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Maximization of the value of the readers can be comprehended in two ways. First, the journal publishes articles that can be directly read or downloaded free of charge at any time, which attracts more readers. Second, the readers can apply the knowledge in clinical practice without delay after reading and understanding the information in their fields. In addition, the readers are encouraged to propose new ideas based on those of the authors, or to provide viewpoints that are different from those of the authors. Such discussions or debates among different schools of thought will definitely boost advancements and developments in the fields. Maximization of the value of the authors refers to the fact that these journals provide a platform that promotes the speed of propagation and communication to a maximum extent. This is also what the authors really need. Maximization of the value of the society refers to the maximal extent of the social influences and impacts produced by the high quality original articles published in the journal. This is also the main purpose of many journals around the world.

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EDITORIAL

Baishideng's century goal: Editing and publishing high-quality articles

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Abstract

Baishideng's goal over the next few years is to edit and publish high-quality articles through the open-access model, to maximize the benefits to members of the editorial board, authors and readers, as well as achieving social and economic benefits.

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INTRODUCTION

Beijing Baishideng BioMed Scientific Co., Ltd was established on November 20, 2007. Its predecessor was The WJG Press, and its main task is to edit and publish biomedical journals. The predecessor to the currently published *Shijie Huaren Xiaohua Zazhi* (*World Chinese Journal of Digestology*) was the *Xin Xiaohuabingxue Zazhi* (*New Journal of Gastroenterology*), which was launched in Shanxi on January 15, 1993. The predecessor of the weekly publication *World Journal of Gastroenterology* was the *China National Journal of New Gastroenterology*, which was launched in Shanxi on October 1, 1995. We are also preparing to publish the *World Journal of Hepatology*, *World Journal of Gastrointestinal Oncology*, *World Journal of Diabetes*, *World Journal of Gastrointestinal Endoscopy*, *World Journal of Stem Cells*, *World Journal of Gastrointestinal Surgery*, *World Journal of Gastrointestinal Pathophysiology*, and *World Journal of Gastrointestinal Pharmacology and Therapeutics* which will be launched successively in Beijing after September 15, 2009. Our goal is to edit and publish high-quality articles through the open-access (OA) model[1], to maximize the benefits to members of the editorial board, authors and readers.

MAXIMIZATION OF THE PERSONAL BENEFITS OF EDITORIAL BOARD MEMBERS

Our editorial board comprises 1126 invited experts in 60 countries, which means that we have access to outstanding scientists around the world, because first-class publications require a first-class editorial team[2,3]. Member of the editorial board should: (1) have attained a high level of scholarship and respect in their respective fields; (2) have a rigorous academic attitude and integrity; (3) participate actively in academic conferences and activities; (4) carry out peer review of six articles for the journal every year; and (5) write a commentary article or organize a series of "Highlight" articles for the journal every 2 years. Maximization of the interests of members of the editorial board is mainly reflected in the following.

Members of the editorial board review articles

(1) Responsibility to their own fields. A member of the editorial board rigorously evaluates a manuscript within a specified time; (2) Responsibility to the author. After agreeing to peer review a manuscript, a member of the editorial board makes a scientific evaluation of its accuracy, clarity, importance and its contribution to the scientific community; and (3) Responsibility to the journal. When a member of the editorial board cannot complete the review within the time set by the journal, he/she should decline the review and explain the reason.

Once a member of the editorial board agrees to review a manuscript, he/she is under an obligation to send their comments to the editorial department within the specified time. If the member of the editorial board cannot complete the review on time, he/she should inform the editorial department and ask whether to return the unreviewed manuscript or request for an extension of time. When a member of the editorial board receives a manuscript involving an apparent conflict of interest with his/her own research work or point of view, the member of the editorial board should decline to review the manuscript and explain the specific conflict of interest. The most common conflict of interest is private or professional relationships between the member of the editorial board and the organization undertaking the research or the author(s) that may affect the impartiality of the review, or competition between the member of the editorial board and those undertaking the research. When a member of the editorial board cannot return a reviewed manuscript to the editor for some reason, he/she can recommend one or two peer reviewers familiar with the topic of the paper to help the editor process the manuscript accurately and quickly. As a qualified member of the editorial board, he/she must abide by certain rules, deal with problems fairly and impartially, and avoid abuse of his/her position[4]. We publish all relevant information, including the conclusion of the peer review and the name and organization of the reviewer, along with the article. This is to control the quality of the peer reviewers, editors and publishers, ensure that each article is published after peer review, and reflect peer reviewers' responsibility.

Members of the editorial board publish commentary articles

Almost all the 1126 members of the *WJG* editorial board are leaders in their own fields. To mobilize the enthusiasm of the editorial board and maximize their interest in the journal, we set ourselves the following tasks. (1) We publish a list of members of the editorial board at a fixed location in the printed and online editions of each issue of the journal; (2) We express thanks to members of the editorial board whose reviewed manuscripts have been accepted, publish relevant information such as the name and organization of the reviewer along with the author's article, and send a PDF copy of the article to them; (3) We also express thanks to members of the editorial board whose evaluated manuscripts have been rejected; (4) We send a summary list of articles published to members of the editorial board each year, and invite them to write commentary articles for the coming year; and (5) We offer 50 US dollars for the author of every "Highlight" or "Observation" article. However, the majority of the members of the editorial board do not take the payment for articles solicited. A Canadian member of the editorial board, Professor H Freeman, University of British Columbia, said at the outset of the "Observation" articles that: "We need the spirit of Bethune to run *WJG* successfully"[5]. Professor Freeman has published 20 commentary articles in the observation column of which he is the chief editor. Scientists contribute to the international community and they do not publish articles for economic gain. *WJG* published a variety of 1203 articles in issues 1-48 in 2008, of which there were 265 commentary articles written by members of the editorial board, which accounted for 22.08% of the published output. According to Journal Citation Reports published from 2006-2007 for *WJG* included in the Science Citation Index Expanded, the top three categories that contributed published articles in terms of the average number of times cited were reviews (8.63), editorials (6.24) and highlights (5.83)[6].

Members of the editorial board are ambassadors for the journal

From 2007 to 2009, the editorial board of *WJG* had 1126 members in 60 countries. Where there are members of the *WJG* editorial board, there are also authors and readers of *WJG*. We decide on candidate members of the editorial board according to their citations in the ISI Web of Knowledge. Whether a person can be finally accepted as a member of the editorial board depends mainly on his/her resumé and job title. Only experts holding a rank higher than associate professor can serve as members of the *WJG* editorial board. International members of the editorial board are ambassadors who help with the global dissemination of our journals. They are also liaison officers who recommend persons in the same field as their own to contribute to *WJG*.

MAXIMIZING THE BENEFIT TO AUTHORS OF PUBLISHING ARTICLES

One of the biggest advantages of open access publishing is that people around the world can have immediate access to peer-reviewed articles *via* the internet. This provides a new platform for the international community to have equitable access to the latest scientific developments, and to play a positive role in advancing scientific knowledge.

Qualifications of authors for publishing articles

We require authors to declare that they all meet the eligibility criteria below, no author who meets these criteria has been left out, and contributors and sponsors have been duly thanked. The eligibility criteria for authors proposed by the international committee of medical journal editors are[7]: (1) authors must have made a significant contribution to the concept and design of the research and the acquisition, analysis and interpretation of data; (2) draft articles and critically revise the article for important intellectual content; and (3) approve the final version of the article to be published.

Editing and publishing quality of published articles

Whether the editorial department of the journals receives high-quality international articles hinges on many factors. The editing and publishing quality of every article published in the journal needs to be guaranteed. Our approach is as follows. (1) We give our journals an international appeal, e.g. containing the word "World"

in the title, to attract international authors to contribute articles; (2) We accept online contributions, so that authors can submit a manuscript from any place that has an internet connection. The system replies automatically or the editorial department returns acknowledgement of the manuscript within 24 h; (3) Members of the editorial board participate in online peer review, which generally takes 2-4 wk; (4) Reject manuscripts or send those that are accepted back to the authors for revision; (5) Edit and ReleaseProcess manuscripts in a scientific manner; (6) Authors revise their articles; (7) Formal acceptance or rejection of manuscripts; (8) In processing manuscripts, the language editor solves common errors, including grammar, syntax, spelling, punctuation and logic; (9) Return manuscripts to the author for final revision; (10) The editorial director organizes the production; (11) Electronic editing and production, including redrawing of charts, graphs and automatic checking by the software Heima; (12) The electronic editor in charge carries out editing and typesetting; the science editor in charge carries out proofreading, including Heima proofreading; (13) The author carries out proofreading; (14) The editorial director and editor-in-chief carry out proofreading; (15) Produce and proofread films; (16) Printing; (17) Create the online edition; (18) The editorial director checks the online edition; and (19) Release the online edition. Every aspect of every article published through the above process is original. For example, charts and graphs are re-drawn, references are re-formatted using software, and Word documents are converted into XML format automatically to avoid human errors. In the entire process from editorial personnel receiving the manuscript to online publishing, we put service and quality first, and aim to establish the reputation of the journal so as to attract international contributors.

Comments accompanying original articles

The accompanying comments for each article are written in an easy-to-understand manner to enable professional scientists, the public and media to understand more broadly the relevance of the article, and to play a role in promoting science to a wider audience. Accompanying comments cover the following. (1) Background information: summarizes relevant background that will give readers a better understanding of the article in an easy-to-understand manner to enable professional readers, the public and the media to understand more broadly relevant knowledge of the article and have a better understanding of the meaning of the article; (2) Research and development: aims to help readers understand how the article relates to the key areas of research in the field; (3) Relevant reports: references to other relevant papers that may help with understanding the article, or as pointers to wider reading on the subject of the article; (4) Inventory of innovations: summarizes the innovative aspects of the article, so that readers can see how the new study differs from and is related to previous work; (5) Main points of application: summarizes the practical applications of the article, and how it may improve existing or develop future applications. Hopefully, this may lead to the article being cited extensively; (6) Glossary: a simple, straightforward and clear explanation of the terms in the article that are important for its understanding, as many readers may not be familiar with these terms of have a full understanding of them; and (7) Peer review: the editorial department summarizes the opinions of the peer reviewers, to demonstrate the significance of the article.

The core interests of authors in publishing articles

Members of the editorial board publish a wide range of commentary articles in the form of invited papers. The purpose of these is to comment on the status quo, progress and problems in fast-developing fields, in an attempt to play a role in guiding clinical or basic research. Other contributing authors submit original articles for publication. The purpose of these is to report the latest developments in scientific research, which are innovative, of a high standard, and have considerable scientific significance in their own field. The core interests and values main purpose of members of the editorial board and contributing authors in publishing articles is to disseminate their peer-reviewed research results around the world, and hopefully, to have them cited by others in the same field, thereby contributing to scientific progress. If journal articles written by members of the editorial board and contributing authors do not achieve the above-mentioned core interests and values, the science editor and peer reviewer's judgment of the quality of the article will be considered to be at fault, which is caused mainly by subjectivity and bias. In this case, subjectivity means that all manuscripts are evaluated by peer reviewers based on their own expertise. Academic research can be controversial, and within a given subject area, the opinions of peer reviewers may be not be exactly same and may even be diametrically opposed. Some peer reviewers are relatively tolerant, while others are relatively harsh. These are all important factors affecting peer reviewers' opinions. Therefore, the review of manuscripts cannot be completely free of subjective assumption. Bias refers to non-academic influencing factors that result from the peer reviewers' different preferences or prejudices, including the authors' nationality, mother tongue, sex and organization for which they work[4]. *WJG* published 2506 articles in 2006-2007, of which 1963 (78.33%) have been cited in another 1597 journals. The self-citation rates for articles in *WJG* in 2006 and 2007 were 22.16% and 11.34%, respectively[6].

International profile of articles published

It is very disappointing for an author to see no reaction after his/her article is published in a journal. A major reason for this is the particular journal's lack of international profile. High-quality journals are an important indicator of a country's achievement in terms of scientific and technological research, therefore, we aim to create high-quality academic journals of global significance to increase our international profile. Influential high-quality scientific journals are an important platform for academic exchange around the world and an effective vehicle for cultivating innovative talent. They

also provide authoritative leadership in their particular discipline and give a lead in developing new areas of research[8]. To achieve these ends, we have adopted an OA publishing model with the following objectives. (1) Release the online edition of *WJG* on time. Up to July 12, 2009, the number of visits was 33 947 050, the maximum number of times an article was viewed was 50 454, and the maximum number of times an article was downloaded in PDF format was 7653; (2) Upload PubMed abstracts on time. From 1998 to 2009, PubMed included 7977 articles from *WJG*. The number of visits by international readers in October 2008 was 76 358; (3) Upload PMC full texts on time. From May 1, 2009 to June 11, 2009, 689 articles were included. The number of dedicated IP addresses visiting articles was 31 491; the number of times articles were downloaded was 22 133; and the number of times XML articles were viewed was 35 479; (4) Upload EurekAlert! press releases on time. A total of 198 *WJG* scientific press releases were published from 2007 to 2009. The number of times press releases were viewed was 441 128; and the maximum number of times a single press release was viewed was 4126. Through the above methods, *WJG* has increased greatly its international profile and has attracted more contributions from international authors. *WJG* published 1203 articles in issues 1-48 in 2008, which originated from 62 countries, and international authors accounted for 70.25% of the articles published. Editing and publishing high-quality articles and distributing them on time to various media is one of the important methods of increasing the international profile of the authors' work. We award 500 RMB to the electronic editor in charge of each issue for publishing on time and have achieved significant results, thus ensuring on-time publication of each issue in various media.

MAXIMIZING THE BENEFITS TO READERS BY OBTAINING KNOWLEDGE

At present, universities and scientific research institutions have to spend vast sums of money to obtain access to academic scientific papers, and many small institutions cannot afford the high fees for access licenses. Implementation of the open access model around the world can increase the access of researchers and the general public to information about achievements in scientific research. The research report compares three kinds of publishing models. The open access model has the greatest advantage because research institutions or those funding the research have paid the publishing costs, therefore, papers can be obtained free of charge[9]. The competitive edge of scientific journals depends on the commitment and loyalty of readers. The more readers, the bigger the market share for any given journal. We must establish a reader-centered service so that they accept and come to value our journals[10]. We make the online edition of 7977 articles published by *WJG* available to readers around the world. The full text of the articles is available for free download and > 20 000 visits are received from readers each day. Readers around the world currently can read and download the latest articles published by *WJG* free of charge, through PubMed and PMC of the United States National Library of Medicine.

CONCLUSION

The only way for Baishideng to achieve a lasting reputation and continue to develop is to publish high-quality articles and make the full text freely available around the world. This will allow us to develop a commercial publishing model that maximizes the interests of the editorial board, authors and readers.

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EDITORIAL

Ascitic fluid analysis for diagnosis and monitoring of spontaneous bacterial peritonitis

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Abstract

Polymorphonuclear (PMN) cell count in the ascitic fluid is essential for the diagnosis and management of spontaneous bacterial peritonitis (SBP). To date, PMN cell count is routinely performed by traditional manual counting. However, this method is time-consuming, costly, and not always timely available. Therefore, considerable efforts have been made in recent years to develop an alternative test for a more rapid diagnosis and monitoring of SBP. The use of urinary reagent strips was proposed to achieve an "instant" bedside diagnosis of SBP. A series of reports evaluated the urine strip test for SBP diagnosis and reported promising results. However, a recent large multicenter study revealed a surprising lack of diagnostic efficacy of the urine screening test for SBP diagnosis. Another method, more recently proposed as an alternative to the manual PMN count, is the measurement of lactoferrin in ascitic fluid, but the data available on the diagnostic value of this test are limited to a single study. However, both urinary reagent strips and ascitic lactoferrin tests are qualitative methods and need, therefore, to be further confirmed by standard cytology of the ascitic fluid. To date, the only quantitative method proposed as a valid alternative to manual PMN counting is automated blood cell counters, commonly used in all laboratories for blood cell counting. Data available in the literature on the diagnostic performance of this method are limited but very promising, and this tool seems to have the potential to replace the manual counting method.

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Key words: Ascites; Ascitic fluid lactoferrin; Automated blood cell counter; Paracentesis; Polymorphonuclear cell count; Urinary reagent strips

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INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is a frequent and severe complication of decompensated cirrhosis. Since its first description in the 1970s, when the mortality rate exceeded 80%, a significant and substantial improvement in the prognosis of SBP has been noted. In more recent prospective studies the mortality rate was reported to be around 20%[1-3]. The widespread use of diagnostic paracentesis, as well as the prompt initiation of empiric antibiotic therapy, based on the results of ascitic polymorphonuclear (PMN) cell count, has contributed to an improvement in the survival of these patients.

DIAGNOSIS AND TREATMENT OF SBP

The percentage of SBP in hospitalized cirrhotic patients with ascites ranges between 10% and 30%[4-9], whereas the prevalence of SBP among outpatients with cirrhosis is markedly lower (approximately 3.5%)[10]. Four practical guidelines and experts' consensus reports on the diagnosis and management of SBP are currently available[11-14], all of which suggest that the diagnosis of SBP should be based on PMN cell count in the ascitic fluid. The detection of a PMN cell count greater than 250 cells/mm^3 is highly suspicious of SBP and provides an indication to initiate empirical antibiotic treatment. Only a few SBP patients, in fact, have typical symptoms suggestive of peritoneal infection such as fever, abdominal pain and a high blood leukocyte count. More often, SBP is suspected when the patient develops signs of hepatic

encephalopathy or a rapid impairment of renal function without any apparent precipitating factor. In addition, in a significant percentage of cases, SBP may be completely asymptomatic and the diagnosis is made only by diagnostic paracentesis. The presentation of SBP recently observed in a consecutive series of hospitalized patients[15] is reported in Table 1. Following hospitalization of any cirrhotic patient with newly diagnosed ascites, even in the absence of symptoms or if admitted for other reasons, a diagnostic paracentesis is therefore advised, aimed at investigating the presence of SBP. To rule out the possible presence of SBP, a paracentesis tap should also be repeated in all ascitic cirrhotic patients with gastrointestinal bleeding or hepatic encephalopathy, and whenever patients develop clinical symptoms and signs of infection, renal impairment or an unexplained worsening of their clinical condition[11].

Following the diagnosis of SBP, ascitic fluid collection and blood cultures should be carried out before initiating antibiotic administration. The commonest organisms isolated in patients with SBP include *Escherichia coli*, Gram-positive cocci (mainly *Streptococcus* species) and *Enterococci*. These organisms account for approximately 70% of all cases of SBP[16,17]. Antibiotic therapy, however, should not be delayed to the moment when microbiological results are available; with conventional culture techniques, the ascitic fluid culture may be negative in up to 60% of patients with SBP[11]. Antibiotic therapy should instead be initiated empirically immediately after evidence of SBP at diagnostic paracentesis, without the need to wait for identification of the causative organism and *in vitro* susceptibility. Cefotaxime is the antibiotic most extensively investigated in patients with SBP[18-20], since it covers 95% of the flora isolated from the ascitic fluid and achieves high ascitic fluid concentrations during therapy[12,21]. The dose of cefotaxime is 4-8 g/d given intravenously for a minimum of five days. Other studies[22-26] have evaluated the efficacy of cephalosporins (such as ceftriaxone and ceftazidime) administered once daily; although data are still limited, they appear to be as effective as cefotaxime. The association between amoxicillin and clavulanic acid also proved to be as effective as cefotaxime, with a similar security profile[27,28]. In patients with uncomplicated SBP (i.e. no gastrointestinal bleeding, hepatic encephalopathy or renal failure), oral treatment with ofloxacin or other quinolones for eight days can be administered[29]. An intravenous → oral step-down schedule with ciprofloxacin (switch therapy) allows completion of the antibiotic treatment at home, once the patient is discharged. This regimen was recently shown to be as efficacious but more cost-effective than intravenous cefotaxime[30]. With the regimen described above, the resolution of SBP is achieved in approximately 90% of cases and 30-d survival is at least 80%[11].

The empirical antibiotic treatment chosen should be continued until symptom resolution or should be modified according to the results of the microbiological test. However, since SBP is often asymptomatic and the ascitic fluid culture outcome is often negative, once again the PMN cell count should be considered as a diagnostic tool to objectively demonstrate the efficacy of the ongoing antibiotic treatment and, finally, resolution of the infection. According to the International Ascites Club guidelines published in 2000[11], a diagnostic paracentesis should be repeated after two days during antibiotic therapy to assess treatment response. A PMN cell count reduction greater than 25% of the initial value was in fact suggested as the main criterion for establishing the efficacy of the antibiotic and the need for switching the therapy. This recommendation is very important, since there have been suggestions that the type and etiology of bacterial infections in cirrhosis may have changed during recent years[31,32]. An increasing incidence of SBP caused by Gram-positive bacteria in cirrhotic patients with ascites has been observed by different authors[33,34]. In addition, an increased frequency of bacteria resistant to multiple antibiotics was shown[35]. Our recent study[15] reported that, in clinical practice, a switch from cefotaxime to another antibiotic was necessary in more than 40% of SBP cases, thus supporting the possibility that the microbial etiology of SBP, as well as sensitivity to antibiotic therapy, is changing. A method aimed at objectively establishing resolution of the infection would be extremely useful; once again, the PMN cell count in the ascitic fluid obtained at diagnostic paracentesis was revealed to be the most reliable tool.

Table 1 Signs and symptoms of SBP at the time of hospital admission in a recently published paper[15]

Patients/No. of episodes of SBP (32/38)	*n*
Clinical manifestations at hospital admission	
Fever	12
Abdominal pain	6
Leukocytosis	11
Hepatic encephalopathy	8
Renal failure	14

THE DETERMINATION OF PMN CELLS IN THE ASCITIC FLUID

Unfortunately, in clinical practice, ascitic PMN cell count is not always possible within a few hours, thus causing an unacceptable delay in the diagnosis and treatment of this potentially fatal infection. At present, PMN cell count is routinely performed by a traditional hematological method with a light microscope in a manual counting chamber. For this method, 10 mL of ascitic fluid are collected in tubes containing ethylenediaminetetraacetic acid (EDTA) and centrifuged at 1500 r/min for 10 min. Nine milliliters of the supernatant are discharged and 40 μL of the remaining ascitic fluid are diluted with 800 μL of Turk's fluid, gently shaken and used to fill the counting chamber. The cells are counted (40 × objective) in one of the nine large squares and the number of white blood cells per cubic millimeter calculated. Another 10 mL sample of ascitic fluid is used for the PMN percentage determination (100 × objective), after centrifugation and May Grünwald-Giemsa staining. This method is presently considered the

Table 2 Results of the urine reagent strips for the diagnosis of SBP

Authors	Samples/SBP (*n*)	Strips/cut-off grade	Sensitivity	Specificity	PPV	NPV
Vanbiervliet *et al*[36] 2002	72/9	Multistix ⩾ 1	100	100	100	100
Castellote *et al*[37] 2003	228/52	Aution ⩾ 3	89	99	98	97
		Aution ⩾ 2	96	89	74	99
Thevenot *et al*[38] 2004	100/9	Multistix ⩾ 3	89	100	100	99
		Combur ⩾ 2	89	100	100	99
Butani *et al*[39] 2004	136/12	Multistix ⩾ 2	83	99	91	98
Sapey *et al*[40] 2005	184/13	Center 1/Nephur ⩾ 1	86	100	100	99
		Center 1/Multistix ⩾ 1	100	100	100	100
		Center 2/Nephur ⩾ 1	100	92.5	75	100
		Center 2/Multistix ⩾ 1	83	96	83	96
Sapey *et al*[41] 2005	245/17	Nephur ⩾ 3	88.2	99.6	93.8	99.1
		Multistix ⩾ 1	64.7	99.6	91.7	97.4
Kim *et al*[42] 2005	75/18	Uriscan ⩾ 2	100	100	100	100
		Multistix ⩾ 3	50	100	100	87
Sarwar *et al*[43] 2005	214/38	Combur ⩾ 3	97.7	89.4	90	97.7
Wisniewski *et al*[44] 2005	47/6	Multistix ⩾ 1	83	83	97	42
Campillo *et al*[45] 2006	443/46	Multistix ⩾ 1	69.6	94.9	64	96
		Combur ⩾ 1	80.4	90.4	52	97.3
		Multistix ⩾ 2	45.7	98	75	93.3
Rerknimitr *et al*[46] 2006	200/42	Combur ⩾ 1	88	81	55	96
		Combur ⩾ 2	63	96	82	81
Braga *et al*[47] 2006	100/9	Combur ⩾ 2	100	98.9	92.3	100
Torun *et al*[48] 2007	63/15	Aution ⩾ 2	93	100	100	98
Gaya *et al*[49] 2007	173/14	Multistix ⩾ 1	100	91	50	100
Nobre *et al*[50] 2008	109/9	Combina ⩾ 2	78	88	37	98
		Combina ⩾ 3	67	98	75	97
Ribeiro *et al*[51] 2007	200/14	Multistix ⩾ 1	86	96	60	99
		Multistix ⩾ 2	71	99	91	98
Nousbaum *et al*[52] 2007	1041/117	Multistix ⩾ 3	45.3	99.2	77.9	96.9

"gold standard" for the evaluation of ascitic PMN count, but this procedure is laborious and time-consuming. This results in the laboratory often providing the PMN count too late in the day, or sometimes even on the next day, when the patient may have progressed from an early stage to a fatal stage of infection. Moreover, the manual method is not always timely available in all hospitals, especially in those small patient care units with limited laboratory facilities, and the method cannot frequently be performed on an emergency basis (at night or on week-ends). This manual system, therefore, often delays the initiation of antibiotic therapy and may expose patients to a higher risk of death.

On the basis of these considerations, considerable efforts have been made in recent years to develop an alternative test for a more rapid diagnosis of SBP. This represents an interesting and promising area of investigation, which could determine the further optimization of SBP management and further improvement in its prognosis. The use of urinary reagent strips-which identify leukocytes by detecting their esterase activity *via* a colorimetric reaction-was proposed to achieve an "instant" bedside diagnosis of SBP. Their employment was tested for the rapid diagnosis of bacterial meningitis, pleural or synovial infections, thus suggesting that they could also be used for the rapid diagnosis of SBP. The first study[36], published in 2002, involved 72 cirrhotic patients, nine of whom had SBP, reported a sensitivity and a specificity of 100% for the urinary strips in the diagnosis of SBP. After this successful result, in the following years a number of reports[37-51] evaluated the urine strip test for the diagnosis of SBP (Table 2). These studies reported promising and encouraging results, with the sensitivity of urinary strips for the diagnosis of SBP ranging between 85% and 100% and the specificity between 90 and 100%. These published studies were, however, limited by the small number of SBP episodes and were restricted to one or two centers. Therefore, the need to validate this method in a larger population of cirrhotic patients in a multicenter study arose. A prospective multicenter (70 centers) study with a total of 2123 paracenteses performed in 1041 patients was published in 2007 by Nousbaum *et al*[52]. Surprisingly, this paper did not confirm the good and promising results of the previous studies, but confirmed a very high specificity for reagent strips in the diagnosis of SBP, and revealed a poor sensitivity of this test (45%). This study, therefore, reported the lack of diagnostic efficacy of the urine screening test and the authors concluded that a routine cytological examination remains mandatory for the diagnosis of SBP. After initial great enthusiasm, the use of reagent strips for SBP diagnosis has been disappointing.

Another method, more recently proposed as an alternative to the manual PMN count, is the measurement of lactoferrin in ascitic fluid. Lactoferrin is released by PMN leukocytes on the activation of these cells and its presence in body fluids is proportional to the flux of neutrophils. It was therefore hypothesized that its use could also be useful in the detection of SBP. The data available on the diagnostic value of this test for SBP diagnosis are limited to a single study[53]. This study, in

Table 3 Results of the automated cell counter for the diagnosis of SBP

Authors	Samples/SBP (*n*)	Automated cell counters	Diagnosis of SBP			
			Sensitivity	Specificity	PPV	NPV
Angeloni *et al*[54] 2003	130/11	Technicon System H*1	94	100	100	99.1
		Coulter	100	92	87	100
Cereto *et al*[55] 2004	133/48	UF-100	98	98	96	99
		ADVIA	100	95	95	100
Riggio *et al*[56] 2008	112/16	Technicon System H*1	100	97.7	94.1	100

which ascitic fluid lactoferrin was measured in a total of 218 ascites samples, reported that the sensitivity and specificity of the assay for SBP diagnosis were 95% and 97%, respectively. However, the quantitative lactoferrin assay used in this study is not commercially available. Qualitative and rapid tests are already commercially available for bedside measurement of lactoferrin concentration in the stool (to distinguish inflammatory from non-inflammatory bowel conditions). The authors hypothesized that qualitative tests able to detect lactoferrin levels in excess of a predetermined level for bedside diagnosis can easily be developed and with limited costs. They concluded that, although further confirmatory studies are needed, ascitic fluid lactoferrin could serve as an initial screening test for the detection of SBP in cirrhotic patients.

It is important to underline that both urinary reagent strips and ascitic lactoferrin tests are qualitative methods and need, therefore, to be further confirmed by standard cytology of the ascitic fluid. These tests could merely be a screening modality providing only a presumptive diagnosis, but they do not have the potential to replace the manual counting method. Moreover, a qualitative test does not have applicability in the clinical management of SBP, since a reduction of more than 25% of the initial PMN cell count value is, according to the current guidelines, the criterion to establish the efficacy of the ongoing antibiotic treatment. To date, the only quantitative methods proposed as a valid alternative to manual PMN counting are automated blood cell counters, commonly and largely used in all laboratories for blood cell counting. They offer accurate and rapid differential counts of leukocytes, including PMN cells. The published data available on the diagnostic performance of this method are limited but very promising (Table 3). The first study[54], which tested an automated cell counter for the diagnosis of SBP, was published in 2003 by our group. This study, in which 130 ascitic fluid samples obtained from 74 cirrhotic patients were analyzed, demonstrated that the use of an automated cell counter provides an accurate determination of ascitic fluid PMN and has a reliable diagnostic accuracy when compared with manual counting. In fact, the automated blood cell counter had a sensitivity of 94% and a specificity of 100%, while positive and negative predictive values were 100% and 99.1%, respectively (Table 3). These findings were also confirmed by Cereto *et al*[55] (Table 3). More recently, a further study[56], in which we analyzed 112 samples of ascitic fluid, showed that the manual and automated methods had good agreement not only in the first diagnostic paracentesis, but also in those performed during the follow-up. These results suggested that the automated cell counter is a useful and reliable tool not only for the diagnosis of SBP, but also in its management for monitoring the therapeutic efficacy of antibiotic treatment, as well as for establishing resolution of the infection. The use of automated PMN counting may provide several advantages for the diagnosis and management of SBP: it is commonly available in all patient care units, it is routinely accessible in emergencies (i.e. at night or on weekends) and provides the results in a few minutes, thus allowing the clinician to quickly decide whether to start empiric antibiotics or to change the ongoing antibiotic therapy. Finally, it is important to remember that all this involves a very low cost (only €1.38 for a single ascitic cell determination). Another, although less important, advantage of the automated cell counting method over urinary reagent strips, the manual method and the ascitic lactoferrin test, is that it is possible to precisely assess the amount of PMN in bloody (for a traumatic tap or a condition inducing bleeding) ascitic fluid. With the other methods, the amount of PMN derived directly from the blood spilled over into the ascitic fluid cannot be differentiated from the amount of PMN due to the infection. A correction factor of 1 PMN per 250 red blood cells has been proposed[11], because this is the maximum expected ratio of PMN to red blood cells normally present in the peripheral blood. With automated cell counters, a measure of the amount of red blood cells and PMN in both the peripheral blood and the ascitic fluid can be obtained simultaneously. The amount of ascitic PMN due to the infection can therefore be calculated by the real PMN-to-red blood cells ratio in the blood and by the real erythrocytes and PMN number in the ascitic fluid.

CONCLUSION

Although further confirmatory data are needed, automated cell counters offer a reliable, easier and quicker PMN count, they may also considerably simplify the diagnostic approach and clinical management of SBP and should be routinely adopted. The automated method has the potential to be an optimal substitute for manual PMN counting and could definitely replace it.

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TOPIC HIGHLIGHT

Damian Casadesus, MD, PhD, *Series Editor*

Surgical resection of rectal adenoma: A rapid review

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Abstract

Transanal excision (TE), endoscopic transanal resection (ETAR) and transanal endoscopic microsurgery (TEM) can be used to remove adenomatous polyps. However, their use is limited by the size or location of the tumor. TE is limited to the lower rectum, TEM offers better access to lesions in the middle and upper rectum, and ETAR is used less frequently than it deserves for resection of rectal lesions.

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Key words: Adenoma; Colorectal surgery; Endoscopy; Gastrointestinal; Microsurgery; Rectal neoplasms

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INTRODUCTION

The risk of carcinoma developing in a colorectal polyp of 1 cm or larger is 2.5% and 8% at 5 and 10 years, respectively[1]. This potential for malignancy of adenomatous colorectal polyps is an indication for their excision and the first-line treatment is endoscopic removal during the diagnostic procedure, which is safe, relatively inexpensive, and associated with the lowest complication rate. When the size and/or location of the tumor limits standard endoscopic resection, a number of different transanal approaches are used to remove adenomatous polyps in the lower rectum, but adenomas in the middle or upper rectum are difficult to remove using standard transanal excision (TE) instruments. Different techniques have been developed for the removal of rectal adenomas that are not amenable to removal during colonoscopy.

TE

TE of lower rectal adenoma, first reported by Parks[2], is one the most frequently adopted approaches for excision of rectal polyps of any dimension, situated as far as 6 cm from the anal verge, and has very few complications, and low recurrence and mortality. In a review of the literature, bleeding after TE was present in up to 10% of the patients, perforation in up to 6%, and anal stenosis in up to 5%, with a mortality rate of 2%[3-6]. Recurrence is variable, at 3%-50% for adenoma from adenoma, and up to 3% for carcinoma from adenoma. In a long series of 117 procedures, Sakamoto *et al*[7] treated 27% of patients for residual disease and 30% for recurrence, with a 10% rate of serious complications. In a recent study, Pigot *et al*[8] obtained better results: they resected apparently benign rectal adenomas from 207 consecutive patients with a 3.6% recurrence rate, eight postoperative complications, and one death. Specific recurrence-free probability was 99.5% at 1 year, 96% at 5 years, and 95% at 10 years.

In the middle and upper third of the rectum, benign lesions are difficult to reach transanally, and standard radical surgical options, such as posterior trans-sphincteric resection, and low anterior resection can be offered to the patient; however, they have been associated with high morbidity, such as anastomotic leakage, sexual dysfunction, and fecal and urinary incontinence.

POSTERIOR APPROACH

The posterior approach (represented by a combination of coccyx removal and partial sphincter division) has been progressively abandoned. It was indicated for extensive lesions in the anterior rectal wall at a distance of 8-10 cm from the anal verge. Variable rates of complications and very high rates of recurrence have been reported. In a

Table 1 Resection of apparent adenoma polyps by ETAR

Ref.	Patients	ETAR	Recurrence	No. of complication patients	Failure	Follow-up (mo)	Related mortality
Tuech *et al*[11]	28	39	2	6	-	60 (2.5-10.5)	0
Beattie *et al*[12]	11	30	0	-	-	-	0
Wetherall *et al*[13]	23	38	2	-	1	4.5 (2-42)	0
Dickinson *et al*[14]	38	62	1	10	-	-	1
Sutton *et al*[15]	60	102	4	-	2	-	0
Bujanda *et al*[16]	13	13	1	2	-	15 (3-24)	-
Tsai *et al*[17]	131	180	27	7	-	60	0
Total	304	464	37	25	3		1

Table 2 Adenoma resected by TEM

Ref.	No. of lesions	Residual adenoma in surgical margin (%)	Local recurrence (%)	Follow-up (mo)
Steele *et al*[20]	77	9	5.1	7.4
Farmer *et al*[21]	36	25	5.6	33
Nakagoe *et al*[22]	9	0	0	d.n.o
Cocilovo *et al*[23]	56	1.7	3.5	d.n.o
Neary *et al*[24]	21	0	4.7	d.n.o
Katti *et al*[25]	58	7	10	34
Vorobiev *et al*[26]	113	-	8.3	29.5
Bretagnol *et al*[27]	148	14.9	7.6	33
Endreseth *et al*[28]	64	20	13	24
Zacharakis *et al*[29]	48	4.2	6.3	37
Schäfer *et al*[30]	33	18	12	36.4
Røkke *et al*[31]	56	10	0	12
Guerrieri *et al*[32]	530	-	4.3	44
McCloud *et al*[33]	75	37.3	16	31
Whitehouse *et al*[34]	146	5.5	4.8	39
Platell *et al*[35]	62	-	2.4	24
Lloyd *et al*[36]	68	9	5.9	28.7
Ganai *et al*[37]	82	10	15	44

d.n.o: Data not obtained.

review of 360 cases treated for benign and malignant lesions, fecal fistula was the most frequent complication in 5%-70% of patients and fecal incontinence in 5%-25%[3,9]. Adenoma recurrence rate was up to 33% and a stoma was necessary in 20%-70% of patients, as a temporary stoma to avoid fecal fistula or as a therapeutic stoma after its appearance[3,9].

ENDOSCOPIC TRANSANAL RESECTION (ETAR)

ETAR was first described in 1977 by Lindenschmidt *et al*[10] and has been used in the resection of rectal adenoma. A review of 304 cases (464 procedures) from seven studies, most of them in the United Kingdom, with probable double recording of patients (Table 1), suggests that ETAR is valuable in the resection of rectal adenoma with low morbidity and mortality. This technique has shown low recurrence, however, it is impossible to know if the resection is complete and the margins are free of tumor after various procedures in the same patient from whom the adenoma is resected piecemeal. ETAR does not require surgical assistance, anesthesia, extreme positioning, new technology or special training. Nevertheless, this technique provides limited resection of mesorectal fat and lymph nodes, limited histopathological information regarding extent of resection, and poor local disease control.

TRANSANAL ENDOSCOPIC MICROSURGERY (TEM)

TEM has been used successfully in the management of rectal adenoma and in selected cases of rectal carcinoma, since it was introduced by Professor Buess in 1984[18]. It has been suggested that TEM enables local excision of adenomas up to 24 cm from the anal verge, and offers a minimally invasive alternative to TE, radical surgery and recurrence from previously used endoscopic resection, with superior endoscopic magnification and illumination, accuracy and complete resection, and secure suture closure.

TEM has produced satisfactory results, with better recurrence rates and low morbidity and mortality. In one of the largest studies of adenoma resection using TEM, the authors reported a 3.4% early postoperative complication rate and 1.2% and 7% recurrence rates after 1 and 5 years, respectively, in a series of 286 cases[19]. In a review of 1682 adenoma resection procedures from 18 studies (Table 2), an average of 11% of the patients were found to have residual adenoma in the surgical margin, 6.3% had recurrence, and the complication rate was up to 11% during a follow-up period of 12 mo in one series, and > 24 mo in the rest of the studies.

The histologically positive resection margin is highly significant in terms of local recurrence rates after adenoma resection. In a review of 18 studies with a minimum follow-up of 12 mo (Table 2), I have found that residual adenoma in the surgical margin varied from 0% to 37.3%. However, the recurrence rate was 0%-15%, predominantly in cases with positive or uncertain resection margins. In three large series, it has been found that adenoma recurrence rate is high in the incompletely resected group (37.7%, 46% and 25%) compared with that in patients with free histological margins after adenoma resection (4.3%, 3% and 3.7%, respectively)[28,33,34]. In another two studies, adenoma extending to the surgical margin of locally excised polyps was found in 25%[21] and 30.7% of cases[38]. The latter was a large series of 238 patients (226 patients treated by TEM and 12 by TE). It is remarkable that, with such high positive-residual-margin rates, the recurrence rates in these studies were only 5.6% after 33 mo follow-up[21] and 3.6% after 67.5 mo follow-up[38], which are lower

than the rates reported in studies with lower positive-residual-margin rates. Røkke *et al*[31] did not observe adenoma recurrence after 12 mo follow-up in a series of patients in whom the residual adenoma in surgical margin was 10%.

Even for recurrent adenoma, TEM has now become an important alternative treatment. Five series have reported the use of TEM in the treatment of recurrent adenoma or residual disease without further recurrence[25,26,32,33,35].

In five studies that have compared adenoma resection with TEM and another procedure, the lower or similar recurrence, residual tumor and early complication rates favor TEM resection[22,38-40]. Late complication rates were higher after TEM because of the increased incidence of transient incontinence in the postoperative period[39,40]. Recently, Moore *et al*[41] have compared rectal adenoma excision with TEM and TE, and concluded that TEM is more effective than the other technique, because they found a significantly higher rate of clear margin with less fragmented specimens after TEM, with low complication and recurrence rates.

CONCLUSION

The transanal approach is a feasible, frequently used technique with few complications and low recurrence and mortality, but its use is limited to the lower rectum. The posterior approach with coccygeal resection, with or without sphincter section, can be mentioned only anecdotally in colorectal surgery. Probably very few surgeons are using it because of its high morbidity and recurrence and the use of colostomy. ETAR is a forgotten minimally invasive technique. It is a simple and safe method and technical expertise has already been acquired on urological conditions such as bladder and prostate tumors, however, it is used less frequently than it deserves for resection of rectal lesions. This is probably because few colorectal surgeons have much enthusiasm for the multiple sessions and piecemeal resection using a resectoscope. TEM offers better access to lesions in the middle and upper rectum, with superior endoscopic magnification and better illumination, superior visualization and pneumorectum, with accurate and complete resection allowing better histological analysis, and secure suture closure. The benefits of TEM are: few complications; low recurrence and mortality; less postoperative pain and use of analgesia; shorter time to the patient being able to walk, sit, eat and defecate; shorter hospital stay; and avoidance of colostomy. These benefits can compensate for the high price of the instruments.

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REVIEW

Secondary hepatic resection as a therapeutic goal in advanced colorectal cancer

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Abstract

Surgery is the only curative option for patients with liver metastases of colorectal cancer, but few patients present with resectable hepatic lesions. Chemotherapy is increasingly used to downstage initially unresectable disease and allow for potentially curative surgery. Standard chemotherapy regimens convert 10%-20% of cases to resectable disease in unselected populations and 30%-40% of those with disease confined to the liver. One strategy to further increase the number of candidates eligible for surgery is the addition of active targeted agents such as cetuximab and bevacizumab to standard chemotherapy. Data from a phase Ⅲ trial indicate that cetuximab increases the number of patients eligible for secondary hepatic resection, as well as the rate of complete resection when combined with first-line treatment with the FOLFIRI regimen. The safety profiles of preoperative cetuximab or bevacizumab have not been thoroughly assessed, but preliminary evidence indicates that these agents do not increase surgical mortality or exacerbate chemotherapy-related hepatotoxicity, such as steatosis (5-fluorouracil), steatohepatitis (irinotecan), and sinusoidal obstruction (oxaliplatin). Secondary resection is a valid treatment goal for certain patients with initially unresectable liver metastases and an important end point for future clinical trials.

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Key words: Colorectal cancer; Liver metastases; Liver resection; Cetuximab; Bevacizumab; Hepatotoxicity

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INTRODUCTION

Globally, one half of the nearly 1 million patients diagnosed with colorectal cancer annually will develop liver metastases during the course of the disease[1,2]. Autopsy findings suggest that approximately 50% of patients who die of colorectal cancer have liver metastases, which are the only site of metastatic disease in approximately 20%-30% of patients[3] and the cause of death in most of these patients[4]. In other types of cancer, liver metastases are a sign of distant dissemination, and surgery is not a curative option[5]. In colorectal cancer, however, portal vein drainage from the gastrointestinal tract to the liver favors metastasis to the liver without systemic dissemination[5]. Surgery therefore provides a potentially curative treatment option for patients with resectable liver disease[6], in contrast with palliative chemotherapy for those with unresectable disease.

One of the most important recent advances in the management of advanced colorectal cancer is the concept of downstaging initially unresectable disease using chemotherapy so that more patients become eligible for potentially curative surgery. Current treatment guidelines[4] and substantial research highlight the importance of increasing the rates of secondary resection in initially unresectable disease. The addition of active targeted therapies, such as cetuximab and bevacizumab, to chemotherapy may further increase secondary resection rates. This article summarizes the current data on secondary resection of initially unresectable liver metastases of colorectal cancer using currently available systemic therapy regimens.

HOW RESECTABILITY MAY BE ACHIEVED

Reported 5-year survival rates following hepatic resection generally range from 25% to 40% (Figure 1)[7-9], though

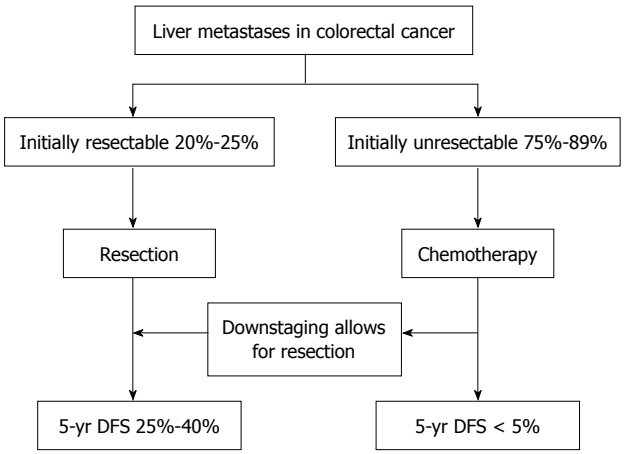

Figure 1 Breakdown of the incidence of liver metastases of colorectal cancer and expected outcomes with current treatment.

rates exceeding 50% have been observed in some studies[1,10,11], and these outcomes may be improved by the use of preoperative or postoperative chemotherapy or both[12,13]. In a recent phase Ⅲ trial, hepatic resection was compared with resection plus perioperative chemotherapy (6 cycles of FOLFOX4 before and 6 cycles after surgery) (Figure 2). Perioperative chemotherapy increased 3-year survival rates significantly in eligible patients (36.2% *vs* 28.1%) and resected patients (42.4% *vs* 33.2%), compared with surgery alone[2].

Unfortunately, 80%-90% of patients with liver metastases are considered to have unresectable disease at the time of diagnosis[4,7]. For them, modern chemotherapy and biologic agents afford median survival times hovering around 15 to 20 mo (Figure 1)[14-20].

Curative surgery remains an option if initially unresectable disease can be downstaged to allow for potentially definitive, ie, secondary, resection. Reported resectability rates after chemotherapy vary widely, depending on the patient population and definition of resectability. In unselected patients with unresectable liver metastases treated with chemotherapy, resection rates of 1% to 26% have been reported[21]. Higher resection rates (approximately 24%-54%) have been seen in patients with disease confined to the liver[21].

Outcomes following secondary resection are comparable to those observed after primary resection, making resectability a high-priority therapeutic goal. In a study of 872 patients with colorectal liver metastases, 701 (80%) were deemed to have unresectable disease. Of these patients, 95 (13%) ultimately underwent secondary resection after downstaging with chemotherapy. The 5-year survival rate in patients undergoing secondary resection was the same as that in patients undergoing primary resection at the same institution for initially resectable disease (34%)[7].

As investigators attempt to refine the use of resectability and collect information about resection as a clinical endpoint, a rough positive indicator of potential improvement in resectability rates is tumor shrinkage activity. It is therefore unsurprising that conversion to resectability correlates with response to

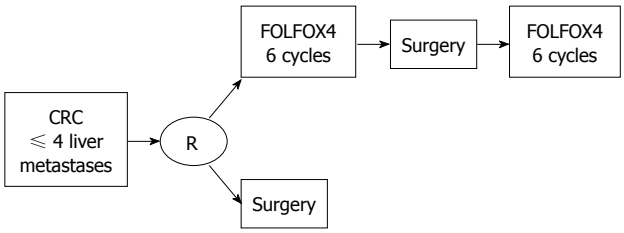

Figure 2 Schema of the EORTC 40 983 of perioperative chemotherapy[2].

chemotherapy[21]. A retrospective review of 305 patients treated with preoperative irinotecan- or oxaliplatin-based chemotherapy followed by resection of liver metastases showed that pathologic response to chemotherapy is an independent predictor of survival[22]. Importantly, these findings support an aggressive approach in which optimal management aims to put curative options within reach by tailoring systemic therapy to achieve the highest response rate possible, particularly in patients with borderline resectable disease.

SECONDARY RESECTION FOLLOWING STANDARD CHEMOTHERAPY

Oxaliplatin- and irinotecan-based regimens

Several combinations of 5-fluorouracil and leucovorin with either oxaliplatin or irinotecan have established efficacy and are accepted as standard treatment for advanced colorectal cancer[4,14,15,17,22,23]. These regimens can also lead to secondary resection in some patients with initially unresectable liver metastases. In unselected populations with advanced colorectal cancer, oxaliplatin-based therapy produces secondary resection rates of 15%-22% and complete resection rates (R0) of 9%-13% (Table 1)[14,23-25]. Among patients treated with irinotecan-based regimens, approximately 9% become eligible for surgery and 7% will achieve complete resection (Table 2)[14,23,26-28].

Comparative data from several studies suggest that oxaliplatin-based regimens may be more effective than irinotecan-based regimens in converting unresectable disease to resectable disease[14,29,30], although this finding has not always been consistent[23]. Importantly in these studies, median survival times for resected patients reach 42-47 mo.

Higher resection rates have been reported in studies of selected patients, such as those with liver metastases only. In a phase Ⅱ trial evaluating FOLFOX4 in patients with unresectable metastases confined to the liver, response rate was 60% and surgery was possible in 40% of patients[25]. Of the 17 patients who underwent surgery, 14 were free of residual disease (R0). The median survival was 26 mo. Additional studies have evaluated irinotecan-based regimens in selected patient populations; resection rates were generally 30%-40%[26-28].

FOLFOXIRI

More recently, several groups have evaluated the

Table 1 Outcomes related to secondary hepatic resection in patients treated with oxaliplatin-based chemotherapy

Study	*n*	Regimen	Response rate (%)	Resection rate (%)	R0 rate (%)	MS (mo)
Unselected populations						
GERCOR[14]	111	FOLFOX6	54.0	22.0	13.0	NYR
Tournigand *et al*[24]	311	FOLFOX4	58.5	17.7	11.3	38.9
Tournigand *et al*[24]	309	FOLFOX7[1]	59.2	15.2	9.4	43.0
Colucci *et al*[23]	182	FOLFOX4	34.0	4.4	NR	NR
Liver metastases only						
Alberts *et al*[25]	44	FOLFOX4	60.0	40.0	33.3	NR

[1]FOLFOX7 × 6 cycles, followed by treatment without oxaliplatin × 12 cycles, followed by FOLFOX7 until PD. MS: Median survival in resected patients; FOLFOX: Oxaliplatin plus infusional 5-fluorouracil/leucovorin (5-FU/LV); NYR: Not yet reached; NR: Not reported.

Table 2 Outcomes related to secondary hepatic resection in patients treated with irinotecan-based chemotherapy

Study	*n*	Regimen	Response rate (%)	Resection rate (%)	R0 rate (%)	MS (mo)
Unselected populations						
Tournigand *et al*[14]	109	FOLFIRI	61.0	9.0	7.0	47
Colucci *et al*[23]	178	FOLFIRI	36.0	5.1	NR	NR
Liver metastases only						
Pozzo *et al*[26]	40	5-FU/LV, irinotecan	47.5	40.0	32.5	NYR
Zelek *et al*[27]	31	5-FU/LV, irinotecan, and HAI of pirarubicin	48.0	35.0	29.0	NYR
Ho *et al*[28]	40	5-FU/LV, irinotecan	55.0	10.0	NR	NR

HAI: Hepatic arterial infusion.

combination of 5-fluorouracil, oxaliplatin, and irinotecan (FOLFOXIRI)[16,31,32], with the rationale of maximizing concurrent exposure to multiple active agents[14,33]. In a preliminary study in 39 patients with unresectable liver metastases, the response rate was 64% and secondary surgery was possible in 23 patients (59%)[32]. Of these patients, 84% achieved R0 resection.

