomization. A double-blinding method was not applied in any of the included studies. The main observational outcomes reported were the overall clearance of stones and stone

Table 1 Overview of study characteristics (mean ± SD)

Ref.	Year	Country	Treatment	Case	Sex	Age (yr)	MFT (mo)	Diameter of stones (mm)	No. of stones	Jadad grade
Minami *et al*[4]	1995	Japan	EPBD	20	13/7	64.0 ± 11.2	21.5 ± 6.2	< 12	No data	4 score
			EST	20	9/11	71.3 ± 14.0	23.1 ± 5.0	< 12		
Bergman *et al*[5]	1997	Holland	EPBD	101	43/58	72.0 ± 11.8	6.2	10.0 ± 5.5	2.0 ± 2.2	6 score
			EST	101	45/56	71.0 ± 11.1	6.1	9.0 ± 3.5	1.0 ± 2.3	
Ochi *et al*[6]	1999	Japan	EPBD	55	34/21	62.6 ± 15.9	23.0 ± 6.3	8.1 ± 3.4	2.1 ± 1.9	4 score
			EST	55	31/24	66.3 ± 14.3	23.0 ± 6.3	8.8 ± 4.2	1.7 ± 1.2	
Arnold *et al*[7]	2001	Germany	EPBD	30	11/19	54.2 ± 18.5	No data	7.0 ± 3.5	1.6 ± 1.1	4 score
			EST	30	13/17	58.5 ± 18.5		10.0 ± 4.7	1.8 ± 1.5	
Yasuda *et al*[8]	2001	Japan	EPBD	35	19/16	69.5 ± 7.3	No data	12.4 ± 3.3	3.7 ± 2.5	4 score
			EST	35	14/21	69.4 ± 7.5		12.3 ± 3.2	3.3 ± 2.5	
Bergman *et al*[9]	2001	Holland	EPBD	16	12/4	73.0 ± 6.8	No data	9.0 ± 2.8	2.0 ± 1.5	6 score
			EST	18	18/0	72.0 ± 2.8		8.0 ± 2.0	2.0 ± 1.5	
Natusi *et al*[10]	2002	Japan	EPBD	70	33/37	64.5 ± 10.6	No data	9.2 ± 3.2	2.7 ± 2.3	4 score
			EST	70	33/37	67.1 ± 8.3		9.7 ± 2.3	2.6 ± 2.3	
Fujita *et al*[11]	2003	Japan	EPBD	138	75/63	66.8 ± 1.3	No data	7.0 ± 3.1	2.4 ± 2.5	5 score
			EST	144	92/52	61.9 ± 1.2		7.7 ± 3.4	2.4 ± 2.9	
Vlaviano *et al*[12]	2003	England	EPBD	103	25/78	60.8 ± 1.3	No data	No data	No data	4 score
			EST	99	35/64	62.0 ± 1.2				
Disario *et al*[2]	2004	United States	EPBD	117	76/41	47.0 ± 19.0	1.6 ± 2.9	6.0 ± 1.6	1.0 ± 16.5	6 score
			EST	120	89/31	54.0 ± 19.0	1.6 ± 0.4	5.0 ± 2.3	1.0 ± 1.5	
Lin *et al*[13]	2004	Taiwan (China)	EPBD	51	28/23	64.0 ± 10.3	16.0 ± 3.0	8 ± 6	No data	4 score
			EST	53	31/22	65.0 ± 10.0	16.0 ± 3.0	8 ± 6		
Tanaka *et al*[14]	2004	Japan	EPBD	16	10/6	67.2 ± 4.7	No data	10.2 ± 3.5	2.0 ± 1.8	5 score
			EST	16	3/13	70.6 ± 6.3		12.4 ± 6.0	2.0 ± 0.5	
Watanabe *et al*[15]	2007	Japan	EPBD	90	51/39	69.1 ± 13.1	No data	8.1 ± 3.2	2.7 ± 2.8	4 score
			EST	90	49/41	70.2 ± 8.1		7.7 ± 2.3	2.5 ± 2.7	
Yasuda *et al*[16]	2010	Japan	EPBD	138	52/92	68.5 ± 11.2	80.3 ± 15.0	6.5 ± 2.1	1.0 ± 2.5	5 score
			EST	144	63/75	71.0 ± 10.3	81.6 ± 15.2	7.0 ± 2.3	1.0 ± 3.8	

M: Male; F: Female; MFT: Mean follow-up time; MD: Mean diameter; EPBD: Endoscopic papillary balloon dilatation; EST: Endoscopic sphincteropapillotomy.

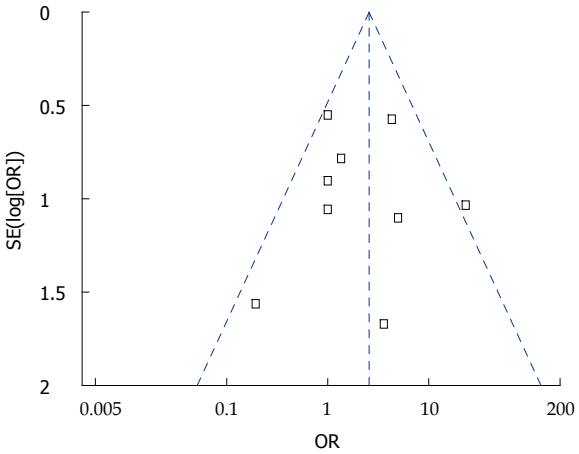

Figure 5 Funnel plot analysis of included studies. X-axis [Peto odds ratio (OR)] represents treatment effects. Y-axis (log[Peto OR]) represents the large sample size. Each square represents an individual study. The dashed line represents 95%CI.

clearance at the first attempt, both of which were reported by all studies. Completed data for the other outcomes was not available.

DISCUSSION

Meta-analysis is an accepted method that can evaluate controversial issues met in clinical practice, by analyzing high quality randomized controlled trials in quantification. Studies from many different countries were included in this analysis, and the differences between EPBD and EST were evaluated systematically to produce meaningful results.

The results indicated that EST increased the overall clearance of stones compared to EPBD, but that the stone clearance at first attempt was similar. It is generally acknowledged that EPBD is less likely to clear big stones than EST because its inflation into the papilla is smaller. In our analysis, however, we have defined the stone diameter as part of the exclusion criteria. Some studies were excluded if the smallest stone was larger than 12 mm[4], 15 mm[6], 20 mm[7], or 14 mm[11]. Two studies were excluded because the total number of stones was more than 10[6] or 5[7]. Stone extraction basket usage was more frequent when patients were treated with EPBD than EST, especially when the diameter of stones was larger than 8 mm[4], 10 mm[5,6], or 12 mm[10].

In the matter of safety, our results suggest that there was no significant difference in short-term complications between EPBD and EST. However, the incidence of long-term pancreatitis was higher after EPBD. This phenomenon may be due to the poor outflow of pancreatic juice caused by hydrops or spasm of the OS after EPBD. The risk of pancreatitis after EPBD is also a highly disputed issue, especially in America. EPBD has been abandoned by many endoscope doctors because they believe

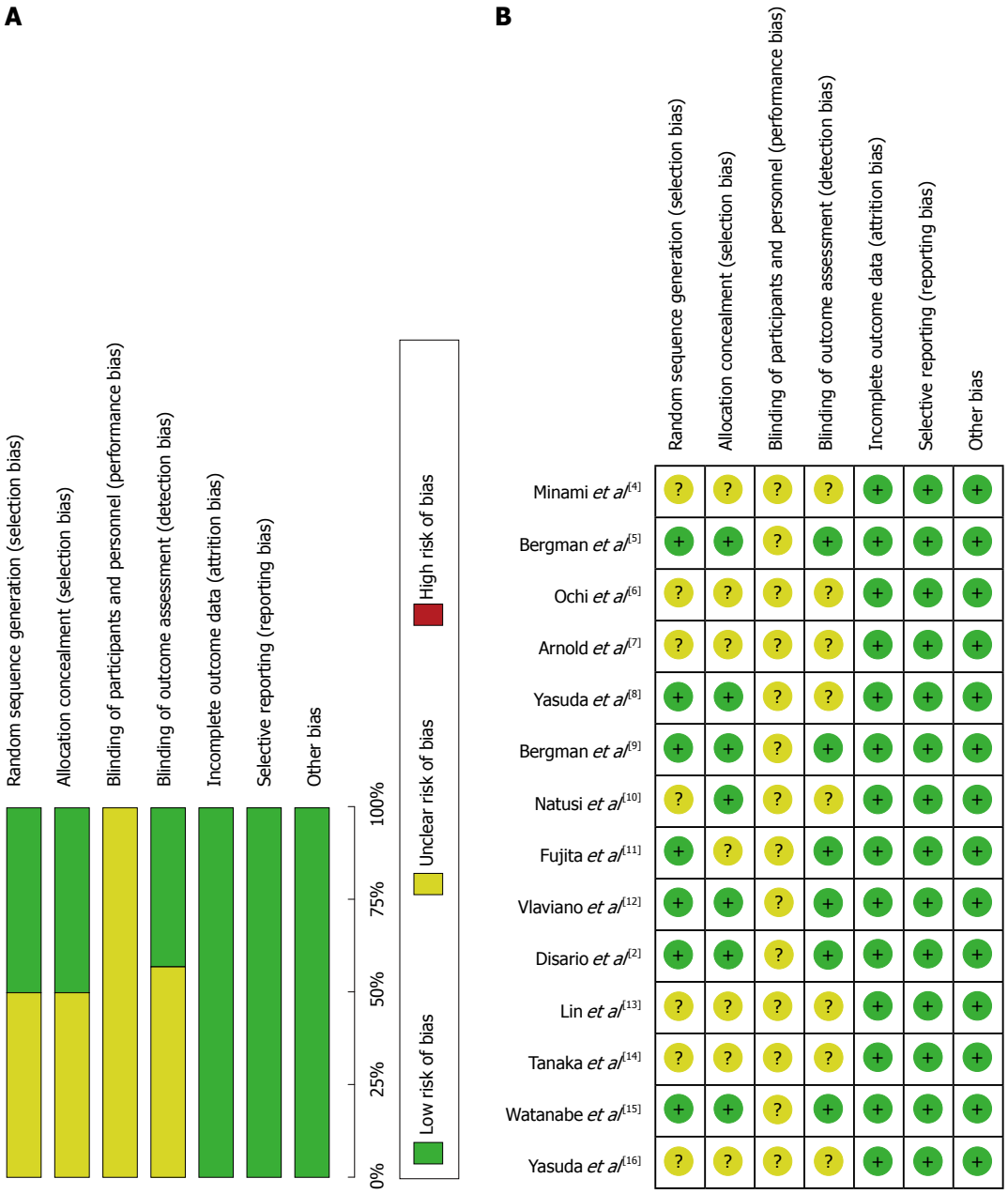

Figure 6 Risk of bias summary (A) and bias assessment for each individual study (B).

that this technology increases risk of long-term pancreatitis[17]. Conversely, most researchers in Asia and Europe maintain that there is no direct consequence between pancreatitis risk and EPBD and report that pancreatitis usually occurs in the light or medium stage[5,6,10,13,14]. A retrospective study by Tsujino *et al*[18] showed that among 1000 patients treated by EPBD, only 48 of them (4.8%) experienced light to moderate pancreatitis and only one patient (0.1%) experienced serious pancreatitis, but was fully recovered 8 mo after treatment.

Compared to EST, the incidence of bleeding during operation was significantly lower in EPBD, possibly due to the protection of the sphincter of Oddi during EPBD that is not incised directly as in EST. This advantage promotes the application of EPBD for patients with poor blood clotting abilities. A previous study of 98 patients who were diagnosed with CBD stones experiencing liver cirrhosis was carried out by Tsujino *et al*[18]. In that report, bleeding only occurred in one patient after the EPBD procedure, even though 31 of these patients were in C-stage of Child-Pugh. Moreover, when faced with atypical anatomy, such as duodenal diverticulum or Billroth-Ⅱ gastrectomy history, EPBD is more suitable than EST because EPBD can expand the OS more easily. In addition, Bergman *et al*[9] has reported that for patients with Billroth-Ⅱ gastrectomy history, the amount of bleeding was significantly decreased after EPBD (EPBD *vs* EST: 2% *vs* 17%, $P = 0.03$).