Two randomized trials have compared FOLFOXIRI with FOLFIRI as first-line therapy for patients with initially unresectable advanced colorectal cancer[16,31]. Although the first study, of 283 patients, found no significant difference between the two treatment groups in response or survival[31], the second study, involving 244 patients, found that FOLFOXIRI significantly increased response rates (60% *vs* 34%) and median survival (22.6 mo *vs* 16.7 mo)[16]. Both studies, however, showed that FOLFOXIRI increased secondary resection rates compared with FOLFIRI. Significantly more patients treated with FOLFOXIRI in the first study were eligible for secondary resection (14 *vs* 6)[31]. This included 14 patients with liver metastases (11 *vs* 3), of whom 11 achieved R0 resection (9 *vs* 2). In the second study, the rate of R0 secondary resection was significantly greater with FOLFOXIRI (15% *vs* 6%) overall and in those with liver metastases only (36% *vs* 12%)[16].

Both randomized trials reported increased toxicity with FOLFOXIRI compared with FOLFIRI, including higher incidences of alopecia, diarrhea, neutropenia, and neurotoxicity, a factor to weigh against the benefits achieved[16,31]. Further investigation is needed to identify new strategies for increasing rates of secondary resection without increasing toxicity associated with standard chemotherapy agents.

IMPROVING ON STANDARD CHEMOTHERAPY: ADDITION OF BIOLOGICS

Two monoclonal antibodies are approved for use in advanced colorectal cancer that have been shown to improve outcomes when combined with chemotherapy. Combining these agents with standard chemotherapy may represent a safe and effective strategy for increasing the proportion of patients eligible for potentially curative surgery.

Cetuximab

Cetuximab blocks the activity of the epidermal growth factor receptor (EGFR) and is approved for use in the United States in combination with irinotecan in patients with irinotecan-refractory disease, or as monotherapy for patients who have failed both irinotecan- and oxaliplatin-based chemotherapy. Adam *et al*[34] assessed the ability of cetuximab-based therapy to downstage patients with unresectable disease and liver metastases refractory to prior chemotherapy to become eligible for surgery with curative intent. A total of 151 patients were treated either completely at the Paul Brousse Hospital (PBH, n = 133) or referred to PBH for hepatectomy after receiving cetuximab-based therapy elsewhere (n = 18). Of the 151 patients, 27 underwent surgery, of whom 20 had received cetuximab plus irinotecan; four, cetuximab plus oxaliplatin; and one, all three agents. Two-thirds of patients who underwent surgery had had at least two prior chemotherapy regimens. Of the 133 patients treated completely at PBH, 9 (7%) underwent surgery, which is encouraging, considering these were heavily pretreated and refractory patients.

Table 3 Randomized trials comparing first-line chemotherapy with or without cetuximab or bevacizumab that report secondary resection rates

Study	*n*	Regimen	Response rate (%)	Resection rate (%)	R0 rate (%)	R0 rate in liver-only disease (%)	PFS (mo)
Cetuximab							
CRYSTAL[37]	559	FOLFIRI	38.7	2.5	1.5	4.5	8.0
	559	FOLFIRI + cetuximab	46.9	6.0	4.3	9.8	8.9
Bevacizumab							
Hurwitz *et al*[38]	411	IFL	34.8	< 2%	NR	NR	6.2
	402	IFL + bevacizumab	44.8	< 2%	NR	NR	10.6
NO16966[39,40]	701	CT[1]	49.0	4.9	NR	11.5	8.0
	699	CT[1] + bevacizumab	47.0	6.3	NR	12.3	9.4

[1]FOLFOX4 or XELOX. PFS: Progression-free survival in the study arm; FOLFIRI: Irinotecan plus infusional 5-fluorouracil/leucovorin (5-FU/LV); IFL: Irinotecan, fluorouracil, and leucovorin; CT: Chemotherapy.

In the first-line setting, an initial phase Ⅰ/Ⅱ trial of 21 patients with unresectable, EGFR-expressing disease who received cetuximab plus 5-fluorouracil, leucovorin, and irinotecan found a 67% response rate with median survival of 33 mo[19]. Five patients (24%) were eligible for secondary resection. A phase Ⅱ study evaluated first-line cetuximab plus FOLFOX4 in 43 patients with unresectable, EGFR-expressing disease[35]. Confirmed responses were seen in 72% of patients and the disease control rate was 95%. Ten patients (23%) underwent potentially curative surgery and nine (21%) had no evidence of residual disease after surgery. Overall, the median survival was 30 mo.

A randomized phase Ⅱ trial comparing FOLFOX4 with or without cetuximab (the OPUS trial) found that cetuximab significantly increases response rates in patients with good performance status when added to standard first-line chemotherapy (49.0% *vs* 36.8%)[36]. Resection rates were higher with cetuximab (6.5% *vs* 3.6%), as were R0 resection rates (4.7% *vs* 2.4%)[36]. Cetuximab was associated with an increase in skin reactions, infusion-related reactions, and hypomagnesemia, but no exacerbation of oxaliplatin-related toxicities.

The CRYSTAL study (n = 1198) investigating the addition of cetuximab to FOLFIRI was the first phase Ⅲ trial to demonstrate that adding a therapeutic monoclonal antibody to standard chemotherapy may improve secondary resection rates (Table 3)[37-40]. Cetuximab significantly increased the overall secondary resection rate (6% *vs* 2.5%), and boosted the rate of complete resection (R0) 3-fold [4.3% *vs* 1.5%; odds ratio 3.0 (95% CI 1.4-6.5)]. As expected, higher rates of R0 resection were seen in the subgroup of patients with disease confined to the liver (approximately 20% of patients in each arm), for whom the addition of cetuximab also appeared to increase R0 resection rates compared with FOLFIRI alone (9.8% *vs* 4.5%) and was associated with a significant increase in progression-free survival (median, 11.4 mo *vs* 9.2 mo)[37].

The second key finding of CRYSTAL revealed that the benefit from cetuximab was concentrated in the subgroup of patients with wild-type K-RAS on their tumors (64.4% of the initial evaluable population); this population experienced a reduction in the risk of progression of 32% (HR = 0.68, P = 0.017), and 16% higher response rate (59.3% *vs* 43.2%, P = 0.0025)[42] compared with those with mutant K-RAS. The effect on patients with disease confined to the liver was also dramatic, with the response rate reaching 77% in the population with K-RAS wild type[41].

These promising observations have propelled more focused investigation on the role of cetuximab as a conversion or downstaging agent. The CELIM study was a randomized phase Ⅱ study (n = 111) in which patients with unresectable liver metastases received cetuximab with either FOLFOX or FOLFIRI. Of 106 patients evaluable for efficacy, a response rate of 74% was reported in both arms combined, 79% in those with K-RAS wild-type tumors. Confirmed response (by second CT scan according to RECIST criteria or by resection) rate was 62% in both arms combined, 70% in those with K-RAS wild-type tumors. The rate of resection was an encouraging 46%, and 34% of patients achieved R0 resections. Nineteen of 75 patients (25%) with unresectable disease at enrollment had resectable disease after 16 wk of therapy (P = 0.021)[42].

Bevacizumab

Bevacizumab binds to vascular endothelial growth factor (VEGF) and is approved for use in combination with 5-fluorouracil-based chemotherapy as first- or second-line treatment for metastatic colorectal cancer. This agent has been shown to improve outcomes when added to first-line chemotherapy for advanced colorectal cancer, increasing response rates by approximately 10% compared with chemotherapy alone and producing median survival rates of approximately 17-24 mo[20,38,43]. Nonrandomized trials evaluating the combination of bevacizumab and oxaliplatin-based chemotherapy as neoadjuvant therapy in patients with resectable disease have also produced promising results[13,44].

Data from phase Ⅲ trials suggest that bevacizumab does not increase secondary resection rates when added to standard chemotherapy (Table 3), both for irinotecan-based and oxaliplatin-based therapy, although the resected patients in bevacizumab are low to extract any firm conclusion[38,39].

A non-randomized, uncontrolled phase Ⅳ study

(first BEAT) prospectively collected data on resection rates following bevacizumab plus various chemotherapy regimens as first-line treatment for advanced colorectal cancer. Of the 1914 evaluable patients, 215 (11.2%) underwent surgery after receiving systemic therapy and 170 (8.8%) achieved R0 resection. Preliminary data suggest a 2-year survival rate of 44% in the entire patient population and 82% in those who achieved R0 resection[40].

Based on the findings described above, it appears that bevacizumab does not compromise the feasibility of secondary resection of metastatic disease; it is unclear, however, whether adding bevacizumab has the potential to improve upon the resectability rates achieved with chemotherapy alone[40].

THE ROLE OF LOCAL THERAPIES IN THE MANAGEMENT OF LIVER METASTASES

Local treatments may be combined with surgery with the potential to improve upon the therapeutic benchmarks achieved with surgery alone. Abdalla *et al*[1] reported on the use of radiofrequency ablation (RFA), alone or in combination with surgery in 158 patients in whom R0 resection was not possible. Outcomes were far superior for the 190 patients who underwent complete resection, with roughly double survival rates at 4 years (65% *vs* 22%-36% with RFA alone or RFA plus surgery). RFA seemed to offer a modest advantage over palliative chemotherapy only.

Stereotactic body radiation (SBRT) is a local treatment approach that may enhance outcomes in patients with liver metastases[45], but it is too early to determine its relative efficacy compared with other modalities. A recent retrospective comparison of outcomes after surgery, RFA, or SBRT in patients whose liver disease recurred after initial partial resection, revealed equivalent outcomes across the three approaches[46]; well-controlled prospective comparisons are lagging.

Finally, selective internal radiation therapy (SIRT), using Yttrium-90 microspheres[47], is also a feasible technology awaiting validation in this disease setting.

TREATMENT DECISIONS, TOXICITY, FEASIBILITY AND OTHER CONCERNS

Considering the importance of achieving resectability as a therapeutic goal, its accurate assessment is key to delineate management strategies, and the availability of increasingly sophisticated diagnostic tests may help optimize evaluation approaches. Positron-emission tomography combined with computed tomography (PET-CT) with [^{18}F]-fluoro-2'-deoxy-D-glucose (FDG) has an important role in staging patients with colorectal cancer, and in ruling out extrahepatic metastases in advanced disease, although prior chemotherapy may lower its sensitivity[48]. MRI is used for further evaluation of the liver in patients with metastatic colorectal cancer. A 2008 study of 20 consecutive patients with colorectal cancer compared whole-body MRI *vs* PET-CT for staging of lymph nodes and distant metastases. Results suggested a preference for PET-CT in diagnosing lymph nodes, but a trend toward superiority for whole-body MRI in detecting metastases in liver, brain, and bone[49]. Finally, intraoperative ultrasound (IOUS) is a standard method to determine resection margin and identify previously undetected tumors[50-52]. Contrast-enhanced IOUS offers greater sensitivity, increasing identification of new metastatic lesions by approximately 20%[50,53,54], and allows more time to find new metastases intraoperatively[51]. Zalinski *et al*[55] developed a new marking technique to help identify resection margins intraoperatively following preoperative chemotherapy: coils are placed behind the deep margin of lesions using a guide needle and CT or ultrasound guidance prior to systemic chemotherapy. The coils are then easily detected during surgery.

The relevance of resectability in disease prognosis recently became clearer. Patients with resectable metastases either hepatic or extrahepatic (but not both) had a 5-year overall survival rate of 54%, compared with 13% for patients with both resectable hepatic and extrahepatic disease and 0% for unresectable metastases, regardless of location[56]. The authors proposed the inclusion of resectability as a criterion to stratify stage Ⅳ colorectal cancer.

The degree of optimization of downstaging strategies in routine clinical practice, however, remains unclear. In a recent review of managed care records of nearly 500 patients with colorectal cancer who underwent hepatic resection, only 20% had received preoperative chemotherapy[57]. It is not known how many patients initially diagnosed with unresectable liver metastases are re-evaluated by a liver surgeon after first-line therapy to determine whether the patient's resectability status has changed. Several authors have emphasized the importance of a multidisciplinary approach to the management of colorectal liver metastases and close cooperation among radiologists, medical oncologists, and surgeons with expertise in liver resection[4,7,58].

Chemotherapy-related hepatotoxicity

Due to recent advances in surgical techniques and postoperative care, the safety of hepatic resection for colorectal liver metastases has improved considerably[1]. Despite the trend toward increasing extensiveness of resection, surgical mortality rates remain less than 5%[8,59]. In addition, use of portal vein embolism (PVE), a well-established and well-tolerated procedure, has been shown to reduce the risk of postoperative liver failure, with a low rate of complications in resection of colorectal liver metastases[60,61].

However, systemic therapy may be associated with liver injury and affect surgical outcomes. Chemotherapy is known to cause pathologic changes in normal liver tissue, modifying gross appearance and impairing parenchymal hemostasis and regenerative capacity[2,12,62]. The impact of these effects on clinical outcomes in patients undergoing hepatic resection is unclear. In the EORTC 40983 trial

Table 4 Liver injury by chemotherapy regimen[12]

Regimen	Sinusoidal dilation[1] (n = 22)			Steatosis > 30% (n = 36)			Steatohepatitis[2] (n = 34)		
	Yes (%)	No (%)	P value[3]	Yes (%)	No (%)	P value[3]	Yes (%)	No (%)	P value[3]
No CT	2	98		9	91		4	96	
FU	0	100	NS	17	83	NS	5	95	NS
IRI	4	96	NS	11	89	NS	20	80	0.001
OX	19	81	0.00001	4	96	NS	6	94	NS
Other	0	100	NS	8	92	NS	0	100	NS

[1]Rubbia: Brandt grade 2 or 3; [2]Kleiner score ⩾ 4; [3]Presence of liver injury characteristic; comparison of each chemotherapy group *vs* no chemotherapy. FU: Fluorouracil; IRI: Irinotecan; OX: Oxaliplatin; NS: Not significant.

(Figure 2), six cycles of preoperative chemotherapy did not increase the rate of surgical mortality compared with surgery alone. Chemotherapy was associated with an increase in reversible postoperative morbidity that was generally within the expected range for hepatic resection (25% *vs* 16%)[2]. Karoui *et al*[63] retrospectively compared outcomes following hepatic resection in 45 patients who received preoperative chemotherapy and 22 who received no preoperative chemotherapy. Treatment with chemotherapy was associated with an increase in postoperative morbidity. Interestingly, morbidity correlated with the number of cycles of preoperative therapy given (⩾ 6 cycles *vs* < 6 cycles), but not the type of chemotherapy used.

Others have reported that different chemotherapy regimens cause different types of liver injury, with varying effects on clinical outcomes (Table 4)[12]. Treatment with 5-fluorouracil-based treatment has been associated with an increase in steatosis, which is associated with increased postoperative morbidity[58,64]. In addition, longer duration of 5-fluorouracil-based therapy (⩾ 9 cycles *vs* 1-8 cycles) was associated with significantly increased incidence of sinusoidal injury (42% *vs* 26%; $P = 0.017$) and postoperative liver insufficiency (11% *vs* 4%; $P < 0.035$), and was an independent predictor of postoperative liver insufficiency ($P = 0.031$; odds ratio, 3.90)[65]. Irinotecan has been associated with steatohepatitis, which in turn was correlated with increased mortality following hepatic resection, particularly due to liver failure[12]. Oxaliplatin therapy does not induce steatosis or steatohepatitis, but increases the risk of developing vascular lesions and sinusoidal obstruction syndrome; a clear link between these effects and postoperative morbidity or mortality rates has not been established[12,66].

Given the varying effects of different chemotherapy agents on liver tissue, both the efficacy and safety of available regimens should be considered when selecting an appropriate regimen for use in an individual patient. It also is advisable limiting the number of cycles of preoperative chemotherapy in order to avoid extensive liver injury, and to perform surgery as soon as possible after achieving resectability.

Cetuximab and hepatic outcomes

The most common adverse event associated with cetuximab treatment is skin reactions. Less common but potentially serious adverse events include infusion reactions, cardiopulmonary arrest, and hypomagnesemia.

There is little data specifically on the effects of cetuximab on the liver in this setting. In Adam *et al*[34], nine patients who became eligible for surgery after cetuximab-based therapy had evidence of liver injury, but this was not attributable to cetuximab. Overall, one patient died postoperatively and approximately 50% of patients had surgical complications.

The addition of cetuximab does not appear to increase the incidence of adverse events typically associated with oxaliplatin-based therapy, such as neutropenia, diarrhea, fatigue, or neurotoxicity[35,36]. In the CRYSTAL trial, the addition of cetuximab to FOLFIRI in the first-line setting did not increase all-cause mortality rates, and there were no deaths attributed to cetuximab. Patients who received cetuximab and FOLFIRI had higher incidences of diarrhea and cetuximab-related skin reactions[37].

Bevacizumab and hepatic outcomes

Treatment with bevacizumab is associated with increased incidence of hypertension and relatively low rates of certain potentially serious events, such as bleeding, gastrointestinal perforation, and arterial thromboembolism[20,38,40]. Recent results have found that bevacizumab reduces the incidence of oxaliplatin-related sinusoidal injury, although the exact mechanism for this effect is unknown[44,65]. It has been suggested that the antiangiogenic effects of bevacizumab can interfere with hepatic regeneration and wound healing, which is relevant to its use in the perioperative setting[13,67]. To avoid surgical morbidity, it is currently recommended to stop bevacizumab therapy at least 6 wk before surgery and resume treatment 28 d or more after surgery, provided that all incisions have healed completely[4,13,67]. Reddy *et al*[68] reported that complications are more prevalent in patients who undergo surgery within 8 wk of stopping bevacizumab, compared with those who discontinue bevacizumab more than 8 wk before surgery (62.5% *vs* 30.4%). In a study of preoperative chemotherapy plus bevacizumab, Gruenberger *et al*[13] reported that when bevacizumab was discontinued about 5 wk before surgery, the resulting rates of complications were similar to that achieved in a historical control group of patients who received chemotherapy alone. Whether the need to discontinue bevacizumab for several weeks perioperatively hinders its ability to allow for complete resection is unknown.

Table 5 Secondary resection in patients with initially unresectable liver metastases

Improve patient selection through early and continued consultation in a multidisciplinary team approach, including close cooperation among a radiologist, medical oncologist, and surgeon with experience in liver resection
Conduct surgical evaluation at baseline and, if disease is initially unresectable, reevaluation at intervals during therapy to determine if conversion to resectability has been achieved
Set appropriate goals of therapy (best response, conversion to resectable disease, or palliation)
Determine length of therapy, with consideration for the risk of potential toxicities
Consider the safety profile of individual agents and the risks of overtreatment, including hepatotoxicity
If the treatment goal is conversion to resection, treat to resectability and not to best response

CONCLUSION

The use of systemic therapy to downstage unresectable liver metastases to achieve resectability offers a curative option with long-term outcomes similar to those achieved with primary resection. Therefore, secondary resection is a valid treatment goal for certain patients with initially unresectable liver metastases and an important end point for future clinical trials (Table 5).

Refinement of available first-line treatment options may increase secondary resection rates. Because response rates correlate with secondary resection rates, aggressive approaches that increase the likelihood of preoperative response seem warranted. The blurring of first-line therapy and neoadjuvant therapy underscores the need for clear definitions of treatment approach, ie, curative versus palliative, and the goals of therapy, e.g. best response, conversion to resectable disease, and palliation. Multidisciplinary involvement in the management of the patient is essential to implement a continued surgical evaluation approach as patients with borderline resectable/unresectable disease undergo systemic treatment.

The benefits of aggressive chemotherapy to downstage liver metastases should be weighed against the potential increase in adverse events, particularly those related to liver injury and surgical outcomes. Different chemotherapy agents have different effects on the liver. To avoid excessive liver injury and postoperative complications, the safety profile of individual agents should be considered when selecting a preoperative regimen; the duration of preoperative chemotherapy should be kept to a minimum; and surgery should be performed as soon as resectability has been achieved.

The addition of targeted therapies to current first-line chemotherapy platforms may improve secondary resection rates. The two leading candidates for this approach are cetuximab and bevacizumab. Data from a randomized trial indicate that cetuximab increases secondary resection rates when added to irinotecan-based therapy. The addition of cetuximab to preoperative chemotherapy does not appear to increase hepatotoxicity or mortality compared with chemotherapy alone. When discontinued at least 6 wk before surgery and reinitiated at least 28 d after surgery, bevacizumab also appears to be a safe treatment option in this setting, and there is some evidence to suggest that bevacizumab may reduce the risk of sinusoidal injury. Current studies evaluating cetuximab, bevacizumab, and both in combination are underway and will help to further refine their role in the preoperative setting.

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Overexpression of β3/γ2 chains of laminin-5 and MMP7 in biliary cancer

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Abstract

AIM: To clarify the clinicopathological significance of laminin-5 γ2 (LNγ2) and β3 (LNβ3) chains and MMP7 expression in biliary tract cancer.

METHODS: We analyzed the association between immunohistochemically detected LNγ2, LNβ3, and MMP7 expression in biliary tract cancer and clinicopathological characteristics. Activity of MMP7 was analyzed by casein zymography. An *in vitro* invasion assay after treatment with MMP7-specific siRNA was performed.

RESULTS: LNγ2 expression was predominantly observed in carcinoma cells at the invasive front. LNγ2 expression was seen in 57% of patients with biliary tract cancer, and was associated with depth of invasion, histologic type, and advanced stage. The expression pattern of LNβ3 was classified into two types: invasive front dominant type (38%) and diffuse type (28%). The invasive front dominant type was associated with histologic type and advanced stage. MMP7 positivity was correlated with LNγ2 or LNβ3 expression but not with clinicopathological characteristics. Active MMP7 detected by casein zymography was correlated with depth of invasion and advanced stage. Downregulation of MMP7 expression by siRNA resulted in a significant decrease in biliary tract cancer cell invasion *in vitro*.

CONCLUSION: Our results suggest that LNγ2 and LNβ3, in conjunction with MMP7, play a key role in the progression of biliary tract cancer.

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Key words: Biliary tract cancer; Laminin-5; Laminin γ2; Laminin β3; MMP7

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INTRODUCTION

Despite recent advances in diagnosis and treatment, the prognosis of patients with biliary tract cancer is still poor. Surgical resection is possible in only a small proportion of patients[1,2]. Consequently, elucidating the biological characteristics of these carcinomas has become necessary to improve the prognosis of patients and to devise better treatment strategies.

Laminins are components of the extracellular matrix (ECM) that contribute to the architecture of the basal lamina surrounding the epithelial cells and mediate cell adhesion, growth, migration, proliferation, and differentiation. Laminins are heterotrimeric glycoproteins composed of three different polypeptide

chains (α, β and γ) arranged in a cruciform structure. A separate gene encodes each polypeptide chain and different combinations of these chains lead to the 15 different laminin isoforms[3-5]. Laminin-5/laminin-332 (LN5), consists of α3, β3, and γ2 chains, which are encoded by three distinct genes (*LAMA3*, *LAMB3*, and *LAMC2*, respectively)[6]. LN5 has been shown to promote the adhesion, migration, and scattering of a variety of cultured cells, mainly through integrin α3β1, more strongly than other ECM proteins[7]. Moreover, in hepatocellular carcinoma (HCC), LN5 reportedly plays an important role in epithelial mesenchymal transition through down-regulation of E-cadherin and translocation of β-catenin into the nuclei[8].

Expression of the three subunits of LN5 is regulated differentially in cancer cell lines and in normal and malignant tissues including HCC[9-11]. Indeed, LNγ2 has been shown to be secreted as a single subunit in gastric cancer[12]. Several lines of evidence suggest that the tumor-derived LNγ2 contributes to invasion of tumor cells. LNγ2 expression has been immunohistochemically detected in various types of carcinomas, such as HCC, colorectum, stomach, and esophagus[9,12-16]. It is notable that LNγ2 has been predominantly detected at the invasive front, where tumor cells with the most aggressive phenotype are localized[17].

Degradation of ECM components is mostly controlled by proteolytic enzymes called matrix metalloproteinases (MMPs)[18]. Specific cleavage of LN5 (γ2 subunit at residue 587) by MMP2 has been shown to induce migration of breast epithelial cells[19]. This altered form of LN5 was found in tumors and in tissues undergoing remodeling, but not in quiescent tissues. LN5 is also converted into a migration-promoting substrate by MT1-MMP[20]. MMP7, also known as matrilysin, is a "minimal domain MMP" that exhibits broad proteolytic activity against components of the ECM and non-ECM[18]. MMP7 is often overexpressed at the invasive front in various types of human cancer and is associated with cancer progression[21,22]. Both LNγ2 and MMP7 are targets of the Wnt/β-catenin pathway[23,24].

Although there are only a few reports regarding LNβ3 expression in human cancer, coexpression of LNγ2 and LNβ3 has been reported in HCC, squamous cell carcinoma of the tongue and colorectal carcinoma and basal cell carcinoma of the skin[9,25,26]. It has recently been reported that human LN5 is a ligand for MMP7 and that a specific cleavage occurs in its β3 chain[27]. These results are interesting because MMP7 is overexpressed in HCC and colorectal carcinoma[18,28]. However, expression of LN5 and MMP7 in biliary tract cancer has not been clearly addressed.

To clarify the possible involvement of LN5 and MMP7 in the progression of biliary tract cancer, we immunohistochemically analyzed these expressions in 61 primary biliary tract cancer. Activity of MMP7 was analyzed by casein zymography. An *in vitro* invasion assay of biliary tract cancer cell lines after treatment with MMP7-specific siRNA was performed.

MATERIALS AND METHODS

Cell lines and tissue samples

Human bile duct cancer cell lines (TFK-1, HuH-28, and MEC) were obtained from Cell Resource Center for Biomedical Research, Tohoku University. Bile duct cancer cell line TKKK and gallbladder carcinoma cell lines TGBC1TKB, TGBC2TKB, and TGBC14TKB were purchased from Riken Cell Bank (Tsukuba, Japan). Cells were cultured in RPMI1640 or DMEM supplemented with 10% fetal bovine serum. Formalin-fixed, paraffin-embedded sections of 61 biliary tract carcinomas (30 extrahepatic bile duct carcinomas, 18 gallbladder carcinomas, 13 carcinomas of the ampulla of Vater) were used for immunohistochemically. Sections containing the most invasive part of each tumor were used. Fresh specimens of extrahepatic bile duct carcinoma (n = 10), gallbladder carcinoma (n = 7), and carcinoma of the ampulla of Vater (n = 3) were obtained from patients who had undergone surgical treatment. Specimens were immediately frozen in liquid nitrogen at the time of surgery and stored at -80℃. Each tissue specimen was used for casein zymography. Histopathological features of the specimens were classified according to the pathological tumor-node-metastasis (TNM) classification system of the International Union Against Cancer. Informed consent was obtained from each subject and the institutional review committee approved the experiments.

Semi-quantitative reverse transcriptase-PCR (RT-PCR) and real-time RT-PCR

Semi-quantitative RT-PCR was carried out as described previously[29]. Total RNA was extracted from cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using SuperScript Ⅲ reverse transcriptase (Invitrogen) with random hexamers. PCR was performed using primers specific for the *LAMA3*, *LAMB2* and *LAMC2* gene and the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene. *GAPDH* served as an internal control of the reaction. Standard curves for semiquantitative RT-PCR were drawn as described previously[30]. All reactions were carried out at least in duplicate and controlled without reverse transcriptase. PCR products were electrophoresed in 2% agarose gels. Real-time RT-PCR was performed using the TaqMan real-time PCR system as described previously[31].

Immunohistochemistry

Immunohistochemistry was carried out as described previously[32]. The antibodies used were as follows: anti-LNα3 rabbit polyclonal antibody (1/100 dilution, Santa Cruz, CA, USA), anti-laminin5 (γ2 chain) mouse monoclonal antibody (1/50 dilution, Chemicon, Temecula, CA, USA), anti-LNβ3 rabbit polyclonal antibody (1/100 dilution, Santa Cruz), and anti-MMP7 mouse monoclonal antibody (1/50 dilution, Daiichi Fine Chemical, Takaoka, Japan). Normal mouse or rabbit

immunoglobulins were substituted for each primary antibody as negative controls. Cytoplasmic expression was defined as positive when immunoreactivity was observed in more than 10% of carcinoma cells. We defined the cells at the deepest invading part of the tumor as the invasive front.

Casein zymography

Casein zymography was performed as previously described with some modifications[28]. Tissue extracts were electrophoresed on 8% polyacrylamide gel containing 1 mg/mL casein. After electrophoresis, gels were washed in 2.5% Triton-X 100 and incubated for 48 h at 37℃ in 50 mmol/L Tris-HCl (pH 7.4), 10 mmol/L $CaCl_2$, 1 mmol/L $ZnCl_2$, and 0.02% NaN_3, followed by staining with 0.1% Coomassie brilliant blue.

siRNA transfection

siRNA transfection was performed as described previously[33]. Levels of MMP7 inhibition were analyzed by RT-PCR and Western blotting. siRNA-transfected cells were used for the *in vitro* invasion assay.

In vitro invasion assay

Assays were performed by the modified Boyden Chamber method as described previously[34]. Assays were also performed with 250 ng/mL of TIMP1, an MMP inhibitor. The results were presented as means ± SD for each sample.

Statistical analysis

Expression was assessed for associations with clinicopathological parameters using the chi-square two-tailed test or Fisher's exact test. A P value < 0.05 was considered statistically significant. A P value between 0.05 and 0.10 was considered as a trend toward an association.

RESULTS

Expression of LNα3, LNβ3 and LNγ2, and MMP7 in cell lines

Expression levels of LNα3, LNβ3 and LNγ2, and MMP7 in cancer cell lines were analyzed using semi-quantitative RT-PCR (Figure 1). LNα3 mRNA was detected in all seven cell lines but at very low levels in TGBC-14TKB. LNβ3 mRNA was detected in six cell lines but at very low levels in MEC and TKKK. LNγ2 mRNA was detected in all seven cell lines but at very low levels in MEC. TFK-1, HuH-28, TGBC-1TKB, and TGBC-2TKB expressed considerable amounts of all 3 chains of LN5. MMP7 mRNA was detected in all seven cell lines. There were no significant correlations between these expression patterns and characteristics of the cell lines. Similar data were observed by real-time RT-PCR (data not shown).

Overexpression of LNγ2, LNβ3, and MMP7 in biliary tract cancer tissues

Expression of LNα3 was detected in normal basement membranes but not in carcinoma cells (data not shown). Figure 2 shows representative results of immunohistochemistry for LNγ2 in biliary tract cancer. In carcinoma tissues, the cytoplasm of carcinoma cells was stained for LNγ2 at levels much stronger than those in normal basement membranes. The cytoplasmic immunoreactivity was often more intense at the invasive front. Cancer cells budding or dissociating from the tumor nests showed intense cytoplasmic staining. Sections with immunostaining signals in over 10% of carcinoma cells, which were observed in 35 (57%) of 61 cases, were judged to be positive for LNγ2. LNγ2 positivity was 67% in extrahepatic bile duct cancer, 50% in gallbladder cancer, and 46% in carcinoma of the ampulla of Vater. Figure 3 shows representative results of immunohistochemistry for LNβ3 in biliary tract cancer. In carcinoma tissues, the cytoplasm of carcinoma cells was stained for LNβ3 at levels much stronger than those in normal basement membranes. The expression pattern of LNβ3 was classified into 2 patterns: invasive front dominant pattern and diffuse pattern. The invasive front dominant pattern and diffuse pattern were observed in 23 (38%) and 17 (28%) of 61 cases, respectively. Positivity for invasive front dominant pattern and diffuse pattern was 47% and 17% in extrahepatic bile duct cancer, 28% and 39% in gallbladder cancer, and 31% and 38% in carcinoma of the ampulla of Vater. Figure 4 shows representative results of immunohistochemistry for MMP7 in biliary tract cancer tissues. MMP7 immunoreactivity was intense at the invasive front in several cases. In general, MMP7 immunoreactivity was diffuse rather than invasive front dominant like LNγ2. Sections with immunostaining signals in over 10% of carcinoma cells, which were observed in 42 (69%) of 61 cases, were judged to be positive for MMP7. MMP7 positivity was 80% in extrahepatic bile duct cancer, 50% in gallbladder cancer, and 69% in carcinoma of the ampulla of Vater.

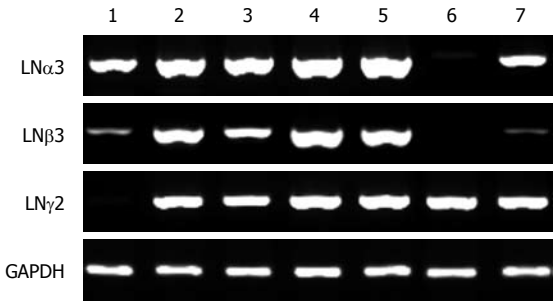

Figure 1 RT-PCR analysis of the *LNα3*, *LNβ3*, *LNγ2* genes in biliary tract cancer cell lines. 1: MEC; 2: TFK-1; 3: HuH28; 4: TGBC1TKB; 5: TGBC2TKB; 6: TGBC14TKB; 7: TKKK.

Association of LNγ2, LNβ3, and MMP7 expression with clinicopathological characteristics

The relationship between LNγ2 positivity and clinicopathological characteristics is summarized in Table 1. LNγ2 positivity was significantly correlated with histologic type (less differentiated type), depth of invasion

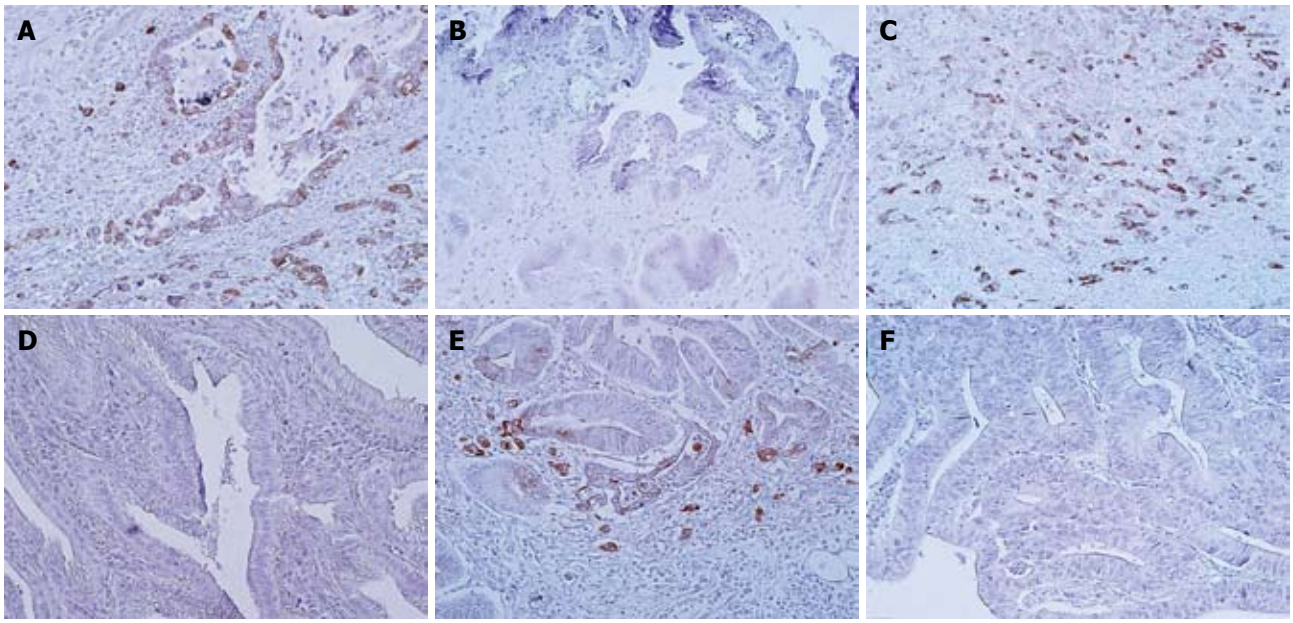

Figure 2 LNγ2 expression in biliary tract cancer tissues. A, B: Extrahepatic bile duct cancer tissues; A: Moderately differentiated tubular adenocarcinoma positive for staining. Note that LNγ2 is strongly positive in tumor cells at the invasive front; B: Papillary adenocarcinoma negative for staining; C, D: Gallbladder cancer tissues; C: Moderately differentiated tubular adenocarcinoma positive for staining; D: Well differentiated tubular adenocarcinoma negative for staining; E, F: Carcinoma tissues of the ampulla of Vater; E: Moderately differentiated tubular adenocarcinoma positive for staining. Note that LNγ2 is strongly positive in tumor cells at the invasive front; F: Well differentiated tubular adenocarcinoma negative for staining.

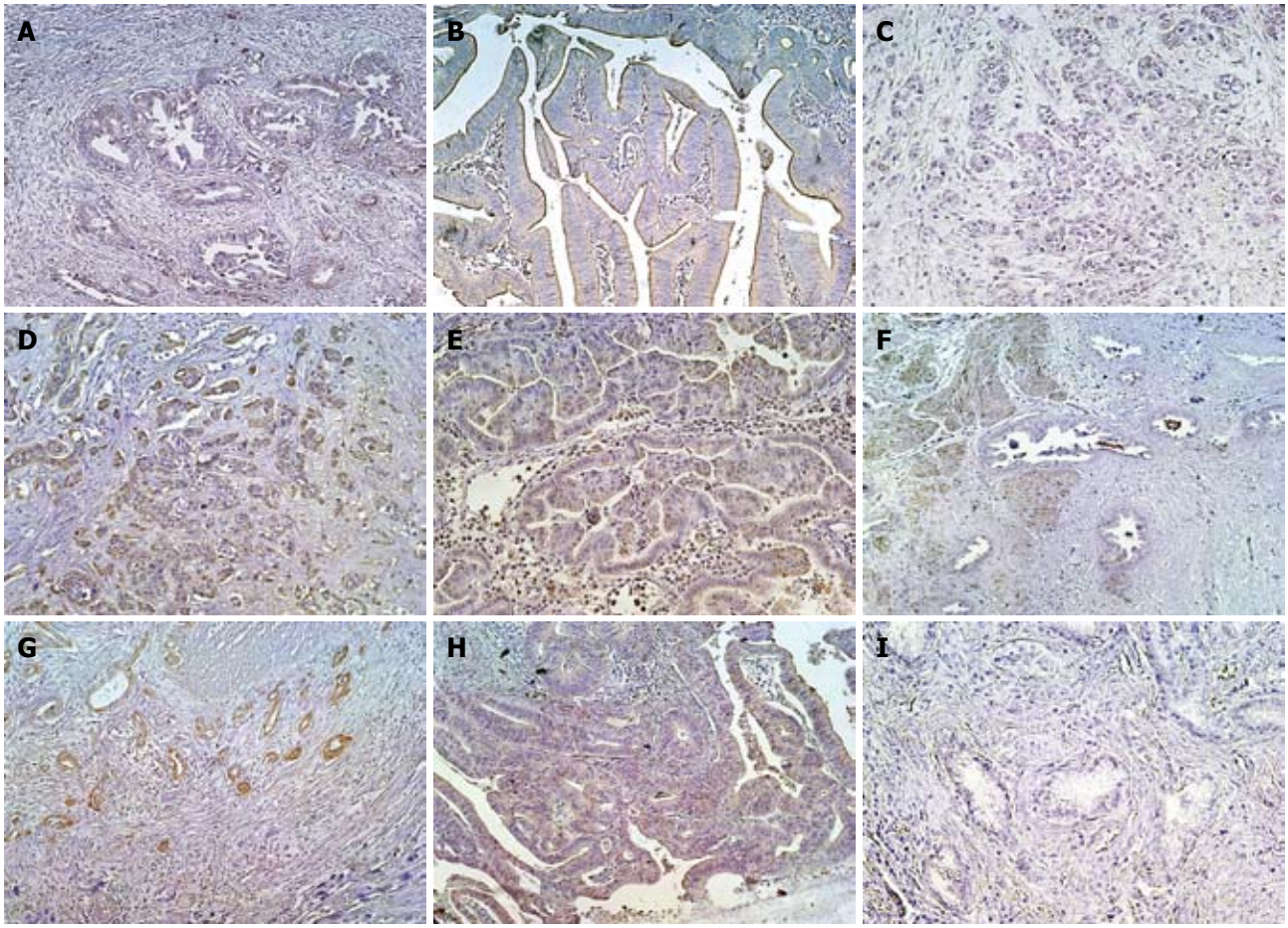

Figure 3 LNβ3 expression in biliary tract cancer tissues. A-C: Extrahepatic bile duct cancer tissues; A: Well differentiated tubular adenocarcinoma positive for invasive front dominant staining; B: Papillary adenocarcinoma positive for diffuse staining; C: Moderately differentiated tubular adenocarcinoma negative for staining; D-F: Gallbladder cancer tissues; D: Moderately differentiated tubular adenocarcinoma positive for invasive front dominant staining; E: Papillary adenocarcinoma positive for diffuse staining; G-I: Carcinoma tissues of the ampulla of Vater; G: Well differentiated tubular adenocarcinoma positive for invasive front dominant staining; H: Well differentiated tubular adenocarcinoma positive for diffuse staining; I: Well differentiated tubular adenocarcinoma negative for staining.

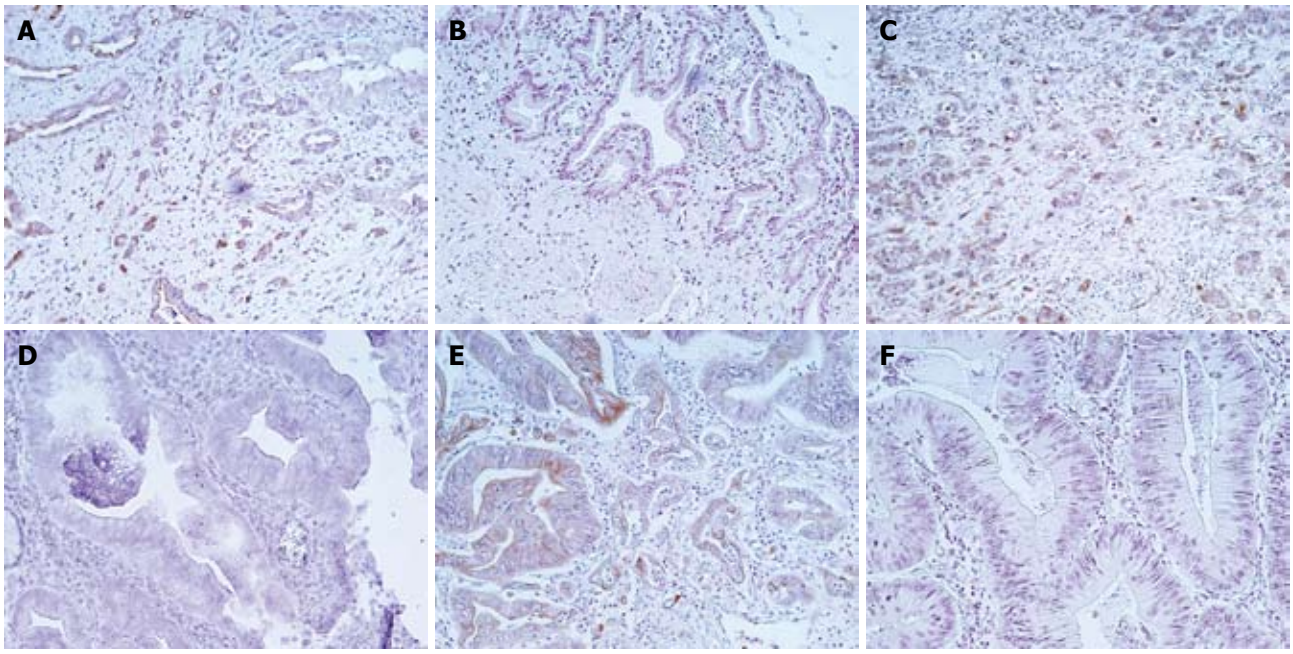

Figure 4 MMP7 expression in biliary tract cancer tissues. A, B: Extrahepatic bile duct cancer tissues; A: Moderately differentiated tubular adenocarcinoma positive for staining. Note that MMP7 is strongly positive in tumor cells at the invasive front; B: Papillary adenocarcinoma negative for staining; C, D: Gallbladder cancer tissues; C: Moderately differentiated tubular adenocarcinoma positive for staining; D: Well differentiated tubular adenocarcinoma negative for staining; E, F: Carcinoma tissues of the ampulla of Vater; E: Moderately differentiated tubular adenocarcinoma positive for staining. Note that MMP7 is strongly positive in tumor cells at the invasive front; F: Well differentiated tubular adenocarcinoma negative for staining.

(invasion into serosa), and advanced stage. The relationship between LNβ3 positivity and clinicopathological characteristics is summarized in Table 2. LNβ3 invasive front dominant pattern was significantly correlated with histologic type (less differentiated type) and advanced stage. There was a tendency that lymph node metastasis was more frequently observed in cases with LNβ3 invasive front dominant pattern than in other cases (P = 0.063). The relationship between MMP7 positivity and clinicopathological characteristics is summarized in Table 3. MMP7 positivity was not significantly correlated with clinicopathological characteristics. The expression of LNγ2, LNβ3 invasive front dominant pattern, and MMP7 were correlated with each other (Tables 4-6).

MMP7 activity detected by zymography

Using casein zymography, the levels of secreted matrilysin were analyzed (Figure 5). Nontumorous tissues secreted neither latent (28 kDa) nor activated (19 kDa) MMP7 activity. Latent and activated forms of MMP7 were detected in 16 (80%) and 12 (60%) of 20 carcinoma tissues, respectively. The activity was eliminated by the addition of the metalloproteinase inhibitor EDTA (data not shown). The activated form but not the latent form was correlated with depth of invasion and advanced stage.

Suppression of cancer cell invasiveness by MMP7 siRNA treatment

In vitro invasion assays after treatment with specific siRNA for the *MMP7* gene were carried out to assess the direct role of the expression of MMP7 in cancer cell invasiveness. Transfection efficiency determined by fluorescein isothiocyanate-labeled oligonucleotide uptake was 84% ± 6% in TFK-1 cells and 86% ± 5% in TGBC-

Table 1 Correlation between laminin γ2 staining and clinicopathologic factors *n* (%)

	Case (n = 61)	Laminin γ2		P value
		Positive (n = 35)	Negative (n = 26)	
Age (yr)				
≤ 65	25 (41)	15 (60)	10 (40)	
> 65	36 (59)	20 (56)	16 (44)	0.47
Gender				
Male	41 (67)	25 (61)	16 (39)	
Female	20 (33)	10 (50)	10 (50)	0.29
Location				
Bile duct	30 (49)	20 (67)	10 (33)	
Gall bladder	18 (30)	9 (50)	9 (50)	
Ampulla of Vater	13 (21)	6 (46)	7 (54)	0.34
Maximum diameter (mm)				
< 30	41 (67)	25 (61)	16 (39)	
≥ 30	20 (33)	10 (50)	10 (50)	0.29
Histologic type				
Tub1 & pap	35 (57)	16 (46)	19 (54)	
Others	26 (43)	19 (73)	7 (27)	0.03
Depth				
Serosa negative	37 (61)	17 (46)	20 (54)	
Serosa positive	24 (39)	18 (75)	6 (25)	0.02
Lymph node metastasis				
Absent	29 (48)	16 (55)	13 (45)	
Present	27 (44)	17 (63)	10 (37)	0.37
Stage				
< Ⅳ	43 (70)	21 (49)	22 (51)	
≥ Ⅳ	17 (28)	13 (76)	4 (24)	0.046

Table 2 Correlation between parameters and the pattern of laminin β3 expression *n* (%)

	Total (*n* = 61)	Laminin β3			*P* value
		Invasive (*n* = 23)	Diffuse (*n* = 17)	Negative (*n* = 21)	
Age (yr)					
≤ 65	25 (41)	9 (36)	8 (32)	8 (32)	
> 65	36 (59)	14 (39)	9 (25)	13 (36)	0.83
Gender					
Male	41 (67)	17 (41)	8 (20)	16 (39)	
Female	20 (33)	6 (30)	9 (45)	5 (25)	0.11
Location					
Bile duct	30 (49)	14 (47)	5 (17)	11 (37)	
Gall bladder	18 (30)	5 (28)	7 (39)	6 (33)	
Ampulla of Vater	13 (21)	4 (31)	5 (38)	4 (31)	0.40
Maximum diameter (mm)					
< 30	40 (66)	18 (45)	10 (25)	12 (30)	
≥ 30	21 (34)	5 (24)	7 (33)	9 (43)	0.27
Histologic type					
Tub1 & pap	35 (57)	9 (26)	14 (40)	12 (34)	
Others	26 (43)	14 (54)	3 (12)	9 (35)	0.02
Depth					
Serosa negative	37 (61)	11 (30)	13 (35)	13 (35)	
Serosa positive	24 (39)	12 (50)	4 (17)	8 (33)	0.18
Lymph node metastasis					
Absent	29 (48)	8 (28)	11 (38)	10 (34)	
Present	27 (44)	14 (52)	5 (19)	8 (30)	0.13
Stage					
< Ⅳ	43 (70)	10 (23)	15 (35)	18 (42)	
≥ Ⅳ	17 (28)	12 (71)	2 (12)	3 (18)	0.003

Table 3 Correlation between MMP7 staining and clinicopathologic factors *n* (%)

	Case (*n* = 61)	MMP7		*P* value
		Positive (*n* = 42)	Negative (*n* = 19)	
Age (yr)				
≤ 65	25 (42)	17 (68)	8 (32)	
> 65	36 (58)	25 (69)	11 (31)	0.66
Gender				
Male	41 (67)	30 (73)	11 (27)	
Female	20 (33)	12 (60)	8 (40)	0.23
Location				
Bile duct	30 (49)	24 (80)	6 (20)	
Gall bladder	18 (30)	9 (50)	9 (50)	
Ampulla of Vater	13 (21)	9 (69)	4 (31)	0.09
Maximum diameter (mm)				
< 30	41 (67)	29 (71)	12 (29)	
≥ 30	20 (33)	13 (65)	7 (35)	0.43
Histologic type				
Tub1 & pap	35 (57)	23 (66)	12 (34)	
Others	26 (43)	19 (73)	7 (27)	0.37
Depth				
Serosa negative	37 (61)	25 (68)	12 (32)	
Serosa positive	24 (39)	17 (71)	7 (29)	0.58
Lymph node metastasis				
Absent	29 (48)	21 (72)	8 (28)	
Present	27 (44)	18 (67)	9 (33)	0.43
Stage				
< Ⅳ	43 (70)	31 (72)	12 (28)	
≥ Ⅳ	17 (28)	10 (59)	7 (41)	0.24

2TKB cells (data not shown). Transfection with siRNA resulted in over 80% inhibition of mRNA and protein expression (data not shown). Transfection with *MMP7*-specific siRNA decreased invasiveness of TFK-1 cells compared with control siRNA-transfected counterparts ($P < 0.01$, Figure 6). This difference was significantly diminished by the addition of TIMP1. Similar results were observed in *MMP7*-specific siRNA-transfected TGBC-2TKB cells (data not shown).

Table 4 Expression of laminin γ2 and MMP7 in biliary tract carcinoma *n* (%)

MMP7 expression	Laminin γ2		*P* value
	Positive (*n* = 42)	Negative (*n* = 19)	
Positive (*n* = 34)	28 (46)	6 (10)	
Negative (*n* = 27)	14 (23)	13 (21)	0.01

Table 5 Expression of laminin β3 and MMP7 in biliary tract carcinoma *n* (%)

MMP7 expression	Laminin β3			*P* value
	Invasive (*n* = 23)	Diffuse (*n* = 17)	Negative (*n* = 21)	
Positive (*n* = 34)	17 (28)	6 (10)	14 (23)	
Negative (*n* = 27)	6 (10)	11 (18)	7 (11)	0.037

Table 6 Expression of laminin β3 and laminin γ2 in biliary tract carcinoma *n* (%)

Laminin γ2 expression	Laminin β3			*P* value
	Invasive (*n* = 23)	Diffuse (*n* = 17)	Negative (*n* = 21)	
Positive (*n* = 42)	18 (30)	8 (13)	9 (15)	
Negative (*n* = 19)	5 (8)	9 (15)	12 (20)	0.036

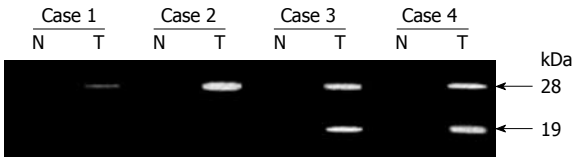

Figure 5 Casein zymography of surgical specimen pairs of biliary tract carcinoma and adjacent nontumor tissue. N and T: Matched samples from nontumor and tumor tissue, respectively.

DISCUSSION

In the current study, LNγ2 positivity in carcinoma cells at the invasive front was immunohistochemically observed in 57% of patients with biliary tract cancer, and was associated with histologic type (less differentiated type), depth of invasion, and advanced stage. LNβ3 invasive front dominant pattern was immunohistochemically observed in 38% of patients with biliary tract cancer, and was associated with histologic type (less differentiated type) and advanced stage. These results suggest that LNγ2 and LNβ3 expression in carcinoma cells at the invasive front contributes to the more aggressive phenotype of carcinoma cells, resulting in the progression of biliary tract cancer.

Preferential expression of LNγ2 and LNβ3 in carcinoma cells at the invasive front and its correlation with tumor progression suggest that these molecules play a role in the acquisition of a migrating and invading epithelial cell phenotype that is a prerequisite for malignancy[13]. Also in metastatic HCC, LN5 was mainly distributed along the tumoral advancing edge[9]. The mechanism underlying the preferential distribution of LNγ2 and LNβ3 at the invasive front in cancer is not known. It is known that activation of cancer-related genes in carcinoma cells affects their associated stromal cells. Certain stromal cell populations lying close to carcinoma cells may be induced to assist the invasion process by signals sent out by the cancer cells, stimulating the synthesis of gene products that facilitate cancer cell invasion and migration[35]. Interactions of carcinoma cells with stromal cells or with the surrounding extracellular matrix at the invasive front may result in an accumulation of LNγ2 and LNβ3 at the invasive front, where they may play a direct role in tumor invasion processes[19].

Although there are only a few reports regarding LNβ3 expression in human cancer, coexpression of LNγ2 and LNβ3 has been reported in HCC, squamous cell carcinoma of the tongue, colorectal carcinoma and basal cell carcinoma of the skin[9,25,26]. Sordat *et al*[14] reported that the heterodimer of the LNγ2 and LNβ3 chains is accumulated in the cytoplasm of dissociating (or budding) tumor cells from neoplastic tubules of colon carcinomas. Since LNγ2 and LNβ3 were not always coexpressed in biliary tract cancer, further analysis is necessary to elucidate the mechanism of overexpression and localization of LNγ2 and LNβ3 in biliary tract cancer. LN5 reportedly plays an important role in epithelial mesenchymal transition through down-regulation of E-cadherin and translocation of β-catenin into the nuclei[8]. It will be interesting to address this issue in biliary tract cancer in the near future.

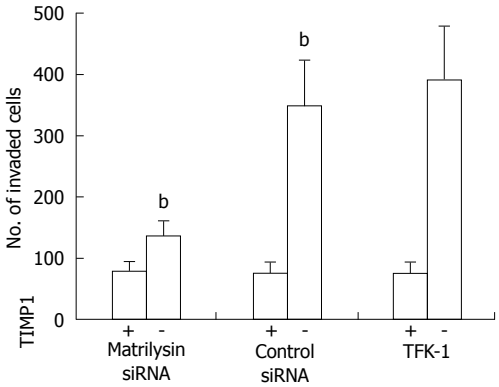

Figure 6 *In vitro* invasion assay with or without TIMP1 (250 ng/mL) in TFK-1 and siRNA transfectants. Each column indicates the means of three experiments; $^{b}P < 0.01$.

In contrast to preferential expression of MMP7 in carcinoma cells at the invasive front in various carcinomas, the expression pattern of MMP7 was diffuse in biliary tract cancer. The mechanism underlying the differential distribution of MMP7 in carcinomas needs to be further analyzed. MMP7 positivity was not significantly correlated with clinicopathological characteristics. However, the expression of LNγ2, LNβ3 invasive front dominant pattern, and MMP7 were correlated with each other. It has been suggested that the controlled up-regulation of gene products is one of the characteristics of invading cancer cells and that these gene products have functions crucial for the invasive phenotype of cancer cells[13]. It is notable that limited proteolysis of LNβ3 by MMP7 increases the cell motility activity of LN5 in colon carcinoma cells[27].

The activated form but not the latent form of MMP7 was correlated with depth of invasion and advanced stage, suggesting that active MMP7 plays an important role in the progression of biliary tract cancer. The implication of up-regulation of MMP7 expression in tumor progression was further substantiated by the *in vitro* invasion analysis. We revealed that down-regulation of MMP7 expression by siRNA resulted in a significant decrease in biliary tract cancer cell invasion *in vitro*, suggesting that up-regulation of MMP7 contributes to the more invasive phenotype of biliary tract cancer cells. Taken together, our results suggest that LNγ2 and LNβ3, in conjunction with MMP7, play a key role in the progression of biliary tract cancer.

COMMENTS

Background

Biliary tract cancers are relatively rare human malignancies involving the gallbladder and/or the bile ducts, but the prognosis is poor. Understanding the molecular biological features of biliary tract cancer progression is necessary for improving the prognosis. The potential role of laminin-5 (LN5) and MMP7 in human cancer is receiving increasing attention.

Research frontiers

Altered expression patterns of LN5, especially the LNγ2 chain, and MMP7 have been correlated with tumor behavior, such as invasiveness, vascularization, metastatic potential, and patients' poor prognosis. However, expression of LN5

and MMP7 in biliary tract cancer has not been clearly addressed. In this study, the authors demonstrate that LNγ2, LNβ3, and active MMP7 play a key role in the progression of biliary tract cancer.

Innovations and breakthroughs

This is the first study to report that invasive front dominant expression of LNγ2 and LNβ3, and active MMP7 play a key role in the progression of biliary tract cancer. Furthermore, our *in vitro* studies suggest that MMP7 plays an important role in biliary tract cancer cell invasion.

Applications

Detection of LNγ2, LNβ3, and active MMP7 could be molecular markers for tumor aggressiveness in biliary tract cancer. Understanding how LNγ2, LNβ3, and active MMP7 are induced and how their expression is blocked may represent a future strategy for therapeutic intervention in the treatment of patients with biliary tract cancer.

Terminology

Laminin: A heterotrimeric glycoprotein composed of three different polypeptide chains (α, β and γ), is a component of the extracellular matrix (ECM) that contributes to the architecture of the basal lamina surrounding the epithelial cells and mediates cell adhesion, growth, migration, proliferation, and differentiation. LN-5/LN-332: LN5, consists of α3, β3 and γ2 chains, and is involved in cell adhesion, migration, and scattering. Altered expression of LN-5 plays an important role in cancer. MMP7: Degradation of ECM components is mostly controlled by proteolytic enzymes called MMP. MMP7, also known as matrilysin, is a minimal domain MMP that exhibits broad proteolytic activity against components of the ECM and non-ECM.

Peer review

This paper reports the expression of LN-5 chains and MMP-7 in biliary cancer. The authors showed that LNγ2 and LNβ3, in conjunction with MMP7, play a key role in the progression of biliary cancer. The study sounds interesting and confirms the role of LN-5 and MMP7 in human cancer.

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ORIGINAL ARTICLES

Peroxisome proliferator-activated receptor-γ is essential in the pathogenesis of gastric carcinoma

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Abstract

AIM: To investigate whether peroxisome proliferator-activated receptor γ (PPAR-γ) is expressed in human gastric carcinoma and whether PPAR-γ is a potential target for gastric carcinoma therapy.

METHODS: PPAR-γ protein in gastric carcinoma was examined by immunohistochemistry. In the gastric carcinoma cell line MGC803, PPAR-γ, survivin, Skp2 and p27 protein and mRNA were examined by Western blotting and real-time reverse transcription-polymerase chain reaction, respectively; proliferation was examined by MTT; apoptosis was examined by chromatin staining with Hoechst 33342 and fluorescence activated cell sorting (FACS). and cell cycle was examined by FACS; the knockdown of PPAR-γ was done by RNA interference.

RESULTS: A high level of expression of PPAR-γ was observed in human gastric carcinoma and in a human gastric carcinoma cell line MGC803. The PPAR-γ agonist 15-deoxy-Δ12,14-prostaglandin J_2 (15d-PGJ_2) inhibited growth, and induced apoptosis and G_1/G_0 cell cycle arrest in MGC803 cells in a concentration-dependent and time-dependent manner. The effect of 15d-PGJ_2 on MGC803 cells was not reversed by the selective and irreversible antagonist GW9662 for PPAR-γ. Furthermore, survivin and Skp2 expression were decreased, whereas p27 expression was enhanced following 15d-PGJ_2 treatment in a dose-dependent manner in MGC803 cells. Interestingly, we also found that small interfering RNA for PPAR-γ inhibited growth and induced apoptosis in MGC803 cells. The inhibition of PPAR-γ function may be a potentially important and novel modality for treatment and prevention of gastric carcinoma.

CONCLUSION: A PPAR-γ agonist inhibited growth of human gastric carcinoma MGC803 cells by inducing apoptosis and G_1/G_0 cell cycle arrest with the involvement of survivin, Skp2 and p27 and not *via* PPAR-γ.

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Key words: Peroxisome proliferator-activated receptor γ; Gastric cancer; Apoptosis; Cell cycle

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INTRODUCTION

Gastric carcinoma is the second most common cancer in the world and kills more than 600000 people annually[1]. At present, the management of gastric carcinoma mainly includes surgery and chemotherapy, but the curative effect of the existing chemotherapeutic drugs is not effective and they have numerous side effects. Many studies have been performed to search for therapeutic targets and drugs capable of preventing and treating gastric carcinoma and other malignancies.

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors, initially described as molecular targets for compounds, which induce peroxisomal proliferation[2]. Up to now, 3 different isotypes of PPARs have been identified in various species, being the products of distinct genes and generally designated as PPAR-α, PPAR-β/δ, and PPAR-γ[2,3]. PPAR-γ has been extensively studied. Similarly to other

members of the nuclear receptor gene family, PPAR-γ is an agonist-activated transcription factor[3,4]. PPAR-γ heterodimerizes with retinoid X receptor to bind to the PPAR response element, leading to the transcription of downstream genes[5]. Several specific agonists have been found, such as the thiazolidinediones (including pioglitazone, rosiglitazone, and troglitazone), 15-deoxy-Δ12,14-prostaglandin-J_2 (15d-PGJ_2), and certain polyunsaturated fatty acids. GW9662 has been shown to be a selective and irreversible antagonist of PPAR-γ, and irreversibly binds within the agonist binding domain through covalent modification of a cysteine residue[6].

PPAR-γ is predominately expressed in adipose tissue and plays a central role in adipocyte differentiation and insulin sensitivity[7]. Recent studies have shown that, in addition to its classic role, PPAR-γ is implicated as a putative therapeutic target for cancer in a variety of tumors as several observations suggest that stimulation of PPAR-γ function may inhibit carcinogenesis and tumor cell growth[8,9]. However, the exact role of PPAR-γ on carcinogenesis and tumor cell growth is still unclear[10-14]. Recent investigations by Morita *et al*[15] and Konturek *et al*[16] have shown that PPAR-γ is implicated in *Helicobacter pylori*-related gastric carcinogenesis, and that PPAR-γ agonists may have potential in a cancer therapeutic role.

We here demonstrate that PPAR-γ may be involved in gastric carcinogenesis, and that the inhibition of PPAR-γ may be of benefit in the treatment of gastric carcinoma.

MATERIALS AND METHODS

Chemicals

PPAR-γ agonist, 15d-PGJ_2 and antagonist GW9662 were purchased from Cayman chemical (Ann Arbor, M1). Other drugs were reagent grade.

Tissue samples

One hundred and thirty eight samples of surgically-resected primary gastric carcinoma tissues, 138 samples of paired adjacent mucosa (2-5 cm from the margin of the gastric carcinoma) and 138 samples of paired normal mucosa (histologically proven) at the surgical margin (at least 5 cm from the margin of the gastric carcinoma) tissues were obtained from the First Affiliated Hospital of Inner Mongolia Medical College in China from October 2004 to January 2005. All gastric carcinoma patients underwent total or subtotal gastrectomy, and no patient received any treatment for cancer before surgery. The patient series included 111 males and 27 females with a mean age of 58.5 years and a median age of 60 years (range, 36-78). After surgery, the gastric specimens were fixed in 10% neutral buffered formalin and embedded in paraffin for immunohistochemistry. The clinical stage of all gastric carcinoma specimens were determined according to new TNM criteria published by the Union International Centre Cancer in 1997. All pathological diagnoses were performed by 2 independent pathologists.

Immunohistochemical staining of PPAR-γ

Sections of paraffin-embedded tissues (4 μm) were mounted on glass slides. The intracellular expression of PPAR-γ was detected using a PPAR-γ specific mAb (sc-7372, E-8, Santa Cruz Biotechnology, CA) with standard non-biotin horseradish peroxidase (HRP) 2-step immunostaining (Zymed) according to the manufacturer's instructions. The immunoreactive products were visualized using 3,3'-diaminobenzidine (DAB)/H_2O_2.

Cell culture and treatment with PPAR-γ agonists and antagonists

We used the human gastric carcinoma cell line MGC803 from Beijing Tumor Institute. It was maintained in RPMI1640 containing 10% fetal bovine serum (FBS) at 37℃ under 5% CO_2. A PPAR-γ agonist or antagonist were added at the time of replating.

Cell survival assay

Gastric carcinoma cells were treated with a PPAR-γ agonist or antagonist dissolved in DMSO for 8, 18 and 24 h in culture medium in 96-well plates. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma] solution was then added to the well with gentle pipetting. After 4 h, the supernatant was removed after centrifugation at 1000 r/min for 5 min at room temperature, and 180 μL DMSO was added to each well to solubilize the crystal products by shaking for 10 min. Absorbance at 570 nm was measured with an ELISA reader. The negative control had medium without serum and cells, and was used as the zero point of absorbance. Cell vitality of the control group was 100%. The inhibitory rate (IR) of cells was calculated according to the equation as follows: IR (%) = [1-(A_{570} nm absorbance in 15d-PGJ_2 treated group/A_{570} nm absorbance in control group)] × 100%. Each assay was performed in triplicate.

Apoptosis analysis

Apoptosis was analyzed by 2 different methods. First, chromatin staining with Hoechst 33342 was carried out. Briefly, MGC803 cells treated with 15d-PGJ_2 were cultured on Laboratory-Tek Chamber Slides for 24 h. The cells were then fixed with methanol for 10 min and rinsed. Chromatin staining was done with Hoechst 33342 (Sigma) to detect nuclear condensation. Secondly, cell apoptosis was measured by fluorescence activated cell sorting (FACS) using the Annexin-FITC Apoptosis Detection Kit according to the manufacturer's protocol. The apoptotic rate was calculated as the percentage of annexin V-positive and propidium iodide (PI)-negative cells divided by the total number of cells in the gated region. The data was pooled from 3 independent experiments.

Cell cycle examination

The effect of 15d-PGJ_2 on the cell cycle was examined with PI staining by FACS using Epics-XL (Beckmam Coulter).

Western blotting analysis

Cell total protein was extracted from MGC803 cells using

CytoBuster™ Protein Extraction Reagent (Novagen). The concentration of the protein was determined with Coomassie brilliant blue G-250. Cell total protein (50 μg) was separated by 12% SDS-PAGE. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride membrane, blocked overnight in Tris buffered saline with 10% skim milk at 4℃. PPAR-γ (Santa Cruz), Skp2 (Zymed), p27 (Santa Cruz) and β-actin (Santa Cruz) were detected using primary mouse mAb, and survivin (Santa Cruz) was detected using primary rabbit polyclonal Ab at a dilution of 1:2000, followed by HRP-conjugated anti-mouse IgG or anti-rabbit IgG at a dilution of 1:1500. Immunocomplexes were visualized using DAB. β-actin was used as the internal control.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from MGC803 cells using TRIzol reagent (Gibco, USA). The resultant RNA samples were quantified using a spectrophotometer at a wavelength of 260 nm. The integrity of the isolated RNA samples was analyzed electrophoretically on agarose gel, followed by staining in ethidium bromide. An aliquot of 2 μg of total RNA from each sample was reversely transcribed and amplified using RT-PCR Enzyme Mix Kit (Sino-American Biotechology Co.). For detection of human PPAR-γ mRNA, a combination of a sense primer (5'-TCTCTCCGTAATGGAAGACC-3') and an antisense primer (5'-GCATTATGAGACATCCCCCAC-3') were used. The reverse transcription was performed for 1 h at 37℃ and amplification was done by initial denaturation at 95℃ for 2 min, 40 cycles of amplification (95℃ for 40 s, 55℃ for 50 s, and 72℃ for 50 s) and a final extension step of 7 min at 72℃ was carried out in a 30 μL mixture containing 2 μg of total RNA, 25 pmol/L each of the sense and antisense primers, 2.0 mmol/L $MgCl_2$, 0.1 mmol/L dNTP, 1.5 μL 20 × buffer without Mg^{2+}, 2 μL RT-PCR Enzyme Mix and double-distilled H_2O. β-actin sense primer, 5'-ATCTGGCACCACACCTTC TACAATGAGCTGCG-3', and antisense primer 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC -3' were used. The reverse transcription was done for 1 h at 37℃ and amplification was performed by initial denaturation at 94℃ for 5 min, 35 cycles of amplification (95℃ for 15 s, 60℃ for 1 min, and 72℃ for 30 s) and a final extension step of 7 min at 72℃ was carried out in a 30 μL mixture containing the same components as above. The length of the β-actin PCR product was 838 bp and was the internal control. The PCR reaction products were separated electrophoretically in a 2% agarose gel and stained with ethidium bromide for 40 min at 80 V, and observed and photographed. All primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.

Construction of small interfering RNA (siRNA) expression vectors

To further elucidate the effect of PPAR-γ in gastric carcinoma, we applied the siRNA targeting approach. We used shRNA™ vector (provided by Wuhan Genesil Biotechnology Co., Ltd.) in which shRNA expression is driven by the U6 RNA promoter to produce small and haired RNA transcripts. We generated 3 different shRNA™ vectors and an empty vector used as a control. The oligonucleotides 5'-GATCCGAACAGAT CCAGTGGTTGCTTCAAGA-CGGCAACCACT GGATCTG-TTCTTTTTTTGTCGACA-3', 3'-GCTTGTCTAGGTCACCAACGAAGTTCTGC CGTTGGTGACCTAGACAAGAAAAAACAG CTGTTCGA-5' were used to generate anti-heparanase shRNA™ pSi1, and oligonucleotides 5'-GATCCGTCAAA-GTGGAGCCTGCATCTTC AAGACGGATGCAGGCTCCACTTTGATTTTT TGTCGACA-3', 3'-GCAGTTTCACCTCGGACGTAG AAGTTCTGCCTACGTCCGAGGTGAAACTAAAA AACAGCTGTTCGA-5' were used to generate anti-heparanase shRNA™ pSi2. Oligonucleotides 5'-GATCCG-ACAACCTGCTACAAGCCCTTCAAGAC GGG-GCTTGTAGCAGGTTGTCTTTTTTGT CGACA-3', 3'-GCTGTTGGACGATGTTCG GGAAGTTCTGCCCCGAACATCGTCCAACAGAA AAAACAGCTGTTCGA-5' were used to generate anti-heparanase shRNA™ pSi3.

Transfection

For the transfection, pSi1, pSi2 and pSi3 (1.0 μg) were added to RPMI1640 medium (without FBS) containing cation liposome vector (2.0 μL) and allowed to incubate for 20 min at room temperature to produce the transfection mixture. The transfection mixture was then added to the cells (1×10^5 per dish) in the serum-free medium. Six hours after the start of transfection, the medium was changed to RPMI1640 medium containing 10% FBS. RNA interference effect was examined by RT-PCR, Western blotting and MTT assay. Cell apoptosis determination was carried out at the final step.

Statistical analysis

The results were expressed as mean ± SD. Statistical analysis was performed using the Student's *t*-test, χ^2 test or one-way ANOVA and subsequent Fisher's LSD test. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS11.0 software.

RESULTS

Enhanced PPAR-γ expression in gastric carcinoma tissue and MGC803 cells

The positive rate of PPAR-γ protein expression was significantly higher in gastric carcinoma (75.0%) than in paired mucosa adjacent to gastric carcinoma (27.5%) and in normal gastric mucosa at the surgical margin of gastric carcinoma (13.0%) ($P < 0.001$). Mucosa adjacent to gastric carcinoma had significantly higher expression than normal gastric mucosa at the surgical margin of gastric carcinoma ($P < 0.05$ and $P < 0.001$) (Figure 1). The rate of PPAR-γ protein expression was positively associated with histologic type and negatively associated with invasive depth, vessel invasion, lymph node metastasis, distant metastasis and clinical stage

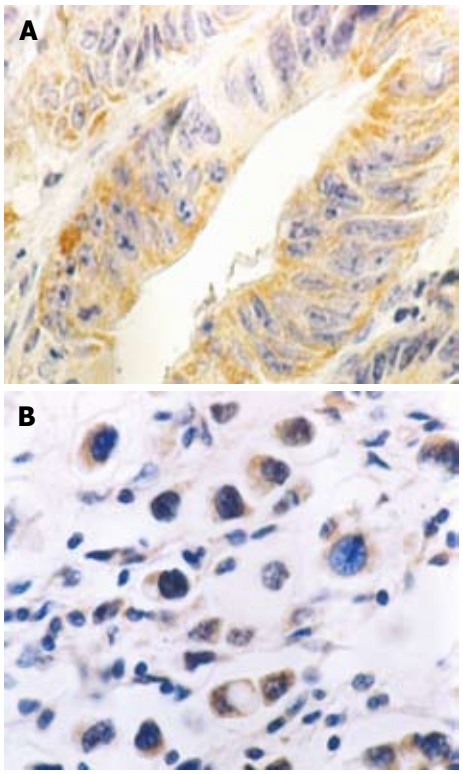

Figure 1 Peroxisome proliferator-activated receptor-γ (PPAR-γ) protein in the cytoplasm of gastric carcinoma cells by immunohistochemistry (3,3'-diaminobenzidine and hematoxylin staining × 400). A: Well differentiated gastric carcinoma; B: Poorly differentiated gastric carcinoma.

Table 1 Correlation of of PPARγ protein expression in gastric carcinoma with clinicopathological features

Clinicopathological features	*n*	PPARγ protein		χ^2	*P*
		+	-		
Histologic subtype	138			24.663	0.000
Intestinal type	63	60	3		
Diffuse type	75	44	31		
Depth of invasion	138			1.183	0.277
Serosal	24	16	8		
Out of serosa	114	88	26		
Vessel invasion	138			0.065	0.799
Absent	120	90	30		
Present	18	14	4		
Lymph node metastasis	138			0.309	0.578
Absent	36	28	8		
Present	102	76	28		
Distant metastasis	138			0.535	0.464
Absent	126	96	30		
Present	12	8	4		
Clinical stage[1]	126			0.017	0.897
Ⅰ-Ⅱ	39	30	9		
Ⅲ-Ⅳ	87	61	26		

[1]Because clinical staging could not be performed because of the lack of accurate records about lymph nodes, data of 12 cases were not included in the statistical analysis.

($P < 0.05$) of gastric carcinoma. The rate of PPAR-γ protein expression was significantly higher in intestinal-type gastric carcinoma (95.2%) than in diffuse-type gastric carcinoma (58.7%) ($P < 0.001$) (Table 1). Both PPAR-γ mRNA and protein showed positivity in the human gastric carcinoma cell line MGC803 (Figure 2).

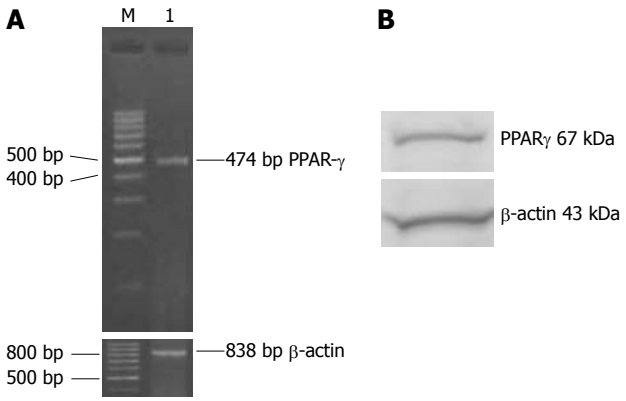

Figure 2 PPAR-γ mRNA and protein expression in gastric carcinoma cell line MGC803. A: PPARγ mRNA expression examined in MGC803 cells by reverse transcriptase-polymerase chain reaction. M: 100 bp DNA marker and 500 bp was the brightest; 1: MGC803 cells. B: PPAR-γ protein expression examined by Western blotting in MGC803 cells.

Effect of 15d-PGJ$_2$, GW9662 and 15d-PGJ$_2$ + GW9662 on growth of MGC803 cells

Treatment with a concentration of 0.01, 0.1 and 1 μmol/L 15d-PGJ$_2$ did not affect MGC803 cell growth. The inhibition of proliferation of MGC803 cells by 5, 10, 20, 30, and 40 μmol/L 15d-PGJ$_2$ was significantly higher than that of 0 μmol/L ($P < 0.001$) (Table 2). Correspondingly, inhibition was also enhanced with increasing 15d-PGJ$_2$ concentrations. Inhibition was 5.98% ± 1.41%, 13.64% ± 0.69%, and 34.64% ± 0.99% after 8, 18, and 24 h, respectively, following 30 μmol/L 15d-PGJ$_2$ addition. It was significantly higher at 18 h than 8 h ($P < 0.001$), and it was significantly higher at 24 h than 18 h ($P < 0.001$); it was enhanced over a prolonged time. Thus 15d-PGJ$_2$ inhibited proliferation of MGC803 cells in a concentration-dependent and time-dependent manner (Figure 3A). The proliferation inhibition rate of MGC803 cells by GW9662 treatment for 24 h, showed no difference between 0.01, 0.1 and 1 μmol/L and 0 μmol/L concentrations (all $P > 0.05$). Inhibition of proliferation was significantly higher at 2.5 and 5 μmol/L compared to 0 μmol/L (all $P > 0.05$). The proliferation inhibition rate of MGC803 cells by 15d-PGJ$_2$ and GW9662 combination treatment for 24 h showed no difference between the 0.01, 0.1 and 1 μmol/L GW9662 and 5, 10, 20, 30 and 40 μmol/L 15d-PGJ$_2$ combination groups and the 5, 10, 20, 30 and 40 μmol/L 15d-PGJ$_2$ group (Table 3).

Effect of 15d-PGJ$_2$ on apoptosis of MGC803 cells

15d-PGJ$_2$ is capable of inducing MGC803 cell apoptosis as seen by morphological observation (Figure 4) and flow cytometry (Figure 5). The apoptosis rate of MGC803 cells of 5, 10, 20, 30, and 40 μmol/L 15d-PGJ$_2$ was significantly higher than that of 0 μmol/L ($P < 0.001$) (Table 1); it was enhanced with increasing concentrations of 15d-PGJ$_2$. The apoptosis rate was 6.58% ± 0.62%, 15.99% ± 1.73%, and 38.23% ± 1.36% at 8, 18 and 24 h, respectively, following 30 μmol/L 15d-PGJ$_2$ addition, and increased with time. Thus, 15d-PGJ$_2$ induced

Table 2 Effect of various concentrations of 15-deoxy-Δ12,14-prostaglandin-J2 (15d-PGJ2) on proliferation, apoptosis and cell cycle of gastric carcinoma MGC803 cells after 24 h [(mean ± SD) %]

15d-PGJ2 concentration (μmol/L)	Proliferative inhibition rate	Apoptosis rate	Cell cycle distribution		
			G_1/G_0	S	G_2/M
0	0.22 ± 0.11	0.37 ± 1.03	31.33 ± 2.31	60.33 ± 1.15	8.33 ± 1.15
0.01	0.21 ± 0.10				
0.1	0.21 ± 0.12				
1	0.23 ± 0.12				
5	7.47 ± 0.49[d]	7.89 ± 1.07[d]			
10	11.49 ± 1.00[d]	11.41 ± 1.47[d]			
20	14.36 ± 1.53[d]	14.27 ± 1.62[d]			
30	38.23 ± 1.36[d]	34.64 ± 0.99[d]	61.33 ± 1.53[d]	36.00 ± 1.00[d]	2.67 ± 0.58[b]
40	57.00 ± 1.24[d]	55.35 ± 1.88[d]			

[b]$P < 0.01$, [d]$P < 0.001$ compared with 0 μmol/L group.

Table 3 Effect of GW9662 in various concentrations on the proliferation of MGC803 cells and on 15d-PGJ2 inhibition of proliferation of MGC803 cells after 24 h [(mean ± SD) %]

15d-PGJ2 concentration (μmol/L)	Proliferative inhibition rate of GW9662 (μmol/L)					
	0	0.01	0.1	1	2.5	5
	0.19 ± 0.11	0.20 ± 0.12	0.20 ± 0.11	0.19 ± 0.12	3.20 ± 0.13[b]	5.29 ± 0.14[b]
5	7.47 ± 0.49	7.49 ± 0.52	7.50 ± 0.50	7.52 ± 0.49		
10	11.49 ± 1.00	11.50 ± 1.00	11.52 ± 1.03	11.53 ± 1.04		
20	14.36 ± 1.53	14.42 ± 1.62	14.44 ± 1.53	14.36 ± 1.53		
30	38.23 ± 1.36	38.33 ± 1.42	38.34 ± 1.36	38.43 ± 1.36		
40	57.00 ± 1.24	57.06 ± 1.34	57.10 ± 1.24	57.12 ± 1.34		

[b]$P < 0.001$ compared with 0 μmol/L group of GW9662.

MGC803 cell apoptosis in a concentration-dependent and time-dependent manner (Figure 3B).

Effect of 15d-PGJ2 on cell cycle of MGC803 cells

15d-PGJ2 at 30 μmol/L significantly increased the proportion of MGC803 cells in the G_0/G_1 phase ($P < 0.001$) and significantly decreased the proportion in the S and G_2/M phases ($P < 0.001$ and $P < 0.01$, respectively) (Figure 6).

Effect of 15d-PGJ2 on survivin, Skp2 and p27 expression in MGC803 cells

Survivin and Skp2 protein were decreased and p27 protein was increased as the concentration of 15d-PGJ2 increased as shown by Western blotting (Figure 7).

Effect of knockdown of PPAR-γ by RNAi

To examine whether the inhibitory effect of PPAR-γ agonist 15d-PGJ2 on the growth of MGC803 cells was *via* a PPAR-γ specific pathway and influenced PPAR-γ expression in the tumor cells, we used the RNAi approach for PPAR-γ. Efficiency of transfection approximate 80%) was evaluated by fluorescence microscopy 48 h after transfection of a vector containing the gene encoding green fluorescent protein (Figure 8). MGC803 cells were transiently transfected with pSi1, pSi2 and pSi3 or empty vectors by cation liposome vector, and the cells were tested for PPAR-γ expression 48 h later. Of the cells transfected with pSi1, pSi2 and pSi3, pSi1 was most effective in silencing PPAR-γ mRNA and protein expression in MGC803 cells, as determined by semiquantitative RT-PCR (Figure 9A) and Western blotting (Figure 9B). PPAR-γ specific RNAi effectively decreased PPAR-γ mRNA and protein level in the MGC803 cells and showed inhibition of cell growth (Figure 10A) and induction of cell apoptosis (Figure 10B).

DISCUSSION

Recently, the potential of PPAR-γ as a target for the prevention and treatment of cancer has been widely studied[17]. However, the potential therapeutic role of PPAR-γ agonists has been questioned, based on contradictory results. In experiments using animal models of colon cancer, PPAR-γ agonists increased the development of colon tumors[10]. This contradictory result was supplemented by a recent report using transgenic mice that expressed a constitutive active form of PPAR-γ in mammary glands, and showed that PPAR-γ signaling accelerated tumor development in mammary glands[18]. The actual role of PPAR-γ in cancer has been complicated by recent findings that PPAR-γ agonists affect cancer cells independent of PPAR-γ[19-22], and silencing of PPAR-γ and PPAR-γ antagonists have been shown to inhibit cancer cell growth[13,14]. To date, the role of PPAR-γ in gastric carcinogenesis remains unclear.

In the present study, PPAR-γ was found to be expressed at higher levels in gastric carcinoma than in paired mucosa adjacent to gastric carcinoma, and in both tissues the PPAR-γ levels were higher than in their

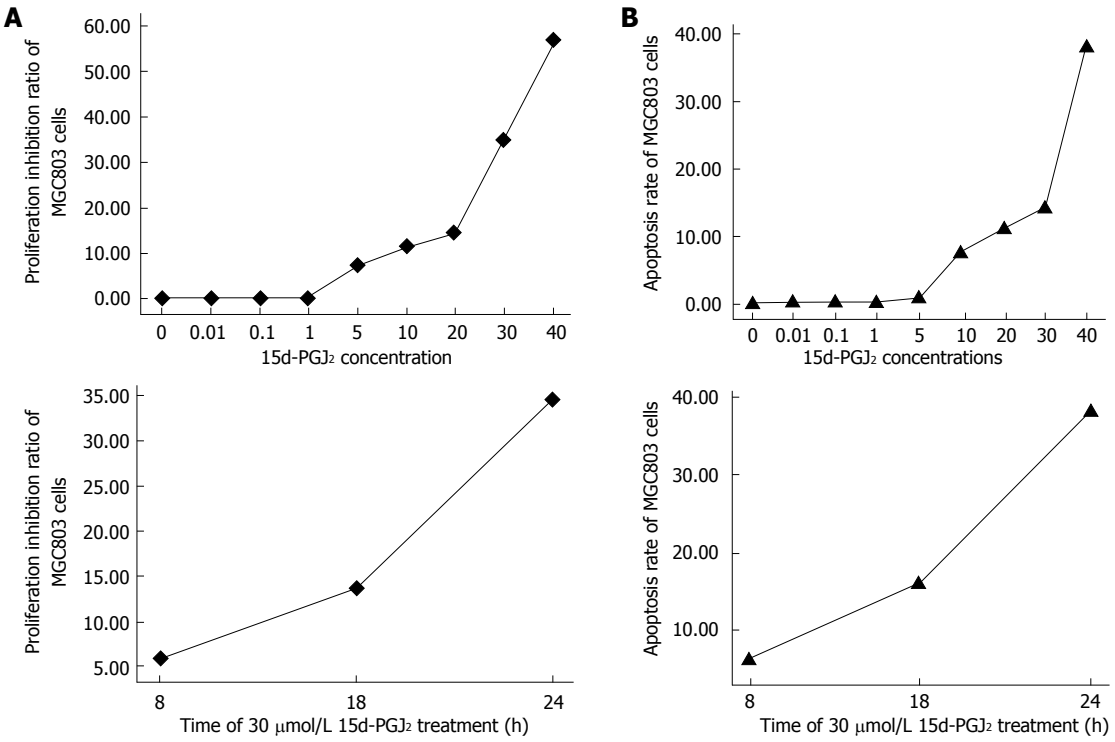

Figure 3 15d-PGJ2 inhibited proliferation (A) and induced apoptosis (B) of MGC803 cells in a concentrationdependent and time-dependent manner.

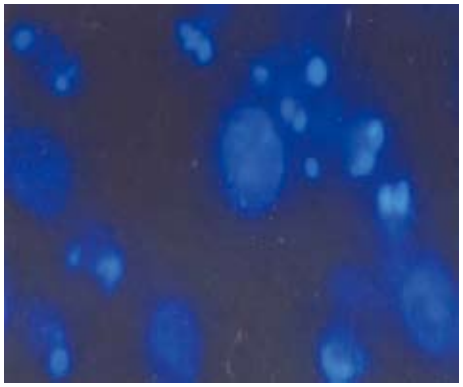

Figure 4 Morphological changes of MGC803 cells 24 h after treatment with 30 μmol/L 15d-PGJ2. Cell shapes were observed by fluorescence microscopy (Hoechst 33342 staining, × 400). Nuclear chromatin condensation, marginalization of nuclear chromatin and half moon formation, nuclear fragments with bright chromatin and apoptotic bodies were easily identified in some cells.

paired normal gastric mucosa at the surgical margin of the gastric carcinoma. Similar effects were also observed in gastric cancer in another study[16]. In addition, we used the siRNA approach to confirm the effect of PPAR-γ on gastric carcinoma. The result showed that silencing PPAR-γ clearly inhibited the growth and induced apoptosis of MGC803 cells. Similar effects were also observed in other cancers such as hepatocellular carcinoma[13]. These results indicated that PPAR-γ may be involved in gastric carcinogenesis.

Here, we clearly showed that the PPAR-γ agonist 15d-PGJ_2 inhibited growth of cultured gastric cancer MGC803 cells, and our study also demonstrated that the PPAR-γ antagonist GW9662 did not interfere with this effect and that 2.5 μmol/L GW9662 inhibited growth of MGC803 cells. Furthermore, PPAR-γ siRNA markedly inhibited the growth of MGC803 cells in our study. These results indicated that 15d-PGJ_2 inhibited growth of cultured gastric carcinoma MGC803 cells by a PPAR-γ-independent pathway. These results also suggested that PPAR agonists may be useful in the chemoprevention or chemotherapy of gastric malignancies. Additionally, some recent studies reported that the biological effect of PPAR-γ agonists is independent of PPAR-γ[19,23-28], but other research concluded that 15d-PGJ_2 inhibited growth of cancer cells and gastric cancer cells by activating the PPAR-γ pathway[29-32]. What is the reason for the discrepancies between our data and the data found in other reports? This may be attributed to the fact that some investigators did not study the effect of PPAR antagonists on tumor cell growth and on the effect of PPAR agonists, as they only used PPAR agonists.

The inhibition of growth of cultured gastric cancer MGC803 cells by PPAR-γ agonists is mainly a result of apoptosis, as evidenced by the data for nuclear condensation by chromatin staining with Hoechst 33342 and annexin V-FITC staining through FACS analysis. Mechanisms may exist by which apoptosis is induced by PPAR agonists. Furthermore, the inhibition of cell growth and induction of apoptosis by PPAR-γ agonists were time- and concentration-dependent.

Survivin is an inhibitor of apoptosis, and is expressed during embryonal development but lacks expression in terminally differentiated adult tissues. Interestingly, it becomes re-expressed in transformed cell lines and in a variety of human tumors, and deserves growing attention as "an ideal target" for cancer therapy. Survivin is involved in the control of apoptosis and directly inhibits caspase 3 and 7 activities. Survivin is mostly

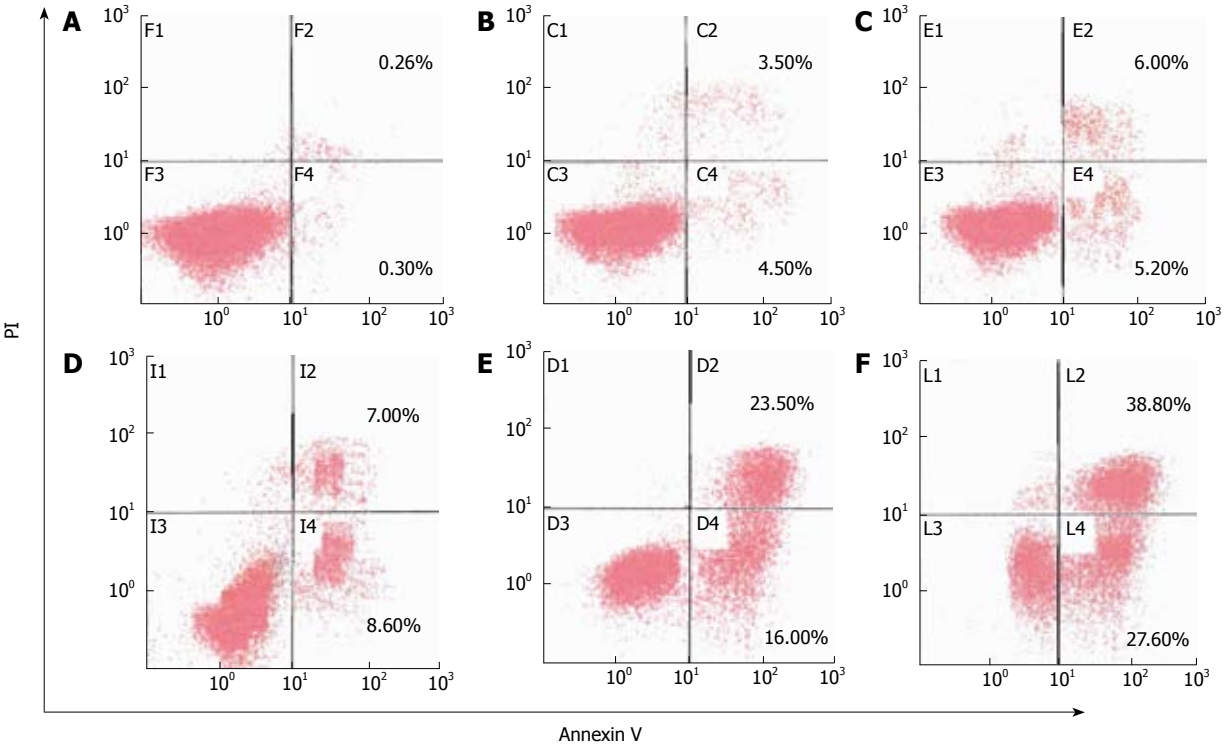

Figure 5 Apoptosis rate of MGC803 cells detected by flow cytometry 24 h after treatment with 15d-PGJ$_2$ at various concentrations. A: 0 μmol/L; B: 5 μmol/L; C: 10 μmol/L; D: 20 μmol/L; E: 30 μmol/L; F: 40 μmol/L.

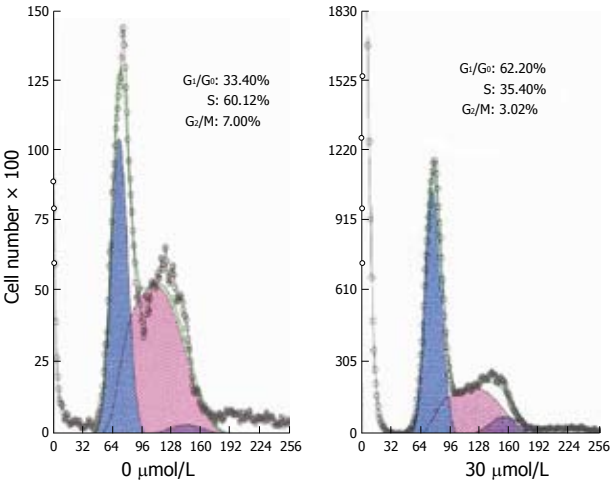

Figure 6 Cell cycle distribution of MGC803 cells by flow cytometry 24 h after treatment with 15d-PGJ$_2$ at various concentrations. 15d-PGJ$_2$ at 30 μmol/L could significantly increase the proportion of cells in the G_0/G_1 phase ($P < 0.001$) and decrease the proportion in the S and G_2/M phases in MGC803 cells ($P < 0.001$ and $P < 0.01$).

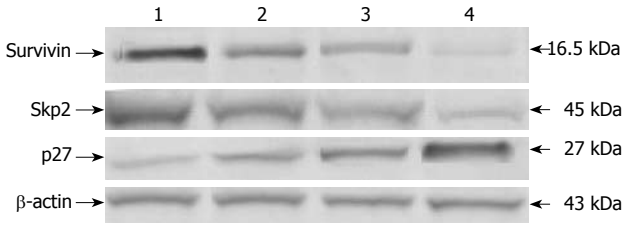

Figure 7 Survivin, Skp2 and p27 protein expression by Western blotting in MGC803 cells 24 h after treatment with 15d-PGJ$_2$ at various concentrations. Lane 1: 0 μmol/L; Lane 2: 5 μmol/L; Lane 3: 20 μmol/L; Lane 4: 30 μmol/L. Survivin and Skp2 protein decreased and p27 protein increased with increasing concentrations of 15d-PGJ$_2$.

expressed in cell cycle G_2/M and lacks expression in G_1/G_0[33]. During tumorigenesis, survivin expression is negatively correlated with apoptosis but positively correlated with proliferation and angiogenesis[34]. Our study showed that the levels of survivin protein were decreased with the increase in 15d-PGJ$_2$ concentration, and were inversely associated with the apoptosis induced by 15d-PGJ$_2$. These data suggest that 15d-PGJ$_2$ may inhibit survivin expression to induce apoptosis in gastric carcinoma MGC803 cells.

Our study showed, by flow cytometry, that 15d-PGJ$_2$ induced MGC803 cell arrest in the G_1/G_0 cell cycle phase and reduced the percentage of cells in the S and G_2/M cell cycle phases. This may be another reason for inhibition of the growth of gastric carcinoma MGC803 cells by 15d-PGJ$_2$. Additionally, this may be in part the reason that 15d-PGJ$_2$ reduced the level of survivin, although we cannot exclude the possibility that 15d-PGJ$_2$ may have a direct effect on survivin expression. In eukaryotes, progression of the cell cycle is controlled by interactions between cell cycle control proteins (cyclins) and their catalytically active cyclin-dependent kinases (CDKs). The activity of each cyclin-CDK complex is in turn regulated by several different mechanisms, the most important being negative regulation by CDK inhibitors[35]. p27 is an inhibitor of cyclin E-CDK2 and cyclin A-CDK2, which drive cells from the G_1 phase to the S phase of the cell division cycle[36,37], and p27 is expressed at its highest level in the G_1/G_0 phase. The amount of p27 is mainly regulated by posttranslational

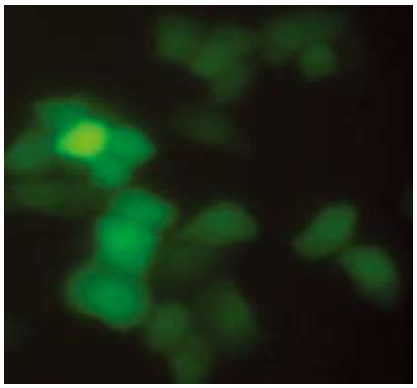

Figure 8 Human gastric carcinoma MGC803 cells observed by fluorescence microscopy 48 h after pSi1 transfection.

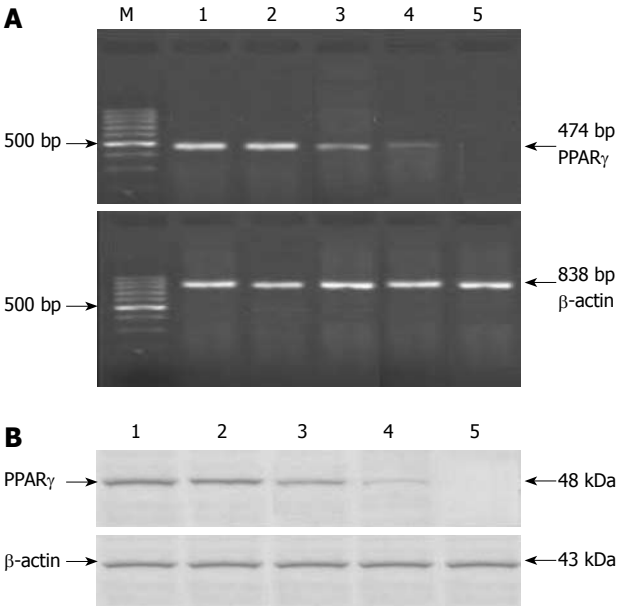

Figure 9 PPARγ after pSi transfection. A: PPARγ mRNA expression in various cells detected by RT-PCR 48 h after transfection; B: PPARγ protein expression in various cells 48 h after transfection as detected by Western blotting. Lane M: 100 bp DNA marker, 500 bp was the brightest; Lane 1: MGC803-N (normal MGC803 cells); Lane 2: MGC803-HK (MGC803 cells transfected by empty vector); Lane 3: MGC803-pSi3 (MGC803 cells transfected by pSi3 vector); Lane 4: MGC803-pSi2 (MGC803 cells transfected by pSi2 vector); Lane 5: MGC803-pSi1 (MGC803 cells transfected by pSi1 vector). The effect of pSi1 was best in silencing PPAR-γ mRNA and protein expression in MGC803 cells.

ubiquitin-proteasome-mediated proteolysis[38]. The cell cycle-dependent degradation of p27 is dependent on phosphorylation at Thr^{187} in the late G_1 phase by CDK2, and Thr^{187} phosphorylation is a necessary prerequisite for the sequential addition of ubiquitin molecules by a ubiquitin ligase complex, SCF^{skp2}, composed of Skp1, Cull, Rbx1, and the F-box protein Skp2[37]. Polyubiquitination of p27 then targets p27 for degradation in the proteasome, thus removing the p27 cell cycle "brake", allowing cells to transition from G_1 to the S phase[39]. Some investigations have shown that Skp2 is a specific substrate-recognition subunit of SCF^{skp2}, the expression of Skp2 is required for the ubiquitination and subsequent degradation of p27 *in vitro* and *in vivo*[40-42], and Skp2 knockout cells exhibit p27 at high levels[43].

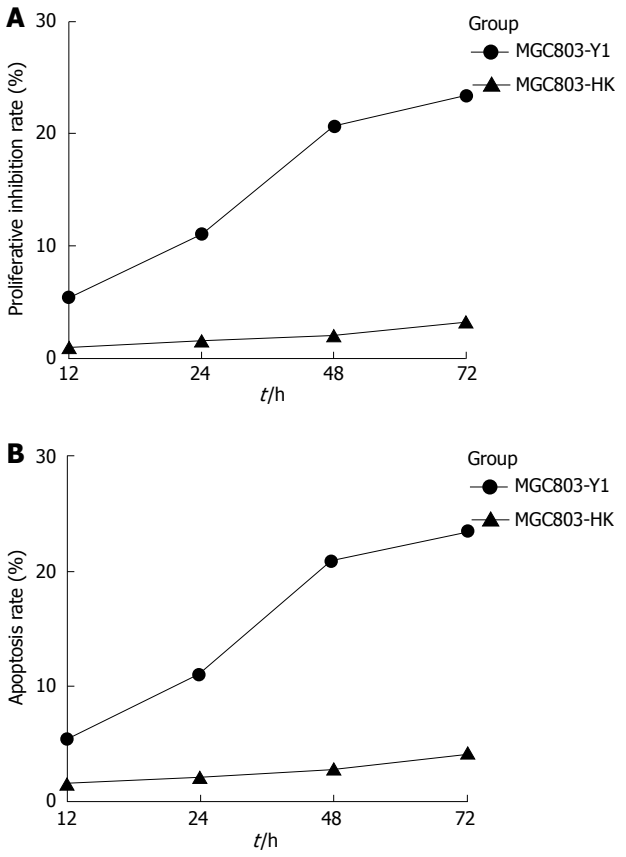

Figure 10 Proliferative inhibition and apoptosis rate of MGC803-Y1 and of MGC803-HK cells after transfection. The proliferative inhibition (A) and apoptosis (B) rate of MGC803-Y1 cells was higher than that of MGC803-HK cells at 12-72 h after transfection.