Based on the results of this analysis, compared to EST, EPBD decreased the incidence of long-term cholecystitis and the recurrence of stones in CBD, but there were no significant differences in the duration of long-

term cholangitis or recurrence of stones. Some research has suggested that EST could lead to some serious side effects, such as exhaustive, non-reversible loss of function of the OS, although pneumobilia and bile regurgitation exists in nearly 50% of patients and biliary bacterial disease exists in nearly all patients[19]. Some complications may be continuously affected by these conditions, such as with acute cholecystitis and cholangitis. Our analysis showed discordant results with long-term cholangitis, but this may be due to the limited sample size and short follow-up.

A study by Natsui *et al*[20] determined that EST was correlated with bacterial colonization in bile duct, which could lead to chronic inflammation and fibrosis of the bile duct system. Research by Kurumado *et al*[21] suggested that destruction of the sphincter of Oddi could even increase the risk of bile duct cancer. A retrospective study by Kojima *et al*[22] found that recurrence of CBD stones was higher after EST than EPBD (17.0% *vs* 6.8%, $P < 0.05$), and the sphincter of Oddi function was protected in 70% patients after EPBD, decreasing the recurrence of stones. Another study reported an 8.8% recurrence rate of stones in 837 patients after 4.4 years of follow-up where mechanical lithotripsy usage and subsequent cholecystolithiasis were considered risk factors for recurrence[18].

This meta-analysis was limited by two factors. First, there was some study selection bias that could not be excluded easily. Secondly, the definition of short-term and long-term complications was not completely consistent. For example, complications that occurred during 24 h[12], 15 d[9,14], and 30 d[2] were all included in short-term complications, while long-term complications included 6 mo[5], 12 mo[8,12], 16 mo[13], 23 mo[6], 30 mo[4,10], 72 mo[14], and 81 mo[16]. These uncontrollable factors may have affected the accuracy of this study to some extent.

In summary, in a comparison of the two treatments, EPBD and EST, the overall stone clearance rate of EPBD was lower and the incidence of pancreatitis with EPBD was higher. EPBD has the advantage of decreasing the incidence of long-term complications, and for patients who have a high risk of bleeding, EPBD may be more suitable.

COMMENTS

Background

To date, endoscopic sphincteropapillotomy (EST) and endoscopic papillary balloon dilatation (EPBD) are the primary choices for the removal of common duct stones. As a replacement for EST, EPBD is a less complicated procedure, has fewer short-term complications, such as bleeding and perforation, and protects the function of the Oddi sphincter. However, a heated debate still remains as to which is the better procedure.

Research frontiers

In this report, a meta-analysis was performed to evaluate the curative effects and safety of EST and EPBD by systematically reviewing newly published randomized trials.

Innovations and breakthroughs

The meta-analysis showed that in comparison with EST, patients in the EPBD group were younger (OR = -1.16,95%CI: -1.49 to -0.84, $P < 0.01$), had lower rate of total stone clearance (OR = 0.64, 95%CI: 0.42 to 0.96, $P = 0.03$), had a greater use of stone extraction baskets (OR = 1.91,95%CI: 1.41 to 2.59, $P < 0.01$), experienced a higher incidence of pancreatitis (OR = 2.53, 95%CI: 1.55 to 4.13, $P = 0.0002$) and a significantly lower incidence of bleeding (OR = 0.12, 95%CI: 0.04 to 0.34, $P < 0.01$) and acute cholecystitis (OR = 0.41, 95%CI: 0.20 to 0.84, $P = 0.02$). The total long-term complication rate was also significantly reduced in the EPBD group (OR = 0.53, 95%CI: 0.36 to 0.77, $P = 0.0008$). These findings also suggest that in patients who experience a greater risk of bleeding, EPBD may be more suitable.

Applications

A greater understanding of the advantages and disadvantages of EST and EPBD treatments for the removal of common biliary duct stones allows clinicians to make appropriate treatment decisions for patients.

Peer review

The authors performed a systematic meta-analysis to investigate the curative effect and safety between EST and EPBD. Compared with EST, in spite of the overall stone clearance rate of EPBD is lower, and the incidence of pancreatitis in the EPBD is higher. EPBD has the advantages to decrease the incidence of long-term complications. For patients experiencing a greater risk of bleeding, EPBD may be more suitable.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3892

World J Gastroenterol 2013 June 28; 19(24): 3892-3894
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Foreign body impaction in the sigmoid colon: A twenty euro bet

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Supported by János Bolyai Research Grant, to Veres G; A Hungarian Scientific Research Fund Grant, No. OTKA-K 105530
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Received: July 23, 2012 Revised: October 26, 2012
Accepted: November 14, 2012
Published online: June 28, 2013

Abstract

Foreign body ingestion is a common clinical problem in early childhood. However, it may occur even in adults, unknowingly. Most ingested foreign bodies entering the stomach pass through the gastrointestinal tract uneventfully. Here we report on a 13-year-old boy who presented with chronic abdominal pain, weight loss and occult gastrointestinal bleeding for 6 mo. Colonoscopy was negative; however, a ballpoint pen was impacted in the sigmoid region. Subsequently, the child admitted swallowing a pen as a 20-euro bet 6 mo previously. Crohn's disease is a chronic relapsing inflammatory gastrointestinal disease. It is often difficult to diagnose due to the fact that there is no single pathognomonic sign or symptom. This case is a description of an adolescent with chronic gastrointestinal symptoms due to a foreign body. Therefore, an ingested foreign body should be included in the differential diagnostic procedure related to gastrointestinal symptoms.

Key words: Foreign bodies; Pen; Differential diagnosis; Crohn's disease; Child

Müller KE, Arató A, Lakatos PL, Papp M, Veres G. Foreign body impaction in the sigmoid colon: A twenty euro bet. *World J Gastroenterol* 2013; 19(24): 3892-3894 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3892.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3892

INTRODUCTION

Foreign body ingestion is a common problem in everyday emergency practice, primarily in children. Fortunately, the majority of the ingested foreign objects pass through the gastrointestinal tract without complications. Foreign body ingestion may present without symptoms[1], and in some cases results in perforation with gastrointestinal bleeding or an obstruction[1]. Rarely, an abscess or a fistula occurs[1]. It has been reported to mimic other diseases (renal stone or irritable bowel syndrome)[1]; however, there are no literature data concerning differential diagnostic difficulties of Crohn's disease (CD) and a foreign body.

CD is a chronic relapsing inflammatory gastrointestinal disease. It is often difficult to diagnose due to the fact that there is no single pathognomonic sign or symptom. Here we report on a case with chronic abdominal symptoms, weight loss and occult bleeding suggesting CD. However, at colonoscopy, a swallowed pen was impacted in the sigmoid region causing the aforementioned symptoms.

CASE REPORT

A 13-year-old boy was admitted to the First Depart-

ment of Paediatrics at Semmelweis University with 10-kg weight loss in the last 6 mo and intermittent colicky abdominal pain. Past medical history revealed that 6 mo previously he was admitted to hospital for 2 d due to acute abdominal pain and vomiting. He was given intravenous fluids and his complaints improved. However, anorexia persisted, abdominal pain returned intermittently, and he lost 10 kg during this period. Stool blood test was positive twice, while stool culture, parasite and assays for *Clostridium difficile* toxins A and B were all negative. Considering the age of the patient, weight loss, chronic abdominal pain and positive stool blood test, as well as negative stool culture, inflammatory bowel disease was suspected and he was referred to our clinic.

Physical examination revealed normal vital signs without any clinical abnormalities. His abdomen was soft, non-tender, without guarding or palpable masses. There was normal sphincter tone, no perianal abscess, skin tag or fistula at rectal examination. Laboratory tests showed normal red blood cell count, white blood cell count and thrombocyte count. C-reactive protein, total protein and albumin were within the normal limits. Abdominal ultrasound showed slight wall thickening in the descending colon. There was no family history of inflammatory bowel disease.

The upper endoscopy was negative. There were no ulcers, no sign of bleeding, and no antral or bulbar lymphonodular hyperplasia. The terminal ileum and the colon appeared normal, confirmed by multiple mucosal biopsies at histology. Surprisingly, at withdrawal of the colonoscope, an impacted foreign body (plastic half-ball pen, Figure 1) was observed embedded in the sigmoid region. The surrounding mucosa was inflamed, with no visible mucosal vessels. The plastic, numbered ballpoint pen could not be removed by Dormia set and polypectomy snare despite several attempts. After the diagnostic procedures, the patient admitted swallowing a half-plastic pen as a 20-euro bet with his friend 6 mo ago. At first, he thought there might have been a connection between the swallowed pen and his symptoms as he had a hospital admission for 2 d due to acute abdominal pain and vomiting (see above). Later, he was embarrassed and hoped that these two events were just a coincidence. He never disclosed his bet.

Plain abdominal X-ray and abdominal ultrasound were performed the next day after endoscopy, but the foreign body could not be visualized. The pen passed through the colon spontaneously hence the control colonoscopy showed no foreign body after 5 d. The patient had no symptoms during 21 mo of follow-up; his weight gain is normal, and there is no occult bleeding.

DISCUSSION

We report on a case of an ingested foreign body as a twenty-euro bet. To our knowledge, this is the first description of a swallowed object causing chronic gastrointestinal symptoms in a paediatric patient. However, ingested foreign bodies are a common event in the paediatric population.

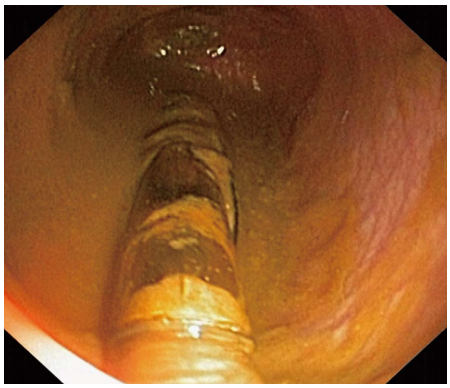

Figure 1 Half-plastic pen impacted in the sigmoid region.

The first recorded paediatric foreign body ingestion was described by Frederick the Great in 1692[2]. Most accidentally ingested foreign bodies go undetected and pass through without any incident. However, 10%-20% require endoscopic removal and 1% or less require surgical intervention[3]. In general, the passing of an ingested foreign body depends on the anatomic conditions of the gastrointestinal tract and on factors related to the ingested foreign body. Long, thin objects as seen in our case are less likely to pass the pylorus or the duodenum[2]. The presenting features vary according to the site and include pancreatitis, hepatic abscess, appendicitis, intussusceptions and irritable bowel syndrome[1,2].

There are only a few reports of foreign bodies imitating CD in the English literature. Ioannidis *et al*[4] reported on a case of incidental toothpick ingestion which caused an ileum fistula and mimicked CD. In addition, a patient presented with recurrent, subacute obstruction and right iliac mass mimicking the presentation of CD[5]. Subsequent lower endoscopy revealed small bowel phytobezoar which passed spontaneously.

To our knowledge, only one paediatric case of an ingested foreign body mimicking CD has been reported[6]; nevertheless, this was an acute event. A 7-year-old boy presented with a 2-wk history of cramping abdominal pain and low grade fever. Colonoscopy revealed an oedematous, friable rectosigmoid junction with a solitary fistula or ulcer. Hydrocortisone enemas were prescribed with minimal improvement. Seven days later his condition became more serious, and a computed tomography scan revealed a right iliopsoas abscess. Repeated colonoscopy showed a toothpick in the lumen of the rectosigmoid colon.

Foreign bodies usually cause acute symptoms of perforation, obstruction or gastrointestinal bleeding. However, our patient had chronic symptoms. This is probably due to the lack of perforation or obstruction. Meanwhile, his symptoms were similar to the reported cases, in which perforation or fistula was diagnosed.

We were presented with a healthy, young adolescent with chronic abdominal pain, weight loss and occult bleeding - classic presenting features of paediatric inflam-

matory bowel disease. Our working hypothesis was CD, though the laboratory tests were negative. Colonoscopy revealed a plastic, half-ball pen embedded in the sigmoid mucosa. The patient concealed swallowing a half-pen as a 20-euro bet with his friend. His subsequent hospital admission due to vomiting and acute abdominal symptoms may be explained as due to gastric irritation. Later the foreign body passed through the upper gastrointestinal tract and impacted in the sigmoid region. We speculate that occult bleeding was caused by the chronic mucosal irritation around the embedded pen. Anorexia and abdominal pain can be explained by the increased bowel peristalsis around the logged pen[7].

The value of imaging studies for an ingested foreign body seems to be questionable based on our case. Hence, plain abdominal X-ray (plastic pen) and abdominal ultrasound (sigmoid localization) could not identify the pen. Nevertheless, the role of imaging studies is crucial to determine the inflammatory reaction in and around the bowel wall and to exclude findings requiring surgical intervention[7].