Skp2 levels are cell cycle regulated, and Skp2 accumulates during the S phase. Inappropriate expression of Skp2 in G_0 cells can promote S phase entry concomitant with loss of p27. Recently, the targeted disruption of Skp2 resulted in an accumulation of p27, and cell cycle arrest in the G_1 cycle. The level of Skp2 protein was decreased, whereas the level of p27 protein was increased with increasing concentration of 15d-PGJ_2 treatment when examined by Western blotting. These data suggested that 15d-PGJ_2 induced MGC803 cell arrest in G_1/G_0 by Down-regulating Skp2 expression and upregulating p27 expression. Furthermore, these results suggested that 15d-PGJ_2 may interfere with ubiquitin-proteasome-mediated proteolysis in gastric carcinoma cells.

In conclusion, our study suggests that PPAR-γ may be involved in gastric carcinogenesis, and that 15d-PGJ_2 may inhibit the growth of human gastric carcinoma MGC803 cells by inducing apoptosis and G_1/G_0 arrest, with the involvement of survivin, Skp2 and p27, but *via* a PPAR-γ-independent pathway.

COMMENTS

Background

Many efforts have been made to search for therapeutic targets and drugs capable of preventing and treating gastric carcinoma and other malignancies. Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors, initially described as molecular targets for compounds, which induce peroxisomal proliferation.

Research frontiers

Three different isotypes of PPARs have been identified in various species, being the products of distinct genes and generally designated as PPAR-α, PPAR-β/δ, and PPAR-γ. PPAR-γ is one that has been extensively studied. As with other members of the nuclear receptor gene family, PPAR-γ is an agonist-activated transcription factor. PPAR-γ heterodimerizes with retinoid X receptor to binding to the PPAR response element, leading to the transcription of downstream genes.

Innovations and breakthroughs

Several specific agonists have been found, such as the thiazolidinediones (including pioglitazone, rosiglitazone, and troglitazone), 15-deoxy-Δ12,14-prostaglandin-J_2 (15d-PGJ_2), and certain polyunsaturated fatty acids. GW9662 has been shown to be a selective and irreversible antagonist of PPAR-γ, and irreversibly binds within the agonist binding domain through covalent modification of a cysteine residue.

Applications

By understanding how PPAR-γ affects the growth of cells, this study may represent a future strategy for therapeutic intervention in the treatment of patients with gastric carcinoma.

Terminology

PPAR-γ receptors are nuclear hormone receptors, which induce peroxisomal proliferation. 15d-PGJ_2 is a specific agonist. Peroxisomal proliferation is thought to be crucial in gastric carcinoma.

Peer review

PPAR-γ receptors are nuclear hormone receptors. The authors examined whether PPAR-γ may affect gastric carcinogenesis, and 15d-PGJ_2 may inhibit the growth of MGC803 cells by inducing apoptosis and G_1/G_0 arrest, but through a PPAR-γ-independent pathway. The result may be useful in treatment of gastric carcinoma.

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BRIEF ARTICLES

Endotoxin receptor *CD14* gene variants and histological features in chronic HCV infection

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Abstract

AIM: To analyze the correlation between CD14 rs2569190/C-159T single nucleotide polymorphism (SNP) and disease progression in chronic hepatitis C.

METHODS: Liver biopsy specimens from a total of 137 and 349 patients with chronic hepatitis C were separately evaluated with respect to necroinflammatory activity (grading) and architectural changes (staging). In one group, further histological lesions characteristic for hepatitis C, hepatitis C virus subtypes, and biochemical parameters of liver disease were also investigated. Samples of genomic DNA were genotyped for the respective SNP by 5′-nuclease assays using fluorescent dye-labeled allele-specific probes.

RESULTS: Genotype distribution did not deviate from the Hardy-Weinberg equilibrium. In the first group, patients homozygous for the variant allele T were found to be younger than C allele carriers (39.6 ± 12.5 *vs* 45.7 ± 11.5, P = 0.008). Among the histological lesions studied, portal lymphoid aggregates were more frequently observed among TT homozygotes than among C carriers (21/37 *vs* 32/100, P = 0.008). The presence of portal lymphoid aggregates was closely correlated with hepatic inflammation (P = 0.003) and with bile duct damage (P < 0.001). The degree of fibrosis, in contrast, was not found to be related to the *CD14* gene C-159T polymorphism.

CONCLUSION: The data suggest a possible relationship between CD14 C-159T polymorphism and the formation of portal lymphoid aggregates, but not liver fibrosis progression in chronic hepatitis C.

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Key words: CD14; Endotoxins; Hepatitis C virus; Inflammation; Lipopolysaccharides; Liver fibrosis; Portal system; Single nucleotide polymorphism

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INTRODUCTION

Hepatitis C virus (HCV), which currently infects about 3% of the world's population (an estimated 210 million people), is a major cause of chronic viral liver disease[1]. Chronic hepatitis C is characterized by mostly mild hepatic inflammatory activity which does, however, hold a significant risk of proceeding to liver cirrhosis and hepatocellular carcinoma[2]. Further characteristic histological alterations may include steatosis, bile duct lesions, and portal lymphoid aggregates[3-5]. While steatosis, for instance, has been shown to be associated with HCV subtype infection and is suggested to be modulated by HCV proteins[4,6], progression of fibrosis in chronic hepatitis C has been attributed to age, gender, or alcohol consumption[7], and to host genetic factors[8]. Different genetic backgrounds have also been found to be associated with the susceptibility to HCV subtype infection[9,10].

As a result of its anatomic links to the gut, the liver is constantly exposed to gut-derived bacterial products, e.g. lipopolysaccharides (LPS), which are suggested to be important cofactors in toxin- or ethanol-induced liver disease by exacerbating ongoing injury[11]. Endotoxemia arises from increased translocation of endotoxins from the gut lumen because of altered intestinal permeability and decreased hepatic clearance capacity[12].

Hepatocytes and Kupffer cells, the resident liver macrophages which play a major role in the clearance of systemic bacterial infection, express the membrane-associated form of the endotoxin receptor CD14 (mCD14) at low levels in comparison to peripheral blood monocytes (reviewed by Schwabe *et al*[12]). Moreover, sinusoidal endothelial cells and activated hepatic stellate cells, the main fibrogenic cell type in the injured liver, also express mCD14[12,13]. mCD14 is anchored by glycophosphatidyl inositol, being part of a cell surface receptor complex which additionally contains the dimerized Toll-like receptor 4 and MD-2 (reviewed by Pålsson-McDermott & O'Neill[14]). Furthermore, it also localizes with TLR3, the double-stranded RNA (dsRNA) receptor, in intracellular compartments enhancing dsRNA sensing and TLR3 signalling[15,16]. In addition to the membranous form, a soluble form of CD14 lacking the glycophosphatidyl inositol anchor is thought to modulate LPS responses *via* local stimulatory (promoting) and systemic anti-inflammatory (competing) mechanisms[17].

Recently, a single nucleotide polymorphism (SNP) within the *CD14* gene, rs2569190/C-159T, has been demonstrated to be associated with the risk of developing liver cirrhosis, but not steatosis or less advanced stages of fibrosis, in patients with alcohol-induced liver disease (ALD)[18-20]. These results were attributed to the finding that, *in vitro*, the T allele is more actively transcribed than the C allele[21], leading consequently to the assumption that the TT carriers' hepatic cells may be prone to enhanced inflammatory reactions after endotoxin exposure. The relationship between rs2569190 TT genotype and liver disease progression, however, was not observed in a study by von Hahn and colleagues, in which the variant allele T was alternatively shown to be solely associated with cryptogenic chronic liver disease[22].

The current study aimed to investigate, in two different cohorts, whether the variant position rs2569190/C-159T within the *CD14* gene is associated with hepatitis C liver disease manifestations, and allowed replicated analysis as is demanded for genetic association studies[23].

MATERIALS AND METHODS

Ethics

The study was approved by the local ethical committee and conformed to the ethical guidelines of the 2000 Declaration of Helsinki. Patients gave their informed consent.

Patients

A total of 137 mainly Caucasian chronic hepatitis C patients (mean age 44.0 ± 12.0 years, median 42 years) who consulted the Liver Unit of the Department of Gastroenterology and Endocrinology at the University Medical Center Goettingen (UMG), Germany, between 1993 and 2006 were enrolled. The chronic nature of infection was proven by detection of HCV-specific antibodies and HCV RNA in the patients' sera using a highly sensitive nested reverse transcription polymerase chain reaction (RT-PCR) over a period of at least 6 mo as described[24]. As part of routine clinical evaluation, liver biopsy procedures were performed and liver disease was confirmed in the course of a defined histological evaluation as described below. Biochemical liver disease parameters, i.e. serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transferase (γ-GT) were recorded in parallel. Patients with concomitant non-hepatitis C viral infections and those with continued alcohol or other drug abuse were excluded.

Samples and data from another 349 chronic hepatitis C patients (mean age 45.8 ± 13.5 years, median 45 years) were kindly provided by the German Network of Competence for Hepatitis (Hep-Net)[25].

Determination of HCV genotype

HCV genotyping was performed for the 137 patients (UMG group) using the Innolipa HCV Ⅱ line probe assay (Innogenetics, Ghent, Belgium).

Histological evaluation

Before the start of therapy, liver biopsies were taken from patients for histological evaluation. In brief, sections (5-10 μm) from formalin-fixed and paraffin-embedded liver biopsies were stained with hematoxylin-eosin, trichrome, and Prussian blue. According to Desmet and colleagues, necroinflammatory activity (grading, score 1 to 3), and structural alterations (staging, score 0 to 4) were scored separately[26]. Other lesions typical of hepatitis C such as degree of steatosis (score 0 to 3), the presence or absence of portal lymphoid aggregates, and the presence or absence of bile duct damage were studied additionally as previously described[4]. Hep-Net samples were independently scored by two experienced pathologists according to the German guidelines with regard to inflammation activity and fibrosis progression[25,26].

Isolation of genomic DNA

Genomic DNA (gDNA) was purified from peripheral blood mononuclear cells (PBMCs) using the QIAamp DNA Mini Kit following the blood and body fluid spin protocol (Qiagen, Hilden, Germany). The concentration and the purity of the DNA isolated from PBMCs were determined spectrophotometrically by reading the absorbance levels at 260 and 280 nm. The integrity of gDNA was ascertained through electrophoresis using a 0.6% agarose gel. Alternatively, when PBMCs were not available, gDNA was purified from a 2 mL sample of serum by means of the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

SNP genotyping by 5'nuclease assay

gDNA (10 ng derived from PBMCs or an aliquot corresponding to 12.5 μL serum) was amplified in a total volume of 20 μL by real-time PCR using the TaqMan® Universal Master Mix (Applied Biosystems, Darmstadt, Germany) and 36 μmol/L of primers each (CD14: forward 5'-CTAGATGCCCTGCAGAATCCTT-3', reverse 5'-CCCTTCCTTTCCTGGAAATATTGCA-3'). Allelic discrimination was achieved by adding 8 μmol/L differentially fluorescence dye-labeled allele-specific minor

Table 1 Epidemiological characteristics of chronic hepatitis C patients with regard to CD14 rs2569190 genotype (UMG group)

	n	CD14 rs2569190 genotype				MAF	*P*
		CC	CT	TT	*P*		
Total number (%)	137	30 (21.9)	70 (51.1)	37 (27)	0.865[1]	0.526	
Gender (Female/male)	60/77	11/19	36/34	13/24	0.214[2]	0.517/0.532	0.795[3]
Age (mean ± SD)		46.5 ± 13.1	45.3 ± 10.8	39.6 ± 12.5	0.008[a]		
HCV subtype							
1a	31	7 (22.6)	17 (54.8)	7 (22.6)			
1b	73	18 (24.7)	38 (52.0)	17 (23.3)		0.500	
1a + 1b	6	1 (16.7)	3 (50.0)	2 (33.3)			
					0.073[b]		0.087[3]
2a	1	0	0	1 (100.0)			
2b	4	2 (50.0)	1 (25.0)	1 (25.0)		0.630	
3a	22	2 (16.7)	11 (50.0)	9 (33.3)			

MAF: Minor allele frequency. [1]Exact test for the Hardy-Weinberg equilibrium; [2]C carriers *vs* TT (χ^2 test); [3]χ^2 test was applied; [a]C carriers *vs* TT (independent samples *t*-test); [b]C carriers *vs* TT, HCV type 1 *vs* non-type 1 infections (χ^2 test).

groove binder probes (CD14: VIC 5'-CCTGTTACGG TCCCCCTG-3', FAM 5'-CTGTTACGGCCCCCCT-3'). Reactions and analyses were carried out in the sequence detection system ABI prism 7000 (Applied Biosystems, Darmstadt, Germany) according to the supplier's instructions.

Statistical analysis

Quantitative parameters were described by mean and standard deviation or median and inter-quartile range, and the Kolmogorov-Smirnov test was applied to investigate whether these distributions were Gaussian. The UMG group and Hep-Net group were compared regarding age using the parametric independent samples *t*-test.

For describing the ordinal and nominal scaled parameters such as gender, HCV subtype, hepatitis activity, fibrosis, steatosis, portal lymphatic aggregates, and bile duct damage, absolute frequencies and percentages were determined. χ^2 tests were applied to investigate the association of these parameters with the genotype or minor allele frequency (MAF).

To avoid bias, data were also stratified by age (< 44 years, and ⩾ 44 years), and parameters which showed significant correlations to the genotype in the univariate analysis were also analyzed using multivariate logistic regression. The results of stratifying and logistic regression were noted in the text and/or the tables where necessary.

All tests were performed two-sided and the level of significance was set at 0.05. The test results were interpreted in an exploratory way because no alpha adjustment for multiple testing was carried out. Statistical analyses were performed with the assistance of Medistat GmbH, Kiel, Germany, using PASW 17 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Epidemiological characteristics

A total of 137 and 349 patients with chronic hepatitis C (UMG and Hep-Net groups, respectively) were genotyped for the bi-allelic SNP within the *CD14* gene, rs2569190/C-159T. The variant allele T in the first group was found to be about as frequent as the wild-type C allele leading to a CC:CT:TT genotype distribution of 30:70:37 and a T allele frequency of 0.526 (Table 1). Hep-Net patients followed a distribution of 109:170:70 leading to a lower MAF of 0.444 (Table 2). MAFs were close to that given for Caucasians in public databases. The genotype distribution in both groups followed the Hardy-Weinberg equilibrium (Tables 1 and 2, respectively).

No significant difference was found between UMG and Hep-Net patients' regarding gender and age (χ^2 test, $P = 0.781$, independent samples *t*-test, $P = 0.177$, respectively). Epidemiological analysis revealed no significant relationships between the patients' CD14 rs2569190 genotype and gender (Tables 1 and 2). However, when analyzing patients' age with regard to the studied SNP genotypes, the UMG patients homozygous for the variant allele T were found to be on average 6.1 years younger than C carrier patients (mean age, 45.7 ± 11.5 years) at the time of liver biopsy taken before the start of therapy (Table 1). This observation, however, was absent in Hep-Net patients (Table 2).

Similar to the European population, most UMG patients were infected with HCV subtype 1b, followed by 1a and 3a. No significant difference was found between the distribution of HCV type 1 and non-type 1 infections among the three SNP genotypes (Table 1).

Biochemical parameters

Before the start of therapy, AST, ALT, and γ-GT serum activities were recorded for UMG patients as indicators of liver injury in chronic hepatitis C. The median levels of AST and ALT showed an increase from the wild-type to the variant type (Table 3). The number of TT patients with markedly elevated serum ALT activities, i.e. greater than two-fold the upper normal limit, was found to be markedly higher than the number of TT patients with normal ALT activities yielding a slightly increased T allele frequency among patients with markedly elevated ALT (χ^2 test, $P = 0.044$) (Table 3). However, after stratification by age, this was no longer significant (data not shown).

Table 2 Epidemiological characteristics of chronic hepatitis C patients with regard to CD14 rs2569190 genotype (Hep-Net group)

	n	CD14 rs2569190 genotype				MAF	*P*
		CC	CT	TT	*P*		
Total number (%)	349	109 (31.2)	170 (48.7)	70 (20.1)	0.828[1]	0.444	
Gender (Female/male)	148/201	47/62	66/104	35/35	0.151[a]	0.459/0.433	0.484[2]
Age (mean ± SD)		47.2 ± 13.5	44.6 ± 13.6	46.5 ± 13.3	0.616[b]		

[1]Exact test for the Hardy-Weinberg equilibrium; [2]χ^2 test was applied; [a]C carriers *vs* TT (χ^2 test); [b]C carriers *vs* TT (independent samples *t*-test).

Table 3 Biochemical serum parameters and the number of patients with elevated parameters in chronic hepatitis C with regard to CD14 rs2569190 genotype (UMG group)

	CD14 rs2569190 genotype				MAF	[2]*P*
	CC	CT	TT	[2]*P*		
AST (median, IQR)	21.0, 15.8-50.5	28.5, 17.0-52.3	32.0, 16.5-76.5			
[1]Number of patients with elevated/normal AST	9/21	29/41	19/18	0.159[a]	0.588/0.481	0.082
ALT (median, IQR)	36.0, 25.8-85.5	46.5, 26.8-87.3	50.0, 34.5-153.5			
[1]Number of patients with elevated/normal ALT	11/19	38/32	23/14	0.171[a]	0.583/0.462	0.044
γ-GT (median, IQR)	35.5, 13.8-58.8	27.0, 14.0-56.3	32.0, 15.5-53.5			
[1]Number of patients with elevated/normal γ-GT	10/20	19/51	11/26	0.934[a]	0.513/0.531	0.781

AST: Aspartate aminotransferase. Upper normal limit is 19 U/mL for males, and 15 U/mL for females; ALT: Alanine aminotransferase. Upper normal limit is 23 U/mL for males, and 19 U/mL for females; γ-GT: γ-glutamyltransferase. Upper normal limit is 28 U/mL for males, and 18 U/mL for females; IQR: Inter-quartile range. [1]Markedly elevated serum activities of transaminases (> two-fold the upper normal limit) were considered; [2]χ^2 test was applied; [a]C carriers *vs* TT. After stratification by age, all *P*-values were non-significant.

Hepatitis C disease activity and progression

Liver biopsy specimens were taken before the start of an interferon-based therapy and evaluated histologically. Hep-Net patients had higher frequencies of advanced degrees of hepatitis activity and fibrosis progression (Tables 4 and 5) (χ^2 test, $P < 0.001$ for both parameters). Both UMG and Hep-Net patients showed no correlation between their CD14 C-159T genotype and hepatitis activity or fibrosis (Tables 4 and 5, respectively). Other lesions typical and more characteristic of hepatitis C, namely the degree of hepatic steatosis, the presence or absence of lymphoid aggregates and bile duct damage, were additionally studied in UMG patients. With regard to the degree of steatosis and bile duct damage, no significant association with CD14 C-159T genotype distribution could be found (Table 4). T allele homozygous patients, however, were found to have portal lymphoid aggregates more frequently than C carriers (21/37 *vs* 32/100, respectively, χ^2 test, $P = 0.008$) (Table 4).

To avoid spurious findings, a separate analysis was carried out to identify other factors which might underlie the formation of portal lymphoid aggregates. No relationship was found between the presence or absence of portal lymphoid aggregates and sex, age, HCV subtype, biochemical parameters, the stage of fibrosis, or the degree of steatosis (logistic regression analysis, data not shown). In accordance with previous studies, however, a significant relationship between the presence of portal lymphoid aggregates and hepatic inflammatory activity (χ^2 test, $P = 0.003$) and bile duct damage (χ^2 test, $P < 0.001$) was found[3,5,27]. Nevertheless, even in the subgroups which had portal lymphoid aggregates with other lesions, a shift towards the T allele was always observed: 15 TT and 13 CT among the 32 patients who had both a high grade of hepatitis activity and portal lymphoid aggregates: (MAF = 0.672 compared to 0.481 for the remaining patients); 13 TT and 17 CT among the 35 patients who presented with both bile duct damage and portal lymphoid aggregates: (MAF = 0.614 compared to 0.495 for the others) (data not shown).

DISCUSSION

Among chronic hepatitis C patients, no evidence was found for a relationship between the endotoxin receptor CD14 rs2569190/C-159T genotype and the progression of liver fibrosis. This finding was obtained by analyzing two different patient cohorts, one derived from the UMG ($n = 137$) (Table 4), the other from the Hep-Net, a Germany-wide collection of samples ($n = 349$) (Table 5). It is in line with previous findings on Caucasian patients[20,22].

By considering the sum of the evidence, the situation of chronic liver disease resulting from HCV infection appears to be different from the situation of chronic liver disease resulting from alcohol consumption: whereas in ALD, progression of fibrosis was shown to be associated with TT genotypes[18-20], in chronic hepatitis C it did not appear to be related to this genetic variation.

Of note, a recent report found the influence of an environmental factor on the association of rs2569190/C-159T and total serum IgE levels in Russian children[28]. Depending on the *Helicobacter pylori* (*H pylori*) infection status, seronegative or seropositive, the T allele was associated with a decreased or an increased IgE serum concentration, respectively[28]. The observation of the lack of an association of rs2569190/C-159T genotype with chronic hepatitis C disease progression, but an

Table 4 Histological features in chronic hepatitis C-infected patients with regard to CD14 rs2569190 genotype (UMG group) *n* (%)

Histological features	CD14 rs2569190 genotype				MAF	[a]*P*
	CC	CT	TT	[a]*P*		
Hepatitis activity						
Mild	19 (25.0)	40 (52.6)	17 (22.4)		0.487	
Moderate	10 (19.2)	24 (46.2)	18 (34.6)	0.172[b]	0.574	0.152
Severe	1 (11.1)	6 (66.7)	2 (22.2)			
Fibrosis						
Absent	7 (38.9)	6 (33.3)	5 (27.8)		0.517	
Mild	14 (20.6)	35 (51.5)	19 (27.9)			
Moderate	5 (19.2)	15 (57.7)	6 (23.1)	0.758[b]		0.727
Marked	3 (21.4)	7 (50.0)	4 (28.6)		0.539	
Cirrhosis	1 (9.1)	7 (63.6)	3 (27.3)			
Steatosis						
Absent	14 (23.3)	28 (46.7)	18 (30.0)		0.528	
Mild	10 (20.8)	26 (54.2)	12 (25.0)	0.695[b]		0.887
Moderate	5 (26.3)	10 (52.6)	4 (21.1)		0.517	
Marked	1 (10.0)	6 (60.0)	3 (30.0)			
Portal lymphoid aggregates						
Absent	22 (26.2)	46 (54.8)	16 (19.1)	0.008[b]	0.464	0.011
Present	8 (15.1)	24 (45.3)	21 (39.6)		0.623	
Bile duct damage						
Absent	21 (23.6)	46 (51.7)	22 (24.7)	0.411[b]	0.506	0.368
Present	9 (18.8)	24 (50.0)	15 (31.3)		0.563	

[a]χ^2 test was applied to compare mild *vs* moderate and severe hepatitis activity, absent, mild *vs* moderate and marked fibrosis and cirrhosis, and absent, mild *vs* moderate and marked steatosis; [b]C carriers *vs* TT.

Table 5 Hepatitis activity (grading) and fibrosis (staging) in chronic hepatitis C-infected patients with regard to CD14 rs2569190 genotype (Hep-Net group) *n* (%)

	CD14 rs2569190 genotype				MAF	[a]*P*
	CC	CT	TT	[a]*P*		
Hepatitis activity						
Mild	19 (27.1)	35 (50.0)	16 (22.9)		0.479	
Moderate	73 (32.2)	113 (49.8)	41 (18.1)	0.513[b]	0.435	0.359
Severe	17 (32.7)	22 (42.3)	13 (25.0)			
Fibrosis						
Absent	4 (57.1)	2 (28.6)	1 (14.3)		0.449	
Mild	35 (28.9)	61 (50.4)	25 (20.7)			
Moderate	44 (32.8)	63 (47.0)	27 (20.2)	0.928[b]		0.837
Marked	18 (27.7)	37 (56.9)	10 (15.4)		0.441	
Cirrhosis	8 (36.4)	7 (31.8)	7 (31.8)			

[a]χ^2 test was applied to compare mild *vs* moderate and severe hepatitis activity, absent, mild *vs* moderate and marked fibrosis and cirrhosis; [b]C carriers *vs* TT.

association with ALD and the dependency of the association on *H pylori* infection status for an atopy-related parameter, suggests that LPS sensitivity depends on both genetic and environmental factors (gene-environment interaction).

The lack of an association between this CD14 SNP and liver disease progression in chronic hepatitis C patients, is also in accordance with a finding by Huang *et al*[29] who, by functional genomic scanning, identified seven SNPs within seven genes which carry the most relevant risk for developing cirrhosis in Caucasian hepatitis C patients, with CD14 not being among them.

In contrast, a significantly higher frequency of rs2569190 T homozygote hepatitis C patients was found among patients with portal lymphoid aggregates (Table 4). As with lymphocyte infiltrations, primary follicles and secondary follicles in the lymph nodes, portal lymphoid aggregates have been described to occur in various patterns ranging from vague lymphoid aggregation to round defined follicles to well-formed follicles with clearly identifiable germinal centers[30]. Their presence has been attributed to the participation of the host's immune system in liver disease pathogenesis[3,5]. To our knowledge, even today, the etiology and relevance of this manifestation for disease development and/or progression remains unclear. The presence of portal lymphoid aggregates was the only histological manifestation found to be related to CD14 rs2569190/C-159T genotypes (Table 4). As reported in other studies, the presence of portal lymphoid aggregates was found to be closely correlated with bile duct damage and the degree of inflammatory activity[3,5,27], but not with sex, age[3,4], or HCV subtype[4]. The finding of a correlation between portal lymphoid aggregates and HCV subtype 1b infection in Chinese[3] but not European patients is obviously related to a different

HCV subtype distribution. The comparison between subtype 1b- and type 2-infected patients, as is possible among Chinese patients, cannot be carried out among European patients because of the low proportion of type 2 infections. Portal lymphoid aggregates have also usually been found in the early stages of liver disease and to have disappeared in cirrhosis[27,31]. Thus, our finding of a positive association of TT status and the presence of portal lymphoid aggregates, on the one hand, and the absence of an association with disease progression, on the other hand, are concordant with that observation.

The relationship between TT genotype and younger age in the UMG group (Table 1) was neither observed among the patients from the Hep-Net group (Table 2) nor among patients from another German study[20]. In contrast to what might have been expected, these three cohorts do differ in demographic and clinical features. For instance, as outlined, the Hep-Net group comprised a higher proportion of patients with greater degrees of hepatic inflammatory activity and also more advanced stages of fibrosis than the UMG group (Tables 4 and 5). The cohort studied by Meiler *et al*[20] was found to differ significantly in age from the Hep-Net group and both in age and gender distribution from the UMG group (data not shown). Von Hahn's cohort was similar to the UMG and Hep-Net groups with regard to gender distribution, however, further demographic analysis, i.e. association of genotypes and age, was not given[22].

In conclusion, in contrast to what was reported for ALD, our analyses did not reveal an increased risk for chronic hepatitis C patients, homozygous for the CD14 C-159T T allele, to develop more pronounced fibrosis but suggested a relationship of this variation in the formation of portal lymphoid aggregates.

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COMMENTS

Background

Progressive hepatic fibrosis develops in patients with chronic liver diseases irrespective of etiology and with a marked inter-individual variability. Different host genetic backgrounds have been shown to act as co-factors in promoting ongoing liver disease progression exacerbated by gut-derived bacterial lipopolysaccharides (endotoxins).

Research frontiers

The variant allele T of a well-known single nucleotide polymorphism (SNP) in the endotoxin receptor *CD14* gene has been reported to be associated with increased alcohol-related liver cirrhosis. In this study, the authors show that the T allele is correlated with the presence of portal lymphoid aggregates rather than being associated with fibrosis progression in chronic hepatitis C virus (HCV) infection.

Innovations and breakthroughs

Recent reports have shown a lack of an association between the T allele and liver fibrosis progression in the context of chronic HCV infection. Genetic association studies, however, require to be replicated. Apart from performing an analysis in two different cohorts, this study is the first to expand the analysis to further histological lesions typical of HCV infection with regard to CD14 rs2569190/C-159T.

Applications

Understanding the mechanisms underlying chronic hepatocellular injury in hepatitis C is important for therapy applications. This study argues for a possible relationship of CD14 rs2569190 T allele in the formation of portal lymphoid aggregates, the presence of which has been attributed to the host's immunological participation in liver disease pathogenesis. Moreover, it excludes a possible role of the variation in promoting fibrosis progression.

Terminology

Liver fibrosis is the excessive accumulation of extracellular matrix proteins including collagen. Cirrhosis is the last stage of fibrosis. A SNP is a variation of one base in the DNA; the nucleotide observed is different from the norm at this position. It occurs with a frequency of > 1% in the normal population. Portal lymphoid aggregates are defined as a densely packed collection of small lymphocytes within the portal tract without or with the formation of a germinal center. Lipopolysaccharide is the main component of the outer cell wall of gram-negative bacteria.

Peer review

This is a valuable population-based association study, which is useful for examining a well-known genetic variation with a role in a common multifactorial disease that may have a strong environmental component. In contrast to the situation in alcoholic liver cirrhosis, the SNP rs2569190/C-159T is not related to chronic HCV-induced fibrosis progression, but to another histological feature, namely, the presence of portal lymphoid aggregates.

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BRIEF ARTICLES

Anti-microbial antibodies in celiac disease: Trick or treat?

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Abstract

AIM: To determine the prevalence of a new set of anti-glycan and anti-outer membrane protein (anti-OMP) antibodies in a Hungarian cohort of adult Celiac disease (CD) patients.

METHODS: 190 consecutive CD patients [M/F: 71/119, age:39.9 (SD:14.1) years], 100 healthy, and 48 gastrointestinal controls were tested for glycan anti-Saccharomyces cerevisiae (gASCA), anti-laminaribioside (ALCA), anti-chitobioside, anti-mannobioside, anti-OMP antibodies and major NOD2/CARD15 mutations. Thirty out of 82 CD patients enrolled at the time of diagnosis were re-evaluated for the same antibodies after longstanding gluten-free diet (GFD).

RESULTS: 65.9% of the CD patients were positive for at least one of the tested antibodies at the time of the diagnosis. Except anti-OMP and ALCA, anti-microbial antibodies were exclusively seen in untreated CD; however, the overall sensitivity was low. Any glycan positivity (LR+: 3.13; 95% CI: 2.08-4.73) was associated with an increased likelihood ratio for diagnosing CD. Significant correlation was found between the levels of anti-glycan and anti-endomysial or anti-transglutaminase antibodies. Anti-glycan positivity was lost after longstanding GFD. Anti-glycan antibody titers were associated with symptoms at presentation, but not the presence of NOD2/CARD15 mutations. Patients with severe malabsorption more frequently had multiple antibodies at diagnosis (P = 0.019).

CONCLUSION: The presence of anti-glycan antibodies in CD seems to be secondary to the impaired small bowel mucosa which can lead to increased antigen presentation. Furthermore, anti-glycan positivity may be considered an additional marker of CD and dietary adherence.

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Key words: Celiac disease; Glycans; Anti-Saccharomyces cerevisiae antibodies; Anti-outer membrane protein antibody; NOD2/CARD15; Gluten-free diet; Presenting symptoms; Bacterial translocation; Crohn's disease

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INTRODUCTION

Celiac disease (CD) is a genetically determined chronic inflammatory disorder with autoimmune components induced by exposure to gluten[1]. The clinical presentation of the disease is highly variable and little is known about the factors that determine the type of symptoms. The inflamed and damaged small bowel mucosa as well as the clinical symptoms shows recovery in most affected subjects after a complete removal of gluten from their diet[2]. The presence of different autoantibodies is a typical feature of CD; however, the exact mechanism behind their production and potential role in the disease pathogenesis has not yet been fully understood[3]. Tissue transglutaminase 2 (TG2) is the main autoantigen of anti-endomysial antibodies (EMA) and anti-TG2/EMA are commonly used for screening and diagnosing CD[4]. More recently antibodies directed against synthetic deamidated gliadin peptides have also been suggested as a reliable tool for diagnosing CD[5]. Most of the other antibodies do not appear to have either high sensitivity or specificity for CD, but they may be associated with specific clinical presentations or extra-intestinal manifestations[6]. As a sign of the cytoskeleton and intercellular tight junction involvement, a high prevalence of IgA anti-actin antibodies was also reported which strongly correlated with the degree of villous atrophy, appearing in more severe forms of the disease[7]. Furthermore, production of anti-actin antibodies was gluten dependent. After strict adherence to gluten-free diet (GFD), they become undetectable within several months indicating that their appearance follows mucosal injury[8,9]. Similarly, also the occurrence of anti-zonulin antibodies was associated with active CD and disappearing during remission[10].

The presence of serological responses to various microbial antigens [e.g. phosphopeptidomannan cell-wall component of anti-Saccharomyces cerevisiae (ASCA), outer membrane porin C transport protein of the *Escherichia coli* (OmpC) or the *Pseudomonas fluorescens* associated protein I2] is characteristic of Crohn's disease and has been intensively studied for its clinical value in this patient group[11]. The occurrence and magnitude of the seroreactivity are associated with complicated small bowel disease and the need for surgical intervention[12,13]. More recently, antibody formations against different glycans, which are common structures in the glycocalyx of pathogenic yeast and bacteria[14], have also been reported in this patient group. Our group demonstrated that with the use of anti-glycan (g)ASCA and a panel of novel anti-glycan antibodies [anti-mannobioside (AMCA), anti-laminaribioside (ALCA), and anti-chitobioside (ACCA)], gave the same conclusions. Additionally, we evaluated the performance of all four anti-glycan antibodies and the traditional combination of ASCA IgG and IgA. Both panels equally identified 59.4% of all Crohn's disease patients. Eighty percent of these identified cases were the same patients while the remaining 10%-10% were detected by only one of the panels[15].

Despite the fact that the exact mechanism behind antibody formation or their possible relevance in the pathogenesis still needs to be elucidated, current data suggest that these markers are not an epiphenomenon related to the inflamed, leaky bowel mucosa[16-18] but reflect a loss of tolerance toward bacterial and fungal flora[19]. Furthermore, the anti-microbial antibodies might represent genetic susceptibility because patients who have positive antibodies often carry mutations in the NOD2/CARD15 gene[11,20,21].

Anti-microbial antibody formation has also been reported in CD. ASCAs remain the most widely investigated antibodies[22-26] in this patient group but increasing experimental data are available on newly discovered antibodies such as anti-I2 or anti-OmpW (*Bacteroides caccae* TonB-linked outer membrane protein)[27,28]. The frequency of ASCA varied from 27% to 59% in various studies, owing to the differences in the number and the age of the patients as well as the commercial assays used for antibody detection. The frequency of seropositivity and serum levels of these antibodies clearly decreased during GFD.

We hypothesized that newly discovered inflammatory bowel disease (IBD)-associated antibodies (including anti-glycan antibodies and anti-OMP) may also be of importance in CD and aimed to determine the prevalence of these antibodies in a Hungarian cohort of adult CD patients in relation to clinical presentation, GFD and NOD2/CARD15 mutations.

MATERIALS AND METHODS

Patients

One-hundred and ninety consecutive, unrelated Hungarian adult patients with biopsy-proven CD (male/female: 71/119, mean age: 39.9 years, SD: 14.1) and 66 of their first degree relatives (siblings, mean age: 37.7 years, SD: 13.9) were investigated. The diagnosis of CD was based on small bowel biopsy showing severe villous atrophy with crypt hyperplasia (Marsh type Ⅲ lesions)[29] and elevated serum levels of antibodies against transglutaminase (TGA) and endomysium (EMA). IgA and IgG class EMA were investigated on human umbilical cord substrate using indirect immunofluorescence method as previously described[30]. TGA were measured by enzyme-linked immunosorbent assay (ELISA) using human recombinant antigen expressed in *Escherichia coli*[31,32].

Of the 190 patients, 82 patients' sera were obtained at the time of diagnosis (Group CD1) and in 30 of these 82 patients further serum samples were re-evaluated for the same antibodies after adherence to long-standing GFD. The median follow-up period between these blood samplings was 28.5 mo [interquartile range (IQR): 18-52]. In the 108 remaining cases the diagnosis of CD had been established prior to this study and they adopted a strict GFD. These 108 patients were divided into two separate groups according to their current TGA and EMA status and dietary compliance at the time of the sampling. Thirty-three patients still had positive EMA and TGA results (Group CD2) and the median duration was here 3.5 mo (IQR: 1-11). The adequate compliance was indicated by reduced antibody titers as compared to those at diagnosis. The remaining 75 patients had

negative EMA and normal TGA titers (Group CD3), median follow up: 21 mo (IQR: 6-85).

Detailed clinical data concerning the clinical presentation of CD at diagnosis were classified as follows: (1) severe generalized malabsorption (presence of at least four of the following five symptoms: diarrhea, abdominal distension, weight loss, anemia, hypoproteinemia); (2) non-specific gastrointestinal symptoms that did not compromise the general condition (diarrhea, constipation, bloating, recurrent abdominal pain or vomiting, esophageal reflux); (3) iron deficiency anemia without major gastrointestinal complaints; (4) dermatitis herpetiformis; (5) silent disease (population screening); (6) other (autoimmune diseases, reduced bone mineral density, liver disease, brain disease). Patients were assigned to one of these major presentation types in a prospective manner, based on clinical and routine laboratory results at diagnosis.

The control group consisted of 100 healthy, ethnically similar, blood donor individuals (male/female: 47/53, mean age: 36.6 years, SD: 9.1) who had normal findings on a thorough medical examination, blood pressure measurements, and routine laboratory tests. A second non-celiac gastrointestinal disease control group consisted of 48 patients with irritable bowel syndrome/diverticulosis without inflammation (male/female: 21/26, mean age 40.4 years, SD: 16.1). In controls, CD was excluded by the negativity of EMA and TGA. None of the control subjects had a family history of CD. Further comparisons were made with the previously published Crohn's disease cohort we investigated for the same antibodies earlier[15].

The study protocol was approved by the Regional and Institutional Committee of Science and Research Ethics of the University of Debrecen (DEOEC RKEB/IKEB: 2600-2007). Informed consent was obtained from all patients and controls.

Anti-microbial antibody assays

gASCA IgG, AMCA IgG, ALCA IgG, ACCA IgA (IBDX®, Glycominds Ltd., Lod, Israel), ASCA IgG, ASCA IgA and OMP IgA (QUANTA Lite™ OMP PLUS, INOVA Diagnostics, San Diego, CA) were measured in sera according to the manufacturers' protocols. The results were presented as arbitrary units, which were calculated based on sample and calibrator optical density. Cut-off levels used for the determination of positivity were according to the manufacturers' guidelines: 50, 100, 60 and 90 U/mL for gASCA IgG, AMCA IgG, ALCA IgG, ACCA IgA, respectively, and 25 U/mL for ASCA IgG, ASCA IgA and OMP IgA.

Detection of NOD2/CARD15 mutations

One-hundred and thirty-four CD patients and 100 healthy control subjects were eligible for NOD2/CARD15 mutation analysis. Major NOD2/CARD15 mutations (SNP8, 12 and 13) were determined as previously described[13] by denaturing high-performance liquid chromatography (dHPLC, Wave DNA Fragment Analysis System, Transgenomic Limited, UK). Sequence variation, observed in the dHPLC profile, was sequenced on both strands to confirm the alteration. Sequencing reactions were performed with the ABI BigDye Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems, Foster City, CA) and samples were sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Statistical analysis

Variables were tested for normality with Shapiro Wilk's *W* test. *t*-test with separate variance estimates, χ^2-test, χ^2-test with Yates correction and likelihood ratio (LR) test were calculated to evaluate differences between various groups of CD patients and controls, as well as within subgroups of CD patients, as appropriate. Sensitivities, specificities, positive and negative likelihood ratios were calculated to determine the predictive power of gASCA, AMCA, ALCA, ACCA, OMP or the combination of these markers in distinguishing between CD and controls. Spearman's rank order correlation was calculated to test the association between anti-glycan/OMP and TGA levels. Two-sided *t*-test for independent samples with separate variance estimates and ANOVA with post hoc Scheffe test were used to analyze the association between anti-glycan antibody titers and clinical symptoms at diagnosis. A P value of < 0.05 was considered as significant. For statistical analysis, SPSS15.0 (SPSS Inc, Chicago, IL) was used with the help of a statistician (Dr. Peter Vargha).

RESULTS

Presence of anti-glycan and anti-OMP antibodies at the time of diagnosis of celiac disease

The frequency and the mean titers of gASCA IgG, AMCA IgG, and ACCA IgA were significantly higher at the time of diagnosis of CD than in healthy and non-celiac gastrointestinal control groups (Table 1, Figure 1). However, the frequency of ALCA IgG and anti-OMP IgA positivity and also the mean titers in the patients were similar to those in control groups. No difference was found between healthy subjects and GI controls based on the presence of these antibodies. For that reason, we only used the healthy subjects as a control group in the subsequent comparisons.

When calculating the sensitivity and specificity of the different markers based on the cut-off values suggested by the manufacturer, 65.9% of the CD patients were positive for at least one of the tested anti-microbial antibodies at the time of diagnosis. Except ALCA, all anti-glycan antibodies were specific for untreated CD. However, the overall sensitivity was low (gASCA: 39.0%, AMCA: 35.4%, ACCA: 37.8%). The above association was further tested by using the LR test. The sensitivity, specificity, positive and negative LR between CD at diagnosis and controls are presented in Table 2. Compared to healthy controls, gASCA, AMCA, and ACCA were associated with a moderate increase in the likelihood of CD, respectively. The positivity of any anti-glycan antibody significantly increased the likelihood for untreated CD (Table 2).

Detailed clinical data on the symptoms at the time

Table 1 Frequency of anti-microbial antibodies in 190 patients with celiac disease and in control groups *n* (%)

	Group 1	Group 2	Group 3	Control	GI control
n	82	33	75	100	48
TGA IgA (U/mL) (median; IQR)	54.3 (20.2-100.0)	32.2 (9.9-73.1)	2.7 (2.2-3.7)	–	–
gASCA IgG positive	32 (39.0)[b,f,j]	12 (36.3)[d,f,l]	10 (13.3)	14 (14)	2 (4.2)
AMCA IgG positive	29 (35.4)[b,f,j,n]	3 (9.1)	1 (1.3)	0 (0)	0 (0)
ALCA IgG positive	7 (8.5)	0 (0)	0 (0)	6 (6)	1 (2.1)
ACCA IgA positive	31 (37.8)[b,f,j,n]	4 (12.1)	3 (4.2)	6 (6)	1 (2.1)
Any glycan positive	54 (65.9)[b,f,j,p]	15 (45.4)[d,f,l]	15 (20)	21 (21)	4 (8.4)
Anti-OMP IgA positive	22 (26.8)	11 (33.3)	23 (30.7)	20 (20)	11 (22.9)

TGA: Antibodies against transglutaminase; Group 1: Celiac patients at the time of diagnosis; Group 2: Celiac patients after starting a gluten-free diet but still with celiac antibody positivity; Group 3: Celiac patients on a long-term strict gluten-free diet; Control: Healthy control; GI control: Non-celiac gastrointestinal disease control. Cut-off levels used for the determination of positivity were according to the manufacturers' guidelines: 50, 100, 60, 90 U/mL and 25 U/mL for gASCA IgG, AMCA IgG, ALCA IgG, ACCA IgA, and anti-OMP IgA, respectively. [b]$P < 0.001$, [d]$P < 0.01$, group 1 or Group 2 *vs* control; [f]$P < 0.001$, group 1 or Group 2 *vs* GI control; [l]$P < 0.001$, [l]$P < 0.01$, group 1 or Group 2 *vs* Group 3; [n]$P < 0.001$, [p]$P < 0.03$, group 1 *vs* Group 2. Using χ^2-test with Yates correction.

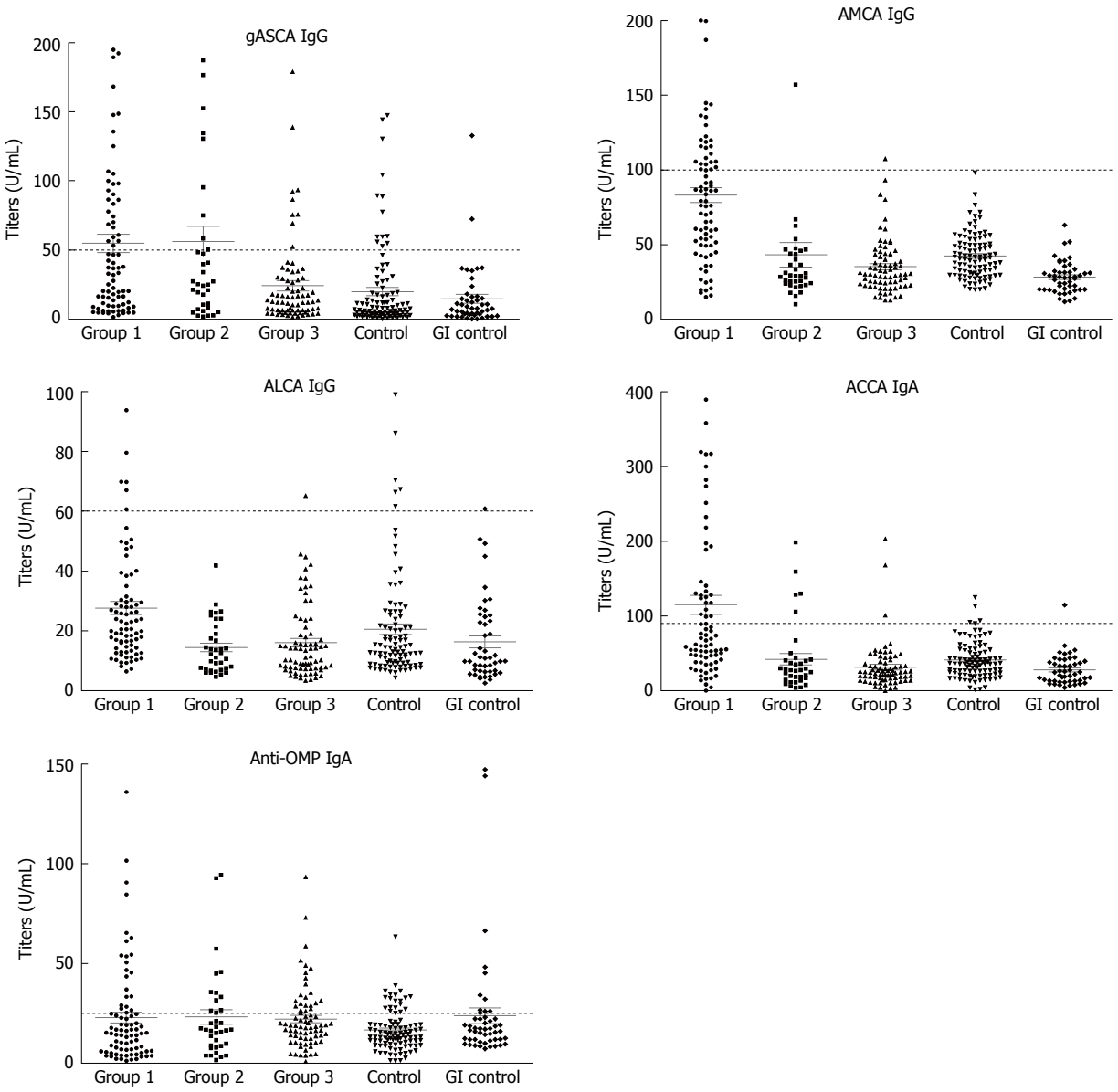

Figure 1 Anti-microbial antibody levels in 190 patients with celiac disease and in control groups. Individual values are shown by black spots. Mean values with standard error bars are indicated in gray. Cut-off values for positivity are pointed out by dotted line and 50, 100, 60, 90 and 25 U/mL for gASCA IgG, AMCA IgG, ALCA IgG, ACCA IgA and OMP IgA, respectively.

Table 2 Predictive power of serological markers for distinguishing between patients with celiac disease at the time of diagnosis and various control groups

	Sensitivity (%)	Specificity (%)	95% CI	
			LR+	LR-
Celiac disease *vs* healthy controls				
gASCA	39	86	2.73 (1.57-4.76)	0.71 (0.59-0.86)
AMCA	35	100	-	-
ACCA	38	94	6.36 (2.76-14.4)	0.66 (0.56-0.79)
Any glycans	66	79	3.13 (2.08-4.73)	0.43 (0.31-0.59)
Celiac disease *vs* non-celiac gastrointestinal controls				
gASCA	39	96	9.36 (2.35-37.4)	0.64 (0.53-0.76)
AMCA	35	100	-	-
ACCA	38	98	18.1 (2.56-128.5)	0.64 (0.53-0.76)
Any glycans	66	92	7.90 (3.05-20.4)	0.37 (0.27-0.51)

Table 3 Association between the titer of anti-microbial antibodies and the leading clinical symptoms at the time of celiac disease presentation (in Group 1, *n* = 78[1])

	Malabsorption	Non-specific gastrointestinal	Anaemia
n	32	34	9
gASCA IgG	54.8 (16.4-99.4)[b]	21.3 (7.1-76.5)	12.2 (8.8-36.7)
AMCA IgG	90.3 (59.5-115.9)[b]	73.8 (52.3-104.4)[a]	44.1 (25.1-55.0)
ALCA IgG	20.6 (16.7-31.2)[b]	23.7 (16.5-39.1)[a]	12.8 (9.9-15.2)
ACCA IgA	103.8 (53.3-192.1)[b]	57.3(37.4-100.5)[a]	26.8 (12.7-53.6)
OMP IgA	14.8 (5.2-33.2)	15.2 (8.2-24.2)	16.2 (2.9-34.1)

[1]Detailed clinical data were not available for 4 patients, data of 3 patients with other symptoms are not shown; [a]$P < 0.005$, [b]$P < 0.01$, between patients with non-specific gastrointestinal symptoms or malabsorption and anaemia by *t*-test for independent samples with separate variance estimates.

Table 4 Occurrence of multiple antibody responses to microbial antigens in untreated celiac disease patients in relation to the number of responses against microbial antigens *n* (%) (*n* = 78)

	0	1	2 to 4	Total
Severe malabsorption	7 (22)	8 (25)	17 (53)	32 (40)
Non-specific gastrointestinal symptoms	13 (38)	12 (35)	9 (27)	34 (44)
Iron deficiency anemia	6 (67)	3 (33)	0	9 (12)
Others	2	0	1	3 (4)
Total	28 (36)	23 (29)	27 (35)	78 (100)

$P = 0.019$ by χ^2-test. Clinical data were not available for 4 patients.