The majority of ingested foreign bodies that reach the stomach pass through the alimentary tract without complication. If not, the management of ingested foreign bodies is dependent on their size, shape, material and location. After imaging studies, endoscopy should be considered as the crucial step in management since it is a potent and safe diagnostic tool. On the other hand, surgical treatment is mandatory in the presence of complications such as abscesses and fistulas[7]. In our patient, we attempted the removal of the embedded pen, but due to the increased possibility of perforation, we did not prevail. Subsequently, the pen was spontaneously passed after the first endoscopy as a result of the previous attempts of removal.

In summary, we report on an adolescent patient with chronic gastrointestinal symptoms due to a swallowed plastic pen that mimicked CD. Therefore, an ingested foreign body should be included in the differential diagnostic procedure related to chronic gastrointestinal symptoms.

ACKNOWLEDGMENTS

We thank Alexandra Horvath for her language editing.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3895

World J Gastroenterol 2013 June 28; 19(24): 3895-3898
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Early-stage primary signet ring cell carcinoma of the colon

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Received: November 22, 2012 Revised: April 15, 2013
Accepted: May 18, 2013
Published online: June 28, 2013

Core tip: Primary signet ring cell carcinoma of the colorectum detected at an early stage is very rare; most cases are detected at an advanced stage. Therefore, its prognosis is poorer than that of ordinary colorectal cancer. We report a case of primary signet ring cell carcinoma of the colon detected at an early stage and provide a review of the literature.

Kim JH, Park SJ, Park MI, Moon W, Kim SE. Early-stage primary signet ring cell carcinoma of the colon. *World J Gastroenterol* 2013; 19(24): 3895-3898 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3895.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3895

Abstract

Primary signet ring cell carcinoma of the colorectum detected at an early stage is very rare; most cases are detected at an advanced stage. Therefore, its prognosis is poorer than that of ordinary colorectal cancer. A 56-year-old Korean man was seen at this hospital for management of signet ring cell carcinoma of the colon. Colonoscopic examination revealed a Ⅱa-like, ill-defined and flatly elevated 9-mm residual tumor in the cecum. Endoscopic mucosal resection was preformed. Pathological examination of the resected specimen revealed signet ring cell carcinoma that had invaded the lamina propria without venous or perineural invasion. Abdominal computed tomography (CT) and positron CT showed no evidence of primary lesions or distant metastasis. An additional laparoscopic right-hemicolectomy was performed; no residual tumor or lymph node metastasis was found. We report a case of primary signet ring cell carcinoma of the colon detected at an early stage and provide a review of the literature.

Key words: Colon carcinoma; Signet ring cell carcinoma; Primary carcinoma; Early stage; Endoscopic mucosal resection

INTRODUCTION

Primary signet ring cell carcinoma is a rare type of colorectal cancer comprising 0.1%-2.6% of all colorectal cancers[1]. Because clinical symptoms tend to occur late in the course of signet ring cell carcinoma, most cases are usually detected at an advanced stage[2]; therefore, its overall survival rate is reported to be poorer than that of ordinary colorectal adenocarcinoma[3,4]. Early diagnosis is important to improve outcomes; however, there is little known about the early stages of signet ring cell carcinoma. In this case report, we describe a case of primary signet ring cell carcinoma of the colon detected at an early stage and provide a review of the literature.

CASE REPORT

A 56-year-old Korean man was seen in the gastroenterology clinic at this hospital for management of signet ring cell carcinoma of the colon.

The patient had been healthy up to the time of his presentation. Ten days before his evaluation at this hospital, the patient saw a gastroenterologist at another hospital and underwent colonoscopy for a health checkup. Colonoscopic examination revealed a Ⅱa-like, ill-defined

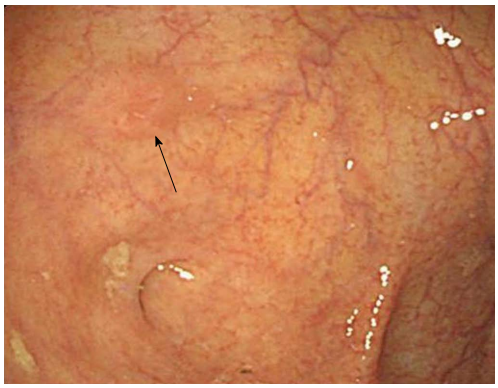

Figure 1 Initial colonoscopic findings (from another hospital). Colonoscopic examination revealed a Ⅱa-like, ill-defined and flatly elevated 5-mm tumor in the cecum (arrow).

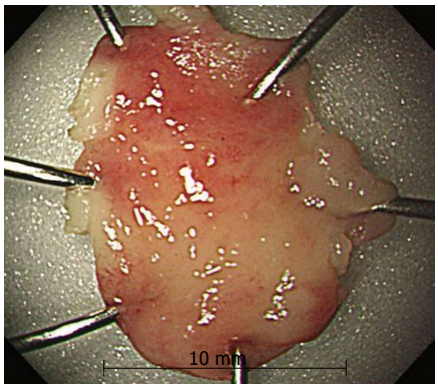

Figure 3 Resected specimen by endoscopic mucosal resection. The resected specimen was 10 mm in diameter.

and flatly elevated 5-mm tumor in the cecum (Figure 1). In addition, several polyps were observed in the ascending colon extending to the transverse colon. Snare polypectomy of the tumor in the cecum was performed incompletely, and a biopsy of several polyps was also conducted. Pathological examination of a biopsy specimen of the cecal tumor showed signet ring cell carcinoma, while biopsy specimens of several polyps showed tubular adenoma with low grade dysplasia. The patient was referred to the gastroenterology clinic at this hospital on October 6, 2009.

At presentation, the patient was active and was experiencing no symptoms. He took no medications and had no known allergies to medications. He drank alcohol, had a history of smoking (20 packs per-year), and did not use illicit drugs. His past history was unremarkable, and there was no family history of cancer. On examination, his body weight was 59.0 kg and height was 154 cm; the vital signs and remainder of the physical examination were normal. The level of carcinoembryonic antigen was 2.26 ng/mL (reference value, < 3.4). Results of a complete blood count, plasma levels of electrolytes, tests of coagulation and kidney and liver function, and a urinalysis were normal. One day later, upper endoscopic examination revealed no primary lesions, and colonoscopic examination revealed a Ⅱa-like, ill-defined and flatly elevated

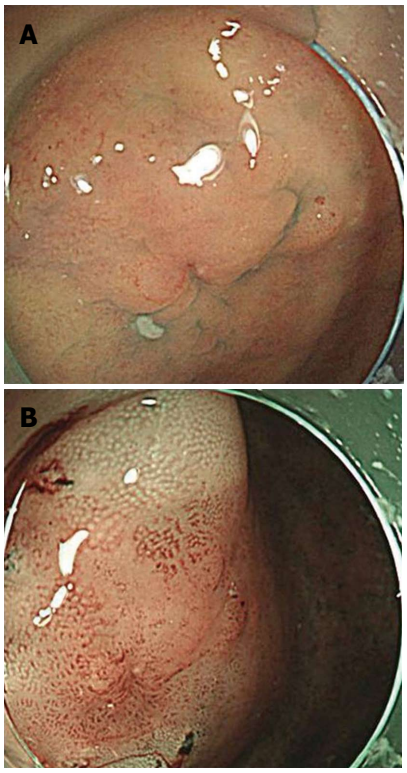

Figure 2 Colonoscopic findings at this hospital. A: Colonoscopic examination revealed a Ⅱa-like, ill-defined and flatly elevated 9-mm residual tumor in the cecum; B: Narrow band imaging shows the lesion more clearly.

9-mm residual tumor in the cecum (Figure 2). In addition, several lateral spreading tumors were observed in the ascending colon extending to the transverse colon. Endoscopic mucosal resection (EMR) of the residual tumor in the cecum was performed. The tumor was successfully removed en bloc by EMR without complications. The resected specimen was 10 mm in diameter (Figure 3). Histologically, the tumor was composed of carcinoma with lymphoid hyperplasia and, favored signet ring cell carcinoma that had invaded the lamina propria without venous or perineural invasion (Figure 4). The cut end of the resected specimen was examined for safety. The other tumors were removed by polypectomy; histologically, the specimens were composed of tubular adenoma with low grade dysplasia. Abdominal computed tomography (CT) and CT scanning with positron-emission tomography (PET-CT) showed no evidence of primary lesions or distant metastasis. Two weeks later, the patient underwent an additional laparoscopic right-hemicolectomy, because of the high incidence of peritoneal metastasis associated with signet ring cell carcinoma and the possibility of recurrence of several lateral spreading tumors. The resected specimen revealed no residual carcinoma at the EMR site and showed no lymph node metastasis (Figure 5).

The patient has been in good health for two years and has shown no recurrence after the operation.

DISCUSSION

Primary signet ring cell carcinoma of the colon and

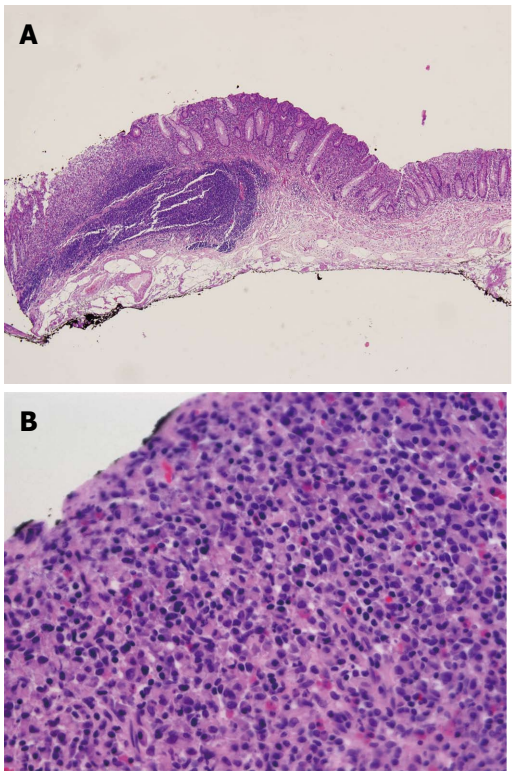

Figure 4 Scanning view of the endoscopic mucosal resection site. Histologically, the resected specimen showed diffusely infiltrated signet ring cells in the lamina propria without venous or perineural invasion. A: hematoxylin/eosin staining, × 40; B: hematoxylin/eosin staining, × 400.

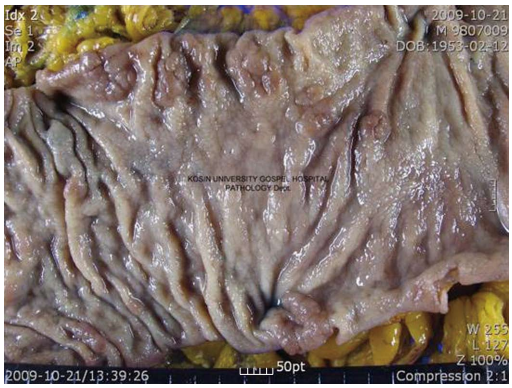

Figure 5 Gross findings. The mucosal surface of the cecum showing an ill-defined irregularly-shaped scar (probably the endoscopic mucosal resection site). The remaining mucosa showing multiple polyps and previous polypectomy sites.

rectum was first described by Laufman and Saphir in 1951[5]. Its characteristics include more advanced stages at presentation[1,4,6], younger age at presentation[3,4], chiefly peritoneal dissemination[1,4,7], lymphatic spread[5,6], few liver metastases[1,4,8], and poor prognosis[1,4,6-8]. Because its clinical symptoms develop late, most cases are usually detected at an advanced stage. Bonello *et al*[9] described three factors for this delay in diagnosis: (1) the rarity of the tumor; (2) intramucosal tumor spread with relative sparing of the mucosa, accounting for minimal symptoms and heme-negative stools; and (3) radiographic tumor resemblance to inflammatory processes. Because most cases are detected at an advanced stage, the prognosis of such tumors is dismal. Median and mean survival times are reported as 20 and 45 mo, respectively[6-8], and 5-year survival rates are between 9% and 36%[1,6,8]. Makino *et al*[10] found that all 17 patients with stage 0/I disease were alive at the latest follow-up evaluation, and the 5-year survival rate of patients with T2 disease was 75.0%. Therefore, to improve outcome, early diagnosis is very important. The patient in this case report was diagnosed with primary signet ring cell carcinoma of the colon at an early stage, and a thorough workup did not reveal any other sites of involvement and the tumor invaded only lamina propria without venous or perineural invasion. A review of the literature revealed that there are only 27 cases of primary signet ring cell carcinoma of the colon and rectum detected at an early stage, including the patient in this case report[11-17]. Of these 27 patients, 22 were males and 5 were females, with a mean age of 57.1 years (range: 6-79 years). The mean size of the tumor was 15.7 mm (range: 2-45 mm). Regarding the location of the tumors, 14 cases were in the right-side of the colon, 7 cases were in the left-side of the colon, and 6 cases were in the rectum. Macroscopically, 10 cases were polypoid type, 4 cases were flat type, and 13 cases were depressed type. Microscopically, 20 cases had submucosal invasion and 7 cases had intramucosal invasion. Makino *et al*[10] reported that all 69 patients with scirrhous and polypoid tumors had stage T3 or T4 disease, while 83.3% of patients with superficial tumors had stage T0 or T1 disease. In this case report, the patient's tumor was located in the mucosa, and the gross shape of the tumor was IIa-like, ill-defined and flatly elevated. Thus, for early detection of signet ring cell carcinoma of the colorectum, we believe that careful observation of easily overlooked lesions such as superficial tumors, flatly elevated, or flatly depressed lesions during colonoscopic examinations is important.