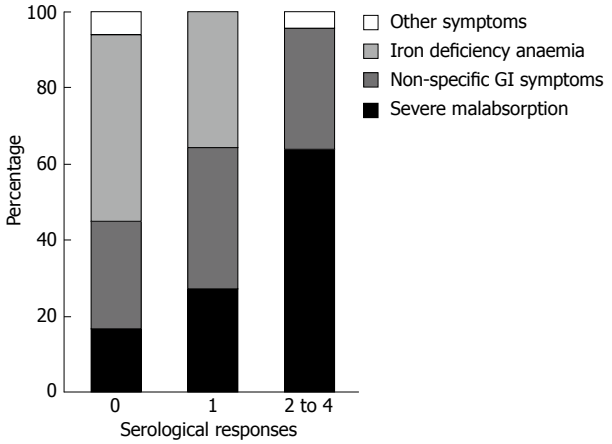

Figure 2 Clinical presentation of celiac disease according to serological response.

of diagnosis in Group CD1 was available in 78 patients out of 82. Of the 78 patients, 32 (41%) presented with severe malabsorption, 34 (43.6%) with non-specific or minor gastrointestinal symptoms, 9 (11.5%) with iron deficiency anemia, and 3 (3.9%) with other symptoms. The titers of the anti-glycan antibodies varied according to the presenting symptoms (Table 3) by 2-sided *t*-test for independent samples with separate variance estimates. If the above association was tested by ANOVA and post hoc Scheffe-test only the association for gASCA ($P = 0.027$) and AMCA ($P = 0.03$) remained significant. Moreover, the clinical presentations of CD were distributed differently according to serological response (Figure 2, Table 4). Patients with severe malabsorption more frequently had multiple antibodies ($P = 0.019$) while in those with non-specific gastrointestinal symptoms or iron deficiency anaemia no seroreactivity or reactivity against only one glycan components was more commonly seen (Table 4). Out of the CD patients with multiple antibodies positivity, 65.4% were diagnosed because of malabsorption, which was significantly higher than in CD patients with another serotype group (0 = 26.9%, or 1 = 34.8%, $P = 0.019$) (Figure 2).

Correlation between anti-glycan and anti-OMP antibodies and TGA or EMA

A significant correlation was found between anti-glycan and TGA levels ($P_{gASCA} < 0.001$, $R = 0.39$; $P_{AMCA} = 0.01$, $R = 0.28$; $P_{ALCA} = 0.006$, $R = 0.23$; $P_{ACCA} < 0.0001$, $R = 0.53$; $P_{antiOMP} = 0.001$, $R = 0.25$ by Spearman's rank order correlation). Similarly, a positive association was found between EMA IgA and gASCA ($P < 0.001$), AMCA ($P < 0.001$), ACCA ($P < 0.0001$), or any-glycan ($P < 0.0001$) but not with anti-OMP positivity.

The effect of strict gluten-free diet on anti-glycan and anti-OMP antibody positivity

In the group of 30 patients who were evaluated both at diagnosis and following a long term GFD (subgroup of Group CD1), initial positivity for anti-glycan antibodies (gASCA in 12, AMCA in 9, and ACCA in 11 patients) observed at diagnosis was lost after GFD. The titer of each antibody decreased significantly after adherence to GFD ($P < 0.001$ for each). Anti-OMP antibody positivity behaved similarly, with all but one of 14 patients positive at diagnosis becoming negative after GFD. The one patient who did not become negative during a 135 month-long period of GFD did in fact decrease during GFD from 33.2 to a borderline positive value of 25.4 units (a positive result is defined as $\geqslant 25$ units).

The level of the different antibodies was also significantly lower after GFD ($P < 0.001$ for each). Figure 3 shows individual anti-glycan and anti-OMP antibody titers at the time of diagnosis and their changes

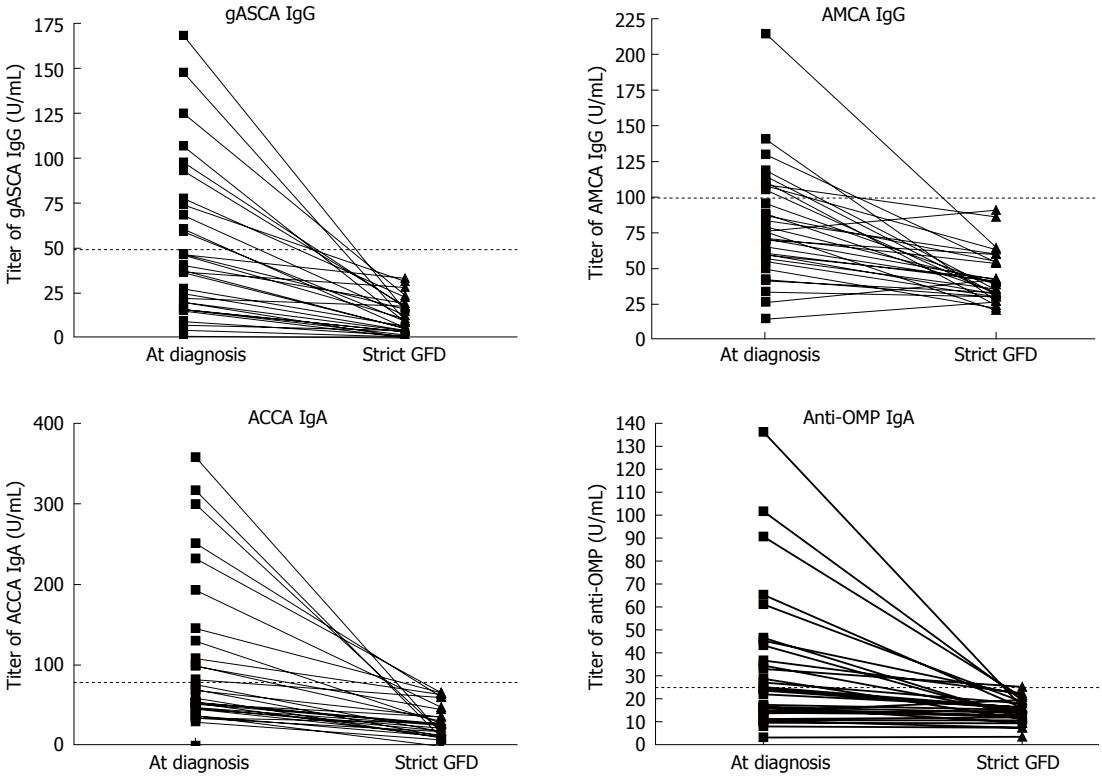

Figure 3 Individual anti-glycan and anti-OMP antibody titers at the time of the diagnosis and their variations after successful adherence of to the gluten-free diet (GFD). Mean follow up period of 49 [10-159] mo (*n* = 30). Dotted lines show cut-off values for positivity.

after successful adherence of to the GFD.

The frequency of antibodies directed against glycans and the mean antibody titers were significantly lower in patients with successful adherence to GFD (Group CD3) than in untreated patients (Group CD1) (Table 2, Figure 1) and did not differ statistically from healthy controls.

Prevalence of NOD2/CARD15 mutations and their association with antibody titers and symptoms at presentation

The prevalence of NOD2/CARD15 mutation in CD (19/134, 14.2%) did not differ from that in the control group (16/100, 16%). Additionally, we did not observe any association between symptoms at presentation or anti-glycan antibody positivity and the presence of NOD2/CARD15 variants (data not shown).

DISCUSSION

This is the first report to investigate the complex associations between a panel of new serological markers, clinical presentation of the disease, and NOD2/CARD15 status in a relatively large cohort of CD patients. Furthermore, direct comparison between the anti-microbial responses in this CD group and our similarly tested previous Crohn's disease cohort[15] can be made to add new pieces to the puzzle of the anti-microbial antibody formation.

In this study, we demonstrated that the presence of anti-glycan antibodies (gASCA, ACCA, and AMCA) are associated with CD at the time of diagnosis. However the prevalence of ALCA and anti-OMP did not differ from the results in the control group. The rate of gASCA positivity (39%) at the time of diagnosis of CD was comparable to the results in CD patients in previous studies[22-26]. Based on previous results a sample size of 42-66 celiac patients and controls would have been needed to confirm the above difference with an alpha error of 5% and a statistical power of 95%. In fact, in the present study, for celiac disease at diagnosis the alpha error was 3% and the statistical power 97%.

We could not concur with the findings of Candelli *et al*[26] and Barta *et al*[33], which showed significant differences in the prevalence of ASCA IgG between CD and Crohn's diseases. In contrast, no significant difference was noted between the two groups (39% *vs* 50.5%, $P = 0.091$). In the present study, except ALCA, the occurrence of other anti-glycan antibodies and their median titers in CD at diagnosis was also similar to those observed in Crohn's disease[13,34] (celiac disease gASCA: 33.1 U/mL, AMCA 79.3 U/mL, ALCA 21.5 U/mL, ACCA 68.4 U/mL *vs* Crohn's disease gASCA: 48.3 U/mL, AMCA 55.5 U/mL, ALCA 25.4 U/mL, ACCA 46.2 U/mL). In addition, the positivity rate for any anti-glycan antibody was also comparable in these patient groups (CD *vs* Crohn's disease: 65.9% *vs* 59.4%, P = NS). In addition, sensitivity, specificity, positive and negative likelihood ratios in celiac disease are comparable to that observed in Crohn's disease. Consequently, in patients with gastrointestinal symptoms, the presence of gASCA, AMCA, or ACCA may not only suggest underlying Crohn's disease but may also be associated with untreated CD. At the same time, and based on our

results, ALCA and anti-OMP proved to be specific but relatively non-sensitive markers for Crohn's disease.

Current data advocate that in both CD and Crohn's disease patients have a primary defect in intestinal permeability that is also shared by a subgroup of relatives. In CD, it is also apparent that the exposure to gluten results in mucosal inflammation and the consequent tissue damage further abrogating the primary gut barrier defect, while gluten removal resolves the enhanced intestinal permeability[35,36]. These gliadin-induced mechanisms are proposed to be the cause of the anti-microbial antibodies formation in the disease and is strongly supported by the association found between anti-glycan markers and TGA or EMA in the present study and also that the antibody status is substantially altered following the introduction of GFD. gASCA and other positive anti-glycan antibodies were entirely lost in our cohort of CD patients, after strict adherence to long-term GFD. These results are concordant with previous findings[20,22]. In the study of Mallant-Hent *et al*[24], ASCA IgG or IgA positivity disappeared in a substantial number but not the all of the 111 patients on a strict GFD (from 28.8% to 8.1%). A possible explanation for this difference could be that the mean follow up period after GFD was longer in our study [49 (10-159) mo *vs* 33 (range 3-113) mo]. These results suggest that as the period of strict GFD increases, so is the greater disappearance of antibody positivity, which will supposedly lead to entire mucosal healing in the small intestine. The higher prevalence of ASCA in adults compared to children further underlines the important role of long-lasting inflammation and consequently antigen exposure in the formation of anti-microbial antibodies.

In the present study, we also established that the kinetics of antibody disappearance is variably sensitive to the length of GFD. Of the anti-glycan antibodies, AMCA and ACCA declined most rapidly, right after the TGA titer started to diminish. In Group CD2, the prevalence of these antibodies had already changed as compared to Group CD1, from approximately 36% to 11%, while the frequency of gASCA and anti-OMP remained unchanged. Among those CD patients who adopted a strict GFD, the duration of GFD was the shortest in this group. In the group of patients with a successful response to GFD (Group CD3), the frequency of gASCA as well as AMCA and ACCA was also lower. At the same time, the overall frequency of anti-OMP did not change, either in group CD2 or in CD3 (Table 1). We showed however, that the level of anti-OMP clearly declined to normal in 13 of the 14 anti-OMP positive CD patients when specific patients in group CD1 were followed (Figure 2). The explanation of this supposedly inconsistency may be that the mean follow-up in both groups CD2 and CD3 was significantly shorter than in those in group CD1 participating in intra-individual longitudinal monitoring, suggesting that anti-OMP requires the longest time to disappear completely and this occurs long after the normalization of TGA and EMA. The differences in the evolution of anti-OMP and anti-glycan antibodies in IBD has also justified our findings in this patient group[15].

We evaluated the possible relationship between serological response and the clinical presentation of the disease. Patients with multiple seroreactivity to glycans, more commonly presented with severe malabsorption as compared to those without any reactivity against any glycan at all (63% *vs* 22%, $P = 0.019$), and accounted for 53% of all malabsorption cases. Among the patient groups, the TGA titer reached the highest value (115.9 U/mL *vs* others: 60.9 U/mL, $P = 0.016$) in those presenting with malabsorption, further supporting enhanced intestinal permeability as a likely component involved in antibody formation. It is well known that the intestinal damage is most pronounced in the malabsorption cases and TGA is a good marker for tissue injury[37]. We must note however, that the number of subjects in different clinical presentation groups were limited, thus further studies with a larger cohort of CD patients are needed to confirm these findings.

Recent data suggest that the presence of anti-microbial antibodies might be linked to genetic susceptibility. In patients with Crohn's disease an association was found between antimicrobial formation and the carriage of mutations in innate immunity receptor genes (NOD2/CARD15 or toll-like receptor)[15,20]. However, in the absence of NOD2 variants in our Crohn's patients' cohort, the gASCA and the any-glycan positivity was also reasonably high (43.5% and 53.7%, respectively). Furthermore, among CD patients in the present study we found that these antibodies occur with the same frequency and magnitude as in patients with Crohn's disease, albeit the occurrence of NOD2/CARD15 mutation was significantly lower. These findings - alongside with the fact that there is no association between TLR4 variants and CD[38,39] - do not support the primary role of genetic predisposition in antibody formation. Nevertheless, the presence of NOD2/CARD15 was associated with an increased antibody formation in Crohn's disease and an apparent link was also reported between increased permeability and NOD2/CARD15 3020insC mutation[40]. We did not observe any association between anti-glycan antibody positivity and the presence of NOD2/CARD15 variants in CD. However, the limited number of subjects carrying NOD2/CARD15 mutations might not have allowed us to recognize significant differences in serological response in this patients group. An inheritable trait of anti-microbial antibody formation is unlikely in CD, since we did not find a higher prevalence of ASCA (9.1% *vs* 14%) and anti-OMP (12.1% *vs* 20%) as compared to the controls in the 66 unaffected, first-degree relatives (siblings) of this cohort.

On the basis of significant similarity in the qualitative and quantitative serological response in the two patients' groups, we hypothesize a similar mechanism for the formation of the anti-microbial antibody formation in both celiac disease and Crohn's disease. The presence of serological response might be the reflection of the sustained exposure to the constituent of the gut microflora due to the enhanced bacterial translocation. The known predisposing factors for bacterial translocation, such as bacterial overgrowth in the small bowel (secondary to intestinal dysmotility)[41-43], the damage to the integrity of the gut

mucosa (secondary to alterations of the local intestinal microvasculature)[44,45], which results in reduced oxygen delivery and an increased formation in oxygen radicals[46] as well as the upregulation of the proinflammatory cytokines, such as tumor necrosis factor α, interleukin-17 or interferon gamma in active lesions[47], and the defective mucosal immunological defense[21,48] are all typical features in both clinical conditions. The significance of the enhanced bacterial translocation out of the small bowel in the anti-microbial antibody formation is further supported by the fact that the presence of the serological response among patients with Crohn's disease is mainly characteristic for those with complicated (stricturing or penetrating) small bowel involvement and is rarely observed in the isolated colonic disease or in patients with ulcerative colitis. At the same time, the recovered gut barrier function protects against the invasion of microbes or their components leading to the cessation of anti-microbial antibody formation. In CD, this process may be justified by the observation that the serological response is a temporary phenomenon. As a result of the discontinuation of gliadin exposure and the subsequent mucosal healing, the antibodies disappear completely. Confirming this aspect of our hypothesis is much more complicated in Crohn's disease. First of all, the pathogenetic processes are not only multifaceted but also less characterized as compared to CD. The complete elimination of the causative agents is not possible. Moreover, no such reliable serological markers are available reflecting the extent of gut inflammation as TGA and anti-actin IgA antibodies in CD. Finally, in terms of the complete loss of microbial seroreactivity, the long-lasting complete remission (without mucosal inflammation) is mandatory but rarely reached in patients with Crohn's disease as compared to CD patients adhering to a strict GFD. In this point of view, findings reporting a lack of solid correlation between disease activity and the presence or the magnitude of seroreactivity[17,49] in Crohn's disease can not be in opposition to our hypothesis any more. The advent of the new biological treatments might answer this unresolved question, since the complete mucosal healing in Crohn's disease can be achieved with this therapy in a greater proportion of cases than with classical drugs. At this moment, however, no data from prospective studies are available addressing the effect of the biological therapy on antibody stability. Our data also call for additional basic research to explore the exact mechanism of immune responses to commensal enteric bacteria as well as the possible clinical significance of the bacterial translocation in the pathogenesis or the complications of these diseases as it is well established in other clinical conditions such as liver cirrhosis, acute pancreatitis or sepsis[50].

In conclusion, our results suggest that ASCA and other anti-glycan antibodies may be considered as additional markers for CD and adherence to a GFD. Furthermore, the presence and the magnitude of response to microbial components is associated with a more severe clinical course but not with mutations in NOD2/CARD15. This seroreactivity may be the consequence of the enhanced bacterial translocation through the impaired small bowel mucosa.

COMMENTS

Background

Anti-microbial antibody formation has been reported in celiac disease. Relatively high positivity rates were observed for the conventional antibodies, for example, anti-Saccharomyces cerevisiae (ASCA), anti-OmpW, and anti-I2, and they were known to decrease after a successful gluten free diet.

Research frontiers

Newly discovered inflammatory bowel disease-associated antibodies (including anti-glycan antibodies and anti-OMP) may also be of importance in celiac disease, however, not studied thus far in the published literature. The presence of anti-microbial antibodies in relation to clinical presentation of the disease and NOD2/CARD15 mutations was also not investigated.

Innovations and breakthroughs

Anti-glycan antibody positivity is a common feature of celiac disease at the time of diagnosis and is lost after long-term gluten-free diet. The positivity rate and titers at diagnosis are as high as observed in Crohn's disease. The presence of anti-glycan antibodies is associated with the presenting symptoms, especially with severe malabsorption but not with mutations in NOD2/CARD15. We did not find a higher prevalence of anti-microbial antibodies in the unaffected, first-degree relatives of this patient cohort.

Applications

The data may add new pieces to the puzzle of the anti-microbial antibody formation and also assist to re-evaluate recently proposed mechanisms. Serological response to various microbial antigens might be considered a universal marker of the enhanced translocation of the gut microflora through the impaired small bowel mucosa both in celiac and Crohn's disease patients.

Terminology

Serology markers: anti-endomysial antibodies, synthetic deamidated gliadin peptides, antibodies against microbial antigens such as cell wall component of Saccharomyces cerevisiae, outer membrane porin C transport protein of the *Escherichia coli* (OmpC) or the Pseudomonas fluorescens associated protein (I2), anti-glycan antibodies: glycan-ASCA (gASCA), anti-mannobioside (AMCA), anti-laminaribioside (ALCA), anti-chitobioside (ACCA).

Peer review

Papp *et al* studied the prevalence of antimicrobial antibodies in celiac disease patients. The most relevant finding is that anti-glycan antibody titers were associated with symptoms at presentation and their positivity was lost after longstanding gluten free-diet as well as patients with multiple anti-glycan antibodies at diagnosis had more frequently severe malabsorption.

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BRIEF ARTICLES

Different patterns of intestinal response to injury after arterial, venous or arteriovenous occlusion in rats

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Abstract

AIM: To investigate the differences in injury patterns caused by arterial, venous or arteriovenous mesenteric occlusion.

METHODS: Male Wistar rats were separated equally into four groups. Occlusion was performed by clamping the superior mesenteric artery (A), the mesenteric vein (V) or both (AV) for 30 min, followed by 60 min of reperfusion. A control group received sham surgery only. Intestinal sections were examined for histological damage and serum tumor necrosis factor-α (TNF-α), endothelin-1 (ET-1), P-selectin, antithrombin Ⅲ (ATⅢ) and soluble intracellular adhesion molecule-1 (ICAM-1) concentrations were measured.

RESULTS: All groups showed significant mucosal injury compared to controls. Furthermore, mucosal injury was significantly more severe in the V and AV groups compared to the A group (3.6 ± 0.55, 3.4 ± 0.55 and 2 ± 0.71, respectively, P = 0.01). ICAM-1 was similarly elevated in all groups, with no significant differences between the groups. P-selectin levels were significantly elevated in the V and AV groups but not the A group (1.4 ± 0.5 ng/mL, 2.52 ± 0.9 ng/mL and 0.02 ± 0.01 ng/mL, respectively, P = 0.01) and ET-1 was significantly elevated in the A and V groups but not the AV group (0.32 ± 0.04 pg/mL, 0.36 ± 0.05 pg/mL and 0.29 ± 0.03 pg/mL, respectively, P = 0.01) compared to sham controls. ATⅢ levels were markedly depleted in the V and AV groups, but not in the A group (29.1 ± 5.2 pg/mL, 31.4 ± 21.8 pg/mL and 55.8 ± 35.6 pg/mL, respectively, P = 0.01), compared to controls. Serum TNF-α was significantly increased in all groups compared to sham controls (1.32 ± 0.87 ng/mL, 1.79 ± 0.20 ng/mL and 4.4 ± 0.69 ng/mL, for groups A, V and AV, respectively, P = 0.01), with higher values in the AV group.

CONCLUSION: Different patterns of response to ischemia/reperfusion are associated with venous, arterial or arteriovenous occlusion. Venous and arteriovenous occlusion was associated with the most severe alterations.

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Key words: Arterial occlusion; Rat; Intestine; Ischemia/reperfusion; Venous occlusion

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INTRODUCTION

Acute mesenteric ischemic disease can be either arterial or venous in etiology. Acute arterial ischemia results

from reduced blood flow caused by mesenteric artery thrombosis or embolism, leading to bowel necrosis and high mortality[1]. Mesenteric vein occlusion is usually due to thrombosis, is less common than arterial occlusion and carries a lesser risk of bowel necrosis and therefore reduced mortality[2,3]. Greater knowledge of the differences between these conditions would aid the search for therapeutic interventions, and also expand our understanding of ischemia/reperfusion (I/R) pathophysiology. This information would also be relevant for transplantation and aortic reconstruction procedures, which are invariably related to periods of I/R[4].

The intestine is very susceptible to ischemic injury. Ischemia causes mucosal damage through the production of substances like oxygen-derived free radicals, pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and the complement system[5]. The pattern of injury has been found to be different after experimental arterial or venous occlusion of the mesenteric circulation followed by reperfusion. Generally, the histological injury grade is thought to be greater after venous occlusion, even though energy metabolism is altered to a lesser extent than in arterial occlusion[6]. However, another study found that venous congestion alone could not cause reperfusion injury, while arterial occlusion could do so easily[7]. This issue could be resolved if the differences between the injury response to these two situations could be found.

During I/R, endothelial functions are altered, resulting in microcirculatory dysfunction which modulates mucosal integrity[8]. Endothelial injury and increased vascular permeability also occur[9]. The vascular endothelium regulates vascular tone, leukocyte adhesion and the coagulation cascade, among other factors. Endothelial function serum marker levels such as intracellular adhesion molecule-1 (ICAM-1), endothelin-1 (ET-1) and coagulation factors have also been found to be altered after I/R. One study showed that ET-1 modulates intestinal I/R injury in rats[10], while another showed that ICAM-1 is overexpressed, possibly contributing to injury by promoting leukocyte infiltration[11]. Another adhesion molecule, P-selectin, is also thought to modulate intestinal I/R injury, through complement deposition and leukocyte interaction[12]. Intestinal I/R also causes hemostatic alterations, namely coagulation induction, thrombin generation, and fibrin deposition, which correlate with the extent of injury[13].

In this study we evaluate the possibility that arterial, venous and arteriovenous occlusion, followed by reperfusion, might cause different types of response to I/R injury in the intestine. These differences were investigated by measuring ET-1, antithrombin Ⅲ (ATⅢ), soluble ICAM-1 and P-selectin serum concentrations, soluble markers of endothelial function, as well as by measuring TNF-α, a cytokine that plays a central role in I/R injury.

MATERIALS AND METHODS

Animal procedures

Animal procedures were performed in accordance with the proper use and care of laboratory animals, approved by the ethics committee of our institution. Experiments were performed on 20 male Wistar rats weighing 200-250 g. Animals were maintained under standard conditions, such as stable room temperature (24 ± 3℃), a 12 h light/12 h dark cycle, and access to commercial rat pellets and water *ad libitum*.

Animal model

Briefly, after pentobarbital sodium anesthesia (Anestesal, Pfizer Inc, Mexico) (35 mg/kg, i.p), a midline laparotomy was performed, and the small intestine was externalized and wrapped in humid gauze. Animals were placed under a heating lamp in order to preserve core body temperature at (37℃). The cecum was occluded using a 2-0 nylon suture to prevent collateral blood flow, and ischemia was induced by obstructing either the superior mesenteric artery, the mesenteric vein before its union with the portal system, or both, with microvascular clamps for 30 min (ischemia), and removed to allow 60 min reperfusion. Immediately after clamping the mesenteric vessels, the intestines were returned to the peritoneal cavity where they remained for the entire duration of the procedure. Occlusion was confirmed by loss of pulsation and characteristic red dark coloring of the intestine in the venous group, or pale pink coloring and loss of pulsation in the arterial group.

Rats were divided in four groups (n = 5). The first group received only sham surgery (Sham), where laparotomy was performed but intestines were only manipulated but not made ischemic. Group A was subjected to arterial occlusion and reperfusion as described above. Group V was subjected to venous occlusion and reperfusion as described above. Group AV was subjected to both arterial and venous occlusion and reperfusion as described above.

Morphological examination

Immediately after concluding the reperfusion period, rats were sacrificed by exsanguination from the aorta and tissue samples were obtained and fixed in 10% neutral buffered formalin overnight. Samples were then embedded in paraffin, and 4 μm-thick sections were stained with hematoxylin and eosin (H/E) and examined under a light microscope by a blinded pathologist. The Chiu score of mucosal injury was used to evaluate the degree of histological damage[14]. The scale consists of values from 0 to 5, where 0 is normal mucosa; 1, development of sub epithelial (Gruenhagen's) spaces; 2, extension of the sub-epithelial space with moderate epithelial lifting from the lamina propria; 3, extensive epithelial lifting with occasional denuded villi tips; 4, denuded villi with exposed lamina propria and dilated capillaries; and 5, disintegration of the lamina propria, hemorrhage, and ulceration.

Inflammatory cell infiltration

Under light microscopy, H/E stained tissue samples were analyzed for leukocyte infiltration by a blinded pathologist. The predominant type was recorded in both mucosa and

muscular layers, and intensity of infiltration was quantified with a numerical scale with values from 0 to 3, where 0, no infiltration; 1, mild infiltration; 2, moderate infiltration and 3, severe infiltration. Two slides and five high power fields per slide were examined and averaged.

Serum levels of ET-1, ATⅢ, P-selectin, ICAM-1 and TNF-α

At the end of the reperfusion period 3 mL of blood was obtained from each rat and left to clot for serum acquisition. Serum levels of ET-1 were determined using a rat ET-1 EIA kit (Immuno-Biological Laboratories, Japan). Serum levels of ATⅢ were determined using a rat ATⅢ ELISA kit (GenWay Biotech, USA). Serum levels of soluble ICAM-1 were determined using a quantikine rat sICAM-1 ELISA kit (R & D Systems, USA). Serum levels of P-selectin were determined using a quantikine rat P-selectin ELISA kit (R & D Systems, USA). These were used as markers of endothelial function. Serum concentrations of TNF-α were determined using a rat TNF-α Elisa kit (PeproTech, Mexico).

Statistical analysis

SPSS 11.0 statistical software (SPSS Inc. Software, Chicago, Illinois, USA) was used to analyze data using one-way analysis of variance (ANOVA) and with the Tukey-Kramer post-hoc test so as to determine comparisons between the groups, and differences between the groups, respectively. The relationships between the variables studied were determined by the calculation of Pearson correlation coefficients. All values are expressed as mean ± SD and $P < 0.05$ was considered statistically significant.

RESULTS

Morphological examination

The sham group showed normal intestinal mucosa (Chiu score 0.2 ± 0.45). All occlusion groups had significantly higher injury scores compared to the sham group (Figure 1, $P < 0.01$). Groups V and AV had the highest injury scores (3.6 ± 0.55 and 3.4 ± 0.55, respectively), showing severe epithelial lifting and severe capillary congestion (Figure 1), with no difference observed between these two groups. However, both the AV and V groups had significantly higher scores compared to group A (2 ± 0.71, $P < 0.01$), which showed moderate lifting and very little congestion.

Inflammatory cell infiltration

Compared to the sham group (1.2 ± 0.45), groups V and AV (2.6 ± 0.55 and 2.6 ± 0.55) both had a significantly higher leukocyte infiltration intensity (Figure 1, $P < 0.05$). Group A (2 ± 0.7) was not statistically different from either the V or sham group. The infiltrate type was similar in all groups, and was composed mostly of neutrophils, with few macrophages or lymphocytes observed.

Serum markers of endothelial function

The sham group had soluble P-selectin serum concentrations of 0.83 ± 0.47 ng/mL. In group A the levels remained barely detectable, and were not statistically different to the sham group (0.02 ± 0.01 ng/mL). Groups V and AV had significantly higher values compared to the sham group (1.4 ± 0.5 ng/mL and 2.52 ± 0.9 ng/mL, respectively, $P < 0.01$), with the latter being significantly higher than the former ($P < 0.05$, Figure 2). ATⅢ serum concentrations were significantly depleted in groups V and AV compared to the sham group (29.1 ± 5.2 pg/mL and 31.4 ± 21.8 pg/mL *vs* 85.3 ± 17.4 pg/mL, respectively, $P < 0.01$), while in group A they remained within the sham group range (55.8 ± 35.6 pg/mL). However, there was no difference between the V or AV groups (Figure 2). No difference was found between ET-1 levels in the sham and AV groups (0.23 ± 0.01 pg/mL *vs* 0.29 ± 0.03 pg/mL, respectively, Figure 2). Levels in both the A and V groups were significantly elevated compared to the sham group (0.32 ± 0.04 pg/mL and 0.36 ± 0.05 pg/mL, respectively, $P < 0.01$), with levels in the V group also being significantly higher that in that in the AV group, but not the A group. Finally, all groups had significantly elevated soluble ICAM-1 serum concentrations compared to the sham group (A: 569.2 ± 170.8 pg/mL, V: 529 ± 191.5 pg/mL and AV: 483.9 ± 41.6 pg/mL *vs* 194 ± 38.9 pg/mL, respectively, $P < 0.01$), but no differences were found between each of these three groups (Figure 2). In summary, venous and arteriovenous occlusion elevated P-selectin, and depleted AT Ⅲ, to a greater extent than arterial occlusion only. Venous and arterial occlusion, but not arteriovenous occlusion, elevated ET-1 levels compared to sham groups, with no differences between them. ICAM-1 levels were elevated in all groups compared to the sham group, with no differences between them.

Table 1 Pearson's correlation coefficient between the variables studied

	TNF-α	ET-1	P-selectin	ICAM-1	ATIII
Chiu Score	0.715[a]	0.714[b]	0.552[a]	0.608[b]	-0.633[a]
TNF-α	x	0.26	0.646[b]	0.337	-0.534[a]
ET-1		x	-0.015	0.655[b]	-0.409
P-selectin			x	0.044	-0.275
ICAM-1				x	-0.532[a]

[a]$P < 0.05$, [b]$P < 0.01$. Data are presented as correlation coefficients (r).

TNF-α serum concentrations

The sham group had TNF-α serum concentrations of 0.11 ± 0.14 ng/mL. In groups A, V and AV the levels were significantly increased compared to sham controls (1.32 ± 0.87 ng/mL, 1.79 ± 0.20 ng/mL and 4.4 ± 0.69 ng/mL, respectively, $P < 0.01$, Figure 2). TNF-α serum concentrations were significantly higher in the AV group compared to both the A and V group, with no differences observed between these two groups.

Linear correlations between variables

Linear correlations were observed between the systemic concentrations of endothelial function markers, TNF-α and injury scores. These correlations are shown in Table 1. All the measured variables, except for ATⅢ, showed a significant positive correlation with the histological injury score. ATⅢ showed a significant negative correlation with

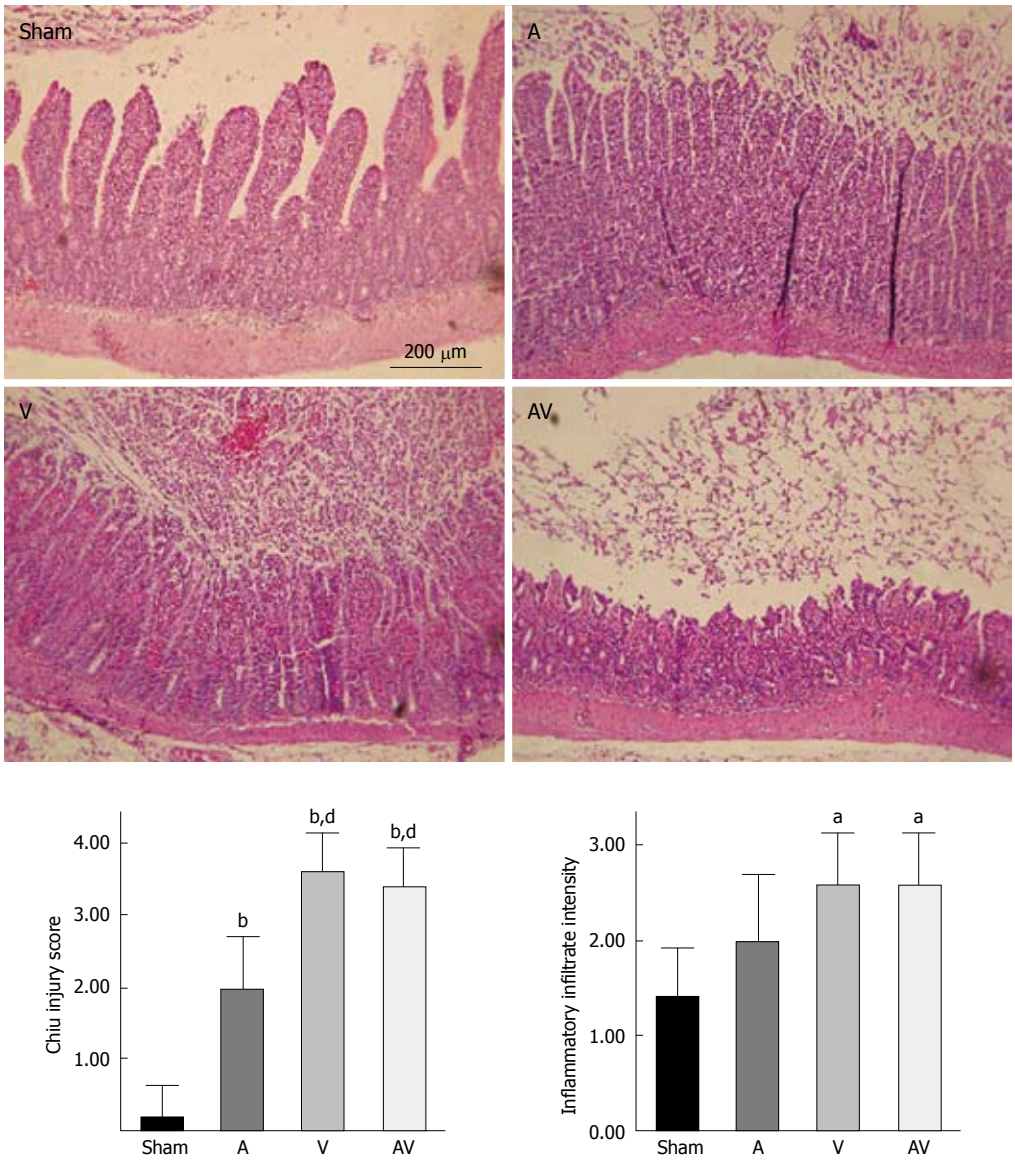

Figure 1 Histological findings (H & E) and injury scores. A: Arterial occlusion and reperfusion group; V: Venous occlusion and reperfusion group; AV: Both arterial and venous occlusion and reperfusion group. $^{a}P < 0.05$ *vs* sham; $^{b}P < 0.01$ *vs* sham; $^{d}P < 0.01$ *vs* A.

the injury score ($r = 0.608$, $P < 0.01$). TNF-α was the variable most strongly correlated with injury score ($r = 0.715$, $P < 0.01$).

DISCUSSION

Ricci *et al*[15] found greater mortality in rats after intestinal arterial versus arteriovenous occlusion. However, Yano *et al*[16] found that the degree of intestinal damage due to venous occlusion was greater than the damage resulting from both arteriovenous and arterial occlusion, with no difference between the latter two procedures. Greater intestinal hemorrhage has also been observed after venous occlusion compared with arterial occlusion[6]. Other studies confirmed these findings, showing that venous occlusion could induce intestinal injury as early as within 5 min, while arterial occlusion could not even at longer time periods[17]. Some of the molecular bases of these differences have been investigated. Venous congestion has been shown to induce increased levels of inflammatory cytokines, in a similar way to arterial occlusion[18]. However, in one I/R study, intestinal malonyldialdehyde levels (as an index of free radicals) were found to be higher in venous occlusion compared to arterial occlusion, despite no obvious difference in tissue injury[19]. In other organs, such as the kidney, renal vein occlusion has been found to induce a stronger inflammatory response (TNF-α, free radicals, but not ICAM-1), neutrophilic infiltration, and greater functional damage, compared to arterial occlusion[20]. In our study, I/R due to venous occlusion was shown to cause greater damage to the intestinal mucosa than arterial occlusion alone, in agreement with the previous studies. However, we found no difference between the type or the intensity of injury between venous and arteriovenous occlusion. Arterial occlusion caused limited but significant histological damage and was associated with little leukocyte infiltration. The pro-inflammatory cytokine TNF-α is one of the main orchestrators of the inflammatory response and a key mediator of injury during I/R[21]. In our study,

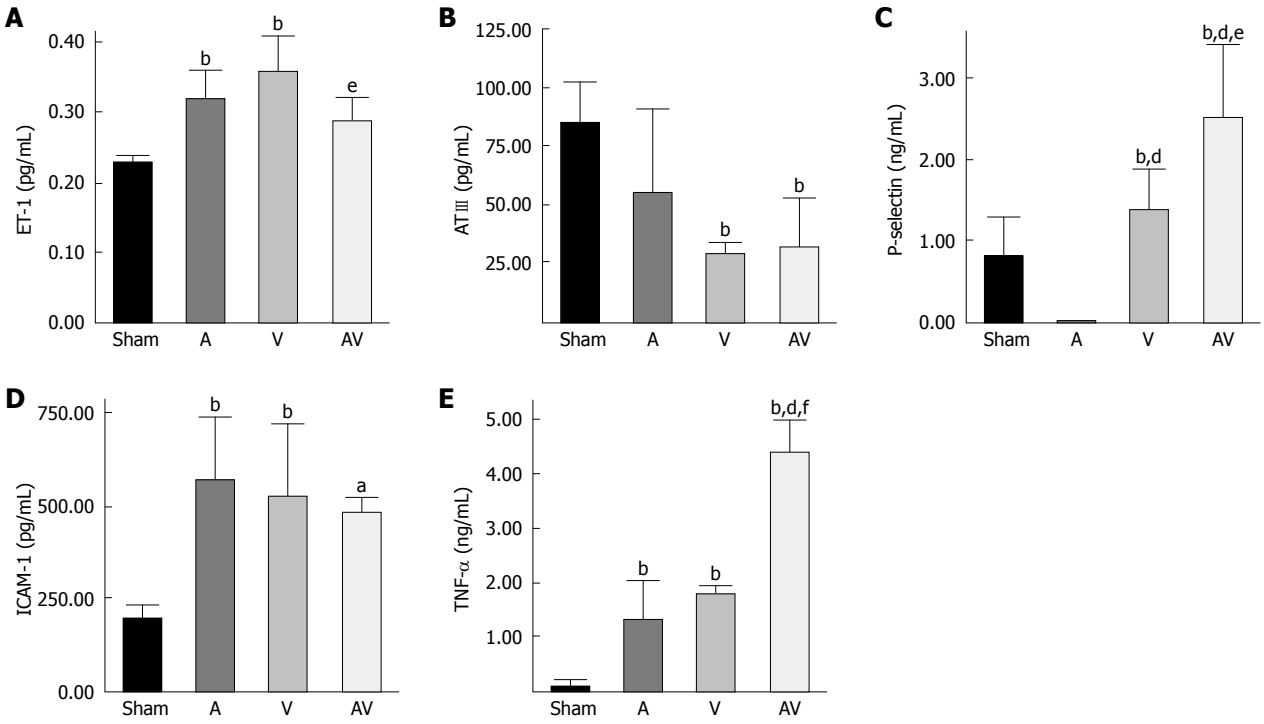

Figure 2 Serum concentrations of endothelial function markers and TNF-α. A: ET-1 serum concentrations; B: ATIII serum concentrations; C: P-selectin serum concentrations; D: Serum levels of soluble ICAM-1; E: TNF-α serum concentrations. $^{a}P < 0.05$ *vs* sham; $^{b}P < 0.01$ *vs* sham; $^{d}P < 0.01$ *vs* A; $^{e}P < 0.05$ *vs* V; $^{f}P < 0.01$ *vs* V.

TNF-α levels were significantly elevated after all three occlusion methods, with the highest levels observed in the arteriovenous occlusion group. TNF-α concentrations were strongly correlated to injury intensity.

Intestinal I/R affects the microvasculature, endothelial cells, and endothelial cell function[8]. ET is a vasoactive substance which induces intense vasoconstriction of blood vessels, and its activation is thought to play an important part in the development of these microvascular changes[22]. ET-1 participates in intestinal I/R injury by modulating leukotriene production[10]. Additionally, it has been demonstrated that ET-1 is involved mainly in post-reperfusion induced vasospasm and damage, and ET receptor antagonists are able to protect against mucosal injury[23]. In agreement with previous reports, we found that I/R due to arterial occlusion induces elevations in ET-1 levels, and further, we demonstrated that venous occlusion has a similar outcome, although there was a tendency for higher levels in the venous occlusion group. Surprisingly, ET-1 elevation was not present when both arterial and venous occlusion was performed simultaneously. Other factors capable of explaining this result might be at play. It is known that reductions in perfusion pressure during mesenteric ischemia cause diminished arteriolar resistance and vasodilation as a compensatory mechanism[24]. While venous occlusion alone causes severe congestion and increased intravascular pressure, arterial occlusion alone might only cause reduced inflow perfusion with normal outflow. Supporting this idea, Tsunada *et al*[18] reported a more pronounced reduction of blood flow and increased subsequent flow recovery after reperfusion with arterial occlusion, compared to venous occlusion. We speculate that arteriovenous occlusion might balance inflow and outflow alterations, resulting in intravascular pressure and tone equilibrium, possibly explaining the absence of ET-1 activation which we observed. Further experiments are required to assess this possibility and the role of ET-1 in regulating post-ischemic vascular tone.

P-selectin is an adhesion molecule which modulates endothelial-leukocyte interactions. During intestinal I/R, P-selectin mRNA expression is upregulated in the microvascular endothelium[25] and P-selectin immunoreactivity is increased in the intestinal mucosa[26]. I/R studies have shown that P-selectin blockade or genetic deficiency result in diminished intestinal mucosa damage after I/R in rodent models, further supporting the idea that P-selectin participates in the pathophysiology of I/R injury[27,28]. In our study, I/R in the arteriovenous occlusion group caused higher elevations of soluble P-selectin serum concentrations than in the venous occlusion group. Moreover, arterial occlusion was unable to alter these values compared to the sham group. P-selectin levels were also found to be correlated to histological injury scores.

The coagulation cascade is promoted by I/R[13]. AT Ⅲ is an endogenous anticoagulation molecule produced in lung, liver and endothelium which limits thrombin formation. Thrombin activation and the formation of thrombin-antithrombin complex is increased after intestinal I/R, contributing to cytokine production and inflammatory injury[29]. Furthermore, these alterations would theoretically result in endogenous ATⅢ depletion. One rodent study found that ATⅢ levels were indeed depleted after intestinal I/R, and that they were restored by protective therapy[30]. Additionally, ATⅢ

administration can protect the intestinal mucosa against I/R injury[31]. We found that I/R due to both venous and arteriovenous occlusion caused ATⅢ depletion whereas arterial occlusion alone did not. In our study, a negative correlation between ATⅢ levels and injury scores was observed, suggesting that depleted ATⅢ levels are indeed reflective of damage intensity. A possible explanation for our results is that the microvascular congestion and venous stasis caused by the venous occlusion component promotes the coagulation cascade to a greater extent than the arterial component during ischemia and reperfusion. Indeed, temporal and histological differences have been found to exist between thrombus generation in arterial and venous endothelium[32]. While stasis seems to be a more important factor in veins, impaired laminar flow and plaque formation seem to be essential for arterial thrombus generation[33]. Furthermore, studies have shown that decreased levels of ATⅢ after major surgery can predict higher complication and mortality rates[34]. Therefore, the differences in ATⅢ depletion between arterial or venous mesenteric ischemia might be of clinical relevance. The physiopathological consequences of these results warrant further investigation.

ICAM-1 is another adhesion molecule that mediates leukocyte reactivity and adhesion to endothelium. Intestinal I/R is also associated with an upregulation of ICAM-1 and subsequent leukocyte infiltration[35]. Systemic ICAM-1 activity is also upregulated, possibly contributing to multisystem failure and remote organ injury[11]. Serum levels of ICAM-1 have been shown to be markedly elevated after intestinal I/R, and low serum concentrations are associated with beneficial outcomes after the administration of protective agents[36]. The inhibition of ICAM-1 with monoclonal antibodies is able to reduce the intensity of the functional and morphological alterations in the intestine subjected to I/R[37,38]. In our study, we found that soluble ICAM-1 serum concentrations were elevated by intestinal I/R, in agreement with the previous reports. However, there was no difference between any of the occlusion methods employed, suggesting that soluble ICAM-1 is non-specifically elevated in arterial, venous or arteriovenous I/R.

In conclusion, we showed that a different pattern of response to I/R characterize different forms of mesenteric occlusion methods. Venous occlusion seems to cause more severe tissue damage and changes in P-selectin and ATⅢ depletion compared to arterial occlusion. This suggests that these molecules are sensitive to changes in the intensity of the histological injury and therefore might be partly responsible for some of the pathophysiological differences between venous and arterial occlusion. Arteriovenous occlusion showed greater tissue damage and TNF-α levels compared to arterial occlusion alone, but only differences in P-selectin and ET-1 were found between them. Finally, arterial occlusion was related to the least severe changes compared to controls, and ICAM-1 values were not different between the different I/R groups.

We discussed the possibility of these differences being dependent on local microvascular factors, but since serum concentrations are indicative of the systemic state, the explanation could lie in differences in the systemic consequences of the occlusion methods employed. Studies on the local and remote tissue expression of these molecules would be helpful in clarifying these issues. However, our results confirm that there are differences in the inflammatory responses associated with venous, arterial or arteriovenous occlusion that could partly explain the molecular bases of the clinical differences between these conditions.

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COMMENTS

Background

Intestinal ischemia can occur when blood flow from the mesenteric vasculature is obstructed. Obstructions can be embolic, atherosclerotic or vasospastic in etiology. Mesenteric veins or arteries can be affected. Ischemia and reperfusion also takes place in transplant procedures and states of hypoperfusion (shock). The pathophysiological differences between venous, arterial or total ischemia of the intestine are not fully understood. In this study, the aim was to investigate these differences using a rat model.

Research frontiers

Ischemia/reperfusion is an important topic of research. The molecular mediators responsible for ischemia/reperfusion injury could be modulated to improve transplantation outcomes.

Innovations and breakthroughs

In this study, it is demonstrated that venous, arterial or arteriovenous occlusion followed by reperfusion, cause different patterns of injury and serum elevations of inflammatory markers.

Applications

To understand the pathophysiological bases of ischemia/reperfusion injury could lead to novel treatments, specifically tailored for conditions of venous, arterial or total ischemia.

Peer review

This is a very interesting study which examines changes in rat intestinal histology and inflammatory serum cytokine markers subsequent to arterial, venous or concomitant arterial and venous occlusion and reperfusion. The authors demonstrated the differences in injury patterns caused by arterial, venous or arteriovenous mesenteric occlusion. This study is well-investigated and has a novel finding.

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BRIEF ARTICLES

Effects of Chinese herbs on salivary fluid secretion by isolated and perfused rat submandibular glands

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Abstract

AIM: To determine whether Chinese herbs (CHs) relieve xerostomia (dry mouth) by increasing salivary secretion.

METHODS: The submandibular glands of Wistar rats were surgically isolated and perfused arterially with buffered salt solution. After control perfusion, recording started 5 min prior to the start of stimulation. After fluid secretion was induced by 0.2 μmol/L carbamylcholine (CCh) in the perfusate for 10 min, Chinese herb (CH) was added in the perfusion for 5 min. CCh was then overloaded at 0.2 μmol/L in the perfusion for 20 min. The volume of salivary fluid secretion was recorded by a computer-controlled balance system.

RESULTS: Saliva secretion formed an initial ephemeral peak at 30 s followed by a gradual increase to a sustained level. CH alone induced no or little saliva in all types of CH selected. During perfusion with CH, overloading of CCh promoted fluid secretion in 15 of 20 CHs. This promotion was classified into four patterns, which were eventually related to the categories of CH: Overall sustained phase was continuously raised (*Yin*-nourishing, fluid production-promoting and heat-clearing agents); The sustained secretion rose to reach a maximum then decreased (*Qi*-enhancing agent); Sustained secretion rose to reach the highest maximum and was then sustained with a slight decline (swelling-reducing, phlegm-resolving and pus-expelling agents); Stimulation of salivary secretion without any added stimulants. Addition of CCh raised the fluid secretion to reach the highest maximum then sharply decreased to a lower sustained level (blood activating agent).

CONCLUSION: The present findings lead to the conclusion that various CHs have different promotional effects directly on the salivary gland.

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Key words: Chinese herbs; Salivary secretion; Submandibular glands; Xerostomia

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INTRODUCTION

Xerostomia (dry mouth) is caused by salivary hypofunction (reduction in salivary fluid secretion). Severe xerostomia induces speech difficulty, infection of the oral cavity and is associated with systemic disease[1]. Salivary hypofunction is associated with aging and with various diseases including diabetes, hypertension, dehydration, drugs, radiation therapy, autoimmune diseases, irradiation, sarcoidosis and many other renal, neurologic and skin diseases[2]. Xerostomia may be transient and/or reversible as in the case of medications but relatively permanent in

the case of autoimmune diseases, damage due to radiation therapy and aging. The present therapeutic procedures for xerostomia are limited and include the supplemental use of artificial saliva and the use of stimulants such as pilocarpine and cevimeline.

Therapeutic use of muscarinic agents is sometimes avoided due to underlying diseases. The use of artificial saliva may bother patients during talking. Clinical research on the gene transfer of aquaporin has recently taken place[3,4], and is still in progress.

For thousands of years, Traditional Chinese Medicine (TCM) has been used in the treatment of xerostomia. TCM does not follow the western style of diagnosis such as whether the symptom is primary or secondary, but is based on the classical theory-Syndrome Differentiation and Treatment. However, both ancient and contemporary practitioners of TCM have no standard classification of xerostomia symptoms, because all herbalist doctors have their own understanding and clinical experiences of syndrome differentiation and herb-choice. Following a comparison of the types of classifications, we can summarize xerostomia symptoms and classify them as follows: *Yin*-deficiency and thin fluid-deficiency; *Yin*-deficiency and dryness-heat; lack of activation due to *Qi*-deficiency; internal block of blood stasis; accumulation of damp-heat; phlegm accumulating with blood stasis[5-7].