Some authors have suggested an association between ulcerative colitis and signet ring cell carcinoma of the colorectum[5,18]. Ojeda *et al*[19] reported that 7 of 60 patients (12%) had both ulcerative colitis and primary colorectal signet ring cell carcinoma. Anthony *et al*[1] found that 4 of 29 patients (14%) had a previous history of inflammatory bowel disease. The diagnosis in 2 of these patients was ulcerative colitis; the other 2 patients were diagnosed with Crohn's disease. The patient in this case report had no inflammatory bowel disease. Although the role of chronic inflammatory bowel disease in the development of this tumor is still undetermined, regular colonic examination of patients with history of chronic inflammatory bowel disease could be important.

A positive family history is a risk factor for ordinary colorectal cancer. However, in accordance with some studies, a positive family history may not be a predictive factor for signet ring cell carcinoma[2,4]. This finding could be attributed to the relatively small number of cases or could represent variability of this type of tumor. The patient in this case report had no family history of colorectal cancer.

Little is known about the early stages of signet ring cell carcinoma. It is unclear whether signet ring cell carcinoma develops from a pre-existing adenomatous polyp or as a so-called *de novo* carcinoma. To our knowledge, only 3 cases of an association between signet ring cell carcinoma and adenomatous polyps have been reported. Hamazaki *et al*[12] reported a case of a 6-year-old boy with a signet ring cell carcinoma in a polyp of the colon, Nakamura *et al*[14] described a case of a 4.5 cm rectal adenoma with multiple foci of signet ring cell carcinoma, and Tandon *et al*[20] reported a case of a sigmoid colon adenoma with a focus of signet ring cell carcinoma. The patient in this case report had several lateral spreading tumors in the ascending colon extending to the transverse colon; however, histologically, none of the specimens were composed of foci of signet ring cell carcinoma. Although controlled studies about the association of adenomas and signet ring cell carcinomas are lacking, it could be possible that progression of an adenoma to signet ring cell carcinoma is occuring.

In the present case report, we have described a rare case of primary signet ring cell carcinoma of the colon detected and treated at an early stage, and provided a review of the literature.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
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doi:10.3748/wjg.v19.i24.3899

World J Gastroenterol 2013 June 28; 19(24): 3899-3903
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Severe irinotecan-induced toxicity in a patient with *UGT1A1*28* and *UGT1A1*6* polymorphisms

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Author contributions: Xu JM designed research; Wang Y analzed the pharmacokinetic and clinical data; Ge FJ and Lin L collected clinical data; Liu ZY analyzed pharmacokinetic data; Xu JM and and Sharma MR wrote the paper; all authors read and approved the final manuscript.
Supported by National Natural Science Foundation Project, Grants No. 30971579; and Capital Development Foundation, No. 2007-2029
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Received: March 8, 2013 Revised: April 24, 2013
Accepted: May 8, 2013
Published online: June 28, 2013

Abstract

Many studies have demonstrated the impact of UGT1A1 on toxicity of irinotecan. In particular, patients bearing UGT1A1*28 (TA 7/7) have a higher risk of severe neutropenia and diarrhea. Based on this, prescribers of irinotecan are advised that patients with UGT1A1*28 (TA 7/7) should start with a reduced dose of irinotecan, although a particular dose is not specified. Research in Asian countries has shown a lower incidence of UGT1A1*28 (TA 7/7), while UGT1A1*6 (A/A) is more often found and is associated with severe irinotecan-related neutropenia. We report here a case of a metastatic colorectal cancer patient who is heterozygous for the *UGT1A1*28* polymorphism (TA 6/7) as well as the *UGT1A1*6* polymorphism (G/A). The patient was treated with FOLFIRI for 9 cycles and underwent two irinotecan dose reductions according to pharmacokinetic data regarding exposure to the active metabolite, SN-38. Simultaneous heterozygous *UGT1A1*28* and *UGT1A1*6* polymorphisms may produce higher exposure to SN-38 and a higher risk of adverse effects related to irinotecan. Additional studies will be necessary to determine the optimal starting dose of irinotecan for patients with both *UGT1A1*28* and *UGT1A1*6* polymorphisms.

Key words: Irinotecan; Toxicity; *UGT1A1*28*; *UGT1A1*6*; Polymorphism

Core tip: This is the first reported case. This patient with heterozygous *UGT1A1*28* and *UGT1A1*6* polymorphisms experienced two dose reductions of irinotecan due to severe toxicity according to pharmacokinetic analyses of SN-38 and SN-38 glucuronide levels. It seems that this patient benefited from a longer treatment duration, suggesting that irinotecan dose individualization for mutant metastatic colorectal cancer patients with heterozygous *UGT1A1*28* or *UGT1A1*6* polymorphisms may be warranted.

Xu JM, Wang Y, Ge FJ, Lin L, Liu ZY, Sharma MR. Severe irinotecan-induced toxicity in a patient with *UGT1A1*28* and *UGT1A1*6* polymorphisms. *World J Gastroenterol* 2013; 19(24): 3899-3903 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3899.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3899

INTRODUCTION

The FOLFIRI regimen, which is composed of 5-fluorouracil (5-FU), leucovorin, and irinotecan, is a commonly

used treatment regimen for metastatic colorectal cancer. UGT1A polymorphisms have been the focus of irinotecan pharmacokinetics (PK) and toxicity research since UGT1A enzymes play a key role in the glucuronidation of the active metabolite of irinotecan, SN-38, to the inactive SN-38G[1-3]. There have been a number of studies to examine the associations of the *UGT1A1*, *UGT1A7*, and *UGT1A9* genotypes and severe irinotecan-induced toxicity, particularly diarrhea and neutropenia[4-7]. The data strongly suggest that the *UGT1A1*28* genotype is associated with severe irinotecan-induced diarrhea and neutropenia[7-9], which led to a change in the irinotecan United States label to recommend dose reduction in patients with lower UGT1A1 activity. The incidence rates of severe neutropenia and diarrhea (grade 3/4) in patients homozygous for UGT1A1*28 (TA 7/7) in Western and Eastern populations were > 30%[10,11]. Interestingly, there are no polymorphisms at the UGT1A1*6 locus reported in the Western population. However, studies in Asian countries indicated that there is a common (35.8%-38.9%) single nucleotide polymorphism at the UGT1A1*6 (G→A) locus that is associated with severe irinotecan-related neutropenia[12-14]. The effects of other *UGT1A7* and *UGT1A9* polymorphisms on irinotecan-related toxicity remain unclear[4,12]. To determine the optimal dose of irinotecan in the FOLFIRI regimen, we are conducting a prospective and multicenter clinical trial in which the dose of irinotecan is adjusted for the specific *UGT1A* genotypes in patients with metastatic colorectal cancer (NCT01523431).

CASE REPORT

A 72-year-old male patient with an adenocarcinoma at the hepatic flexure of the colon underwent right hemicolectomy. Several mo later, the patient developed metastases in the liver, bilateral lungs and mediastinal lymph nodes. Liver biopsy confirmed metastatic disease from colon cancer. Routine genotyping showed that the patient was heterozygous for the *UGT1A1*28* polymorphism (TA 6/7) as well as the *UGT1A1*6* polymorphism (G/A). The patients had normal liver function and renal function. He was treated with the standard FOLFIRI regimen: a 90-min intravenous (*iv*) infusion of irinotecan (Camptosar, Pfizer) (180 mg/m^2); an *iv* infusion of leucovorin (400 mg/m^2); followed by 5-FU by *iv* bolus (400 mg/m^2) and continuous *iv* infusion (2400 mg/m^2) over 46 h; this regimen was repeated every 2 wk. Concurrently, a 5-mL heparinized blood sample was collected before irinotecan administration, at 1 and 1.5 h during the infusion and at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, and 48 h after the termination of the drug infusion. After the first treatment cycle, the patient suffered grade 4 neutropenia, grade 3 diarrhea, grade 2 fatigue, and grade 2 mucositis. The area under the curve (AUC) of SN-38, the active metabolite of irinotecan, was 929 ng/mL per hour (Figure 1), which was 4-fold that of the mean AUC for wild-type patients treated with the standard FOLFIRI regimen

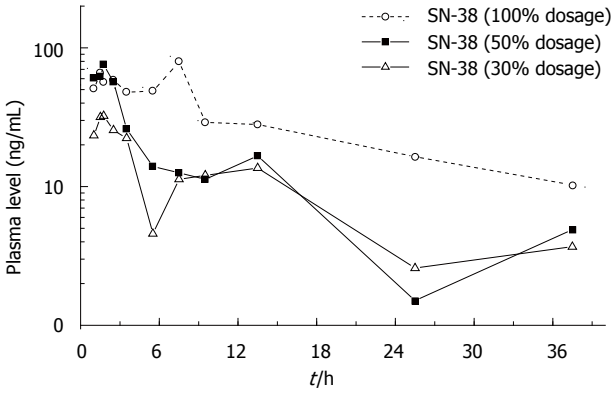

Figure 1 Plasma concentration-time profiles of SN-38 in different dose levels of irinotecan.

Table 1 Area under the curve of irinotecan, SN-38 and SN-38G for this patient at various doses of irinotecan, compared with a group of patients with wild type UGT1A1 at the standard dose of irinotecan

Irinotecan dose (mg/m^2)	AUC$_{irinotecan}$ (ng/mL per hour)	AUC$_{SN-38}$ (ng/mL per hour)	AUC$_{SN-38G}$ (ng/mL per hour)	AUC$_{SN-38G}$/AUC$_{SN38}$
180	26838	929	3000	3.2
90	12790	476	2014	4.2
54	9488	328	2096	3.4
		UGT1A1 wild type patients[1]		
180	6321 ± 3993	234 ± 185	645 ± 353	3.3 ± 2.1

[1]Treated with standard FOLFIRI regimen at our cancer center (n = 38, mean ± SD). AUC: Area under the curve.

at our cancer center. The AUC ratio of glucuronidated SN-38G/SN-38 was 3.23, which was similar to that of wild-type patients (Table 1). Therefore, we reduced the irinotecan dose by 50% (to 90 mg/m^2) but maintained the doses of 5-FU and leucovorin in subsequent cycles. The second round of pharmacokinetic analysis showed that the AUC of SN-38 was 476 ng/mL per hour, which was more than 2-fold that of the mean AUC for wild-type patients. After the second cycle, there was no neutropenia and only grade 1 diarrhea. Moreover, computerized tomography (CT) and magnetic resonance imaging scans showed a partial response in the lung and liver metastases, respectively, and the response was confirmed after two additional cycles (Figure 2). However, after the fifth cycle, the patient developed recurrent neutropenia and additional doses of irinotecan were held. His Eastern Cooperative Oncology Group performance status had worsened from 0 to 1. He received two cycles of capecitabine (2000 mg/m^2 divided *bid* for 2 wk on, 1 wk off) as maintenance therapy, and his CT showed stable disease (SD). However, the patient discontinued therapy for 1 mo because of grade 3 mucositis and grade 3 diarrhea. A few mo later, he experienced disease progression in the liver and lung and his weight had decreased from 54.5 to 47 kg over 3 mo. We decided to reinitiate the FOLFIRI regimen. The irinotecan dose was reduced by

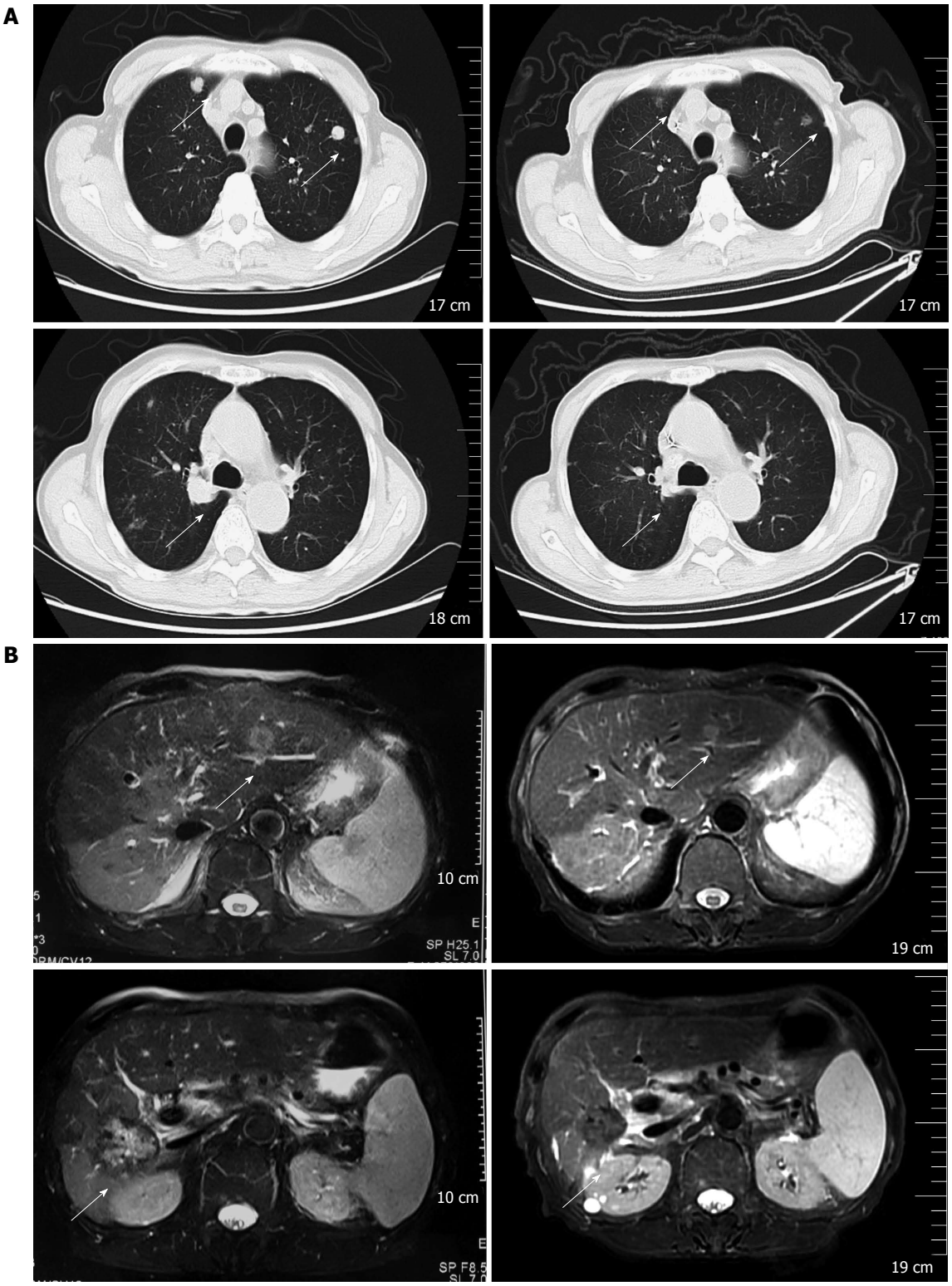

Figure 2 Computerized tomography scan (A) and magnetic resonance imaging (B). Before treatment (left figure) and after treatment (right figure) of the lung and the liver metastases, respectively; the response was confirmed after two additional cycles.

70% (to 54 mg/m^2), while the 5-FU dose was reduced by 30%. The third round of pharmacokinetic analysis showed that the AUC of SN-38 was 328 ng/mL per hour, which is close to the mean AUC with a standard dose in wild-type patients. After two additional cycles, he had SD but experienced grade 3 neutropenia and grade 2 diarrhea. CT scan showed disease progression after two additional cycles of chemotherapy.

DISCUSSION

Recent studies in Asian countries have indicated that

the polymorphism in UGT1A1*6 has a similar effect as UGT1A1*28 on irinotecan-induced toxicity and PK[12,15,16]. However it is unclear whether simultaneous heterozygous *UGT1A1*28* and *UGT1A1*6* (TA 6/7 + G/A genotype) polymorphisms may have significantly more side effects and impact on PK of SN38. Irinotecan PK are determined by multiple metabolizing enzymes, whereas the saturation of enzymatic reactions is affected by other factors, such as age and creatinine clearance[17]. The patient in the present study, who carried both polymorphisms, experienced serious toxicity after one cycle of a standard irinotecan dose, which corresponded to an SN-38 exposure (AUC) that was 4-fold higher than in wild-type patients. It has been reported that low dose irinotecan-induced toxicity is not associated with *UGT1A1* polymorphisms[18]. In the present study, however, toxicity recurred even after reducing the irinotecan dose by 50%, and the AUC of SN-38 was still 2-fold higher than the mean in wild-type patients who received standard dose irinotecan. Even after a 70% dose reduction, the AUC of SN-38 was close to the mean in wild-type patients who received standard dose irinotecan. This observation suggests that the PK were still affected by the polymorphisms in the *UGT1A1* gene even at relatively low doses. Whether dosing and AUC may be associated with efficacy is still unclear; however, data from Toffoli *et al*[7] suggest that higher doses and higher AUC may be associated with higher efficacy in patients with mutant metastatic colorectal cancer.

When faced with intolerable toxicity, oncologists typically reduce the dose, delay treatment, or discontinue treatment, any or all of which may reduce the treatment duration and affect the progression-free survival and overall survival of patients. The patient in this report underwent two irinotecan dose reductions during the course of 9 treatment cycles with FOLFIRI, as well as 2 cycles of capecitabine as maintenance therapy. The duration of disease control, including breaks, was 7 mo, which suggests that the patient benefited from the dose reductions. The AUC ratio of SN-38G/SN-38 did not decrease with additional treatment cycles, suggesting that patients with UGT1A1 polymorphisms may not experience the irinotecan-induced inhibition of UGT1A1 and corresponding decrease in the AUC ratio of SN-38G/SN-38 that has been observed by Hirose *et al*[16] in wild-type patients. To our knowledge, the present case is the first report to adjust the irinotecan dose twice based on the patient's *UGT1A1* genotype and according to PK characteristics. Additional studies will be necessary to determine the optimal starting dose of irinotecan for patients with both *UGT1A1*28* and *UGT1A1*6* polymorphisms and to determine how this genotype may influence efficacy.

In summary, simultaneous heterozygous *UGT1A1*28* and *UGT1A1*6* polymorphisms may produce higher exposure to SN-38 and a higher risk of adverse effects related to irinotecan. Additional studies will be necessary to determine the optimal starting dose of irinotecan for patients with both *UGT1A1*28* and *UGT1A1*6* polymorphisms.

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P- Reviewer Ladero JM **S- Editor** Gou SX
L- Editor O'Neill M **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3904

World J Gastroenterol 2013 June 28; 19(24): 3904-3910
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Pathological diagnosis is maybe non-essential for special gastric cancer: Case reports and review

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Supported by The Science and Technology Development Project of Guangdong Province, No. 2011B031800240 and No. 2012B031800389

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Received: November 21, 2012 Revised: April 17, 2013
Accepted: May 8, 2013
Published online: June 28, 2013

Abstract

Histopathological results are critical for the diagnosis and surgical decision regarding gastric cancer. However, opposite opinions from radiology and pathology can sometimes affect clinical decisions. The two cases reported in this article were both highly suspected as gastric cancer by clinical manifestations and radiologic findings, although both showed negative results in the first biopsy examination. One was confirmed as gastric cancer by the time of the 6th biopsy, while the other was still negative even after 8 biopsies. With a definite pathologic result and the agreement of the patient for the latter case, both of them finally received surgery. Postoperative pathological examination revealed findings that were the same as Borrmann type Ⅳ gastric cancer. We believed that duplicate biopsies under radiologic guidance were necessary for highly suspected gastric cancer cases in the absence of a definite pathology result, and patients should be under close follow-up. We propose that, if gastric cancer is highly suspected when typical radiology changes of widely diffuse gastric parietal lesions suffice to exclude lymphoma and other similar situations, and even in absence of a positive biopsy result, a diagnostic laparotomy under laparoscopy and even radical gastrectomy may be reasonably performed by an experienced gastric cancer center with the agreement of the patient after being decided by a multidisciplinary discussion team.

Key words: Gastric cancer; Pathology; Diagnosis; Borrmann type Ⅳ

Core tip: Histopathological diagnosis is the diagnostic gold standard of gastric cancer required by medical ethics and practice guideline. However, cases with repeated suspected false negative pathological results always concern medical practitioners a great deal. This article might illustrate a possible standard process for these cases. We propose that, if gastric cancer is highly suspected when typical radiology changes of widely diffuse gastric parietal lesions suffice to exclude lymphoma and other similar situations, and even in absence of a positive biopsy result, surgery could be perform with the agreement of the patient after being decided by a multidisciplinary discussion team.

Song W, Chen CY, Xu JB, Ye JN, Wang L, Chen CQ, Zhang XH, Cai SR, Zhan WH, He YL. Pathological diagnosis is maybe non-essential for special gastric cancer: Case reports and review. *World J Gastroenterol* 2013; 19(24): 3904-3910 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3904.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3904

INTRODUCTION

Gastric cancer is one of the most common cancers of the digestive system, and shows the highest morbidity and mortality among all digestive malignancies in China. Early detection, diagnosis, and treatment are of significant importance in improving patient cure rate and 5-year survival. Gastroscopic biopsy remains one of the primary means for the screening of the disease, and at the same time the gold standard of diagnosis[1]. However, biopsy accuracy relies on samples obtained from gastrofiberscopy so much that doctors would be confused when a pathologic examination showed a negative result inconsistent with radiologic findings and other results. Thus, treatment is often delayed due to medical ethics considerations, which require a definite pathological diagnosis. Both cases we are reporting in this article were negative in repeated biopsies but suggested as gastric cancer by radiologic and gastrofiberscopy examination. We intend to demonstrate the clinical decisions for similar patients.