In order to choose herbs, all herbalist doctors obey the principle of therapy "Supply the deficiency, Reduce the excess". Because all practitioners of Chinese Medicine have their own preferences for particular herbs, overall, they are likely to choose herbs for xerostomia as follows: *Qi*-enhancing agents, Body fluid-regenerating agents, *Yin*-nourishing agents, Heat-clearing agents, Dampness-eliminating agents, Blood-activating agents, and Phlegm-resolving agents[8-11].

Several thousand types of Chinese herbs are now in clinical use. By combining our previous findings[12] with reports by other herbalist doctors, we chose the following twenty herbs: *Yuzhu* (YZ, *Rhizoma polygonati odorati*[13,14]), *Shihu* (SH, *Herba dendrobii*[15,16]), *Shashen* (SS, *Radix glehniae*[17,18]), *Maimendong* (MD, *Radix ophiopogonis*[10,18]), *Tianmendong* (TD, *Radix asparagi*[17,18]) as *Yin*-nourishing agents; *Gegen* (GG, *Radix puerarie*[5]), *Wumei* (WM, *Fructus mume*[10,19]) as Body fluid-regenerating agents; *Shengdihuang* (SD, *Radix rehmanniae*[19,20]), *Xuanshen* (XS, *Radix scrophulariae*[17,18]), *Chishao* (CS, *Radix paeoniae rubra*[5,21]) as Heat-clearing agents; *Huangqi* (HQ, *Radix astragali*[8,14]), *Taizishen* (TZS, *Radix pseudostellar iae*[8]), *Gancao* (GC, *Radix glycyrrhizae*[18,22]) as *Qi*-enhancing agents; *Danshen* (DS, *Radix salviae miltiorrhizae*[17,18]) as Blood-activating agents; *Zaojioci* (ZJC, *Fructus gleditsiae abnormalis*[19]), *Tianhuafen* (THF, *Radix trichosanthis*[20,23]), *Ziyuan* (ZY, *Radix asteris*[24,25]) as Phlegm-resolving agents; *Taoren* (TR, *Semen persicae*[13,24]), *Chuanshanjia* (CSJ, *Squama manis*[23]) as Body mass-softening and resolving agents; *Dandiquionyu* (DDQY[26]) as a mixture.

However, the effects of these CHs have not been evaluated quantitatively and their mechanisms have not been widely investigated. In particular, it is unknown whether the effects of these CHs act directly on the salivary glands or indirectly *via* neural or hormonal controls.

The present study examines whether these CHs have a direct effect on the salivary gland to increase salivary fluid secretion. Effective CHs can induce fluid secretion solely or accelerate fluid secretion by muscarinic agents such as carbachol. For this purpose, we used isolated and arterially perfused submandibular salivary glands from rats. The results provided us with quantitative data on how much CHs can accelerate salivary secretion.

MATERIALS AND METHODS

Materials

Granular extracts of the Chinese herbs (CHs): YZ, SH, SS, MD, TD, SD, XS, CS, GC, WM, HQ, TZS, GG, DS, TR, ZJC, ZY, THF and CSJ were provided by Tian Jiang Pharmaceutical Co., Ltd., Jiang Yin, China. The preparations were in strict standard control according to the industry standards.

Just before each experiment, the extract equivalent to 10 g of each CH was dissolved in distilled water by ultrasonic wave concussion. After centrifugation at 5000 r/min for 10 min, the supernatant was collected and adjusted to 0.5 g/mL as the CH stock solution. Before use, the stock solution was diluted 100 times into the perfusate to obtain a final concentration of 5 g/L. Then, this CH-containing perfusate was filtered through a 0.22 μm pore size filter (Sterivex-GV, Millipore, MA, USA). The clinical dose of CH ranges from 10 to 50 g/person because the recipe is a mixture of several CHs (each CH dose is 5 g). For experimental convenience, we used an average dose of 25 g/animal for each experiment. Assuming that all CHs will move to the systemic circulation (5 L for 60 kg body weight), the concentration of CHs in the blood will be 5 g/L. We took this as the concentration of CH in the perfusion buffer.

DDQY has been proved effective in promoting saliva secretion. The ingredients of DDQY are as follows: *RenShen* 5 g, *ShengDi* 20 g, *FuLing* 10 g, *DanShen* 15 g, *MaiDong* 10 g. All the ingredients were added with 200 mL distilled water and were decocted twice for half an hour each time and the solution after each decoction was mixed and filtered. The final solution obtained was 96.7 mL. For convenience, we blended the solution with distilled water until the CH concentration of the stock solution was 0.5 g/mL. All the above ingredients were provided by JingQuan Group Chinese Traditional Medication Decoction Pieces Co., Ltd., AnHui, China.

Salts, glucose, carbamylcholine chloride (carbachol, CCh) were from Sigma, MO, USA. A fluorine-fiber tube (EXLON™) was purchased from Iwase Co. Ltd. (Atsugi, Japan).

Methods

Wistar rats were fed a standard pellet diet and water *ad libitum*. The rats were anesthetized with pentobarbitone sodium (50 mg/kg body weight, by intraperitoneal injection). The submandibular glands (120-180 mg) were surgically isolated as previously described[12] and the

attached sublingual gland was removed after ligation of the feeding arteries, draining vein and sublingual duct. The extralobular main duct from the submandibular gland was cannulated with a fluorine-fiber tube (0.3 mm × 0.5 mm O.D.) for sampling. The artery distal to the glandular branch was cannulated with a stainless steel catheter (26G) connected to the infusion line for perfusion. The vein from the gland was cut free. The gland was isolated and transferred to an organ bath (37℃), where the arterial catheter was connected to the perfusion apparatus. The drained venous effluent was continuously removed.

The glands were perfused arterially at a rate of 2 mL/min using a peristaltic pump (Cole-Palmer) to supply enough oxygen even without a specific oxygen carrier during the secretory period[12]. The perfusion fluid was a buffered salt solution of the following composition (mmol/L): Na^+, 145; K^+, 4.3; Ca^{2+}, 1.0; Mg^{2+}, 1.0; Cl^-, 148.3; glucose, 5.0. This solution was buffered at pH 7.4 with 10 mmol/L HEPES. The solution was prepared from stock solutions for each experiment and placed in a reservoir where it was equilibrated with 100% oxygen at 37℃.

To measure fluid secretion rate, the ductal cannula was filled with perfusate buffer and the tip placed under the water surface in a cup on an electronic balance (minimum digit was 0.1 mg, Shimadzu AEG-220), avoiding any contact with the bottom of the cup. Then, when salivary secretion started, the cumulative secreted mass could be measured. Cumulative weight was automatically measured every 3 s and transferred to a computer. The rate of fluid secretion was calculated from time-differentiation of the cumulative volume of saliva assuming a saliva specific gravity of 1.0.

Following control perfusion for longer than 20 min, recording was started 5 min prior to the start of stimulation. Fluid secretion was induced by the addition of 0.2 μmol/L carbamylcholine (CCh) in the perfusate. Following control stimulation with CCh for 10 min, CCh was removed from the perfusion line by perfusion with control perfusate for 5 min. Then only the CH was added to the perfusion for 5 min. This was to observe the effect of only the CH. After CH perfusion for 5 min, CCh was overloaded at 0.2 μmol/L in the perfusion for 20 min. To observe the accelerative effect of the CH, the dose of CCh used was 0.2 μmol/L to induce a moderate fluid secretion (c.f. 1.0 μmol/L is a supramaximal dose). The digital noise was smoothed by taking the moving average of every 11 data sets.

Statistical analysis

To reduce the variance among individual glands, the value of the flow rate was shown as mean ± SE. The statistical significance of the changes were assessed by double-tailed Student's *t*-test in a comparison between the control values (4.95-5 min) and the data sets obtained at 19.95-20 min, 24.95-25 min, 29.92-30 min and 34.95-35 min, along the time course. The induction of salivary secretion by CH only was determined by a comparison between the data-sets at 9.95-10 min and 14.95-15 min. *P*-value < 0.1 were considered statistically significant.

RESULTS

Control stimulation with CCh

The isolated perfused rat submandibular gland had almost no spontaneous fluid secretion. To standardize secretion in each gland, carbachol (CCh) stimulation was applied for 5 min. This initial phase included an initial transient increase and a pre-sustained stage of fluid secretion around 5 min from the start of CCh stimulation (71.4 ± 8.5 μL/g per min). After washing with CCh for 5 min, CHs were applied for 5 min to check if the single application of the CH could induce fluid secretion. The period of 5 min allows the CHs and secretagogues to fully circulate in the gland. Because physiological neural reflex stimulation was applied to the gland under circulation of CH, we overloaded CCh on the CH circulated gland to determine the promotional effect of CH. Figure 1A shows the CCh-induced fluid secretion without addition of CHs. A single application of CCh showed a sustained plateau phase with a slight hump after the initial phase of secretion. For statistical examination of the promotional effect we compared how much the percentage of fluid secretion changed at 5, 10, 15 and 20 min from the start of the second CCh stimulation. The control stimulation of CCh showed no statistical difference to that of the 5-min value for the first stimulation (Figure 1A, $P > 0.05$).

Yin-nourishing agents

YZ, SH, SS, MD and TD induced no secretion on single application (Figure 1B). Pre-loading of YZ did not increase the initial transient response to CCh (Figure 1B and Table 1). The initial transient peak (37.2 ± 12.7 μL/g per min at 16.2 min, $n = 6$) and the maximal response (68.1 ± 12.4 μL/g per min at 23.7 min) were similar to control values (36.9 ± 7.0 μL/g per min at 1.1 min) and the value at 4.85 min (71.6 ± 7.6 μL/g per min). Pre-loading of MD and TD increased the secretory response to CCh (Table 1). The initial transient peak (50.9 ± 5.4 μL/g per min at 16.2 min, $n = 6$; 41.4 ± 6.0 μL/g per min at 17.55 min, $n = 5$) was higher than the control response to CCh (36.9 ± 7.0 μL/g per min at 1.1 min; 36.6 ± 12.5 μL/g per min at 1.55 min), and the maximal response (82.2 ± 6.1 μL/g per min at 23.05 min; 73.2 ± 3.6 μL/g per min at 27.2 min) was also higher than the control response to CCh (72.3 ± 7.7 μL/g per min at 23.05 min; 64.1 ± 10.7 μL/g per min at 5.35 min). During CCh stimulation, fluid secretion was maintained at a higher level. On the other hand, pre-loading of SH did not change the responses to CCh. Pre-loading of SS decreased the responses to CCh (Table 1). The initial transient peak (25.3 ± 5.8 μL/g per min at 16.4 min, $n = 6$) and the maximal response (43.8 ± 5.3 μL/g per min at 27.35 min) were lower than the control value (45.4 ± 6.1 μL/g per min at 1.1 min) and the value at 5.3 min (70.4 ± 11.5 μL/g per min).

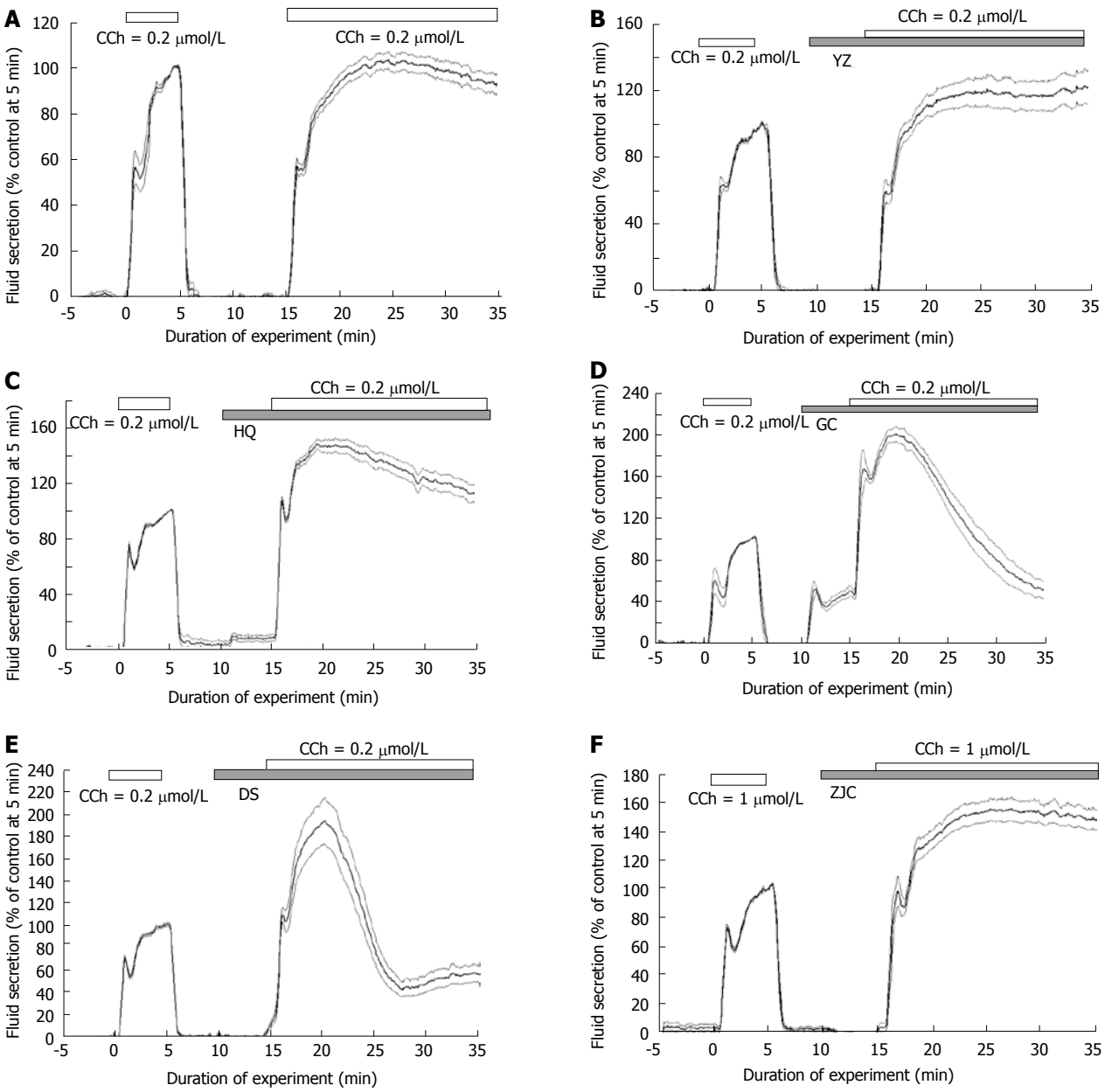

Figure 1 Time course of fluid secretion during stimulation. A: 0.2 μmol/L carbamylcholine (CCh); B: YZ + 0.2 μmol/L CCh; C: HQ + 0.2 μmol/L CCh; D: GC + 0.2 μmol/L CCh; E: DS + 0.2 μmol/L CCh; F: ZJC + 0.2 μmol/L CCh. The values (%) were normalized by the value at 5 min from the first CCh stimulation (indicated by an open bar on the top of the graph). After washing the CCh for 5 min, Chinese herbs (CHs) were added in perfusion (indicated by a shaded bar). After another 5 min, the second CCh stimulation was added (an open bar, in Figure 1A without CH) to CHs perfusion. The second CCh stimulation was applied (open bar). The values for fluid secretion were statistically unchanged at 5, 10 and 15 min after the start of the second stimulation. The overall sustained phase was continuously raised. The average values (bold line, n =16 from 8 glands in A; n = 12 from 8 glands in B; n = 12 from 6 glands in C; n = 10 from 5 glands in D; n = 12 from 6 glands in E; n = 10 from 5 glands in F) and the mean of standard error (mean ± SE, thin line) are shown. n: The number of sampling points.

Body fluid-regenerating agents

GG and *WM* did not induce secretion on a single application. Pre-loading of GG decreased the responses to CCh (Table 2). The initial transient peak (45.5 ± 8.8 μL/g per min at 16.5 min, n = 6) was lower than the control initial transient peak (59.3 ± 7.9 μL/g per min at 1.1 min) The maximal response (68.2 ± 13.4 μL/g per min at 21 min) was also lower than the control response at 5 min (83.0 ± 11.4 μL/g per min). After reaching a maximum, the flow rate was slowly decreased to 52.9 ± 16.0 μL/g per min at 35 min even during sustained stimulation. Thus, GG did not show a promotional effect on CCh-induced fluid secretion. Pre-loading of WM increased the secretory response to CCh (Table 2). The initial transient peak (61.3 ± 16.2 μL/g per min at 16.2 min, n = 6) was higher than the control response (53.9 ± 16.1 μL/g per min at 1.1 min), and the maximal response (89.8 ± 15.6 μL/g per min at 25.95 min) was also higher than the control response (72.3 ± 7.7 μL/g per min at 5.4 min). During CCh stimulation, the fluid secretion was maintained at a higher level (83.2 ± 10.2 μL/g per min at 35 min).

Heat-clearing agents

SD, XS and *CS* did not induce secretion on a single application. Pre-loading of SD and XS decreased the

Table 1 Fluid secretion induced by CCh with/without *Yin-nourishing* CHs in SMG

Time (min)	YZ (n = 12)		SS (n = 12)		MD (n = 12)		TD (n = 10)	
	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min
(1) 4.95-5	63.8 ± 11.5	100.6 ± 0.5	69.4 ± 7.0	99.8 ± 0.4	68.3 ± 5.9	100.3 ± 0.3	64.7 ± 5.3	100.9 ± 0.4
(2) 19.95-20	65.3 ± 8.7	112.4 ± 6.3^b	42.4 ± 3.4^a	65.4 ± 6.4^a	77.7 ± 3.8	122.0 ± 11.1^a	62.0 ± 2.6	103.2 ± 11.6
(3) 24.95-25	67.0 ± 7.9	118.9 ± 8.1^a	45.2 ± 3.4^a	69.7 ± 6.7^a	80.8 ± 4.1^b	125.4 ± 9.3^a	75.0 ± 2.8^b	124.6 ± 13.9^b
(4) 29.95-30	65.8 ± 7.5	117.7 ± 8.8^b	40.6 ± 3.6^a	62.8 ± 7.5^a	80.7 ± 4.7^b	123.6 ± 6.9^a	77.0 ± 2.7^a	127.6 ± 13.5^b
(5) 34.95-35	67.9 ± 7.5	122.5 ± 10.2^a	32.3 ± 4.9^a	49.4 ± 8.3^a	78.7± 5.3	119.2 ± 6.0^a	75.4 ± 3.5^b	125.3 ± 14.5^b
(6) 9.95-10	-0.6 ± 0.4	-0.9 ± 1.1	0.8 ± 1.2	1.3 ± 1.7	0.1 ± 0.4	0.2 ± 0.6	-0.3 ± 0.5	-0.1 ± 1.1
(7) 14.95-15	-0.5 ± 1.4	-0.9 ± 3.4	-1.2 ± 0.5	-1.7 ± 0.5	-1.3 ± 0.81	-1.2 ± 1.2	-0.5 ± 0.3	-0.8 ± 0.4

Fluid secretion (FS) induced by (CCh) with/without Chinese herbs (CHs) in SMG. Fluid secretion was expressed in an absolute value (FS in μL/min per g wet weight of the gland) and % of the control value obtained at 5 min from the start of CCh perfusion (mean ± SE). n: The number of sampling points. Column (1) shows the average and SE from data set obtained at 4.95 and 5 min from the start of CCh application. Columns (2)-(5) show the values during addition of CH on CCh stimulation. Columns (6)-(7) show the values 5 min after CCh washing (6) and that under CH addition without CCh (7). t-tests between the control and the values at a fixed time during addition of CH on CCh stimulation are shown. The control and the values at a fixed time during addition of CH on CCh stimulation are shown $^bP < 0.1$, $^aP < 0.05$ *vs* FS at 4.95-5 min. YZ: *Yuzhu*; SS: *Shashen*; MD: *Maimendong*; TD: *Tianmendong*.

Table 2 Fluid secretion induced by CCh with/without body *fluid-regenerating/heat-clearing* CHs in SMG

Time (min)	GG (n = 12)		WM (n = 12)		SD (n = 12)		CS (n = 12)	
	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min
(1) 4.95-5	82.9 ± 7.2	99.9 ± 0.2	66.9 ± 9.7	107.0 ± 3.8	58.5 ± 5.7	97.7 ± 0.3	66.4 ± 6.9	100.6 ± 0.5
(2) 19.95-20	64.0 ± 8.1^b	78.4 ± 6.8^a	80.9 ± 10.1	116.2 ± 4.2	51.7 ± 6.0	86.9 ± 5.1^a	86.0 ± 5.6^a	140.1 ± 10.4^b
(3) 24.95-25	60.4 ± 8.5^a	76.5 ± 8.6^a	88.4 ± 9.9	130.0 ± 6.5^a	56.2 ± 4.9	95.4 ± 3.2	101.2 ± 4.7^a	168.0 ± 14.5^b
(4) 29.95-30	55.4 ± 8.2^a	70.4 ± 8.4^a	85.3 ± 7.3	129.3 ± 8.7^a	54.5 ± 3.4	94.2 ± 4.4	106.7 ± 5.5^a	179.4 ± 17.9^b
(5) 34.95-35	50.8 ± 8.2^a	63.7 ± 8.0^a	83.2 ± 6.6	127.5 ± 9.6^b	49.7 ± 3.2	85.8 ± 3.4^a	109.6 ± 6.3^a	184.64 ± 18.5^b
(6) 9.95-10	1.6 ± 0.7	2.2 ± 1.2	-0.9 ± 0.3	-1.1 ± 0.3	-0.4 ± 0.7	-1.1 ± 1.1	-0.9 ± 0.4	-1.3 ± 0.6
(7) 14.95-15	-1.7 ± 0.6^c	-2.4 ± 1.0^c	-0.7 ± 0.2	-1.1 ± 0.3	-1.7 ± 1.4	-3.5 ± 2.3	0.6 ± 1.6	1.8 ± 3.1

t-tests between the control and the values at a fixed time during addition of CH on CCh stimulation are shown $^bP < 0.1$, $^aP < 0.05$ *vs* FS at 4.95-5 min, $^cP < 0.05$ *vs* FS at 9.95-10 min. GG: *Gegen*; WM: *Wumei*; SD: *Shengdihuang*; CS: *Chishao*.

responses to CCh (Table 2). The initial transient peak of SD (34.4 ± 7.5 μL/g per min at 16.2 min, n = 5) and XS (42.9 ± 7.9 μL/g per min at 16.2 min, n = 6) were similar to or lower than the control values (34.7 ± 7.3 μL/g per min at 1.15 min; 61.0 ± 13.6 μL/g per min at 1.15 min), whereas the maximal response of both SD and XS (56.9 ± 7.6 μL/g per min at 24.5 min; 61.2 ± 14.2 μL/g per min at 21.5 min) were lower than the control maximal value (72.3 ± 7.7 μL/g per min at 5.1 min; 81.0 ± 15.2 μL/g per min at 5 min). The response gradually declined during stimulation. Thus, SD and XS did not show a promotional effect on CCh-induced fluid secretion. On the other hand, pre-loading of CS increased the secretory response to CCh (Table 2). The initial transient peak (57.9 ± 7.4 μL/g per min at 17.2 min, n = 6) was higher than the control response (44.7 ± 8.5 μL/g per min at 1.25 min), and the response increased 86.0 ± 8.7 μL/g per min at 20 min, which was also higher than the corresponding response to single stimulation with CCh (66.6 ± 10.8 μL/g per min at 5 min). During CCh stimulation, fluid secretion continued to increase to the highest value, 109.2 ± 9.9 μL/g per min at 35 min.

Qi-enhancing agents

HQ did not induce statistically significant fluid secretion (comparison between the values at 10 min and 12 min). Pre-loading of HQ enhanced the secretory response to CCh (Figure 1C and Table 3). Both the initial transient peak (85.9 ± 6.8 μL/g per min at 16 min, n = 6) and the maximal response (116.9 ± 5.1 μL/g per min at 19.4 min) were higher than the control CCh stimulation (60.9 ± 7.3 μL/g per min at 1 min and 80.3 ± 5.7 μL/g per min at 5.15 min, respectively). After reaching a maximum, fluid secretion declined slowly to a similar level to that of the control response (Figure 1C and Table 3). *Taizishen (TZS)* did not induce secretion on a single application. Pre-loading of TZS increased the secretory response to CCh (Table 3). Both the initial transient peak (71.1 ± 16.2 μL/g per min at 17.25 min, n = 5) and the maximal response (104.9 ± 13.7 μL/g per min at 24.05 min) were higher than the control CCh stimulation (43.5 ± 6.3 μL/g per min at 1.2 min and 72.2 ± 7.8 μL/g per min at 5.05 min, respectively). After reaching a maximum, fluid secretion declined gradually. *Gancao* (*GC*) induced a significant secretion even on a single application (Figure 1D) with an initial transient peak (27.0 ± 4.7 μL/g per min at 11.5 min, n = 5) and sustained secretion (25.6 ± 4.6 μL/g per min at 15 min). Pre-loading of GC promoted the secretory response to CCh (Figure 1D and Table 3). Both the initial transient peak (85.1 ± 13.2 μL/g per min at 16.65 min) and the maximal response (102.3 ± 14.4 μL/g per min at 19.4 min) were higher than the control CCh stimulation (39.0 ± 3.5 μL/g per min at 1.15 min and 62.6 ± 7.8 μL/g per min at 5.15 min, respectively). After fluid secretion increased to reach a value double the level of stimulation without GC,

Table 3 Fluid secretion induced by CCh with/without *Qi-enhancing/Blood-activating* CHs in SMG

Time (min)	HQ (n = 12)		TZS (n = 10)		GC (n = 10)		DS (n = 12)	
	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min
(1) 4.95-5	79.1 ± 3.5	99.6 ± 0.1	80.3 ± 7.0	101.1 ± 0.5	51.8 ± 4.3	101.7 ± 0.6	71.6 ± 8.0	101.9 ± 1.8
(2) 19.95-20	115.5 ± 3.6[a]	146.8 ± 4.4[a]	95.7 ± 8.8	122.7 ± 7.0[a]	101.5 ± 8.9[a]	204.4 ± 6.8[a]	126.5 ± 11.5[a]	194.8 ± 20.4[a]
(3) 24.95-25	107.6 ± 4.2[a]	137.2 ± 6.0[a]	103.6 ± 8.3[a]	135.3 ± 9.5[a]	69.2 ± 6.2[a]	144.0 ± 14.1[a]	50.3 ± 10.4	75.0 ± 11.7[a]
(4) 29.95-30	96.6 ± 3.5[a]	123.6 ± 6.0[a]	100.5 ± 7.9[b]	132.0 ± 11.0[a]	38.3 ± 4.7[a]	82.3 ± 15.3	31.3 ± 3.7[a]	50.7 ± 8.1[a]
(5) 34.95-35	86.9 ± 3.8	112.0 ± 6.1[a]	91.7 ± 7.1	121.6 ± 10.2[b]	24.0 ± 3.5[a]	50.0 ± 7.9[a]	35.1 ± 3.0[a]	56.9 ± 7.3[a]
(6) 9.95-10	2.2 ± 2.5	2.2 ± 2.6	-0.2 ± 0.3	-0.6 ± 0.2	-0.2 ± 0.2	-6.3 ± 3.5	0.7 ± 0.5	1.5 ± 1.0
(7) 14.95-15	5.9 ± 2.0	7.4 ± 2.3	-0.4 ± 0.3	-0.1 ± 0.5	25.4 ± 2.7[c]	50.5 ± 4.7[c]	7.4 ± 1.8[c]	12.0 ± 3.1[c]

The control and the values at a fixed time during addition of CH on CCh stimulation are shown [b]$P < 0.1$, [a]$P < 0.05$ *vs* FS at 4.95-5 min, [c]$P < 0.05$ *vs* FS at 9.95-10 min. HQ: *Huangqi*; TZS: *Taizishen*; GC: *Gancao*; DS: *Danshen*.

Table 4 Fluid secretion induced by CCh with/without *Phlegm-resolving/Body mass-softening*/resolving CHs in SMG

Time (min)	ZJC (n = 10)		ZY (n = 12)		TR (n = 10)		CSJ (n = 10)	
	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min
(1) 4.95-5	67.0 ± 7.9	100.5 ± 0.7	63.5 ± 6.4	101.7 ± 0.3	53.8 ± 4.4	102.1 ± 0.8	46.2 ± 5.6	100.3 ± 0.4
(2) 19.95-20	85.9 ± 7.0[b]	135.2 ± 6.3[a]	70.8 ± 2.9	121.0 ± 8.5[a]	57.6 ± 4.2	111.0 ± 5.9	49.8 ± 6.7	107.2 ± 2.7[a]
(3) 24.95-25	98.3 ± 7.8[a]	154.7 ± 7.1[a]	83.8 ± 4.7[a]	141.0 ± 7.6[a]	66.0 ± 5.4[b]	126.0 ± 8.5[a]	55.4 ± 8.3	118.8 ± 8.0[a]
(4) 29.95-30	97.1 ± 7.8[a]	152.4 ± 6.4[a]	85.6 ± 5.6[a]	142.9 ± 7.1[a]	63.5 ± 5.2	121.9 ± 8.1[a]	54.1 ± 7.6	117.0 ± 8.9[b]
(5) 34.95-35	94.1 ± 7.4[a]	148.1 ± 6.9[a]	80.5 ± 5.0[a]	134.3 ± 6.5[a]	59.9 ± 5.4	114.3 ± 7.2[b]	50.8 ± 7.3	109.9 ± 9.2
(6) 9.95-10	1.6 ± 1.0	2.5 ± 1.4	1.4 ± 1.5	3.5 ± 3.1	-1.2 ± 0.7	-2.4 ± 1.5	0.5 ± 0.6	0.2 ± 0.9
(7) 14.95-15	1.0 ± 0.5	2.2 ± 1.3	-0.9 ± 0.3	-1.5 ± 0.6	-2.2 ± 1.0	-4.5 ± 2.0	2.3 ± 1.4	3.4 ± 1.8

The control and the values at a fixed time during addition of CH on CCh stimulation are shown [b]$P < 0.1$, [a]$P < 0.05$ *vs* FS at 4.95-5 min. ZJC: *Zaojioci*; ZY: *Zi-yuan*; TR: *Taoren*; CSJ: *Chuanshanjia*.

secretion then declined gradually to half the maximal value (24.0 ± 5.3 μL/g per min at 35 min).

Blood-activating agents

DS induced significant secretion even on a single application (Figure 1E) with a long delay of 3-4 min (8.0 ± 2.9 μL/g per min at 15 min, n = 6) and then increased markedly to a maximum and thereafter declined (data are not shown, as the overload of CCh was started at 15 min in the present series of experiments). Pre-loading of DS promoted the secretory response to CCh (Figure 1E and Table 3). Both the initial transient peak (76.2 ± 11.5 μL/g per min at 16.05 min) and the maximal response (124.0 ± 17.5 μL/g per min at 19.3 min) were higher than the control CCh stimulation (52.1 ± 10.0 μL/g per min at 1.05 min, 72.3 ± 12.6 μL/g per min at 5.15 min) as shown in Figure 1E. Fluid secretion then increased to reach a value double the level of stimulation without DS, and declined gradually to half this value (36.6 ± 6.1 μL/g per min at 35 min).

Phlegm-resolving agents

ZJC did not induce secretion on a single application (Figure 1F). Pre-loading of ZJC increased the secretory response to CCh (Figure 1F, Table 4). The initial transient peak (60.9 ± 7.1 μL/g per min at 16.8 min, n = 5) and the maximal response (99.1 ± 11.8 μL/g per min at 25.3 min) were higher than the control CCh stimulation (45.2 ± 7.0 μL/g per min at 1.2 min, 65.8 ± 12.5 μL/g per min at 5.25 min) as shown in Figure 1F. Fluid secretion tended to decline gradually (93.9 ± 11.8 μL/g per min at 35 min) during stimulation. *THF* and *ZY* did not induce secretion on a single application. Pre-loading of THF and ZY (Table 4) increased the secretory response to CCh. The initial transient peak (45.1 ± 9.8 μL/g per min at 16.25 min n = 6; 55.7 ± 11.0 μL/g per min at 16.55 min, n = 6) were higher than the control CCh stimulation (43.3 ± 13.2 μL/g per min at 1.15 min, 45.3 ± 9.3 μL/g per min at 1.2 min); the maximal response (77.0 ± 11.0 μL/g per min at 26.3 min; 86.9 ± 9.7 μL/g per min at 28.95 min) was also higher than the control CCh stimulation (65.4 ± 15.4 μL/g per min at 5.15 min, 63.9 ± 10.6 μL/g per min at 5.3 min). Fluid secretion tended to decline gradually (73.2 ± 10.0 μL/g per min at 35 min; 80.5 ± 8.0 μL/g per min at 35 min) during stimulation.

Body mass-softening and resolving agents

TR and *CSJ* did not induce secretion on a single application. Pre-loading of TR increased the secretory response to CCh (Table 4). The initial transient peak (47.6 ± 8.6 μL/g per min at 16.4 min, n = 5) was higher than the control CCh stimulation (43.5 ± 6.3 μL/g per min at 1.1 min). However, the maximal response (66.1 ± 8.6 μL/g per min at 1.1 min) was similar to the control CCh stimulation (71.8 ± 7.6 μL/g per min at 5.25 min). Fluid secretion was sustained at the same level during stimulation. Pre-loading of CSJ did not increase the secretory response to CCh (Table 4). The initial transient peak (38.3 ± 8.6 μL/g per min at 16.65 min, n = 5) was similar to the control response (39.0 ± 3.5 μL/g per min at 16.65 min), and the maximal response (53.3 ± 12.7 μL/g per min at 25.75 min) was lower than the control response (71.8 ± 7.4 μL/g per min at 5.2 min). Because the

control values for fluid secretion in response to CCh were lower in the series using CSJ, the percentage change (118.8 ± 8.0% at 25 min) showed promotion by CSJ (Table 4).

Herb mixture

DDQY did not induce secretion on a single application. Pre-loading of DDQY increased the secretory response to CCh. The initial transient peak (40.9 ± 9.8 μL/g per min at 16.35 min, $n = 5$) and the maximal response (78.0 ± 7.9 μL/g per min at 27.45 min) were higher than the control CCh stimulation (25.9 ± 4.7 μL/g per min at 1.45 min, 51.3 ± 8.7 μL/g per min at 5.25 min). Fluid secretion tended to decline gradually (74.2 ± 4.7 μL/g per min at 35 min) during stimulation.

DISCUSSION

Selection of CHs which possibly increase salivary fluid secretion

Disorders of salivary secretion are the result of various causes and mechanisms in modern medicine, while in TCM, saliva is a body fluid and its deficiency is related to the deficiency of *Yin*-fluid, the vigor of internal heat and the dysfunctional distribution of *Yin*-fluid. TCM regards the body as a whole, and holds that the normal secretion of saliva needs the generational effect of *Qi* as well as it's promotional effect. Once there is dysfunctional secretion of body fluid, the oral cavity cannot be moistened, and new pathologic features such as phlegm and excessive fluid will occur, leading to stagnation, which incurs another secondary injury which we call internal block (e.g. blood stasis),

In the treatment of secretion disorders, Body fluid-regenerating, *Yin*-nourishing, and Heat-clearing agents are commonly used and are reported in the literature[9,10]. *Qi*-enhancing agents are often prescribed simultaneously[8]. In chronic patients with a long disease course, Blood-activating agents are usually used, and sometimes even stasis-resolving[17,18], hard mass-softening and block-dispelling agents[19,23]. In conclusion, the therapeutic concept of TCM is to identify suitable herbs which can resolve the pathologic status of patients so as to reach a new balance, which is called Syndrome Differentiation and Treatment.

Classification of responses

It is interesting to find in our experiments that the promotional patterns of CH on secretion were classified into four patterns, which were eventually related to the categories of CH: overall sustained phase was continuously raised (*Yin*-nourishing, fluid production-promoting and heat-clearing agents). Although they belong to different CH types, they are closely correlated in the TCM theory, that is, *Yin*-nourishing and fluid production-promoting agents are similar in function, while heat-clearing agents can reserve body fluid which is reduced by internal heat; Sustained secretion rose to reach a maximum then decreased, this pattern was observed in HQ and TZS. These are *Qi*-enhancing agents; Sustained secretion rose to reach the highest maximum then was sustained with a slight decline (swelling-reducing, phlegm-resolving and pus-expelling agents). These 3 types of herbs have one thing in common, in that they all aim to dispel pathologic features inside the body. It is possible that they may have a similar promotional pattern; Stimulation of salivary secretion without any added stimulants. The addition of CCh promoted fluid secretion to reach the highest maximum then inhibited secretion to a lower sustained level (blood activating agent DS and GC). GC, a *Qi*-enhancing herb, is usually used as a conciliatory agent, and is quite different from the common *Qi*-enhancing agents such as HQ.

The present findings at an organ level lead to the conclusion that various CHs have different promotional mechanisms and target sites. There are scientific essences in the TCM theory and its herb classifications, however further investigations are needed, including dose-dependency of the promotional pattern of each CH.

CHs which did not show potential in promoting salivary fluid secretion

The isolated and arterially perfused salivary gland can induce fluid secretion either by neural stimulation and/or by arterial application of secretagogues. If there is no stimulation then no secretion occurs. In the present study, SH, XS, GG, SS, and DH did not show any potential in promoting fluid secretion in the salivary gland. This suggests no salivary promotional effects directly on the salivary glands by SH, XS, GG, SS, and DH. However, we have to examine the dose-response of these CHs, then we might expect to see that these CHs have indirect effects on the neural system and/or hormonal system, and indirectly affect salivary fluid secretion.

Possible mechanisms for the promotion of salivary fluid secretion by CHs

Fluid secretion by salivary glands has been recognized as the transcellular movement of electrolyte/water at secretory endpiece cells (or acinar cells). Recently, the paracellular component of fluid secretion was also taken into account. The initial fluid secretion in response to CCh is estimated mainly due to transcellular fluid movement, whereas 60%-70% of fluid secretion is due to the paracellular component during the sustained stimulation period 1 min after the start of stimulation[27]. The initial transient peak of fluid secretion is thought to be due mainly to transcellular fluid secretion. Whereas, fluid secretion during the sustained phase depends both on transcellular and paracellular components.

One possible mechanism for the promotion through transcellular movement is activation of receptors to increase cytosolic Ca^{2+} which is mobilized from Ca^{2+} store/Ca^{2+} entry. In addition, the activation of transporters for Cl^- entry is possible. Cl^- channels allow Cl^- release across luminal membrane, and the activation of Na^+/K^+ ATPase can increase Na^+-coupled Cl^- entry. The activation of unknown receptors can also play a role in these possible mechanisms.

In paracellular mechanisms, opening of junctional complexes is possible. In addition, increases in unknown

driving forces related to paracellular fluid transport needs further study.

Research to clarify the mechanisms of promotion is tightly linked to the search for control points of fluid secretion mechanisms. We could check cytosolic Ca^{2+} and Na^{+}-coupled Cl^{-}-entry in transcellular mechanisms by measuring the ouabain sensitive component of oxygen consumption. We could also determine the junctional complexes in paracellular mechanisms by measuring the secretion of fluorescent dye which cannot enter the cell. These possible mechanisms require further study.

COMMENTS

Background

Xerostomia (dry mouth) is caused by salivary hypofunction (reduction in salivary fluid secretion). Traditional Chinese Medicine (TCM) has been used in the treatment of xerostomia. The aim of the present study was to determine if Chinese herbs (CHs) had a direct effect on the salivary gland to increase salivary fluid secretion.

Research frontiers

The present therapeutic procedures for xerostomia are limited and include the supplemental use of artificial saliva and the use of stimulants. TCM is an effective therapy with few side effects. However, there is little research on the effects of CHs on salivary secretion. Our study aims to identify the effects of CHs on salivary secretion.

Innovations and breakthroughs

By combining our previous findings with reports by other herbalist doctors, we chose twenty herbs and used isolated and arterially perfused rat submandibular salivary glands to examine if CHs had direct effects on the salivary gland to increase salivary fluid secretion. The results provided us with quantitative data how much CHs could accelerate salivary secretion.

Applications

The quantitative results of this study showed how much CHs could accelerate salivary secretion. This information could possibly provide a theoretical guide for choosing herbs to relieve xerostomia in TCM practices.

Peer review

The manuscript by Murakami *et al*, describes the results of studies on the effect of several types of traditional Chinese herb remedies, commonly used to relieve the effects of dry mouth disease (xerostomia), on salivary fluid secretion in the perfused rat submandibular salivary gland. It has some merits as it addresses the problem of xerostomia, a condition affecting quite a large segment of the world population including millions of elderly women.

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BRIEF ARTICLES

Soluble intercellular adhesion molecule-1, D-lactate and diamine oxidase in patients with inflammatory bowel disease

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Abstract

AIM: To study the levels of serum soluble intercellular adhesion molecule-1 (sICAM-1), plasma D-lactate and diamine oxidase (DAO) in patients with inflammatory bowel disease (IBD), and the potential clinical significance.

METHODS: Sixty-nine patients with IBD and 30 healthy controls were included in this study. The concentration of sICAM-1 was detected with enzyme-linked immunosorbent assay, the level of D-lactate and DAO was measured by spectroscopic analysis, and the number of white blood cells (WBC) was determined by routine procedure.

RESULTS: The levels of sICAM-l, DAO, and WBC in IBD patients were significantly higher than those in the control group (P < 0.01). sICAM-l in IBD patients was found to be closely related to the levels of DAO and D-lactate (212.94 ± 69.89 *vs* 6.35 ± 2.35, P = 0.000), DAO 212.94 ± 69.89 *vs* 8.65 ± 3.54, P = 0.000) and WBC (212.94 ± 69.89 *vs* 7.40 ± 2.61, P = 0.000), but no significant difference was observed between patients with ulcerative colitis and patients with Crohn's disease. The post-treatment levels of sICAM-l, D-lactate and WBC were significantly lower than before treatment (sICAM-l 206.57 ± 79.21 *vs* 146.21 ± 64.43, P = 0.000), (D-lactate 1.46 ± 0.94 *vs* 0.52 ± 0.32, P = 0.000) and (WBC 7.24 ± 0.2.33 *vs* 5.21 ± 3.21, P = 0.000).

CONCLUSION: sICAM-1, D-lactate and DAO are closely related to the specific conditions of IBD, and thus could be used as a major diagnostic index.

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Key words: Inflammatory bowel diseases; Intercellular adhesion molecule-1; D-lactate; Diamine oxidase

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INTRODUCTION

The barrier function of the intestinal mucosa is of vital importance in inflammatory bowel diseases (IBD). Analysis of this function provides an important basis in the diagnosis of intestinal mucosal barrier dysfunction. Therefore, the importance of a timely, correct assessment of intestinal mucosal barrier function cannot be overestimated in judging the patient's disease state, estimating the prognosis and determining a comprehensive treatment program. However, direct observation of the intestinal barrier function involves many difficulties, so observation mostly needs to be made indirectly.

D-lactate, a metabolic end product of gastrointestinal bacteria, can be produced by many of these bacteria. As mammals do not have an enzyme system capable of its decomposition, D-lactate will enter the blood when the intestinal barrier function is damaged, and as they do

not have D-lactate dehydrogenase, a rise in the level of D-lactate can be detected when the permeability of the intestinal mucosa is increased. Therefore, examination of peripheral blood can reveal the degree of damage of the intestinal mucosa and the change in its permeability[1]. Diamine oxidase (DAO) is a highly active intracellular enzyme in the cytoplasm of the upper chorial cells of the intestinal mucosa. In cases where the intestinal mucosal epithelial cells and barrier function are damaged, the release of DAO is increased, and DAO enters the extracellular space, lymphatic vessels and bloodstream, thus increasing the level of DAO in the plasma. As the activity of DAO is stable, its concentration in the blood can reflect the damage and restoration of the intestinal cavity[2].

Overactivation of leukocytes is an important pathological process of IBD. The stagnation and infiltrative exosmosis of leukocytes depend on the expression and function of the intracellular adhesion molecules (ICAMs) of leukocytes and endotheliocytes at the inflammatory location.

ICAMs are a type of glycoprotein synthesized by cells and assembled on the cell surface or secreted to the cell epimatrix, and can promote the adhesion between cells or between cells and the epimatrix. The role of ICAM-1, belonging to the immunoglobulin superfamily, in the development of IBD, has been receiving more and more attention in recent years. The ICAM-1 of normal tissues is usually expressed at low levels in vascular endothelial cells, and in mononuclear macrophagocytes in the intestinal mucosal lamina propria and lymph. In the intestinal tissues of patients with IBD, the expression and distribution of ICAM-1 is significantly increased and is closely related to the degree of inflammation of the tissues[3]. The adhesive molecules in vascular endothelial cells, leukocytes or other cells can be swallowed into the cells, or peel off into the blood circulation, becoming soluble intercellular adhesion molecule-1 (sICAM-1). The post-translation product of the mRNA of some cells, are possibly not expressed on the cellular surface, but are directly secreted into the blood and is thus another important source of sICAM-1[4,5]. The increase in sICAM-1 in the serum is a marker of the damage or activation of endotheliocytes. Therefore, the level of the sICAM-1 in the serum is a significant index in the detection of some diseases[6,7].

In this research, sICAM-1, D-lactate and DAO of 69 patients with IBD were measured quantitatively with the objective of assessing their changes and clinical significance.

MATERIALS AND METHODS

Materials

Subjects: Test group including 69 patients, aged 18-60 years, with IBD hospitalized in our department [41 cases of ulcerative colitis (UC), 27 males and 13 females; 19 cases of Crohn's disease (CD), 11 males and eight females]. All patients were diagnosed by enteroscopy and were given the standard treatment set by the National Symposium on IBD, 2000 for 15 d before reassessment. Thirty healthy blood donors are used as control group.

Reagents and apparatus: Human serum sICAM-1 enzyme-linked immunosorbent assay (ELISA) kit (purchased from Boehringer Mannheim, Germany); D-lactate standard solution and D-lactic acid dehydrogenase, O-dianisidine, cadaverine dihydrochloride, horseradish peroxidase and DAO standard solution (all purchased from Sigma). The following analytical reagents were prepared in the laboratory: methotrexate injection (purchased from Zhejiang Wanma Pharmaceutical Ltd.); sulfasalazine tablets (purchased from Shanghai Sanwei Pharmaceutical Ltd.); superoxide dismutase and myeloperoxidase kit (purchased from Nanjing Jiancheng Biological Institute).

Major analysis apparatus: 721 spectrophotometer (Shanghai Sophisticated Scientific Instruments Ltd.).

Methods

A 3 mL sample of venous blood was collected from each subject of the test and control groups. After the sample was injected into dry test tubes and the serum was separated centrifugally, the serum was stored at -20℃ for examination.

The level of sICAM-1 in the serum was detected with an ELISA, the plasma level of D-lactate was determined, after the plasma was deproteinized with perchloric acid, using enzyme-coupled UV-spectrophotometry[8]; the serum DAO was determined by spectrophotometry as by Luk *et al*[2]; the number of white blood cells (WBC) was detected routinely.

Statistical analysis

All the data are presented as mean ± SD. Statistical methods used included the *t*-test, analysis of variance and linear correlation analysis. Statistical software SPSS (version 10.0) was employed for data analysis.

RESULTS

Comparison between the test group and the control group

As is shown in Table 1, the levels of sICAM-1 (206.57 ± 79.21 *vs* 107.25 ± 52.41), D-lactate (1.46 ± 0.94 *vs* 0.82 ± 0.47), DAO (9.91 ± 5.64 *vs* 2.04 ± 0.95), and WBC (7.24 ± 2.33 *vs* 4.82±1.46) in IBD patients were significantly higher than those of the control group ($P < 0.01$).

Comparison of WBC, sICAM-1, DAO and D-lactate between the 49 UC cases of UC and 19 CD cases

As is shown in Table 2, no distinct difference was demonstrated in the levels of all four substances in the UC group and CD group ($P > 0.05$). Analysis of the levels of WBC, sICAM-1, DAO and D-lactate of the test group demonstrated that sICAM-1 was related to the levels of DAO, with the correlation coefficient being 0.321 and $P < 0.01$, and also to the level of D-lactate, with the correlation coefficient being 0.412, and $P < 0.01$.

Table 1 Comparison of the values of WBC, serum sICAM-1, DAO and plasma D-lactate between the test IBD group and control group (mean ± SD)

Group	*n*	WBC ($\times 10^9$/L)	sICAM-1 (ng/mL)	DAO (U/mL)	D-lactate (μg/mL)
Test	69	7.24 ± 2.33	206.57 ± 79.21[a]	9.91 ± 5.64[a]	1.46 ± 0.94[a]
Control	30	4.82 ± 1.46	107.25 ± 52.41	2.04 ± 0.95	0.82 ± 0.47

[a]$P < 0.01$ *vs* control group. sICAM-1: Soluble intercellular adhesion molecule-1; DAO: Diamine oxidase; WBC: White blood cells; IBD: Inflammatory bowel disease.

Table 2 Comparison of the values of the 4 substances in the ulcerative colitis (UC) group and Crohn's disease (CD) group (mean ± SD)

Group	*n*	WBC ($\times 10^9$/L)	sICAM-1 (ng/mL)	DAO (U/mL)	D-lactate (μg/mL)
UC	49	7.40 ± 2.61	212.94 ± 69.89[a]	8.65 ± 3.54[a]	6.35 ± 2.35[a]
CD	20	7.29 ± 2.25	208.31 ± 51.05[b]	8.58 ± 2.49[b]	6.32 ± 2.23[b]
Control	30	4.82 ± 1.46	107.25 ± 52.41	2.04 ± 1.35	0.82 ± 0.17

[a]$P < 0.01$ *vs* control group, [b]$P < 0.01$ *vs* control group.

Table 3 Comparison of WBC, sICAM-1, DAO and D-lactate of the IBD group before and after treatment (mean ± SD)

IBD	WBC ($\times 10^9$/L)	sICAM-1 (ng/mL)	DAO (U/mL)	D-lactate (μg/mL)
Pre-treatment	7.24 ± 2.33	206.57 ± 79.21	9.91 ± 5.64	1.46 ± 0.94
Post-treatment	5.21 ± 3.21	146.21 ± 64.43[a]	6.42 ± 2.18[a]	0.52 ± 0.32[a]

[a]$P < 0.01$ *vs* pre-treatment.

Comparison of WBC, sICAM-1, DAO and D-lactate of the test group before and after treatment

The levels of WBC, sICAM-1, DAO and D-lactate of the test group were examined after the subjects were treated and the IBD was shown to be improved substantially by clinical symptoms and enteroscopy. As is shown in Table 3, the post-treatment levels of sICAM-1 (206.57 ± 79.21 *vs* 146.21 ± 64.43), D-lactate (1.46 ± 0.94 *vs* 0.52 ± 0.32), DAO (9.91 ± 5.64 *vs* 6.42 ± 2.18) and WBC (7.24 ± 2.33 *vs* 5.21 ± 3.21) were significantly lower than before treatment ($P < 0.01$).

DISCUSSION

In case of an acute attack of IBD, the structure and function of the intestinal mucosa of the patient can be seriously damaged, resulting in intestinal barrier dysfunction and thus release of intestinal bacteria, and may even induce multi-organ failure thus endangering life.