CASE REPORT

Case 1

A 50-year-old male presented on May 15, 2009, and was admitted due to recurrent upper abdominal discomfort associated with acid reflux, hiccups, and weight loss for 3 mo. Two months previously, it was suggested that he have an abdominal computed tomography (CT) scan in another hospital, and an obviously thickened antral wall was discovered. The CT report considered a differential diagnosis of gastric cancer and primary gastric lymphoma. The patient then had his first gastroscopy examination. Gastroduodenoscopy did not find any apparent ulcer of the stomach, but did show stenosis of the antrum and pyloric. The biopsy result was antral mucosal inflammation. Finally, the patient was treated as gastritis for 2 mo, but no alleviation of discomfort was achieved. Thus, he came to our hospital for further treatment and was suggested to have further examinations after admission. General blood tests that included alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), tumor marker-CA125 (CA125), tumor marker-CA19-9 (CA19-9), and squamous cell carcinoma antigen revealed nothing abnormal. A second abdominal CT scan in our hospital found circular thickening of the antrum stomach wall and retention of gastric contents (Figure 1). Possible gastric cancer was considered. But after a consultation of biopsy slides of the previous scan, we had the same comments. Therefore, a second gastroscope biopsy was conducted in our hospital, only to find chronic inflammation of antral mucosa and local fibrous tissue proliferation, rather than any malignant cells. Since the patient was highly suspected for Borrmann type Ⅳ gastric cancer by our multidisciplinary discussion team (MDT), a third biopsy was performed with endoscopic ultrasound as guidance. We sampled 15 tissue cores by electric biopsy forceps through holing in the thickening antrum stomach wall. Endoscopic ultrasonography also supported that the thickening antral wall was a typical change of malignant lesions. However, pathological diagnosis reported chronic inflammation of the antral mucosa without any atypical cells (Figure 1). The patient was discharged for another month of continuous gastritis treatment, which failed to bring about any improvements. One month later, the patient came back for a fourth gastroscope biopsy, but with immunohistochemical examination this time. As before, the report showed chronic inflammation of the antral mucosa and local fibrous tissue proliferation. Immunohistochemistry results were: suspicious atypical cells with M-CEA (±), S-100 (-), actin (-), Syn (-), CD117 (-), CD56 (-), glandular epithelium CK (-), small lymph L26 (+), CD79a (+), the LCA (+), CD3 (+), the tissue cells CD68 (+) and plasma cells CD38 (+). Although there was no definite pathologic result of gastric cancer, Borrmann type Ⅳ gastric cancer was strongly suspected. Therefore a further examination of positron emission tomography-CT (PET-CT) was performed and reported. It showed uneven diffuse thickening of the antrum stomach wall, with antrum metabolic imaging not supporting this malignant change and no abnormal metabolic lesions or standard uptake value (SUV) occurring in the other tissues. Conventional medical management seemed to be ineffective and the patient gradually felt worse and worse. On July 29th, he underwent alimentary tract barium meal examination, and the result indicated it was most probably primary gastric lymphoma or antrum cancer with submucosal infiltration, besides eosinophilic gastritis. The patient was discharged from hospital again because we failed to get a positive biopsy result supporting the diagnosis of cancer. On August 30th, one month after discharge, he was asked to have an abdominal CT and gastroscopy examination, which was the suggestion from the MDT last time. The sixth gastroscopy biopsy result confirmed it was gastric antrum signet-ring cell (Figure 1) carcinoma, accompanied with immunohistochemical stain results: signet ring-like cells CK (+), CEA (+). Abdominal CT examination reported gastric antrum cancer with perigastric lymph nodes enlargement. A surgery decision was made soon after the confirmation.

During laparotomy, we found the antropylorus where lesions located was thickened, and the perigastric lymph nodes, especially those of the lesser curvature, were obviously enlarged from the 0.5 to 2.5 cm. In addition, multiple metastatic nodules were detected on the omentum. There were no findings of metastasis to other organs within the abdominal cavity and no dissemination of the peritoneum was observed. Radical distal gastrectomy (D2 lymphadenectomy) was therefore performed. Postoperative pathological examination showed that gastric poorly differentiated adenocarcinoma infiltrated the whole gastric wall, and with myenteric nerve invasion (Figure 1) and lymph nodes metastasis (12/27). The patient was finally diagnosed as having antrum signet ring cell carcinoma (T3N2M0, ⅢB period, Borrmann type Ⅳ). Eight courses of postoperative adjuvant chemotherapy treatment followed by the XELOX plan were initiated since

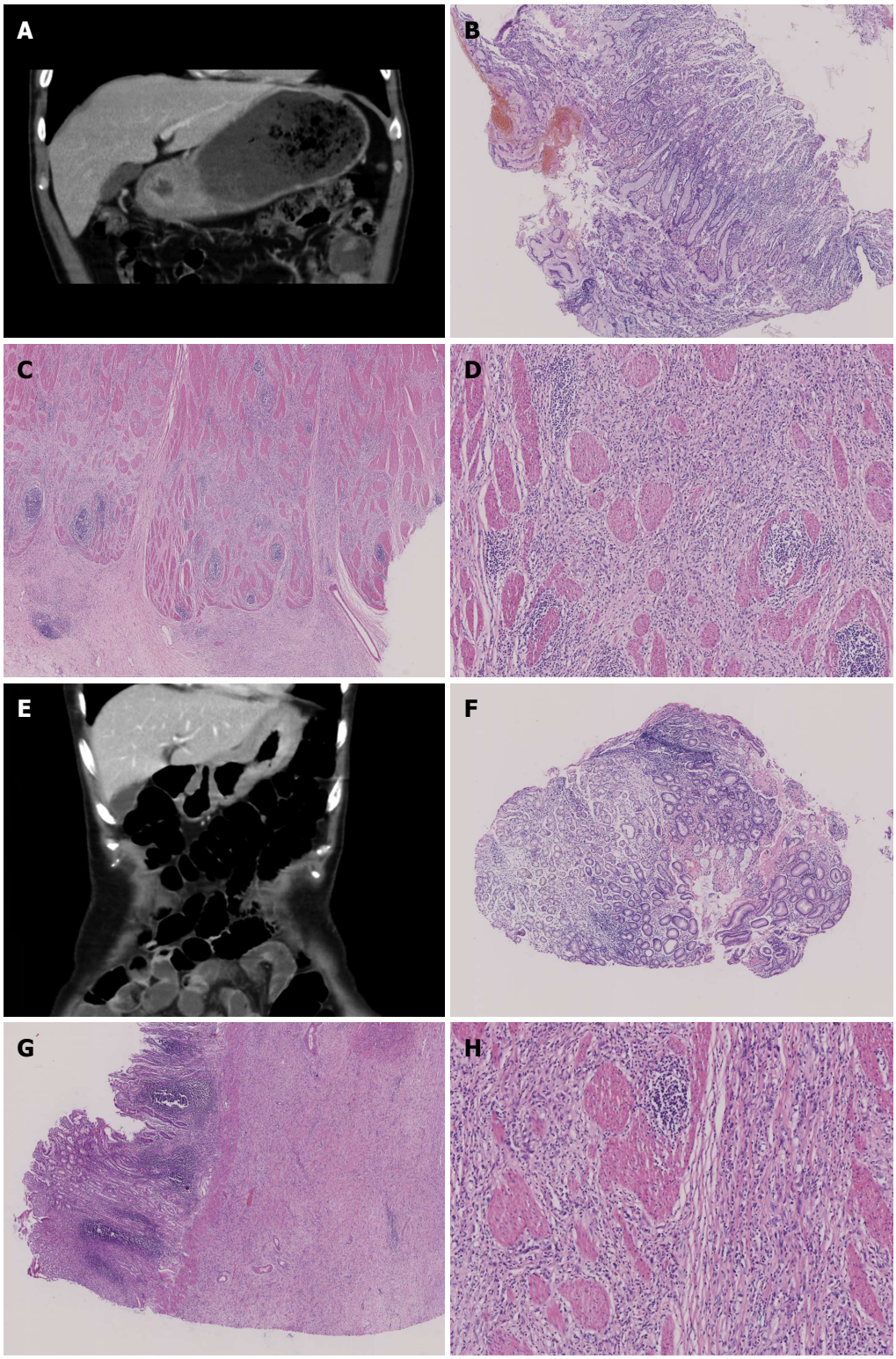

Figure 1 Computerized tomography and pathological findings. A: Diffuse thickened antral detected by computerized tomography (CT) scan; B: Repeated false negative slides without cancer cells detected for the previous 5 biopsies [hematoxylin-eosin (HE) stain, × 40]; C: Positive slide with cancer cells observed by the 6th biopsy (HE stain, × 100); D: Postoperative slide with cancer cells observed, many signet ring-like cells can be observed in the upper-right quadrant of the slide (HE stain, × 200); E: Diffuse thickened fundic and gastric body detected by CT scan; F: Repeated false negative slides without cancer cells detected for 8 biopsies (HE stain, × 40); G: Postoperative slide with cancer cells observed and the whole gastric wall infiltrated (HE stain, × 100); H: Fibrosis stomach wall, scattered or small focal distributed signet ring-like cells could be observed (HE stain, × 200).

the fourth week after surgery. Our follow-up data show that the patient is still alive.

Case 2

A 52-year-old female had been suffering from abdominal

dull pain with intermittent melena and weight loss for 11 mo, and her medical history showed nothing special. Before she came to us, she had been in a local hospital three times for the same complain, and received five gastrofiberscopy examinations with biopsies, since the doctors strongly suspected she had gastric cancer. Unfortunately, every biopsy result remained negative, as did the upper gastrointestinal barium meal examination. The barium meal examination regarded it as gastritis due to the imaging findings: a thick mess of the gastric body mucosa folds was detected but no signs of niche or filling defect, gastric motility and tension were well, and barium could go through the pyloric canal smoothly. However, views of the gastrofiberscopy reports were unified: hypertrophy of gastric body mucosal folds; gastric cancer, and duodenal ulcers was considered. Due to the previous ineffective medical management and being unqualified for any further examination or treatment at the local hospital, the patient was referred to our hospital. General blood tests, including tumor markers AFP, CEA, CA125, CA19-9 and squamous cell carcinoma antigen, were performed after admission, which all showed results within the normal ranges. Gastric endoscopic ultrasonography reported a differential diagnosis of adenoma and lymphoma due to the thickened stomach wall of the fundus and gastric body. The patient was suggested to have a sixth biopsy. Under a gastroduodenoscopy, this time in our hospital, we found the gastric mucosa had obvious congestion, edema, was stiff, and the gastric folds were thicker and harder than normal. A biopsy was then performed. Pathological findings showed that no adenocarcinoma was found with hematoxylin-eosin stain, a small amount of abnormal glands were noticed under cytokeratin immunohistochemical stain, M-CEA (-), and a particularly high positive rate of Ki-67 was not seen in the samples. Pathology failed to diagnose the cancer. An abdominal CT examination revealed a diffusely thickened and stiff stomach wall of the gastric fundus and body, a fixed stomach shape, obvious changes to the mucosa of the thickened stomach wall during the contrast enhanced phase, and a number of enlarged lymph nodes in the lesser curvature and retroperitoneal area (Figure 1). The CT report stated possible lymphoma. However, gastric cancer was highly suspected according to our experience and the patient's clinical manifestation. A seventh deep layer biopsy was therefore carried out, but only to disappointingly show a negative result again. After deliberation by the MDT, including experts from departments of imaging, ultrasound diagnosis, pathology, surgery, and internal medicine, we insisted it to be Borrmann type Ⅳ stomach adenocarcinoma that showed hypertrophic gastric lesions, and that conventional biopsy methods would always display a low positive rate. We decided to take endoscopic ultrasound guided fine-needle aspiration for the eighth biopsy. We made two punctures deep into the submucosa and muscularis at the stomach wall of the lower segment of the gastric body for samples where the endoscopic ultrasound showed disappearing gastric structure. Unfortunately, our pathologist told us that cancer cells were still not detected in the samples, only normal gastric mucosa epithelium and some lamina propria glands. A liquid-based cytology test was also negative (Figure 1). However, according to our experience, Borrmann type Ⅳ stomach cancer was strongly suspected and surgery was recommended after an MDT discussion. Naturally, the patient refused to have surgery without a definite histological diagnosis. So a PET-CT examination was followed. It reported that the stomach wall of the gastric fundus and body were obvious thickened to a maximum of 1.5 cm. Abnormal ^{18}F-fluorodeoxyglucose (^{18}F-FDG) uptake was also observed, with a maximum SUV of approximately 3.1. The thickened gastric wall and slightly active metabolic imaging indicated the possibility of poorly differentiated adenocarcinoma. Considering that the patient's clinical symptoms were aggravating, surgery was again recommended, and she agreed to undergo surgery and a laparotomy was performed.

During the laparotomy, we found the stomach wall was thickened overall and the tumor, as large as 14 cm × 9 cm, had invaded the serosa, accompanied by the enlargement of the perigastric lymph nodes. The center of the transverse colon, omentum, the spleen, and partial left adrenal were all invaded. Enlarged retroperitoneal lymph nodes were palpable. Adhesion or ascites was not detected, and there were no findings of metastasis to the rest organs in the abdominal cavity. So we decided to perform gastric cancer extended radical mastectomy (radical total gastrectomy, splenectomy, partial resection of the left adrenal, D2 lymphadenectomy, and Roux-en-Y esophagojejunostomy). Postoperative pathological examination showed that the stomach wall tissue was fibrotic with small focal distributed signet ring-like cells (Figure 1), CK (+), M-CEA (+), CK20 multifocal (+), poorly differentiated adenocarcinoma infiltrated the whole stomach wall, no cancer cells were found in the Splenic tissue, transverse colon, surgical margins or omentum, lymph nodes metastasis (0/32). She was diagnosed with gastric signet ring cell carcinoma (T4aN0M0, ⅡB stage, Borrmann type Ⅳ). As with the former case, the patient was suggested to receive postoperative adjuvant chemotherapy treatment, and is still alive.

DISCUSSION

Histopathological diagnosis is the diagnostic gold standard, as well as the treatment basis of gastric cancer due to its high specificity and negative predictive value[2,3]. Since a definite preoperative pathological result is required by medical ethics and practice guidelines, we could not carelessly make clinical decisions only according to our experience. But it is definite that there no test methods that can be 100% confirmed for a diagnosis. Thus, when the biopsy results didn't agree with the patient's clinical manifestations and other examination results (such as CT, endoscopic ultrasound, PET-CT, *etc*., in our article), both patients were suggested to have repeated

Table 1 Gastroscope statistics from the database of the First Affiliated Hospital *n* (%)

Items	Total	F (-) 1st	(+) 1st	F (-) 2nd	(+) 2nd	F (-) ⩾ 3	(+) ⩾ 3
Borrmann Ⅳ	194	21 (10.8)	173 (89.2)	10 (5.2)	11 (5.7)	6 (3.1)	4 (2.1)
Early type	165	11 (6.7)	154 (93.3)	1 (0.6)	10 (6.1)	0 (0.0)	1 (0.6)
Other types	1388	4 (0.3)	1384 (99.7)	0 (0.0)	4 (0.3)	0 (0.0)	0 (0.0)
Total	1747	36 (2.1)	1711 (97.9)	11 (0.6)	25 (1.4)	6 (0.3)	5 (0.3)

F (-) 1st: False negative diagnosis of the 1st biopsy; (+) 1st: True positive diagnosis of the 1st biopsy; 2nd: The 2nd biopsy; ⩾ 3: More than 3 times of biopsies.

gastroscopic biopsies to create a proper medical plan for MDT discussion.

Even as the only diagnostic gold standard for gastric cancer, gastroscopy biopsies still have a certain percentage of false negative (positive) rates. A prospective study on 1331 cases by Tatsuta *et al*[2] reported a false negative rate of 3.7% and a false-positive rate of 0.6% for gastroscopy biopsies. The data derived from a statistics of the patients' postoperative pathology, autopsy and clinical follow-up etc. According to the study, early gastric cancer, Borrmann type Ⅳ gastric cancer, and leiomyosarcoma were the main causes of false negative cases, and the false positive situations were all caused by active ulcer. Therefore, if the biopsy outcomes were suspected to be false negative (positive), repeated examinations for confirmed diagnosis are necessary. That was why we were so hesitated to make surgery decisions without a pathological diagnosis for both cases. However, at the same time, we were very anxious that the increasing number of biopsies would naturally increase the risk of bleeding at the biopsy site, rather than improve accuracy. A study by Choi *et al*[3] thus recommended 3-4 biopsies from visible tissue through endoscopy to make a correct pathologic diagnosis. When 6 or more biopsy specimens were obtained, diagnostic accuracy would reach 100%. But they also admitted the special difficulty for Borrmann type Ⅳ gastric cancer and insisted that if the negative results were accompanied with a malignant impression under endoscopic examination, a re-biopsy should be performed with careful targeting, which were the measures we performed in the cases reported. Similarly, statistics from the database of our hospital, including 1747 gastric cancer patients (Table 1), revealed a general false negative rate of 2.1% for the initial pathological diagnosis, but a second biopsy followed by multiple and deep sampling could mostly bring a definite outcome. However, we found it difficult to have a positive result, even with careful targeting, if the second biopsy was still negative. Among the false negative cases, Borrmann type Ⅳ gastric cancer covered the highest false negative rate, and the cases we reported were the most notable two. Borrmann type Ⅳ gastric cancer often calls for several biopsies before a confirmatory diagnosis, due to its peculiar biological properties in which a submucosal spread of malignant cells is present without a mucosal lesion. Malignant cells could not be obtained by subsequent repeated endoscopic biopsy. However, repeated biopsies with the guide of CT and ultrasound might increase the accuracy[4-7]. Ahn *et al*[4] insisted that if cases were strongly suspected malignant prior to surgery, and surgery is being considered, endoscopic stomach mucosal resection should be recommended despite any development of stomach perforation or complications. In fact, the risk and uselessness of repeated biopsies were our inevitable concern. Thus, further noninvasive measures, though not the gold standard but of great importance, were under consideration for diagnosis. Upper gastrointestinal imaging, especially for the double contrast barium-air test, is one of the most commonly-used methods for the diagnosis of gastric cancer. Park *et al*[8] even pointed out that upper gastrointestinal imaging was superior to gastrofiberscopy for the diagnosis and localization of Borrmann type Ⅳ gastric cancer. Practically, alimentary tract barium meals were suggested in case 1 and the result supported this point. But most doctors would hardly be persuaded by this, especially when it is controversial in comparison to other, more credible, results (such as pathology and CT). In addition, PET-CT was suggested and performed in both patients. However, the use of PET-CT in the diagnosis of gastric cancer and evaluation of lymph nodes metastasis is still controversial[9]. The reported diagnosis accuracy varies from 60% to 95% in different studies[10-13]. For cases in which ordinary CT did not prompt a diagnosis of gastric cancer, we could not carelessly make a diagnosis of cancer simply according to the high gastrointestinal ^{18}F-FDG uptake[14,15]. Since such situations could also happen in normal physiological conditions, gastritis as well as stomach ulcers could also show false positive results. Furthermore, being related to the rich mucin content, Borrmann type Ⅳ gastric cancer is often associated with a false negative result. It should be distinguished from mucinous adenocarcinoma, signet ring cell carcinoma, and poorly differentiated adenocarcinoma that are also low in FDG uptake[9,11]. The two cases we reported also suggest the limitations of PET-CT in the diagnosis of Borrmann type Ⅳ gastric cancer. Case 1 was with a false negative result, though the result of case 2 proved to be true postoperative. However, interfering by the CT report of possible lymphoma and the negative pathologic results, even the MDT felt the decision was tough.

Actually, reasonable treatments determined by a pathologic result were a real concern of doctors, and the reason for the repeated biopsies. Borrmann type Ⅳ gastric cancer usually shows an extensive diffuse thickened stomach wall through a CT scan, but so do stomach sarcoma, gastrointestinal stromal tumors, hiatal hernia, gastric lymphoma, benign gastric ulcer, benign tumor, and

gastritis. Combined medical methods such as endoscopic ultrasound, and upper gastrointestinal contrast, would make it easier for the differentiation of most of the diseases mentioned, although gastric lymphoma was the one we mainly concerned about. Since gastric lymphoma also originated from the submucosa, it would firstly invade and grow with a wide lesion. Therefore, gastric lymphomas often have false negative biopsy results, and are also difficult to differentiate from gastric cancer through CT and endoscopic ultrasound. The differential diagnosis of gastric cancer from primary gastric lymphoma is especially important, since gastric cancer mainly depends on surgical treatment combined with radiotherapy and chemotherapy as a supplement at present. However, it is not the first choice for primary gastric lymphoma. Patients of mucosa associated lymphoid tissue lymphoma could mostly be cured simply by *Helicobacter pylori* (*H. pylori*) eradication, local radiotherapy, or chemotherapy[16]. For invasive gastric lymphomas, such as the diffuse large B-cell lymphoma patients, if they were at an early stage, chemotherapy combined with local radiotherapy would be suggested instead of surgical treatment. Patients' life quality would be improved during the course of treatment[17]. Surgical therapy would be suggested only when meeting with the failure of gastric retention treatment, focal lesion, or uncontrollable complications such as serious perforation and bleeding[18]. Therefore, both patients of our reported cases, with imaging findings of diffuse gastric wall thickening, were suggested to have immunohistochemistry tests at the same time as the biopsy, so that we could make a conclusion according to the immunohistochemistry result and the specific lymphoma phenotype[19]. ^{18}F-FDG PET-CT also played an important role in the differentiation and diagnosis of gastric lymphoma, but it is more valuable for a therapeutic evaluation[20], while CT and endoscopic ultrasonography were superior in evaluating gastric lesions and lymph node status[7,21] and promising a more accurate guide for the biopsy. Fan *et al*[22] concluded that gastric cancer lesions often covered less than 50% of the stomach wall under a CT scan, but usually more than 75% if it were gastric lymphoma. Furthermore, enlarged perigastric lymph nodes tend to be in one area, but for gastric lymphoma there would be multiple enlarged areas. Interesting, they pointed out that this might indicate a diagnosis of lymphoma if it were associated with the enlargement of the retroperitoneal lymph nodes inferior renal hilus[22]. However, it seemed unsuitable for our cases. Needless to say, a confirmed diagnosis of gastric lymphoma also depends on the biopsy results. But similar findings under gastrofiberscopy of superficial ulcers or protuberant submucosal mess in the stomach often lead to a misdiagnosis of adenocarcinoma or benign ulcers. According to a study by Guzicka-Kazimierczak *et al*[23], endoscopic ultrasound-guided biopsies, multiple biopsies, and *H. pylori* tests would contribute to a diagnosis. However, it seemed not helpful for our cases either.

The two difficult cases we reported were both highly suspected as Borrmann type Ⅳ gastric cancer by the MDT preoperative, but cancer cells could not be detected in early biopsies. A confirmed diagnosis of cancer was mainly as a result of the deterioration of the clinical course. Since, for case 1, the patient was finally pathologically diagnosed as having cancer only in after the sixth biopsy after more than 6 mo from admittance, while the case 2 patient had to undergo surgery to find the truth, because her clinical symptoms were aggravating.

In conclusion, a definite preoperative pathological diagnosis of Borrmann type Ⅳ gastric cancer is difficult in many cases due to its characteristic submucosa originated development process. Repeated endoscopic biopsies and deep biopsy guided by CT and endoscopic ultrasonography that reaches the proper area of the stomach may be necessary. Compared with histopathology radiology (such as CT, PET-CT, upper gastrointestinal imaging), ultrasonography, and endoscopy are not the diagnostic gold standard for gastric cancer, and diagnostic value can not be ignored. Especially when cancer cells could not be detected despite repeated biopsies, the results of the methods mentioned above should be considered together with the clinical manifestations of patients. For those with radiology performance of widely diffuse lesions of the stomach wall, it may be highly characterized by Borrmann type Ⅳ gastric cancer. But surgery decisions should be carefully made in case of primary gastric lymphoma, which might benefit more from a non-surgical treatment method. We propose that, if gastric lymphoma could be excluded for such cases, even in the absence of any positive biopsy results supporting the diagnosis of gastric cancer, a diagnostic laparotomy and even radical gastrectomy may be reasonably performed by an experienced gastric cancer center with the agreement of the patient after being decided by a multidisciplinary discussion team.

ACKNOWLEDGMENTS

We thank Peng ZP and Chen WF from the pathology department of the First Affiliated Hospital of Sun Yat-sen University for pathological support and Xu CC from the Zhongshan Ophthalmic Center of Sun Yat-sen University for editorial assistance.

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P- Reviewers Guo JM, Rodriguez DC **S- Editor** Gou SX
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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i24.3911

World J Gastroenterol 2013 June 28; 19(24): 3911-3914
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Traumatic rupture of a type Ⅳa choledochal cyst in an adult male

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Received: February 16, 2013 Revised: March 26, 2013
Accepted: April 10, 2013
Published online: June 28, 2013

Abstract

Choledochal cyst (CC) is a rare, congenital anomaly of the bile ducts. We describe a 26-year-old male patient who was transferred to our hospital with a reported traumatic rupture of cystic liver lesions following a fall. At the time of injury, the patient experienced severe abdominal pain. He was found to have peritonitis and abdominal hemorrhage, which is quite rare. Laparotomy revealed 3000 mL fluid consisting of a mixture of blood, bile and inflammatory effusion in the peritoneal cavity. The liver, gallbladder, spleen, stomach, duodenum, small intestine, and colon appeared normal. A large cystic mass was discovered near the porta hepatis. This mass, which connected to the hepatic bifurcation and gallbladder had a 5 cm rupture in the right wall with active arterial bleeding. Abdominal computed tomography (CT) and emergency laparotomy revealed rupture of a huge type Ⅳa CC. The patient was successfully managed by primary cyst excision, cholecystectomy, and Roux-en-Y end-to-side hepaticojejunostomy reconstruction. The postoperative course was uneventful and the patient was discharged on the 12th day of hospitalization. Four weeks after surgery, abdominal CT scan showed pneumatosis in the intrahepatic bile duct, and intrahepatic dilatation which decreased following adequate biliary drainage. The patient has remained well in the close follow-up period for 9 mo.