Presently, whether IBD is active is comprehensively judged primarily from clinical symptoms, erythrocyte sedimentation rate, C-reactive protein, endoscopic observations and pathology. This method can, in most cases, determine the activity of IBD quite accurately. However, for a small number of patients, those with the disease in the early stage and those with recurrence of the disease, the biochemical features are not conspicuous enough and endoscopic examination fails to display the acute activity of IBD, thus missing the opportunity of treatment because of the lack of a timely diagnosis; therefore, working out a set of monitoring indexes which are more sensitive is of substantial importance to clinical work.

The permeability of the intestinal mucosa has been used for the assessment of the development and prognosis of some diseases, such as CD, UC, *etc*. The prospective research by Tibble *et al*[9] showed that 12 mo's duration of the alteration in intestinal mucosal permeability worsens the disease, with the sensitivity and specificity being 84% and 61%, respectively. In addition, this index can also be used in prediction of the recurrence of the disease. The multi-parameter regression analysis of 47 cases of intensive care patients[9,10] demonstrated that the intestinal mucosal permeability is the only index which can predict the development of multi-organ function failure syndrome in critical patients.

Injury is a process of inflammation, during which the adhesion of leukocytes and the damage to intestinal endotheliocytes will increase the level of DAO and D-lactate. In this study, the serum DAO and D-lactate of the 69 patients with IBD before treatment were higher than those of the control group ($P < 0.01$), but no distinct difference was observed between the UC group and the CD group. The levels of DAO and D-lactate after treatment saw a marked decline. This offers proof to the fact that the levels of DAO and D-lactate, which reflect the changes in the disease condition, increase with activity of IBD but decline when the damage to the intestinal mucosa is restored after treatment.

Sans *et al*[11] found that sICAM-1 increased in the inflammatory intestinal mucosa of patients with IBD and was related to the concentration of sICAM-1 in the blood. The research by Mack *et al*[12] revealed that when

the expression of endotheliocyte ICAM-1 was lowered, the interaction between endotheliocytes and leukocytes weakened, thus alleviating the inflammatory activity. Taniguchi *et al*[13] relieved the symptoms of colitis induced by dextran sulphate sodium by adopting preventive treatment using anti-ICAM-1 monoclonal antibody.

In our research, it was found that the content of DAO and D-lactate of IBD patients were markedly higher than those of the control group, showing that damage to a certain degree existed in the intestinal mucosa of IBD patients in the acute stage ($P < 0.01$), and the permeability increased. In addition, the post-treatment content of both DAO and D-lactate decreased, indicating that the permeability had somewhat improved.

It was also demonstrated in the research that the IBD patients' leukocyte number and sICAM-1 level before treatment were markedly higher than those of the control group ($P < 0.01$) but markedly declined after treatment ($P < 0.01$). This testified to the fact that the sICAM-1 level was obviously related to the condition of the disease. The research on anti-intracellular adhesion molecule monoclonal antibody in the treatment of IBD also pointed to the role of sICAM-1 in IBD.

To sum up, the tissue damage caused by the adhesion between leukocytes and endotheliocytes is one of the common pathologies in IBD produced by bacteria and cytokines. As high expression of sICAM-1 is the important molecular basis for the increase of the adhesion between leukocytes and endotheliocytes, blocking this adhesion has become an effective preventive and therapeutic method; therefore, sICAM-1, DAO and D-lactate in the serum can be utilized as important monitoring indexes in the treatment of IBD, and sICAM-1 may be a promising molecular indicator of the activity of IBD.

COMMENTS

Background

The barrier function of the intestinal mucosa is of vital importance in inflammatory bowel diseases (IBD). Detection of this function provides an important basis in the diagnosis of intestinal mucosal barrier dysfunction.

Research frontiers

The importance of a timely correct assessment of the intestinal mucosal barrier function cannot be overestimated in judging the patient's disease state, estimating the prognosis and determining a comprehensive treatment program. However, direct observation of the intestinal barrier function involves many difficulties, so the observation mostly needs to be made indirectly.

Innovations and breakthroughs

Presently, whether IBD is active is comprehensively judged primarily from clinical symptoms, erythrocyte sedimentation rate, C-reactive protein, endoscopic observations and pathology. This method can, in most cases, determine the activity of IBD quite accurately. However, for a small number of patients, those with the disease in the early stage and those with recurrence of the disease, the biochemical features are not conspicuous enough and endoscopic examination will fail to display the acute activity of IBD, thus missing the opportunity of treatment because of the lack of a timely diagnosis; therefore, determination of a set of monitoring indexes which are more sensitive is of substantial importance to clinical work.

Applications

Serum soluble intercellular adhesion molecule-1 (sICAM-1), plasma D-lactate and diamine oxidase can be used as very important diagnostic indices, and sICAM-1 may be a useful molecular marker of the activity of IBD.

Peer review

In this manuscript, Song *et al* report that serum levels of sICAM-1, D-lactate, and diamine oxidase are increased in IBD patients. Although the sample numbers tested seem to be relatively low, I feel that this manuscript contains potentially attractive data that would be of benefit to both clinical and basic scientific fields of IBD.

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BRIEF ARTICLES

Barrier-focused intervention to increase colonoscopy attendance among nonadherent high-risk populations

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Abstract

AIM: To increase attendance for colonoscopy among nonadherent high-risk individuals for colorectal cancer (CRC) screening in China.

METHODS: During the first 12 mo without intervention, only 428 of the 2398 high-risk subjects attended a scheduled colonoscopy examination. The 1970 subjects who did not attend for CRC screening were enrolled in the present study. Prior barrier investigation was performed to ascertain the reasons for nonadherence. A barrier-focused intervention program was then established and implemented among eligible nonadherent subjects by telephone interviews and on-site consultations. The completion rates of colonoscopy during the first 12 mo without intervention and the second 12 mo with intervention were compared. Variations in the effect of the intervention on some high-risk factors and barrier characteristics were analyzed using logistic regression.

RESULTS: 540 subjects who were not eligible were excluded from the study. The colonoscopy attendance rate was 23.04% (428/1858) during the first 12 mo without intervention, and 37.69% (539/1430) during the second 12 mo with intervention (P < 0.001). Logistic regression analysis showed that the intervention was more effective among subjects with only objective barriers (OR: 34.590, 95% CI: 23.204-51.563) or subjects with some specific high-risk characteristics: first-degree relatives diagnosed with CRC (OR: 1.778, 95% CI: 1.010-3.131), personal history of intestinal polyps (OR: 3.815, 95% CI: 1.994-7.300) and positive result for immunochemical fecal occult blood testing (OR: 2.718, 95% CI: 1.479-4.996).

CONCLUSION: The barrier-focused telephone or on-site consultation intervention appears to be a feasible means to improve colonoscopy attendance among nonadherent high-risk subjects for CRC screening in China.

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Key words: Colorectal cancer; Screening; Intervention; Colonoscopy; Attendance

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INTRODUCTION

In 2002, colorectal cancer (CRC) was the fourth most common cancer diagnosed worldwide in men and the third in women, over 1 000 000 new cases were diagnosed, and more than 500 000 deaths resulted[1]. CRC screening can reduce mortality and incidence[2-5]. Data from 1991 to 2004 for the United States show that advances in prevention, early detection, and treatment of cancer have resulted in an approximately 14% decrease

in overall cancer mortality, with remarkable declines for lung, colorectal, breast and prostate cancer[6]. This evidence demonstrates that changes in lifestyle and/or cancer screening have been responsible for the decline in cancer mortality and incidence. The data from Chinese CRC screening practice have also shown decreased mortality and incidence rate in Jiashan and Haining[7,8]. A two-step screening method has been recommended for community-based CRC screening in China: immunochemical fecal occult blood testing (iFOBT) and investigation by questionnaire of high-risk factors, followed by colonoscopy examination.

Although attendance rate for colonoscopy examination is crucial to the screening effect, it has remained disappointing in our urban CRC screening. Low attendance for CRC screening has also been found in other urban areas in China[9]. Therefore, it is necessary to establish effective CRC screening intervention methods to increase attendance. The reasons for low attendance[10-16] can be categorized into subjective or objective barriers. In this study, we evaluated the effects of a barrier-focused intervention program on colonoscopy attendance among nonadherent high-risk subjects undergoing community-based CRC screening in an urban area.

MATERIALS AND METHODS

CRC screening protocol in China

The protocol for CRC mass screening in China is as follows[17]. Primary screening: subjects aged 40-74 years, who are positive for one or more of the following items are considered to be at high-risk for CRC: (1) immunochemical fecal occult blood testing (iFOBT); (2) first-degree relatives (FDRs) with CRC; (3) personal history of cancer or colorectal polyps; (4) two or more of the following items: (Ⅰ) chronic diarrhea; (Ⅱ) chronic constipation; (Ⅲ) mucous and bloody stools; (Ⅳ) personal history of appendicitis or appendectomy; (Ⅴ) personal history of chronic cholecystitis or cholecystectomy[18]; (Ⅵ) history of psychiatric trauma (e.g. divorce or death of relatives). Secondary screening: subjects who are considered to be at high risk for CRC should undergo colonoscopy examination.

Enrollment

From the end of July 2006, we conducted population-based CRC screening according to Chinese screening guidelines in Xiacheng District, Hangzhou, China. All participants provided written informed consent. The 2398 subjects who were positive for the questionnaire and/or iFOBT were regarded as the high-risk population and were invited to follow-up colonoscopy. Only 428 (17.85%) high-risk subjects had attended a scheduled colonoscopy examination by the end of July 2007. The remained 1970 nonadherent high-risk subjects who did not attend colonoscopy examination were enrolled as the target population for our intervention. The cost of screening and intervention were supported by our research funding.

Table 1 Main barriers to colonoscopy among high-risk subjects (n = 407)

Barriers	n (%)
Subjective barriers	
"I am not at risk for CRC": no symptoms	185 (45.45)
Fear of finding cancer and subsequent surgery	10 (2.46)
Fear of some complications related to colonoscopy	9 (2.21)
Other personal health concerns[1]	9 (2.21)
Embarrassment	2 (0.49)
Total	215 (52.83)
Objective barriers	
Intolerance of pain or discomfort	112 (27.52)
Busy on working days	50 (12.29)
Intolerance of bowel preparation or diet control	14 (3.44)
Inconvenience and complexity of colonoscopy procedure	14 (3.44)
Transportation problems	2 (0.49)
Total	192 (47.17)

[1]Subjects preferred to focus on current health problems, such as diabetes, and fractures. CRC: Colorectal cancer.

Barrier investigation

To obtain in-depth information about the reasons for nonadherence, we selected 500 subjects from the target population and conducted questionnaire surveys by telephone or on-site interviews about the barriers to adherence. The questionnaires contained some acknowledged barrier options. Barriers were classified as subjective or objective. Only the most influential barrier was recorded. Four hundred and seven valid questionnaires were obtained. Table 1 lists the main barriers among high-risk nonadherent subjects.

Population classification

We planned to classify the target population into three groups for later evaluation of intervention effects. (1) Subjects with one or more subjective barriers (such as "I am not at risk for CRC"), with or without objective barriers. At least, the subjective barriers prevented them from attending colonoscopy examination. (2) Subjects with one or more objective barriers (such as intolerance of pain), without subjective barriers. They recognized the screening benefits but the objective barriers prevented them attending. (3) Subjects who simply forgot about or missed their colonoscopy examination. They were prepared to undergo colonoscopy.

Establishment and implementation of intervention

Based on the results of barrier investigation, a barrier-focused intervention program was established. The program included general and special measures. The general measures were performed among all eligible subjects and the special measures were performed selectively on each subject according to his/her specific barriers (Table 2). For subjects with subjective barriers, an explanation and education were used. For subjects with only objective barriers, the aim was mainly to adopt definite goals to improve objective conditions. For subjects without barriers, only general measures were used.

The intervention program was started at the end

Table 2 Barrier-focused intervention program

Barriers	Special measures	General measures
Subjective barriers	Explanation and education	
"Not at risk for CRC": no symptoms	Explain benefits and importance of screening, and give results of previous screening	Answering questions about the screening
Fear of finding cancer	Explain that early detection of CRC leads to good prognosis	Reminding of upcoming appointments
Fear of complications or perforation	Explain that complications in colonoscopy examination are rare	Arranging on-site consultations from the physician and epidemiologists if necessary
Embarrassment	Ensure that the patient's confidence will be well protected	Providing guidance
Objective barriers	Improving objective condition	
Intolerance of pain	Arranging painless colonoscopy[1]	
Lack of time on working days	Arranging examination at weekends	
Intolerance of bowel preparation	Simplifying bowel preparation	
Inconvenience and complexity of colonoscopy procedure	Providing guidance	

[1]Painless colonoscopy: Intravenous general anesthesia was performed during colonoscopy.

Table 3 Baseline characteristics of eligible subjects (n = 1430)

Characteristics	n (%)
Age (yr)	
40-59	687 (48.04)
60-74	743 (51.96)
Sex	
Male	511 (35.73)
Female	919 (64.27)
High-risk factors	
FDRs diagnosed with CRC	240 (16.78)
Personal history of intestinal polyps	105 (7.34)
Personal history of other cancers	136 (9.51)
Positive for questionnaire[1]	839 (58.67)
Positive for iFOBT	241 (16.85)
Characteristics of barriers	
With subjective barriers	646 (45.17)
With objective barriers	714 (49.93)
Without barriers	70 (4.90)

[1]Positive for questionnaire refers to having ⩾ 2 of the following six items: (1) chronic diarrhea; (2) chronic constipation; (3) mucous or bloody stools; (4) personal history of appendicitis or appendectomy; (5) personal history of chronic cholecystitis or cholecystectomy; (6) history of psychiatric trauma. iFOBT: Immunochemical fecal occult blood testing.

of August 2007. The 1970 subjects received a series of telephone interviews or on-site interviews. The prevention care managers carried out the following: (1) explained the study purpose and obtained consent; (2) assessed eligibility by inquiring about patient disease history, and it was suggested that those with a disease history should be evaluated by specialists to make sure that they would tolerate the colonoscopy examination; (3) identification of the specific individual barriers to colonoscopy; (4) implementation of intervention measures predesigned to reduce these barriers; (5) arranged on-site consultations, during which the oncology physician and epidemiologists answered questions and tried to persuade subjects to participate in the colonoscopy examination; and (6) scheduled the colonoscopy appointments if the subjects consented.

The prevention care managers reminded the subjects by telephone 2 d ahead of the examination and provided guidance on the day of examination. Colonoscopy was performed by gastroenterologists in the endoscopy units of local hospitals, and the results were retrieved via manual review of the medical records.

The intervention process was terminated in the following situations: (1) the subject attended a scheduled colonoscopy; (2) the subject definitely refused our invitation; and (3) the subjects were lost to follow-up after at least three calls. The intervention program was terminated at the end of August 2008.

Statistical analysis

The χ^2 test was used to evaluate statistical differences between the colonoscopy completion rates. Logistic regression analysis was used to evaluate the variation in the effects of the intervention on different characteristics of barriers and among subgroups with different high-risk factors. All data were analyzed using SPSS version 16.0.

RESULTS

Baseline characteristics

Five hundred and forty subjects were excluded from the study because of death (n = 16), medical unfitness to undergo colonoscopy (n = 154), movement from the community (n = 260), or other reasons (n = 110). Table 3 provides the baseline characteristics.

Intervention effect

Twenty-five subjects were lost to follow-up during the intervention, and we assumed that these subjects refused the colonoscopy invitation. The above 540 subjects were excluded from the study. The colonoscopy attendance rate was 23.04% (428/1858) during the first 12 mo without intervention, and 37.69% (539/1430) during the second 12 mo with intervention (P < 0.001). Among the eligible 1430 subjects, attendance rate post-intervention was 5.11% (33/646) for subjects with subjective barriers, 62.32% (445/714) for those with objective barriers, and 87.14% (61/70) for those with no barriers.

As shown in Table 4, the intervention was more effective for subjects with only objective barriers (OR:

Table 4 Variation in intervention effects (n = 1430)

Characteristics	Adjusted OR	95% CI	*P* value
Age (yr)			
40-59	1.000	0.643-1.133	0.273
60-74	0.853		
Sex			
Male	1.000		
Female	0.955	0.706-1.292	0.767
High-risk factors			
FDRs diagnosed with CRC	1.778	1.010-3.131	0.046
Personal history of intestinal polyps	3.815	1.994-7.300	0.000
Personal history of other cancers	1.444	0.745-2.799	0.277
Positive for questionnaire[1]	1.589	0.925-2.727	0.093
Positive for iFOBT	2.718	1.479-4.996	0.001
Characteristics of barriers			
With subjective barriers	1.000		
With objective barriers	34.590	23.204-51.563	0.000
Without barriers	132.421	59.709-293.681	0.000

[1]Positive for questionnaire refers to having ⩾ 2 of the following six items: (1) chronic diarrhea; (2) chronic constipation; (3) mucous or bloody stools; (4) personal history of appendicitis or appendectomy; (5) personal history of chronic cholecystitis or cholecystectomy; (6) history of psychiatric trauma. FDRs: First-degree relatives.

34.590, 95% CI: 23.204-1.563) and those without barriers (OR: 132.421, 95% CI: 59.709-293.681), compared to those with subjective barriers. The intervention was also more effective for subjects with FDRs diagnosed with CRC (OR: 1.778, 95% CI: 1.010-3.131), personal history of intestinal polyps (OR: 3.815, 95% CI: 1.994-7.300) or positive results for iFOBT (OR: 2.718, 95% CI: 1.479-4.996).

DISCUSSION

In previous studies on CRC screening intervention, higher attendance was achieved by some intervention practices such as health-care-provider-directed intervention[19], telephone support[20,21], psychoeducational intervention[22,23], tailored guidance[24,25], patient-physician communication[26], motivational interviewing[27], physician reminder[28,29], community volunteers[30], and mailed brochure[31]. However, one-sided intervention methods may not achieve a satisfactory effect among nonadherent high-risk subjects.

In this study, we first identified the main barriers to colonoscopy examination, and then established a multifaceted barriers-focused intervention program that targeted objective and subjective barriers. The results indicated that the intervention program effectively increased the completion rate of colonoscopy among nonadherent high-risk subjects. The attendance rate of colonoscopy screening significantly increased during the intervention compared with the first 12 mo without intervention (23.04% *vs* 37.69%, $P < 0.001$), which reduced more effectively the mortality and incidence in the screening area, because more positive lesions were detected[2,32]. Moreover, the targeted subjects during the intervention were nonadherent and did not respond to the examination invitations during the first 12 mo, which made the intervention more difficult.

There were several barriers for any one subject, which made evaluation of each specific intervention measure complicated and inaccurate. We simplified the evaluation by classifying the target population into three behavioral groups according to their respective barriers. Colonoscopy completion rate in the population with objective barriers was higher than that in those with subjective barriers (62.32% *vs* 5.11%), which indicates that measures that target objective barriers are more effective. The colonoscopy completion rate in the population without barriers was as high as 87.14%, which indicates that only general intervention measures achieve satisfactory results among these individuals. Logistic regression also showed that the intervention was more effective for subjects with objective barriers and with no barriers compared to those with subjective barriers. Intervention measures that target subjective barriers should be improved to further increase the uptake rate.

Previous studies on barriers to CRC screening have noted that younger age is a predictor of receiving a physician recommendation for screening[33,34]. However, in our study, logistic regression showed that there was no significant difference in intervention effects between middle-aged (40-59 years) and aged (60-74 years) populations. This may have resulted from the differences between our study population and those of previous studies in terms of ethnicity, socioeconomic status and other sociodemographic characteristics.

Our findings suggested that several high-risk factors were associated positively with intervention effect. The intervention was more effective among subjects with a history of intestinal polyps. Most of these subjects had been advised by their physicians to take regular examinations, which may have contributed to their higher compliance with our intervention. Subjects with positive results for iFOBT showed better compliance. The perceived value of different high-risk factors may explain partially the variation in intervention effect. According to the medical knowledge of high-risk subjects, some items in the questionnaire (e.g. personal history of appendicitis or appendectomy, history of psychiatric trauma) seem to be less specific to CRC than iFOBT is. This may reduce the perceived importance of questionnaire investigation and cause the subjects to consider iFOBT more valuable in CRC screening. However, further investigation is needed to confirm this explanation. The poor compliance of subjects with positive questionnaire results highlighted the importance of tailoring education programs to address questionnaire investigation of CRC. Poor participation in CRC screening in FDRs of patients with CRC has been reported previously[35]. In our study, the subjects with FDRs diagnosed with CRC showed slightly better compliance. The possible reason was that the physicians who diagnosed and treated the index patients also explained the increased risk for the FDRs and advised CRC screening to increase their awareness and attendance.

Our study had several strengths: (1) we recruited

nonadherent high-risk subjects among whom intervention for colonoscopy examination has not been widely studied before; (2) the sample number was large, the consent rate was high, and few were lost to follow-up; and (3) the community setting of the study provided ready access to the subjects.

There were also some limitations to the study: (1) there was no comparison group, and one group pre- and post-test was studied; (2) it did not have saturation coverage of the communities, and it may not have included completion of all colonoscopy examinations, because the duration of the intervention was short; and (3) the population classification was based on self-reported barriers, which may have potential bias.

In summary, this barrier-focused intervention in nonadherent high-risk subjects may increase colonoscopy attendance in urban CRC screening. We found that our intervention in individuals with subjective barriers was not as effective or practical as in those with objective barriers. The next steps are to modify the intervention methods to better overcome subjective barriers. The ultimate goal is to apply the multifaceted barriers-focused intervention to other urban CRC screening regions in China.

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COMMENTS

Background

Colonoscopy examination is regarded as the gold standard for mass screening of colorectal cancer (CRC). Although attendance rate for colonoscopy examination among high-risk populations is crucial for screening success, it has remained disappointing for CRC screening. Therefore, it is necessary to establish effective screening intervention methods to increase colonoscopy compliance.

Research frontiers

In previous studies on CRC screening intervention, higher attendance was achieved by some intervention practices such as health-care-provider-directed intervention, or telephone support. However, one-sided intervention methods may not achieve a satisfactory effect among nonadherent high-risk subjects. Many studies have reported that subjective or objective barriers prevent high-risk subjects from undergoing colonoscopy examination, which indicates that barrier-focused intervention might be effective. However, such barrier-focused intervention has not been reported previously.

Innovations and breakthroughs

This is believed to be the first study to establish a barrier-focused intervention program and evaluate its effects on colonoscopy attendance in CRC screening. Moreover, in this study, nonadherent high-risk subjects were recruited, among whom, intervention for colonoscopy examination has not been widely studied before.

Applications

The study suggests that barrier-focused intervention is an effective method and may be used to increase colonoscopy attendance in other CRC screening regions.

Terminology

Barrier-focused intervention in CRC screening is an intervention method that aims to increase colonoscopy attendance by removing the barriers that prevent the subjects from undergoing colonoscopy examination. Barrier-focused intervention must be tailored or individualized because every subject may have different barriers.

Peer review

The authors performed barrier-focused intervention to increase colonoscopy attendance among nonadherent high-risk populations in China. The colonoscopy attendance rate was 23% during the first 12 mo without intervention and 38% during the second 12 mo with intervention. The authors concluded that the barrier focused telephone or on-site consultation intervention appears to be a feasible means to improve colonoscopy attendance among nonadherent high-risk populations for CRC screening. Improving colonoscopy attendance in CRC screening is an important issue, and the results of this study are interesting.

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BRIEF ARTICLES

Prognostic impact of dissected lymph node count on patients with node-negative gastric cancer

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Abstract

AIM: To investigate the long-term effect of the number of resected lymph nodes (LNs) on the prognosis of patients with node-negative gastric cancer.

METHODS: Clinical data of 211 patients with gastric cancer, without nodal involvement, were analyzed retrospectively after D2 radical operation. We analyzed the relationship between the number of resected LNs with the 5-year survival, the recurrence rate and the post-operative complication rate.

RESULTS: The 5-year survival of the entire cohort was 82.2%. The total number of dissected LNs was one of the independent prognostic factors. Among patients with comparable depth of invasion, the larger the number of resected LNs, the better the survival ($P < 0.05$). A cut-point analysis provided the possibility to detect a significant survival difference among subgroups. Patients had a better long-term survival outcomes with LN counts ⩾ 15 for pT1-2, ⩾ 20 for pT3-4, and ⩾ 15 for the entire cohort. The overall recurrence rate was 29.4% within 5 years after surgery. There was a statistically significant, negative correlation between the number of resected LNs and the recurrence rate ($P < 0.01$). The post-operative complication rate was 10.9% and was not significantly correlated with the number of dissected LNs ($P > 0.05$).

CONCLUSION: For node-negative gastric cancer, sufficient number of dissected LNs is recommended during D2 lymphadenectomy, to improve the long-term survival and reduce the recurrence. Suitable increments of the dissected LN count would not increase the post-operative complication rate.

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Key words: Stomach neoplasms; Lymph nodes; Gastrectomy; Lymphadenectomy; Prognosis

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INTRODUCTION

Gastric cancer is a common malignancy worldwide. Despite its declining incidence, mortality of gastric cancer remains high[1]. It is generally accepted that a higher survival rate benefits from a standardized pattern of lymph node dissection[2-4]. Many studies favor an extended lymphadenectomy at the time of potentially curative gastrectomy for node-positive gastric cancer, and the risk of long-term death tends to decrease when the number of resected lymph nodes increases to about 25[5-7]. However, there are few studies on how many nodes should be removed and examined when performing a radical gastrectomy for node-negative gastric cancer. Therefore, the aim of this retrospective study was to evaluate the long-term effect of the number of resected lymph nodes (LNs) on the prognosis of patients with node- negative gastric cancer.

MATERIALS AND METHODS

Materials

Between January, 1995 and March, 2004, 211 patients diagnosed with primary gastric cancer were treated with curative resection at the Department of Oncology,

Affiliated Union Hospital of Fujian Medical University, Fuzhou, China. The surgical procedure was defined as curative when no grossly visible tumor tissue (metastasis or LN involvement) remained after resection and when the resection margins were histologically normal. There were 164 males and 47 females whose age ranged from 29 to 84 years (57.4 ± 11.3 years). All patients received a D2 dissection according to the Japanese Classification of Gastric Carcinoma[8]. Lymph nodes were meticulously dissected from the en bloc specimens, and the classification of the dissected LNs was determined by specialist surgeons who reviewed the excised specimens after surgery, based on the Japanese Classification of Gastric Carcinoma[8]. All resected lymph nodes were proven to be without nodal involvement. The tumors' size was 4.2 ± 2.1 cm. Patients were divided according to the primary site of gastric cancer: 75 upper third (U) tumors; 40 middle third (M) tumors; and 96 lower third (L) tumors. Based on the Japanese Classification of Gastric Carcinoma[8], there were 69 at stage pT1, 58 at stage pT2, 62 at pT3 and 22 at pT4. As far as the histological grades were concerned, there were 55 differentiated and 156 undifferentiated cases. Total gastrectomy, proximal subtotal gastrectomy and distal subtotal gastrectomy were carried out in 99, 15 and 97 cases, respectively. A total of 4649 LNs were taken for histological examination, and the median number of LN per patient was 22 (range, 6-48; mean 22.7 ± 7.0). Routine follow-up consisted of physical examination, laboratory tests (including measurement of carcinoembryonic antigen, CA19-9 and CEA levels), chest radiography, and abdominopelvic ultrasonography or computed tomography (CT). At the early stage (pT1), patients were followed every 6 mo during the first 2 years, and then yearly beyond the third year; at more advanced stages (pT2 or greater), follow-up was every 3 mo during the first year, every 6 mo or yearly beyond the second year, for a total of 5 years. Endoscopy was performed every 6 mo or yearly. All surviving patients were followed for more than five years. The survival time was the time from diagnosis until the last contact, the date of death, or the date that the survival information was collected. The rate of follow-up visits was 92.9%, with 196 cases involved.

Methods

Patients were stratified into five groups, based on the total number of LNs removed, as follows: < 15 LNs (18 cases), 15-19 LNs (49 cases), 20-24 LNs (63 cases), 25-29 LNs (44 cases) and ⩾ 30 LNs (37 cases). The statistical analysis was performed with the Statistical Package for Social Science (SPSS), version 13.0 for Windows. Actuarial survival was determined *via* the Kaplan-Meier method, with univariate comparisons between groups through the log-rank test. Cox regression was used for multivariate analysis, with a backward elimination model for all covariates. Spearman rank correlation coefficient was used to analyze the relationship between the number of dissected LNs and recurrence rate. The correlation between the number of resected LNs and post-operative complication rate was analyzed with logistic regression model. Significance of differences was assumed at $P < 0.05$.

Table 1 Univariate analysis of variables in patients with node-negative gastric cancer

Characteristics	*n*	5-year survival rate (%)	χ^2	*P* value
Gender			1.887	0.170
Male	164	81.7		
Female	47	84.0		
Age (yr)			0.619	0.431
< 60	109	84.2		
⩾ 60	102	79.9		
Tumor size (cm)			4.538	0.033
⩽ 4	94	88.9		
> 4	117	76.8		
Tumor location			1.681	0.432
Upper	75	80.1		
Middle	40	81.7		
Lower	96	84.0		
Depth of invasion			27.698	0.000
T1	69	92.4		
T2	58	83.9		
T3	62	76.4		
T4	22	57.8		
Pathology			0.212	0.645
Differentiated	55	88.6		
Undifferentiated	156	80.0		
Surgical type			0.219	0.640
Total gastrectomy	99	80.5		
Subtotal gastrectomy	112	83.6		
Number of resected LNs			45.219	0.000
0-14	18	43.2		
15-19	49	76.8		
20-24	63	84.5		
25-29	44	90.6		
⩾ 30	37	94.5		

Table 2 Multiple stepwise regression analysis with the Cox proportional hazards model

Characteristics	β	SE	Wald	*P*	RR	95% CI
Tumor size (cm)	0.223	0.319	0.487	0.485	1.249	0.668-2.335
Depth of invasion			8.802	0.032		
T2 *vs* T1	0.789	0.483	2.666	0.103	2.200	0.854-5.670
T3 *vs* T1	0.945	0.464	4.138	0.042	2.572	1.035-6.391
T4 *vs* T1	1.466	0.500	8.605	0.003	4.334	1.627-11.545
Number of resected LNs	-0.641	0.141	20.628	0.000	0.527	0.399-0.695

β: Coefficient of regression.

RESULTS

Univariate survival analysis

The five-year overall survival rate of the entire cohort was 82.2%. The clinicopathological variables tested in the univariate analysis are shown in Table 1. Factors influencing the 5-year survival rate were tumor size ($P = 0.033$), depth of invasion ($P < 0.001$) and number of resected LNs ($P < 0.001$). The covariates age ($P = 0.431$), gender ($P = 0.170$), tumor location ($P = 0.432$), pathological types ($P = 0.645$) and type of gastrectomy ($P = 0.640$) had no significant influence on the survival.

Multivariate survival analysis

Multiple survival analysis was calculated by the Cox's proportional hazard regression model. The prognostic

Table 3 Five-year overall survival by stage subgroups and total number of resected LNs *n* (%)

Depth of invasion	*n*	OS					*P*
		0-14	15-19	20-24	25-29	⩾ 30	
T1	69	4 (37.5)	19 (83.9)	20 (88.9)	17 (100.0)	9 (100.0)	0.003
T2	58	4 (25.0)	14 (70.1)	20 (89.4)	11 (90.9)	9 (100.0)	0.005
T3	62	7 (57.1)	12 (72.7)	19 (83.5)	10 (88.9)	14 (92.3)	0.022
T4	22	3 (33.3)	4 (75.0)	4 (50.0)	6 (66.7)	5 (80.0)	0.045
Total	211	18 (43.2)	49 (76.8)	63 (84.5)	44 (90.6)	37 (94.5)	0.000

OS: Overall 5-year survival rate.

Table 4 Overall survival by total LN count and cut-point analysis per each stage subgroup

Depth of invasion	⩽ 14 *vs* ⩾ 15		⩽ 19 *vs* ⩾ 20		⩽ 24 *vs* ⩾ 25		⩽ 29 *vs* ⩾ 30	
	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*
pT1-2	19.953	0.000	13.595	0.000	5.249	0.022	2.216	0.137
pT3-4	11.916	0.001	14.196	0.000	12.262	0.000	6.483	0.011
Total	32.824	0.000	26.679	0.000	16.109	0.000	7.572	0.006

Table 5 Impact of number of resected LNs on recurrence rate *n* (%)

Depth of invasion	No. of resected LNs	*n*	Recurrence rate[2]	*r*	*P*
pT1-2	0-14	8	5 (62.5)	-0.253	0.001[1]
	15-19	33	11 (33.3)		
	20-24	40	8 (20.0)		
	25-29	28	5 (17.8)		
	⩾ 30	18	1 (5.6)		
pT3-4	0-14	10	8 (80.0)	-0.405	0.000[1]
	15-19	16	9 (56.2)		
	20-24	23	8 (34.8)		
	25-29	16	4 (25.0)		
	⩾ 30	19	3 (15.8)		

[1]Significance of differences was assumed at $P < 0.01$; [2]Recurrence within 5 years after surgery.

factors considered at univariate analysis were analyzed first by stepwise regression, including tumor size, depth of invasion and number of resected LNs. As a result, there were two independent, statistically significant prognostic parameters: depth of invasion ($P = 0.032$) and number of resected LNs ($P < 0.001$). The risk ratios and their 95% confident interval were listed in Table 2.

Impact of total LN counts by univariate survival analysis

The five-year survival, based on T category, showed considerable variations with increasing counts of resected LNs. An obvious trend toward better survival was observed for higher numbers of resected LN (Table 3).

Cut-point survival analysis

A cut-point analysis was performed to determine the numeric LN value that determines the greatest actuarial survival difference between pT1-2 and pT3-4 subgroups. We selected the ability to detect differences between groups based on the magnitude of the log-rank test χ^2 statistic. Results for all relevant cut points and stage subgroups are listed in Table 4. The cut-point analysis yielded the greatest survival difference at the levels of 15 (pT1-2), 20 (pT3-4) and 15 (entire cohort).

Impact of the number of resected LNs on recurrence rate

Within 5 years after surgery, a recurrence was confirmed by clinical, radiological examination or reoperation in 62 patients, with an overall recurrence rate of 29.4%. The number of patients with peritoneal and lymph nodes recurrence were 28 and 13 respectively. The most common site of haematogenous recurrence was the liver, occurring in 12 patients. Other patterns of recurrence were bone recurrence in 5 patients and lung recurrence in 4 patients. The relationship between the number of resected LNs and recurrence rate was analyzed by Spearman rank correlation coefficient (Table 5). For either pT1-2 or pT3-4 subgroups, there was a statistically significant, negative correlation between the number of dissected LNs and the recurrence rate ($P < 0.01$).

Correlation between number of resected LNs and post-operative complication rate

Twenty-three of the 211 patients (10.9%) experienced postoperative complications. These included pancreatitis (4 cases), anastomotic leakage (3), intra-abdominal abscess (4), postoperative ileus (1), stenosis (3), countercurrent esophagus (2), pancreatic leakage (2) and pneumonia (4). Logistic regression analysis revealed that the number of dissected LNs was not significantly correlated with the post-operative complication rate ($P = 0.214$).

DISCUSSION

It is commonly regarded that lymph node metastases is one of the most important prognostic factors in patients with gastric cancer after curative operation[9,10]. Bruno *et al*[11] pointed out that patients with node-negative gastric cancers have clinicopathological features similar to those with early gastric cancer and they showed also how those patients have a better prognosis than those with node-positive gastric carcinoma. The 5-year survival rate of patients with node-negative gastric carcinoma was 89.5%, based on the work by Wu *et al*[12], whereas, based on our present data, this rate is

82.2%. The only potentially curative treatment for this disease is complete surgical resection, with an en bloc LN dissection. D2 lymph node dissection can increase the long-term survival of gastric cancer patients with lymph node metastases and it has become a standard surgical procedure for curative treatment in Korea and Japan[13]. D2 lymph node dissection requires not only a specific anatomical extension (i.e. it must include both perigastric and suprapancreatic nodes), but also a certain number of resected LNs (at least 15). The number of dissected LNs is closely related to the postoperative pathologic staging and the prognostic assessment. Bouvier *et al*[14] investigated 749 cases with gastric cancer, and the results showed that the risk of misclassification became significant, with an error rate of 47.1% in the pathology reports, when fewer than 10 lymph nodes were examined. They concluded that, in the latter cases, staging is not reliable. Karpeh *et al*[15] reviewed 1038 patients with gastric cancer who had undergone gastrectomy with curative intent. They found that removal of $\geqslant$ 15 LNs appears to provide a considerable survival advantage, in comparison with the removal of < 15 LNs. In an attempt to improve staging accuracy, it was recommended that a minimum of 15 lymph nodes be examined. Based on our data, the median number of total LNs examined was 22 (mean 22.7 ± 7.0) per patient. In addition, the number of LNs dissected emerges as one of the most important prognostic indicators. Our present study shows that, with comparable depth of invasion, patients with a larger number of dissected LNs have a better survival rate ($P < 0.05$), consistent with the results of Smith *et al*[16]. The contribution of negative LN counts to the prognosis of patients is partly due to LN micrometastases. In patients without LN metastases identified by HE staining, about 20% had LN micrometastases[17]. Thus, it can be concluded that an extended lymphadenectomy contributes to decrease the number of residual tumor cells, leading to better prognosis. However, it is unclear how many LNs need to be removed in a D2 dissection in patients with node-negative gastric cancer. Giuliani *et al*[18] suggested that patients with node-negative gastric cancer should undergo adequate lymphadenectomy to permit examination of $\geqslant$ 23 LNs, which would allow accurate identification of prognostic variables. From the cut-point analysis, we suggest that, to be adequate, the resection, at the time of a D2 radical dissection, should involve 15 LNs for pT1-2 and 20 LNs for pT3-4, and this based on an accurate survival estimates in patients with node-negative gastric cancer.

Despite improved prognosis, as a result of early diagnosis, radical operations and the development of adjuvant therapy[9,19,20], death from gastric cancer is still almost entirely due to recurrent disease. About 5% of patients with early gastric cancer and 50% of patients with advanced gastric cancer die with recurrent disease, as reported by Böhner *et al*[21]. In a large set of follow-up data from Japan, among 14 000 patients with recurrent gastric cancer, the rate of loco-regional recurrence following potentially curative resection was 25.6%[22]. This may be due to the insufficient anatomical extension of the dissection at the time of the first surgery, including an insufficient gastrectomy or insufficient lymphadenectomy. The randomized Dutch gastric carcinoma study compared D1 and D2 lymphadenectomy and appeared to observe a significant reduction of the loco-regional recurrence rate after a more extended lymphadenectomy: following D1 lymphadenectomy, 36% of patients died with local recurrence, compared with 27% when a D2 lymphadenectomy was performed ($P < 0.05$)[23]. Our present study shows that the overall recurrence rate is 29.4% within 5 years after surgery. The Spearman rank correlation coefficient analysis revealed that there was a statistically significant, negative correlation between the number of dissected LNs and the recurrence rate ($P < 0.01$). A lower recurrence rate was observed in cases with higher total LNs, indicating that a sufficient lymphadenectomy contributed to reduce the number of residual tumor cells. While correlating the number of dissected LNs with the post-operative complication rate, Zilberstein *et al*[24] did not find any difference between the average number of dissected LNs in patients with and without complications. These authors suggested that the higher morbidity and mortality rates were not due to the extended lymphadenectomy. Those rates may be more likely correlated with the level of experience in gastroesophageal surgery, with the presence of an appropriate learning curve for radical gastrectomy and with the experience in the management of postoperative complications. The post-operative complication rate in our study was 10.9% and the logistic regression analysis showed that the number of dissected LNs did not correlate with the post-operative complication rate ($P > 0.05$). According to our results, it appears that a suitable increment of the number of dissected LN would not increase the post-operative complication rate if the surgery is performed by a trained surgical team.

COMMENTS

Background

Many studies have favored an extended lymphadenectomy at the time of a potentially curative gastrectomy for node-positive gastric cancer, and the risk of long-term death tends to decrease when the number of resected lymph nodes increases to about 25. However, few studies have assessed the relative contribution of the number of total resected lymph nodes to the outcome in patients with node-negative gastric cancers.

Research frontiers

It is generally accepted that a higher survival rate benefits from a standardized pattern of D2 radical dissection, which requires the removal of Group 1 and Group 2 lymph nodes (LNs). The number of dissected LNs is strongly correlated with patients' survival.

Innovations and breakthroughs

The authors retrospectively reviewed 211 patients with node-negative gastric cancer, who were treated with D2 radical resection at a hospital in Fujian between 1995 and 2004, to assess how the number of dissected LNs may affect their survival rate.

Applications

The authors suggest that importance should be attached to removing a sufficient number of LNs in order to improve the long-term survival and reduce the recurrence rate of patients with node-negative gastric cancer. Moreover, suitable increments of the number of dissected LNs would not increase the post-operative complication rate.

Peer review

The authors are to be congratulated on this paper, recording the effect of the number of lymph nodes resected in a large number of patients (211) with node negative gastric cancer and a remarkable survival rate of 82.2% at 5 years. The number of lymph nodes resected influenced the prognosis of the patients with node negative gastric cancer. This finding has important information for gastrointestinal surgeons.

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BRIEF ARTICLES

Tacrolimus dosage requirements in living donor liver transplant recipients with small-for-size grafts

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Abstract

AIM: To investigate the tacrolimus dosage requirements and blood concentrations in adult-to-adult right lobe living donor liver transplantation (AALDLT) recipients with small-for-size (SFS) grafts.

METHODS: During January 2007 and October 2008, a total of 54 cases of AALDLT with an observation period of 6 mo were enrolled in this study. The 54 patients were divided into two groups according to graft-recipient body weight ratio (GRBW): SFS grafts group (Group S, GRBW < 0.8%, n = 8) and non-SFS grafts group (Group N, GRBW ⩾ 0.8%, n = 46). Tacrolimus 12-hour blood levels and doses were recorded during weeks 1, 2, 3 and 4 and months 2, 3, 4, 5 and 6 in group S and group N. Meanwhile, acute rejection rates, liver and renal function test results, and the number of potentially interacting medications were determined at each interval in the two groups. A comparison of tacrolimus dosage requirements and blood levels were made weekly in the first month post-surgery, and monthly from months 2 to 6.

RESULTS: There were no differences in the demographic characteristics, acute rejection rates, liver and renal function test results, or the number of potentially interacting medications administered between the two groups. The tacrolimus dosage requirements in group S were significantly lower than group N at 2 wk (2.8 ± 0.4 mg/d *vs* 3.6 ± 0.7 mg/d, P = 0.006), 3 wk (2.9 ± 0.7 mg/d *vs* 3.9 ± 0.8 mg/d, P = 0.008), 4 wk (2.9 ± 0.8 mg/d *vs* 3.9 ± 1.0 mg/d, P = 0.023) and 2 mo (2.8 ± 0.7 mg/d *vs* 3.8 ± 1.1 mg/d, P = 0.033). Tacrolimus 12-h trough concentrations were similar between the two groups at all times except for 2 wk post-transplantation, when the concentrations were significantly greater in group S recipients than in group N recipients (11.3 ± 4.8 ng/mL *vs* 7.0 ± 3.8 ng/mL, P = 0.026).

CONCLUSION: SFS grafts recipients have significantly decreased tacrolimus dosage requirements compared with non-SFS grafts recipients in AALDLT during the first 2 mo post-surgery.

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Key words: Blood concentration; Liver transplantation; Living donor; Small-for-size graft; Tacrolimus

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INTRODUCTION

With the shortage of cadaveric donor organs, living donor liver transplantation (LDLT) is generally accepted for end-stage liver disease. In LDLT, the graft size is inevitably small and requires regeneration, especially in small-for-size (SFS) grafts. Various studies have indicated that liver regeneration and function return rapidly in both donor and recipient after transplantation[1-3]. However, several studies have shown that living donor liver transplant

recipients required smaller doses of tacrolimus compared with deceased donor liver transplant patients[4,5], which indicated that liver regeneration could affect the metabolism of tacrolimus in LDLT. The liver is a large metabolic pool which metabolizes many drugs including immunosuppressant drugs in humans. Tacrolimus, a calcineurin inhibitor, is predominantly metabolized in the liver by cytochrome P450 3A4 (CYP3A4)[6]. Kishino *et al*[7] reported that inter- and intra-individual variations in CYP3A4 activity were caused by differences in the actual ratio of graft volume (GV) to standard liver volume (SV) and donor age. Furthermore, Fukatsu *et al*[8] reported that graft hepatic weight was significantly correlated with clearance of tacrolimus. One possible explanation for the high variability of the optimal tacrolimus dose and its pharmacokinetics is the difference in graft size. In recent years, LDLT in adult patients with SFS grafts has become increasingly accepted[9]. However, questions related to this technique have arisen: What are the tacrolimus dosage requirements in SFS grafts which require adequate liver regeneration? Does liver regeneration of SFS grafts have any impact on tacrolimus metabolism? Are there any differences in tacrolimus dosage requirements between SFS grafts and non-SFS grafts in LDLT? The answers to these questions are unknown because there are few studies on tacrolimus dosage requirements in LDLT with SFS grafts in the existing literature. Hence, the purpose of this study was to determine the tacrolimus dosage requirements and blood concentrations in adult-to-adult right lobe living donor liver transplantation (AALDLT) recipients with SFS grafts.

MATERIALS AND METHODS

Inclusion and exclusion criteria

Patients were included in this analysis if they were 18 years or older, had their transplantation performed at West China Hospital, received tacrolimus as basic immunosuppression and were followed up for at least 6 mo post-surgery. Exclusion criteria: (1) Patients receiving dual liver grafts. (2) Patients who had undergone combined liver and kidney transplantation. (3) Patients who were followed up for less than 6 mo and who were lost to follow up. (4) Patients who underwent retransplantation. And (5) patients who received cyclosporine as basic immunosuppression.

Recipients and donors

According to the inclusion and exclusion criteria, the study enrolled 54 AALDLT recipients and 54 donors who underwent right lobe hepatectomy at West China Hospital of Sichuan University during January 2007 and October 2008.

The 54 donors consisted of 34 males and 20 females. Donor age ranged from 19 to 53 years (mean, 30.9 ± 8.5 years). The relationships of the donors to the respective patients were: four fathers, three mothers, 11 offspring, 16 brothers, eight sisters, five wives, two husbands and five friends.

All the recipients underwent LDLT using right lobe graft without a middle hepatic vein. Of the 54 recipients, 45 (83.3%) were male and 9 (16.7%) were female. The average age of the recipients was 43 (27-64) years. Indications for transplantation were: chronic hepatitis B with liver cirrhosis (18 cases); fulminant hepatic failure (six cases); hepatocellular carcinoma (28 cases) and other causes (two cases). The pre-transplantation MELD scores of the recipients were 1-13 in 30 patients, 14-24 in 16 patients, and ⩾ 25 in eight patients.

According to the GRBW, the 54 AALDLT recipients were divided into two groups: group S (GRBW < 0.8%), eight cases and group N (GRBW ⩾ 0.8%), 46 cases. Written informed consent was obtained from both donors and recipients before surgery, and all the AALDLTs were approved by the Ethics Committee of West China Hospital.

Immunosuppression protocols

Basic postoperative immunosuppression consisted of corticosteroids and tacrolimus (Prograf; Fujisawa, Osaka, Japan). Supplemental immunosuppression consisted of mycophenolate mofetil (for benign diseases) or azathioprine (for malignant diseases). Initial steroid tapers consisted of 1000 mg of intravenous (IV) methylprednisolone intraoperatively, followed by a 7-d taper (50 mg Q6h day 1, 40 mg Q6h day 2, 30 mg Q6h day 3, 20 mg Q6h day 4, 20 mg Q8h day 5, 10 mg Q6h day 6 and 10 mg Q8h day 7) to 20 mg of oral prednisone once daily. The corticosteroid was withdrawn from all patients within 3 mo after transplantation. Initial suspected or biopsy-proven rejections were treated with two 500-mg IV methylprednisolone boluses on consecutive days, followed by a 7-d taper to 20 mg of oral prednisone once daily.

Severe or steroid-resistant rejections were treated with a 7- to 14-d course of muromonab-CD3 (Orthoclone OKT3; Orthobiotech Products, Raritan, NJ, USA). Initial tacrolimus doses were 0.05-0.1 mg/kg per day divided into twice-daily dosing. Tacrolimus doses were adjusted to achieve a target 12-h trough concentration of 10-15 ng/mL for the first 3 mo post-transplantation, followed by 5-10 ng/mL thereafter. Tacrolimus 12-h trough concentrations were analyzed by the IMx assay (Abbott Laboratories, Chicago, IL, USA).

Data collection

Tacrolimus daily doses and 12-h trough blood levels were recorded in both group S and group N at the following intervals after transplantation: weeks 1, 2, 3 and 4 and months 2, 3, 4, 5 and 6. At each interval, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB), albumin (Alb), and creatinine (Cr) levels were recorded. Tacrolimus 12-h trough concentrations, AST, ALT, TB, Alb and Cr were measured twice weekly in the first month post-surgery, and weekly from month 2 to 6. At each interval, values recorded for dose, level, and respective liver function and kidney function test results represent the mean value over the past days. Meanwhile, acute rejection rates and the number of potentially interacting medications were determined at each interval in the two groups. Body weight was also recorded for each patient at each interval.

Table 1 Patient demographics

	Group S	Group N	*P*
Recipients' age (yr)	41.9 ± 11.6	43.4 ± 7.3	NS (0.695)
Donors' age (yr)	28.6 ± 4.4	31.8 ± 9.5	NS (0.246)
Sex (Male/female)	6/2	39/7	NS (0.864)
MELD score			NS (0.911)
1-13	5	25	
14-24	2	14	
⩾ 25	1	7	
Indications for LDLT			NS (1.000)
Cirrhosis	3	15	
HCC	4	24	
FHF	1	5	
Others	0	2	
Rejection	1/8	4/46	NS (0.567)
Body weight	66.6 ± 7.6	61.7 ± 9.8	NS (0.244)
Biliary anastomosis			NS (0.484)
Roux-en-Y	1 (12.5)	3 (7)	
Choledochocholedochostomy	7 (87.5)	43 (93)	

NS: Not significant.

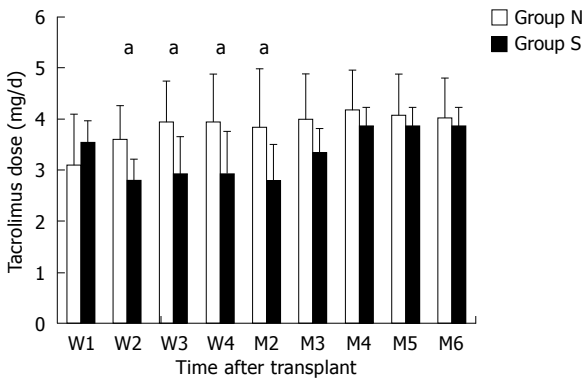

Figure 1 Tacrolimus dosage requirements (week 1 to month 6). $^aP < 0.05$.

Statistical analysis

Quantitative descriptive data were expressed as mean ± standard deviation (SD) or median (minimum to maximum). Qualitative descriptive data were expressed as percentages. Fisher's Exact, χ^2, Student's *t* and rank tests were used for statistical analysis. Tacrolimus doses and 12-h trough concentrations were compared weekly for the first month post-transplantation. For months 2-6 post-transplantation, tacrolimus doses and 12-h trough concentrations were compared monthly. ALT, AST, TB, Alb, and Cr were also compared between the two groups over the 6-mo period. All statistical analyses were performed using SPSS version 16.0 for Windows statistical software (SPSS Inc., Chicago, IL, USA). Differences with a *P* value < 0.05 were considered significant.