Key words: Biliary tract; Choledochal cyst; Trauma; Rupture; Peritonitis; Hemorrhage

Core tip: Choledochal cyst (CC) is a rare, congenital anomaly of the bile ducts. We describe a young man who was transferred to our hospital with a reported traumatic rupture of liver cystic lesions. The patient was found to have peritonitis and abdominal hemorrhage, which is quite rare. Abdominal computed tomography and emergency laparotomy revealed rupture of a huge type Ⅳa CC. The patient was successfully managed by primary cyst excision, cholecystectomy, and Roux-en-Y end-to-side hepaticojejunostomy reconstruction.

Duan YF, Yang B, Zhu F. Traumatic rupture of a type Ⅳa choledochal cyst in an adult male. *World J Gastroenterol* 2013; 19(24): 3911-3914 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i24/3911.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i24.3911

INTRODUCTION

Choledochal cyst (CC) is a rare congenital disease characterized by single or multiple dilatations of the intra and/or extrahepatic biliary tree. There is a higher incidence of CC in females. In CC, spontaneous perforation is observed in 1.8%-7% of all cases. Nevertheless, reports of traumatic rupture of CC are extremely rare. Herein, we describe a young man who was transferred to our

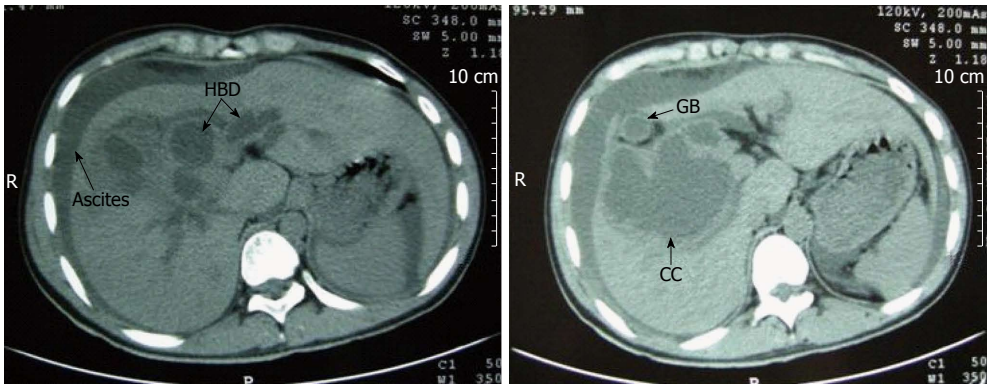

Figure 1 Abdominal computed tomography shows ascites, a normal-sized gallbladder, dilatation of the intrahepatic bile duct, and a huge choledochal cyst. GB: Gallbladder; HBD: Intrahepatic bile duct; CC: Choledochal cyst.

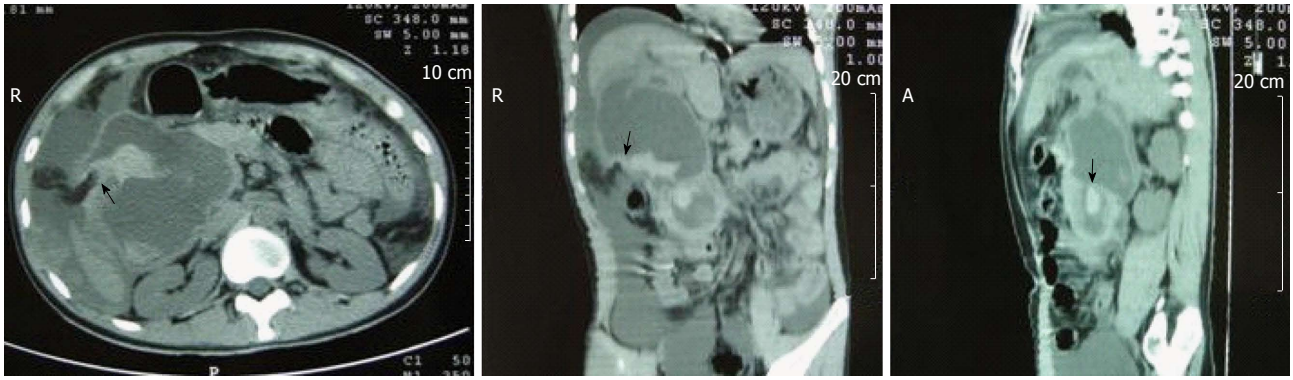

Figure 2 Abdominal computed tomography shows a rupture in the right wall of the choledochal cyst with hemorrhage (arrow).

hospital with a reported traumatic rupture of liver cystic lesions. The patient was found to have peritonitis and abdominal hemorrhage. Abdominal computed tomography (CT) and emergency laparotomy revealed rupture of a huge type Ⅳa choledochal cyst. The patient was successfully managed by primary cyst excision, cholecystectomy, and Roux-en-Y end-to-side hepaticojejunostomy reconstruction.

CASE REPORT

A 26-year-old male patient fell in a bathroom and suffered an impact to the abdomen. At the time of injury, the patient experienced severe abdominal pain. He was immediately admitted to a local hospital. On ultrasound examination, there was moderate free fluid in the peritoneal cavity, especially around the liver, and multiple cystic lesions in the liver. A plain radiograph of the chest was normal, and a plain radiograph of the abdomen revealed no gastrointestinal perforation. Abdominal paracentesis was performed with bloody fluid aspirated from the peritoneal cavity.

Two hours after injury, he was transferred to our hospital with a reported traumatic rupture of liver lesions. The patient's past medical history was unremarkable with the exception of transient and recurrent mild abdominal pain in childhood. His blood pressure was 100/65 mmHg and heart rate was 126 beats/min. Physical examination revealed tenderness and guarding of the whole abdomen. The results of laboratory examination were as follows: white blood cells: 9.88 × 10^9/L, neutrophils: 69.4%, hemoglobin: 105 g/L, and hematocrit: 0.31.

An urgent abdominal CT scan revealed ascites and a normal-sized gallbladder, dilatation of the intrahepatic bile duct, and a 13 cm × 10 cm × 9 cm cyst with hemorrhage in the common bile duct region, extending from the porta hepatis down to the level of the pancreatic head (Figures 1 and 2). He was taken emergently to the operating room with a presumed diagnosis of CC rupture.

Laparotomy revealed 3000 mL fluid consisting of a mixture of blood, bile and inflammatory effusion in the peritoneal cavity. The liver, gallbladder, spleen, stomach, duodenum, small intestine, and colon appeared normal. A large cystic mass was discovered near the porta hepatis and was dissected circumferentially. This mass, which connected to the hepatic bifurcation and gallbladder had a 5 cm rupture in the right wall with active arterial bleeding. It was confirmed to be a congenital CC, type Ⅳa, according to Todani's classification[1].

Because intraperitoneal inflammation was moderate, a complete cyst excision with cholecystectomy and Roux-en-Y end-to-side hepaticojejunostomy reconstruction were performed. The postoperative course was uneventful and the patient was discharged on the 12th day of hos-

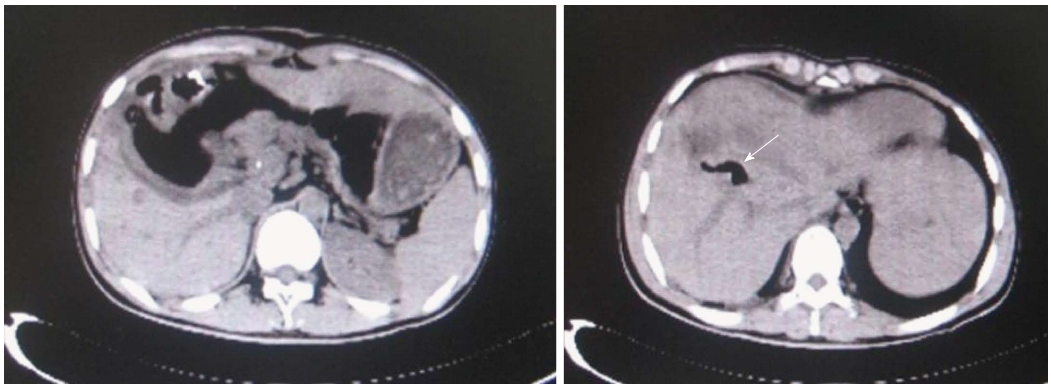

Figure 3 Abdominal computed tomography shows pneumatosis in the intrahepatic bile duct after cyst excision, cholecystectomy and Roux-en-Y hepaticojejunostomy reconstruction (white arrow). The intrahepatic dilatation tends to reduce in size following sufficient biliary drainage.

pitalization. Histological sections of the surgical margins and the operative specimen did not reveal malignancy. The patient has remained well in the close follow-up period for 9 mo. Four weeks after surgery, abdominal CT scan showed pneumatosis in the intrahepatic bile duct, and intrahepatic ductal dilatation, which was reduced following adequate biliary drainage (Figure 3).

DISCUSSION

Choledochal cyst is a rare congenital disease characterized by single or multiple dilatations of the intra and/or extrahepatic biliary tree. Although the incidence in the Western population is 1 in 100000-150000 live births, it is remarkably higher in Asian countries, particularly in Japan, where these cysts can be found in up to 1 in 1000 live births[2]. The incidence is higher in females, with a male/female ratio of 1:3[3]. These cysts are typically a surgical problem of infancy and childhood, however, the diagnosis is delayed until adulthood in nearly 20% of patients[4]. In CC, spontaneous perforation is observed in 1.8%-7% of all cases[5]. Nevertheless, reports of traumatic rupture of CC are extremely rare. As far as we know, only a few cases have been described in the literature[6-13].

The classical triad of jaundice, right upper quadrant mass, and abdominal pain is present in only a minority of patients[14]. Other presenting features of CC are cholangitis, pancreatitis, and biliary peritonitis from cyst rupture[5]. However, our patient was found to have peritonitis and abdominal hemorrhage, which is quite rare.

Ultrasound is the initial examination of choice in suspected CC[15]. Nevertheless, inherent limitations (*e.g.*, gas in the bowel, intraperitoneal inflammation, *etc.*) and the radiologist's skill may affect diagnosis. CT is required when the distal common bile duct is not visualized due to bowel gas. This imaging technique is excellent for detecting cystic lesions in the right upper abdomen, assessing their range and providing information regarding the impact of CC on the surrounding structures. Magnetic resonance cholangiopancreatography (MRCP) is the non-invasive imaging modality of choice for biliary pathology, and may offer as much as endoscopic retrograde cholangiopancreatography. However, MRCP is not suitable in patients who are unable to hold their breath for a few seconds, a requisite for breath-hold MRCP sequences.

Surgical treatment of CC should be recommended to reduce the risk of serious complications, such as cholangitis, pancreatitis, rupture, portal hypertension, cirrhosis, and cholangiocarcinoma[15]. In patients with type Ⅳ cysts without preoperative or intraoperative evidence of liver cirrhosis or biliary malignancy, resection of the entire portion of the extrahepatic cystic dilatation is currently recommended[16,17]. Reconstruction at the hepatic duct bifurcation is indicated. In rare cases where cyst rupture occurs, previous reports have speculated that a portal dissection and biliary reconstruction may be hazardous in the presence of bile peritonitis. These reports recommended primary management with temporary external drainage, by percutaneous transhepatic cholangiodrainage or by open placement of a T-tube[18,19]. Once the patient has recovered and has been thoroughly evaluated, a complete cyst excision, cholecystectomy, and hepaticojejunostomy can be performed[20]. However, this requires long-term maintenance of a T-tube and a second laparotomy. Furthermore, complications may occur in the interim. However, bile is aseptic, and even if bile drains into the abdominal cavity from a CC rupture, the possibility of this condition developing into generalized peritonitis is slight because such peritonitis will be chemical. In our patient, chronic inflammation induced thickening of the CC and fibrous adhesion with adjacent tissues, thus no shrinkage of the cyst cavity was observed following rupture of the CC which was excised. In recent years, due to progress in imaging diagnosis, it has become possible to diagnose the condition early. Therefore, in our opinion, treatment methods must also change. The case reported here demonstrated the feasibility of primary cyst excision and biliary reconstruction.

In conclusion, traumatic rupture of type IVa CC in an adult male is rare. A thorough preoperative diagnostic workup, with CT and/or MRCP, will guide surgeons to an appropriate operative strategy. Primary complete cyst excision, cholecystectomy and Roux-en-Y hepaticojejunostomy reconstruction are the preferred management options.

ACKNOWLEDGMENTS

The authors thank Dr. Xiao-Dong Li and Dr. Yan Tan for their technical support.

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World Journal of Gastroenterology

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World J Gastroenterol 2013 June 28; 19(24): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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Name of journal
World Journal of Gastroenterology

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

Launch date
October 1, 1995

Frequency
Weekly

Editorial office
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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]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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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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