RESULTS

The demographics of group S and group N cohorts are listed in Table 1. There were no differences between the two groups with regard to recipients' age, donors' age, sex, and MELD score. In addition, there were no differences between the two groups in indications for transplantation or number of treated rejection episodes. There were no differences between the two groups with regard to the number of patients administered an interacting drug, total number of courses of an interacting drug, or duration of therapy (Table 2). Mean body weight for SFS grafts recipients undergoing AALDLT was slightly higher (66.6 kg) than for non-SFS grafts recipients undergoing AALDLT (61.7 kg, *P* = 0.244 not significant). The proportion of group S who underwent Roux-en-Y anastomosis was greater than that in group N, however, not statistically significant (12.5% *vs* 7%, *P* = 0.484).

Table 2 Medication interactions

Medication interaction factors	Group S	Group N	*P*
Patients administered interacting drugs	2/8	7/46	NS
Total number of treatment courses	4	9	NS
Interacting drugs			
Fluconazole	1	3	NS
Felodipine	1	2	NS
Lansoprazole	2	4	NS
Average length of therapy (d)	7	9.5	NS
No. of treatment courses on postoperative day			NS
1-30	3	5	
31-60	1	3	
61-180	0	1	

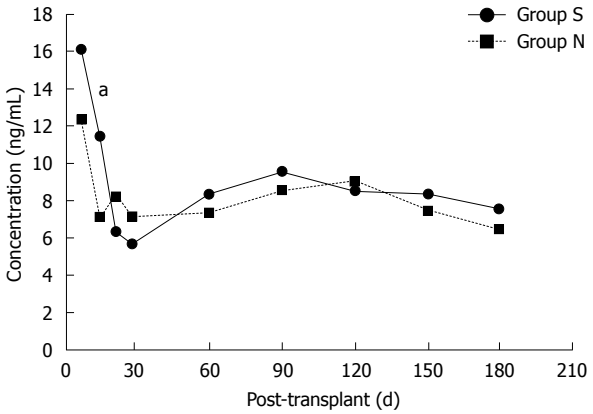

Figure 2 Tacrolimus 12-h trough level. $^aP < 0.05$.

Tacrolimus dosage requirements for each group over the first 6 mo post-transplantation are shown in Figure 1. There was no significant difference in doses during the first week. The tacrolimus dosage requirements in group S were significantly lower than those in group N at 2 wk (2.8 ± 0.4 mg/d *vs* 3.6 ± 0.7 mg/d, *P* = 0.006), 3 wk (2.9 ± 0.7 mg/d *vs* 3.9 ± 0.8 mg/d, *P* = 0.008), 4 wk (2.9 ± 0.8 mg/d *vs* 3.9 ± 1.0 mg/d, *P* = 0.023) and 2 mo (2.8 ± 0.7 mg/d *vs* 3.8 ± 1.1 mg/d, *P* = 0.033). At 3, 4, 5 and 6 mo post-transplantation, there was no statistically significant difference between the two groups with regard to tacrolimus dosage requirements.

Despite having lower dosage requirements at 2, 3 and 4 wk and 2 mo post-transplantation, tacrolimus 12-h blood concentrations were not significantly different between the two groups except for week 2 post-transplantation (11.3 ± 4.8 ng/mL *vs* 7.0 ± 3.8 ng/mL, *P* < 0.05, Figure 2). In

Table 3 Liver function and kidney function test values

	Month					
	1	2	3	4	5	6
Group N						
ALT (IU/L)	115.9	67.9	43.6	39	38	37.7
AST (IU/L)	52.7	47	34.9	39.3	36.1	38.9
TB (μmol/L)	23.1	18.5	17.2	14.9	17.6	17.9
Alb (g/L)	35.1	40.7	42.9	41.2	43	42.7
Cr (μmol/L)	55.5	55.1	59	55.7	54.1	52.3
Group S						
ALT (IU/L)	124.2	78.2	46.8	41.5	34.4	39.7
AST (IU/L)	62.6	53.4	42.5	39.9	38.8	39.3
TB (μmol/L)	33.2	22.5	19.6	18.1	16	15.6
Alb (g/L)	33.6	39.7	42.3	42.7	43.9	42.9
Cr (μmol/L)	56.9	58.3	61	61.7	64.5	63

addition, there were no significant differences between the two groups with regard to ALT, AST, TB, Alb, or Cr over the entire study period (Table 3).

DISCUSSION

LDLT has emerged as an effective alternative strategy to overcome donor organ shortage. In recent years, LDLT in adult patients with SFS grafts has become increasingly accepted[9]. With SFS grafts, it is considered that reduced functional liver mass is a necessity for adequate liver regeneration. Nevertheless, the liver is a large metabolic pool which metabolizes many drugs including immunosuppressant drugs in humans. Tacrolimus (FK506) is a potent immunosuppressive agent that is widely used in organ transplantation[10]. Because FK506 has serious side-effects (such as nephrotoxicity, hypertension and neurotoxicity) and a narrow therapeutic window (5-20 ng/mL)[11], the desired blood concentration should be achieved as soon as possible. However, tacrolimus dosage requirements and blood concentrations in LDLT with SFS grafts have not yet been reported.

Ours results indicated that tacrolimus dosage requirements were substantially reduced in AALDLT recipients with SFS grafts compared with non-SFS grafts recipients during the first 2 mo post-transplantation despite having similar 12-h trough concentrations. Beyond 2 mo post-transplantation, dosage requirements between the two groups were similar.

The reason for the relatively low tacrolimus dosage requirements in SFS grafts recipients during the first 2 mo post-transplantation is not clear. However, there are several possibilities. We analyzed pharmacokinetic variables between group S and group N (such as absorption, volume of distribution, and clearance) to explain the dosage requirement difference. Tacrolimus dosage requirements may be reduced by increased absorption, decreased volume of distribution, or decreased clearance. Neither increased absorption nor decreased volume of distribution in group S was a possible explanation. The only difference in group S and group N that could impact on absorption was the greater proportion of AALDLT recipients with a Roux-en-Y anastomosis. The proportion of group S who underwent Roux-en-Y anastomosis was greater than that in group N (12.5% *vs* 7%), but this was not statistically significant ($P = 0.484$). Moreover, a Roux-en-Y anastomosis is associated with decreased immunosuppressive absorption[12]. The volume of distribution of immunosuppressants was larger rather than smaller in group S because of their heavier body weight (SFS grafts recipients were 4.9 kg heavier than non-SFS grafts recipients).

Another explanation for the lower tacrolimus dosage requirements in group S might be reduced immunosuppressive clearance, which may be explained by two possible mechanisms. The first possible mechanism is that as the graft size in group S was smaller than in group N, it did not meet the demands of metabolism. SFS grafts have a relatively small liver mass because the GRBW is less than 0.8% and severe ischemic injuries caused by decreased hepatic arterial inflow or even hepatic artery thrombosis can occur[13-15]. Fukatsu *et al*[8] reported that graft hepatic weight was significantly correlated with clearance of tacrolimus in adult patients who had undergone LDLT, and Sugawara *et al*[16]reported that the optimal tacrolimus dose was best correlated with GV/SV ratio. Lee *et al*[17] reported that the clearance of tacrolimus was related to GRWR and the clearance of tacrolimus was decreased in patients with a small graft. Kishino *et al*[18] reported that the mean $T_{1/2}$ of tacrolimus in patients with SFS grafts (GV/SV ratio: smaller than 50%) was significantly ($P < 0.05$) longer than that in patients with GV/SV larger than 51%. These results suggest that graft size is important and could influence the clearance of tacrolimus. The reason why graft size can influence the clearance of tacrolimus is not very clear. CYP3A4 content and activity in liver grafts may partially explain the problem. Tacrolimus is mainly metabolized by CYP3A4 in the liver and intestine[6,19]. Powis *et al*[20] reported that CYP3A4 content and activity in human liver tissue decreased rapidly after surgical removal, and Kishino *et al*[7] reported that inter- and intra-individual variations in CYP3A4 activity were caused by differences in GV/SV ratio and donor age. In other words, the reduced CYP3A4 content and lower CYP3A4 activity in SFS liver grafts could partially explain the difference in tacrolimus dosage requirements between group S and group N.

Another explanation for reduced immunosuppressive clearance in SFS liver grafts may be related to several cytokines and growth factors. Several studies[21,22] have revealed that a SFS liver graft retained the capacity to regenerate faster by modulation of the expression pattern of HGF, IL-6 and TGF-βl immediately after LDLT and both the regeneration rates and the levels of cytokines and growth factors were higher in SFS liver grafts than normal sized liver grafts. These markers have also shown an ability to decrease cytochrome P450 3A activity in the liver in both mice and humans[23-25], which may decrease its ability to metabolize drugs. In other words, there were many more cytokines and growth factors (such as HGF, IL-6) in SFS liver grafts, which could decrease the clearance of tacrolimus.

In order to eliminate the impact of drug interactions, the potential medications which could affect tacrolimus level were determined in the two groups. It was noted in several studies that fluconazole, felodipine and lansoprazole could elevate tacrolimus blood level by inhibiting the activity of CYP3A4[26-28]. In the present study, the majority of patients in both groups were administered interacting drugs in the first month post-transplantation. However, there were no differences between the two groups with regard to the number of patients administered an interacting drug, total number of courses of an interacting drug, or duration of therapy. As a result, the impact of drug interactions was balanced in the two groups.

To make a rough determination of hepatic function and kidney function in group S and group N, we measured serum AST, ALT, TB, Alb, and Cr levels at each interval post-transplantation in both groups of patients. AST, ALT, and TB reflected the hepatic metabolic function while Alb was responsible for the synthetic function of the liver. AST, ALT, and TB levels were slightly greater at almost all follow-up intervals and Alb level was slightly lower in group S in the first 3 mo post-transplantation, although there was no statistical significance. This could not explain the difference in tacrolimus dosage requirements between group S and group N, however, more sensitive measures of hepatic function, i.e. galactose, caffeine, indocyanine green and monoethylglycinexylidide clearance test, should be performed to confirm the hypothesis.

There are several limitations to this study. Firstly, the sample size was small in group S. In addition, we did not study tacrolimus pharmacokinetics in this analysis which may have strongly affected the difference in the tacrolimus dosage requirements between the two groups.

In conclusion, AALDLT recipients with SFS grafts have significantly decreased tacrolimus dosage requirements compared with non-SFS grafts recipients during the first 2 mo post-surgery, in spite of having similar tacrolimus concentrations. We recommend that relatively low initial doses of tacrolimus should be administered to these patients.

COMMENTS

Background

Several studies have shown that living donor liver transplant recipients required smaller doses of tacrolimus compared with deceased donor liver transplant patients, which indicated that liver regeneration could affect the metabolism of tacrolimus in living donor liver transplantation (LDLT). However, there are few studies in the existing literature on tacrolimus dosage requirements in LDLT with small-for-size (SFS) grafts which require adequate liver regeneration.

Research frontiers

In recent years, LDLT in adult patients with SFS grafts has become increasingly accepted. The administration of immunosuppressants for SFS grafts has not been explicitly reported due to limited studies. In this field, the research goal was to determine the optimal initial tacrolimus dose for LDLT recipients with SFS grafts.

Innovations and breakthroughs

Several studies have shown that living donor liver transplant recipients required smaller doses of tacrolimus compared with deceased donor liver transplant patients. This is the first study to compare tacrolimus dosage requirements in patients who received a graft with adequate hepatic mass and patients who received a SFS graft in LDLT.

Applications

This study revealed that relatively low initial doses of tacrolimus should be administered to patients who receive a SFS graft in LDLT, which is a good guideline to follow for the rational administration of tacrolimus in future LDLT recipients with SFS grafts.

Terminology

SFS graft: SFS liver graft, this is generally considered when a graft has a graft-recipient body weight ratio (GRBW) less than 0.8% in LDLT. GRBW: Graft-recipient body weight ratio.

Peer review

The current paper is well-written and informative.

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CASE REPORT

Celecoxib-induced cholestatic liver failure requiring orthotopic liver transplantation

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Abstract

Selective cyclooxygenase-2 (COX-2) inhibitors are widely used due to their efficacy and good safety profile. However, recent case reports have described varying degrees of liver injuries associated with the use of COX-2 inhibitors. We report the case of a patient who developed acute cholestatic hepatitis progressing to hepatic failure requiring liver transplantation, following a 3-d course of celecoxib for treatment of generalized muscle aches and pains. The clinical presentation, the laboratory data, as well as the liver histopathology were supportive of the putative diagnosis of drug induced liver injury.

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Key words: Celecoxib; Cholestatic hepatitis; Liver failure; Liver transplantation

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INTRODUCTION

The primary effect of non-selective non-steroidal anti-inflammatory drugs (NSAIDs) is to inhibit cyclooxygenase (prostaglandin synthase), thereby impairing the ultimate transformation of arachidonic acid to prostaglandins, prostacyclin, and thromboxanes. The extent of enzyme inhibition varies among the different NSAIDs. At least two isoforms of cyclooxygenase enzymes have been described. While cyclooxygenase-1 (COX-1) is constitutively expressed in most normal tissues, cyclooxygenase-2 (COX-2) expression is predominantly induced during inflammation and tissue injury[1]. Most of the side effects associated with the use of non-selective NSAIDs are thought to be due to inhibition of COX-1. Therefore, selective COX-2 inhibitors have been developed in order to minimize some of the NSAID-associated adverse effects. Celecoxib is a widely used COX-2 inhibitor with high levels of patient acceptability and satisfaction, which may result from its combination of efficacy and relatively benign adverse effect profile. The daily recommended adult dose ranges between 100 and 800 mg/d for various clinical indications including osteoarthritis, ankylosing spondylitis, rheumatoid arthritis, chemoprevention of familial polyposis, primary dysmenorrhea, and acute pain[2].

Recent reports described liver injuries in association with COX-2 inhibitors ranging from acute liver failure[3-6] to varying degrees of transient cholestatic[7-10] and/or hepatocellular injuries[11,12]. We report a case of celecoxib-associated acute cholestatic hepatitis progressing to liver failure requiring transplantation.

CASE REPORT

A 52-year-old Caucasian female was in her usual state of health until she developed generalized muscle aches and pains 1 d after performing yard work. Over the next 3 d, she took a total of eight 200 mg-tablet of the prescription drug Celecoxib. On the 3rd day, she developed fatigue, loss of appetite, intense pruritus and dark-brown ("coke") colored urine. The patient's symptoms progressively worsened over the next 3 d, and 1 wk after the use of

Table 1 Laboratory values

	AST	ALT	AP	GGT	Bilirubin	INR
One year before	22	18	78	33	0.4	1.0
D 1-3	Celecoxib (eight 200 mg-tablet)					
D 10	104	258	700	262	10.8	1.0
D 24	220	297	889	711	15	1.0
D 48	442	509	1427	895	35	1.0
D 51	152	167	1024	573	38	2.6
D 54	Orthotopic liver transplantation					
1 mo (post OLTx)	16	27	570	116	1.7	1.1
6 mo (post OLTx)	36	28	204	109	0.3	0.9

AST: Aspartate aminotransferase (17-59 IU/L); ALT: Alanine aminotransferase (21-72 IU/L); AP: Alkaline phosphatase (38-126 IU/L); GGT: Gamma glutamyl transpeptidase (0-65 IU/L); Bil: Total bilirubin (0.2-1.3 mg/dL); INR: International normalized ratio (0.8-1.2).

celecoxib, she presented to the local emergency room. She denied abdominal pain, nausea, vomiting, but did endorse a weight loss of five pounds.

The patient worked as a registered nurse. Past medical history was positive for a needle stick injury from an HIV/HCV co-infected patient one year earlier, for which she was evaluated at employee health. Her liver function tests (LFTs) were normal (Table 1), and the serologies for HIV, hepatitis B and C were negative at the time of needle stick and at subsequent follow-up visits at three, seven and 10 mo. The patient was single and not sexually active. She denied any alcohol intake, smoking, use of aspirin, or over the counter medications, herbals, and illicit drugs. She denied recent travel or sick contacts.

On physical exam, she was afebrile and jaundiced with mild right upper quadrant tenderness. Initial laboratory studies revealed abnormal LFTs with predominantly cholestatic pattern (Table 1). Serum urea nitrogen and creatinine levels were normal. Blood count differential was notable for peripheral eosinophilia with an absolute eosinophil count of 760/mL. Platelets count, INR and serum albumin were within normal limits.

Repeat testing at a 2-wk follow-up revealed worsening LFTs (Table 1). Antibodies to hepatitis A, B and C and Epstein-Barr virus, Cytomegalovirus and Herpes simplex virus were negative. Iron studies, an autoimmune panel (Anti-nuclear antibodies, anti-smooth muscle antibodies, liver-kidney-microsomal antibodies and immunoglobulins), anti-mitochondrial antibodies, alpha-one anti-trypsin, and ceruloplasmin were within normal limits. A CT scan of abdomen with intravenous contrast revealed normal liver morphology with no focal lesions and no biliary duct dilatation; the hepatic and portal veins were patent. Ultrasound guided liver biopsy showed ductopenia with lobular foam cell change and cholestasis along with periportal fibrosis and no evidence of bridging fibrosis (Figure 1A and B). The patient was started on 500 mg of ursodiol twice daily, oral fat-soluble vitamins, and prednisone 40 mg daily, tapered progressively over 2 wk for presumed drug induced liver injury (DILI).

On a return visit 2 wk later, the patient showed no improvement in her symptoms and was complaining of worsening pruritus and an additional 10-pound weight loss. Lab tests at this time revealed a total bilirubin of 35 mg/dL and acute renal failure with BUN of 89 and creatinine of 7.5 (Table 1). The patient was admitted to the medical intensive care unit. She was challenged with intravenous fluids with improvement in her creatinine to 3.9 mg/dL. However, the patient's clinical condition continued to deteriorate with a new development of lethargy. The INR increased to 3.4 despite subcutaneous injections of Vitamin K, and the total bilirubin peaked at 51 mg/dL. The patient underwent orthotopic liver transplantation, 54 d after the initial ingestion of celecoxib.

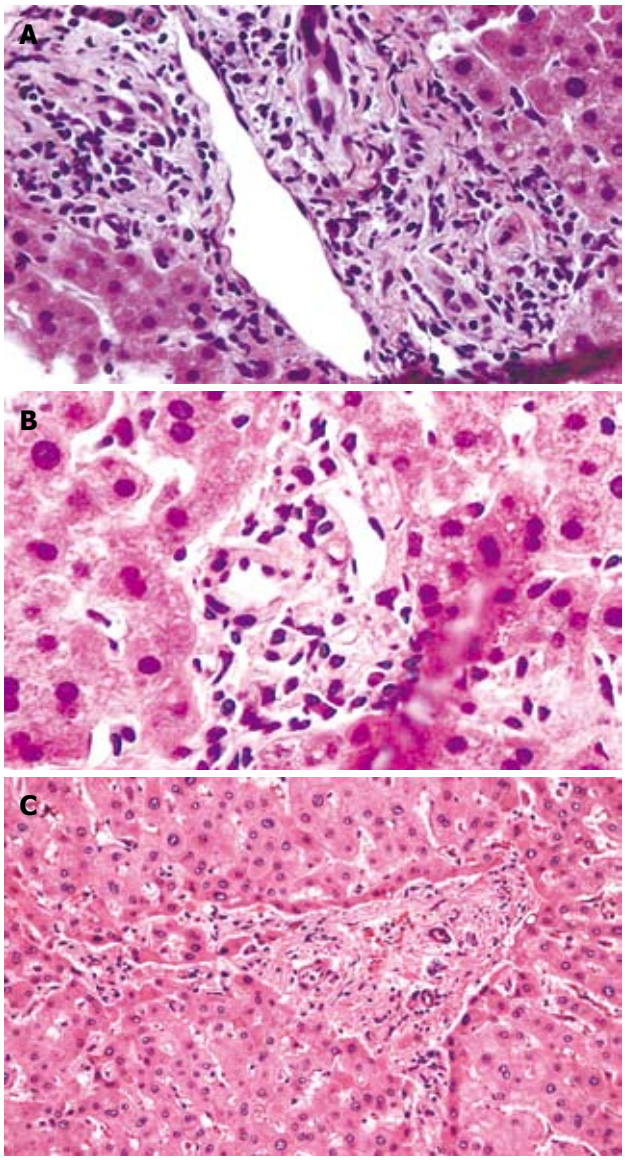

Figure 1 Hepatic histology. A: Relatively large portal tract containing bile ducts with increased nuclear to cytoplasmic ratio, eosinophilic transformation of the cytoplasm, nuclear hyperchromasia, and uneven nuclear spacing; B: Small portal tract containing the hepatic artery and portal vein, but there is no bile duct. Five of twelve interlobular portal tracts in this biopsy lacked bile ducts; C: Hepatectomy specimen: loss of interlobular bile ducts in most of the small portal tracts, more advanced portal and periportal fibrosis with short fibrous septa. (Haematoxylin & Eosin stain, × 100).

Sections through the hepatectomy specimen showed severe intrahepatic cholestasis, prominent ductopenia,

and degenerative changes of the duct epithelial cells involving most of the small bile duct branches; the large bile ducts showed little to no evidence of duct injury. More advanced portal and periportal fibrosis was noted (Figure 1C). The postoperative course was uneventful. On follow-up visits at 1 and 6 mo, the patient remained clinically stable and had normal LFTs (Table 1).

DISCUSSION

Many NSAIDs and COX-2 inhibitors have been associated with hepatotoxicity varying from transient cholestatic[7-10] and hepatocellular injury[11,12] to fulminant hepatic failure[3-6]. The incidence of COX-2-associated hepatotoxicity is extremely difficult to estimate accurately and the current literature is based on case reports of side effects. Several cases of liver injury associated with celecoxib use have been reported. Self-limited cholestatic hepatitis was reported in most of the cases with resolution of symptoms after stopping celecoxib[7-10].

Liver failure associated with COX-2 inhibitor use has been reported with Nimesulide and rofecoxib[3-6]. Fulminant liver failure requiring orthotopic liver transplantation after celecoxib use has been recently reported in one case[3]. To our knowledge, our case is the second documented case of celecoxib-induced liver failure requiring liver transplantation. Interestingly, our patient developed a liver injury after a 3-d course of celecoxib when compared to the 2-wk course in the other reported case of celecoxib-induced liver failure[3].

Several factors support the diagnosis of drug-induced liver injury (DILI) secondary to celecoxib as the most likely etiology for our patient's liver failure. These include: the acute presentation following celecoxib use; the significant eosinophilia in the blood and on the liver biopsy; and finally, the exclusion of other etiologies for liver failure.

To further assess the likelihood of DILI, we used the Roussel Uclaf Causality Assessment Method/Council for International Organizations of Medical Sciences (RUCAM/CIOMS) scoring system[13]. The criteria for scoring include: time to onset of the reaction, course of the reaction, risk factor(s) for drug reaction, concomitant drug(s), non drug-related causes, previous information on the drug, and response to re-administration. Using this system, the causality of the drug in our case is classified as "probable", with a score of 8. A more recent DILI diagnostic scale uses temporal relationship between drug intake and the onset of clinical picture, exclusion of alternative causes, extrahepatic manifestations, intentional or accidental re-exposure to the drug, previous reports in the literature of cases of DILI associated with the drug[14]. On a scale of -6 to 20, the probability of the diagnosis of DILI is expressed as: definite $\geqslant$ 17, probable = 14-17, possible = 10-13, unlikely = 6-9, and excluded $\leqslant$ 6. Our patient scored 15, hence the likelihood of DILI is "probable".

The underlying mechanisms for NSAIDs-induced liver injury are poorly understood. The adverse effects are due to either host dependent idiosyncratic reactions or dose dependent intrinsic reactions. Idiosyncratic reaction, which is the most common type, is mediated by either an immunological mechanism or abnormalities in drug metabolism[15]. The etiology of DILI in our patient is most likely an idiosyncratic reaction as she developed her symptoms after taking a relatively short course of celecoxib, well within its recommended daily adult dose.

In conclusion, we present a case of cholestatic hepatic injury following a short course of celecoxib progressing to liver failure and requiring liver transplantation. Clinicians should be aware that despite its better safety profile for gastrointestinal side effects compared to nonselective NSAIDs, celecoxib may be associated with severe hepatotoxicity.

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CASE REPORT

Combined hepatocellular and cholangiocellular carcinoma presenting with radiological characteristics of focal nodular hyperplasia

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Abstract

Combined hepatocellular and cholangiocellular carcinoma (cHCC-CC) is a rare tumor type containing unequivocal elements of both hepatocellular carcinoma and cholangiocarcinoma that are intimately mixed. Although these tumors are usually considered to be more related to hepatocellular carcinoma than to cholangiocarcinoma, they sometimes, in contrast to hepatocellular carcinoma, contain a significant amount of fibrous stroma. This might in some cases explain atypical radiological features. We report a case of a cHCC-CC in a 47-year-old female that resembled focal nodular hyperplasia on Magnetic Resonance Imaging. Correlation of imaging and serum levels of α-fetoprotein and CA19.9 can help to make the correct diagnosis preoperatively.

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Key words: Combined hepatocellular; Cholangiocellular carcinoma; Magnetic resonance imaging

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INTRODUCTION

The two major subtypes of primary liver cancer are hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC). Combined hepatocellular and cholangiocellular carcinoma (cHCC-CC) is a rare tumor type containing unequivocal elements of both HCC and CC that are intimately mixed[1,2]. It accounts for 1.0%-14.2% of all primary liver carcinomas[3]. Initial misdiagnosis was reported in several patients. The main diagnostic techniques for cHCC-CC include serum markers, imaging and histological analysis.

CASE REPORT

A 47-year-old woman, who was admitted to the radiology department of our hospital, presented with a 2 mo history of general illness, slight weight loss and a palpable mass in the right upper abdomen. Ultrasound and contrast-enhanced computed tomography (CT) of the abdomen were performed in another hospital. Ultrasound of the upper abdomen revealed a heterogeneous hypoechoic tumor in the liver. The CT scan showed a heterogeneous contrast-enhanced mass in segments 5 and 6 of the liver. Her past medical history included an appendectomy and hepatitis B positivity for more than 15 years. She was considered a so called healthy carrier of hepatitis B.

Magnetic Resonance Imaging (MRI) was performed in our hospital. On MRI a focal liver lesion inferior in the right lobe was visualized. The mass was homogeneous hypointense on T1 (Figure 1C and G) and slightly hyperintense on T2 (Figure 1A and B). The dynamic contrast-enhancement with gadolinium chelate (Magnevist®, Baeyer HealthCare Pharmaceuticals, Düsseldorf, Germany) established a relatively hypervascular heterogeneous nature with late enhancement of the central scar (Figure 1D-H). The contrast-enhancement

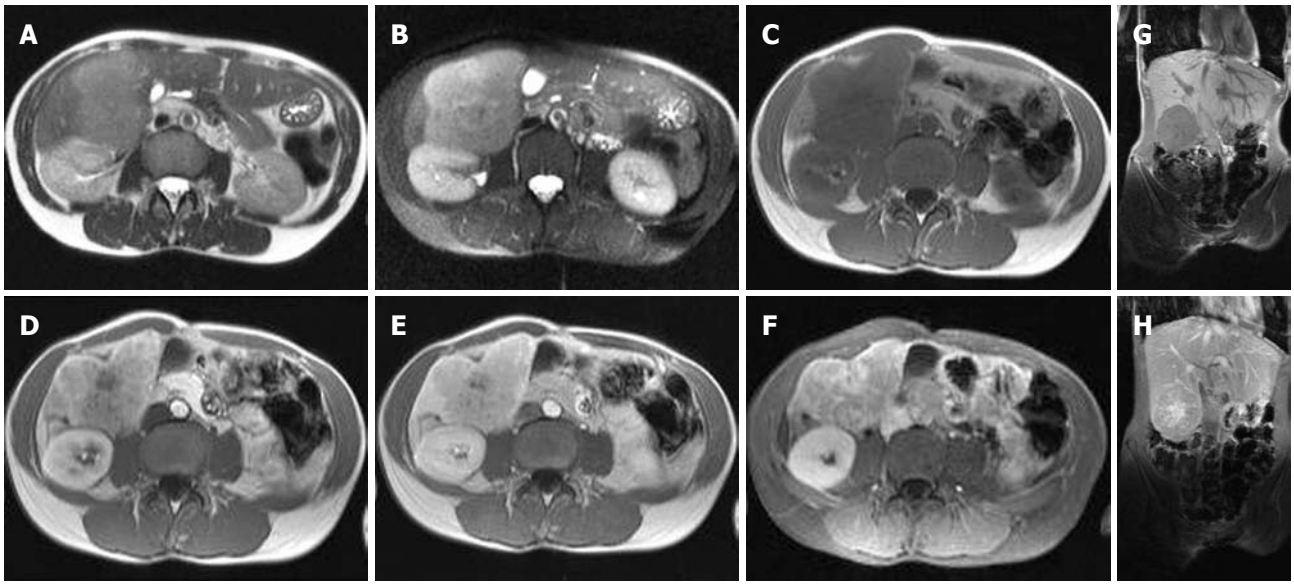

Figure 1 MRI images of combined hepatocellular and cholangiocellular carcinoma. Axial T2 (A) and T2 fat saturated (B) MRI images showed a slightly well circumscribed lesion. The mass is hyperintense in comparison to the normal liver. Axial T1 (C) MRI images demonstrated a hypointense lesion. Early arterial (D), late arterial (E) and venous (F) MRI images displayed a hypervascular heterogeneous enhancement with central scar. The coronal T1 (G) image showed a hypointense lesion, while the coronal T1 MRI image after Gadolinium contrast (H) demonstrated a heterogeneous early enhancement of the lesion with delayed uptake in the scar.

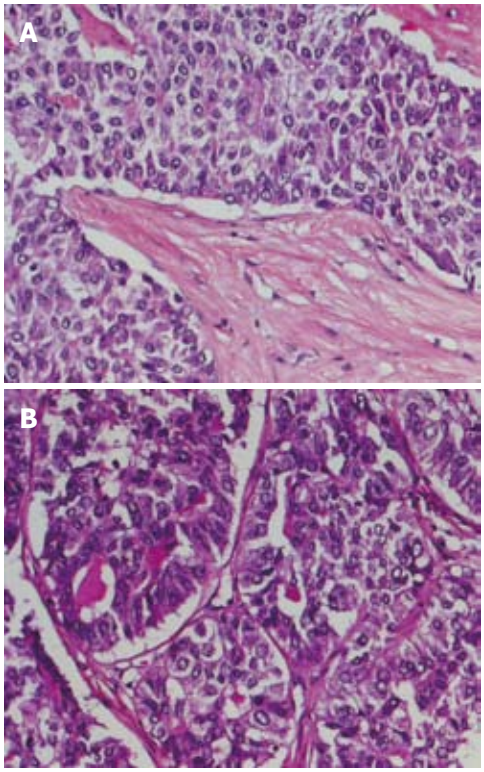

Figure 2 Histopathological images of combined hepatocellular and cholangiocellular carcinoma. A: Nests of tumor cells with trabecular architecture, reminiscent of hepatocellular differentiation, separated by bands of fibrous tissue; B: Focally tumor cells formed glandular structures with mucinous secretory material, compatible with cholangiocellular differentiation.

and morphologies were consistent with focal nodular hyperplasia. Laboratory results, however, revealed an elevated α-fetoprotein level of 154 000 ng/mL.

Right hepatectomy was performed. A white firm tumoral mass of 8.5 cm in diameter and one satellite nodule of 1 cm were identified during macroscopic examination of the resection specimen. The non-tumoral liver did not display signs of cirrhosis macroscopically. Microscopically, the tumor consisted of nests of tumor cells separated by broad bands of fibrous tissue, focally resembling a fibrous scar (Figure 2A). In most tumor cell nests, tumor cells displayed a trabecular growth pattern. Trabecular structures were several cell layers thick, in contrast to normal liver tissue in which trabeculae are only 1 to 2 layers in thickness. Focally, however, glandular structures containing secretory material were observed (Figure 2B). Tumor cells arranged in trabecular structures displayed positivity for HepPar1 and α-fetoprotein and immunohistochemistry with a polyclonal anti-CEA antibody revealed the presence of canalicular structures. These features are consistent with hepatocellular differentiation. Where tumor cells formed glandular structures, the presence of neutral mucin could be demonstrated. The secretory material displayed alcian blue positivity and was positive in a PAS-diastase reaction, compatible with cholangiocellular differentiation. Moreover, areas of glandular differentiation displayed positivity for CA19.9 and MUC1, while areas of hepatocellular differentiation were negative. In addition, positivity for CK7 and CK19 was observed in areas of glandular growth pattern, while areas with trabecular growth pattern displayed most often only positivity for CK8 and CK18. These histological features together with the immunohistochemical profile are consistent with a diagnosis of cHCC-CC.

DISCUSSION

cHCC-CC is a rare primary liver tumor type containing unequivocal elements of both hepatocellular carcinoma and cholangiocarcinoma[1,2]. It accounts for 1.0%-14.2% of

all primary liver carcinomas[3]. In 1949, the first description of cHCC-CC was reported by Allen *et al*[4]. Some studies found that this tumor type is more frequently diagnosed in patients with chronic hepatitis and liver cirrhosis[5], although this could not be confirmed in other studies[1]. Most studies report that patients with cHCC-CC show similar clinical and pathological features as hepatocellular carcinoma patients, including a mean age onset in the sixth decade, male predominance, a high incidence of hepatitis B virus infection, underlying chronic liver disease and elevated serum α-fetoprotein levels[6]. Several reported patients were initially misdiagnosed[7]. Various factors, such as an atypical enhancing pattern on imaging, the small size of one of the components, and the presence of a tumor composed of intermediate tumor cells, were found to be the causes of misdiagnosis[8]. The tumor remains usually generally clinically silent and patients present with advanced illness[6]. A simultaneous elevation of serum levels of CA 19-9 and α-fetoprotein in patients with a liver mass on imaging may suggest the presence of cHCC-CC[7]. Ultrasound usually demonstrates a round or ovoid hypoechoic mass with a central hyperechoic area[9]. On contrast-enhanced CT, cHCC-CC lesions are divided into 3 classes of enhancement patterns. The first group of lesions, namely cHCC-CC resembling hepatocellular carcinoma, shows hyperenhancement in the early phase and hypoenhancement in the late phase due to washout of contrast (Type I lesions). The second group, Type Ⅱ lesions, demonstrates peripheral enhancement in the early as well as in the late phase. Type Ⅲ lesions present an area of hyperenhancement in the early phase and an area of slight delayed enhancement in the late phase[10]. The cHCC-CC lesions resembling cholangiocarcinoma, however, enable low attenuation in the arterial phase and low, iso- or high attenuation in the venous phase[11]. Preoperatively cHCC-CC is, however, usually only considered when a liver tumor has CT features of both hepatocellular carcinoma and cholangiocellular carcinoma[12]. In addition, Toh *et al*[13] reported a hypodense tumor with vague contrast enhancement associated with elevation of α-fetoprotein levels and multiple regional lymphadenopathies as a cHCC-CC. Finally, cHCC-CC has also been reported as presenting as a hypervascular tumor associated with increased serum CEA and CA 19-9[14].

On MRI, cHCC-CC has been reported to show low signal intensities on T1-weighted images and high signal intensities on T2-weighted images[15]. In the arterial phase, it has been described that the T1-weighted images describe an enhancing mass nearly isointense to the surrounding parenchyma with a central necrotic non-enhancing zone[16].

Final diagnosis of cHCC-CC can only be made by histological examination. Extensive sampling of tissue is required in order not to miss the diagnosis in case one of the tumor compounds represents only a minor component of the tumor. Surgery is the only effective treatment for this tumor. The median survival time following cHCC-CC surgery has been reported to be 48 mo[17]. Partial hepatectomy with lymph node dissection can result in 5-year survival rates exceeding 50% in patients with early stage disease[18]. Prognostic factors of poor outcome of cHCC-CC are distant metastasis, major vascular branch invasion, regional lymph node involvement, bilobar distribution, multiple tumors, tumor necrosis and ascites[19].

In conclusion, preoperative diagnosis of cHCC-CC remains difficult. It is of great importance to choose appropriate imaging, such as MRI, in combination with serum α-fetoprotein and CA 19.9 levels. In addition, extensive tissue sampling is required in order to not miss this diagnosis in cases where one of the two components is of small size. Most studies show that this tumor type has similar geographical distribution and age and sex distribution as hepatocellular carcinoma. Some authors, however, have reported a worse prognosis as compared to patients with pure hepatocellular carcinoma.

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CASE REPORT

Sustained virologic response following HCV eradication in two brothers with X-linked agammaglobulinaemia

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Abstract

X-linked agammaglobulinaemia (XLA) is a humoral immunodeficiency syndrome characterized from childhood by the absence of circulating B lymphocytes, absent or reduced levels of serum immunoglobulin and recurrent bacterial infections. For many affected patients, regular treatment with immunoglobulin is life saving. Hepatitis C viral (HCV) infection acquired through contaminated blood products is widely described in this patient cohort. The natural history of HCV infection in patients with XLA tends to follow a more rapid and aggressive course compared to immunocompetent individuals. Furthermore, standard anti-viral therapy appears to be less efficacious in this patient cohort. Here we report the cases of two brothers with XLA who contracted HCV through contaminated blood products. They were treated with a six month course of Interferon alpha-2b and Ribavirin. We report a sustained virologic response five years after completing treatment.

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Key words: Hepatitis C virus; X-linked agammaglobulinaemia; Immunodeficiency; Viral hepatitis; Cirrhosis; Hepatocellular carcinoma

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INTRODUCTION

Infection with the hepatitis C virus (HCV) is characterized by low grade hepatitis which may progress to cirrhosis and hepatocellular carcinoma over many years. The mortality associated with HCV infection is adversely affected by several factors including: the age of the patient at initial infection, ongoing alcohol consumption, intra-venous drug abuse and the viral genotype[1,2]. Furthermore, the natural history of HCV infection in immunocompromised patients appears to follow a more aggressive course leading to rapid development of cirrhosis and hepatocellular carcinoma.

X-linked agammaglobulinaemia (XLA) is an inherited immunodeficiency disease caused by mutations in the gene coding for Bruton's tyrosine kinase (BTK) and occurs with a frequency of 1 in 250 000 males[3]. Abnormal gene expression prevents B lymphocyte differentiation and maturation in the bone marrow leading to absence of circulating antibody-producing plasma cells[4]. Furthermore, abnormalities in T cell function have been demonstrated in patients with XLA[5]. The diagnosis of XLA may be made clinically when the following criteria are met: recurrent bacterial infections in a male infant, absence of circulating peripheral B cells and more than one male in the family affected in different generations.

Early reports of HCV infection in hypogammaglobulinaemic patients suggest a severe and rapidly progressive course[6-9]. Initial attempts at treatment with interferon alpha demonstrated poor efficacy in maintaining virologic response and made little impact on the mortality and morbidity of those with a rapidly progressive course. Here we report the cases (diagnosis, management and follow-up) of two brothers with XLA who acquired HCV infection through infected blood products.

CASE REPORT

C.D. and J.D. are brother's aged 27 and 30 years,

respectively. They both suffered from recurrent lower respiratory tract infections as infants. Their younger brother died of pseudomonal meningitis and septicaemia as an infant. Their clinical history raised suspicion for an underlying immunodeficiency syndrome, which was confirmed when both brothers were found to be deficient in serum B cells and immunoglobulin in early childhood. Genotyping for BTK mutation was carried out and a diagnosis of XLA was established in each case. Subsequently, their cousin, who had a similar clinical history, was also diagnosed with XLA.

Both brothers were commenced on gammaglobulin infusions in 1985. This was comprised of fresh frozen plasma pooled from donors, which was not initially screened for viral contaminants. In 2002, routine biochemical analysis revealed raised liver function tests in J.D. and a full liver screen was carried out. Initial screening tests for anti-HCV including Ortho HCV ELISA Test System and Recombinant Immunoblot Assay-3 were negative. Subsequently, PCR for HCV RNA was carried out and was positive for HCV genotype 3 infection. C.D was then screened and also found to be HCV antibody negative, but PCR positive for genotype 3 infection. The baseline viral loads were 1 723 102 copies/mL and 52 352 copies/mL for J.D. and C.D., respectively. Neither brother had risk factors for HCV infection other than their previous treatment with intravenous gammaglobulin. The results of a full infectious, metabolic and auto-immune liver screen were otherwise negative in both patients. The baseline clinical data for each patient is summarized in Table 1.

Both patients underwent a liver biopsy, which showed chronic active hepatitis. J.D. had stage 1 fibrosis with mild to moderate inflammatory activity, while C.D. had stage 0 fibrosis with mild inflammation. Both brothers were treated with a 24-wk of Pegylated Interferon alpha-2b and Ribavirin which they completed in June, 2003. There were no significant complications during treatment. Both patients were treated with antibiotics for respiratory tract infections during treatment, but did not require admission to hospital. Their viral load became undetectable 4 wk into therapy and both patients remain PCR negative five years post treatment. J.D. currently receives 35 g of Flebogamma and C.D. receives 40 g, every 3 wk, which is well tolerated by both.

Table 1 Baseline clinical data for both patients

	Age at diagnosis (yr)	Baseline viral load	Baseline ALT (IU/L)	Liver histology	Treatment	Time to PCR negative (wk)
C.D.	27	52 352 copies/mL	37 (< 42)	Mild inflammation	Peg-Interferon alpha-2b + Ribavirin	< 4
J.D.	30	1 723 102 copies/mL	87 (< 42)	Stage 1 fibrosis + moderate inflammation	Peg-Interferon alpha-2b + Ribavirin	< 4

DISCUSSION

Immunoglobulin therapy is widely used for the treatment of immune deficiency syndromes. Unfortunately, several outbreaks of HCV infection secondary to immunoglobulin replacement therapy have been documented in recent times. Although the true prevalence of HCV infection is difficult to estimate in this population, large studies estimate the incidence at approximately 8.3%, a number far greater then that in the immunocompetent population[10]. Unfortunately, the diagnosis of HCV in this high risk population remains problematic for several reasons. Firstly, the poor sensitivity of traditionally used screening tests including measurement of serum aminotransferase and detection of anti-HCV antibody means that a diagnosis of HCV infection will be missed unless PCR for viral RNA is carried out. Secondly, as there is no set of international guidelines or recommendations for screening this patient cohort, there is wide variation in the screening tests employed between different centers. Finally, despite significant developments regarding viral safety for immunoglobulin preparations, clear product documentation is often absent, making early detection of HCV infection and identification and tracing of exposed individuals difficult.

The clinical course of HCV infection in patients with hypogammaglobulinaemia is unclear. Some reports suggest a rapidly progressive course with poor patient outcomes[8,11], while others demonstrate the more usual, slowly progressive course, at least in the short term[12,13]. A less aggressive clinical course has been observed in patients with XLA compared with those with combined variable immunodeficiency[14], although this remains a source of debate[15]. The variability between different trials remains unexplained. The duration of infection remains as an important predictor of patient outcome. Undoubtedly other co-morbidities including alcohol consumption, intra-venous drug abuse and co-infection with hepatitis B (HBV) adversely impact on the clinical course also. It is noteworthy that Italian patients appear to follow a more benign course following HCV infection than other populations[13,16], suggesting a role of HCV viral load and genotype in the genesis of liver disease.

Early attempts to treat HCV infection with interferon-α monotherapy in patients with primary immunodeficiency have been disappointing[6,8]. However, more recent data demonstrates sustained virologic responses (SVRs) of 54%[13], and between 35%-40% approximately[14,15], in similar patients with acute and chronic HCV infection respectively. Due to the small number of patients involved, it is difficult to determine the influence of viral load and viral genotype on treatment response. Improved treatment outcomes are seen however in patients with a shorter duration of infection and when combination therapy (Interferon and Ribavirin) is used. The excellent treatment response seen in our patients may relate to multiple factors including a favourable genotype, the young age of each patient, minimal liver fibrosis and the absence of other co-morbidities. The duration of infection is unknown as the infected batch was never identified. Although patients are considered to have achieved SVR when they remain PCR

negative six months post completion of treatment, we delayed the reporting of our patients in case of late relapse.

In conclusion, our report raises a few important points. Firstly, all patients who received intravenous immunoglobulin during periods when routine screening was not carried out, should be tested for HCV infection. The importance of measuring HCV RNA rather than looking for HCV antibodies in this patient cohort can not be overstated. Indeed, serological tests failed to make a diagnosis of active HCV infection in both our patients. Secondly, the successful outcome of treatment in both patients highlights the limited role of humoral immunity in the mechanisms of HCV clearance. Finally, despite pessimistic initial reports, the diagnosis and treatment of HCV infection in patients with XLA can lead to excellent long-term outcomes.

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CASE REPORT

Cavernous mesenteric lymphangiomatosis mimicking metastasis in a patient with rectal cancer: A case report

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Abstract

Lymphangioma usually occurs in children and usually involves the skin. Mesenteric lymphangioma is extremely rare in adults. Typically, lymphangioma appears on computed tomography (CT) as a lower attenuation of a cystic mass, however, some cases appear to be a solid mass. We describe the CT and 18F-FDG positron emission tomography/CT appearance in a case of jejunal and mesenteric cavernous lymphangiomatosis mimicking metastasis in an adult patient with rectal cancer.

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Key words: Lymphangioma; Lymphangiomatosis; Mesentery; Small intestine; Computed tomography; 18F-FDG positron emission tomography/computed tomography

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INTRODUCTION

Gastrointestinal and mesenteric lymphangioma or lymphangiomatosis are extremely rare in adults[1-3]. A lymphangioma usually appears as a partially septated, cystic mass on imaging studies including ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI). CT is a basic imaging modality for detecting and evaluating various malignancies. Occasionally, lymphangioma may appear as a solid or infiltrative soft tissue mass on CT because of its microcystic nature or if there is intracystic debris or hemorrhage. The non-cystic appearance of mesenteric lymphangioma may be confused on CT with metastasis or a malignant tumor, especially in patients with malignancy. 18F-FDG positron emission tomography (PET)/CT may have an important role for staging malignancy because hypermetabolic tumor cells actively uptake 18F-FDG[4]. This report describes the CT and 18F-FDG PET/CT appearance of a mesenteric and jejunal lymphangiomatosis mimicking metastasis in an adult patient with rectal cancer.

CASE REPORT

A 71-year-old man was referred for evaluation of a rectal cancer detected on colonoscopy and CT during an evaluation of hematochezia. He had a 5-year history of taking medication for underlying angina. Rectal examination showed a hard mass in the rectum. The standard laboratory tests were normal. Rectal cancer was seen as segmental and irregular wall thickening in the distal rectum with several enlarged perirectal lymph nodes on contrast-enhanced CT (Figure 1A). The soft-tissue density of the nodular mass and hazy attenuations in the jejunal mesentery (Figure 1B) were overlooked initially on either CT scans or on surgery. The patient underwent laparoscopic abdominal transanal resection and ileostomy. Adjuvant chemotherapy and ileostomy closure were performed during the year following surgery. Follow-up, contrast-enhanced CT revealed nodular jejunal and adjacent mesenteric masses in contrast to the barium-filled jejunum; the masses measured approximately 8 cm × 6 cm in width (Figure 1C). Under the suspicion of mesenteric and jejunal wall metastasis from the rectal cancer, 18F-FDG PET/CT was performed, but no remarkable FDG uptake was seen (Figure 1D).

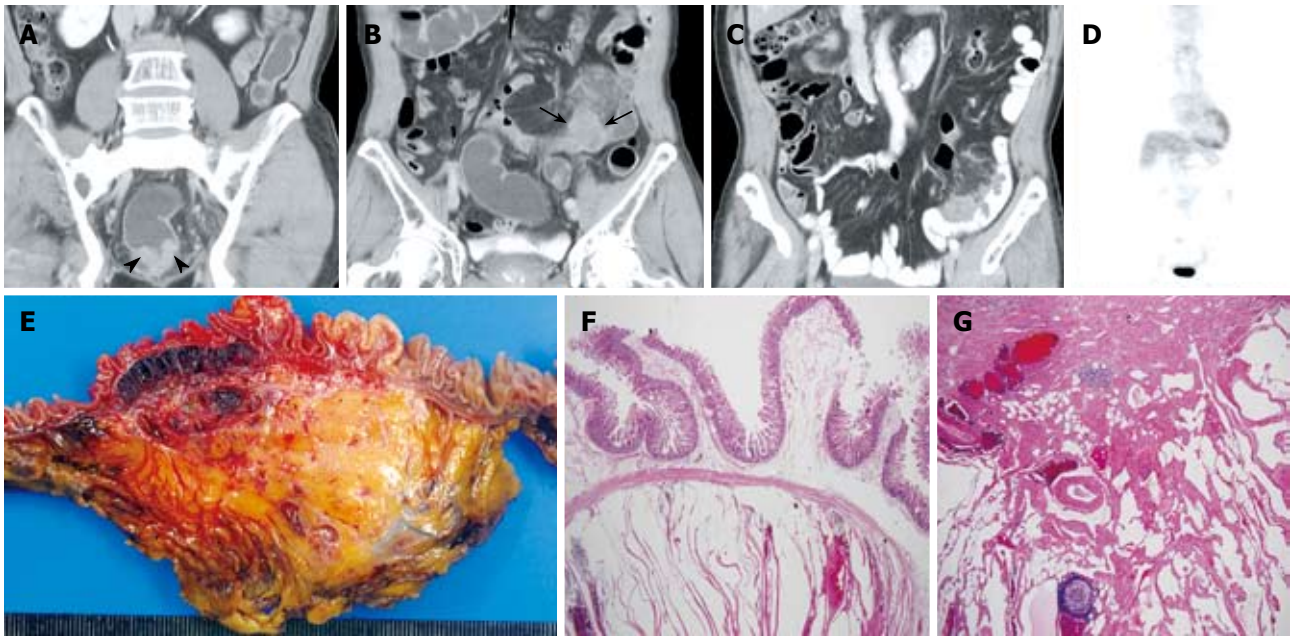

Figure 1 A 71-year-old man with rectal cancer and mesenteric lymphangioma. A, B: Contrast-enhanced, coronal CT images show irregular and concentric rectal wall thickening (arrow heads), a nodular soft-tissue-density mass, and hazy strands in the jejunal mesentery (arrows); C: Follow-up, contrast-enhanced, coronal CT obtained one year after laparoscopic rectal cancer resection, reveals clear demarcation of the jejunal nodular lesions and infiltrative soft tissue masses in the adjacent mesentery. No remarkable jejunal obstruction was found; D: 18F-FDG PET shows no 18F-FDG uptake in the jejunum or mesenteric lesions; E: The cut section of the jejunum reveals a dark-red, multiloculated, cystic lesion measuring 8.0 cm × 5.0 cm in the mucosa to the subserosa; F, G: Histopathologic view of the tumor shows numerous, multiloculated, cystically dilated lymphatic spaces lined by attenuated endothelium in the entire jejunal wall (HE stain, × 40) and adjacent mesentery (HE, × 20).

Pathological examination of a resected segment of the jejunum revealed a macroscopic, dark red, multiloculated cystic lesion measuring 8.0 cm × 5.0 cm from the mucosa to the subserosa (Figure 1E). Microscopically, it showed numerous multiloculated, cystically dilated spaces lined by attenuated endothelium and involved mucosa to subserosa. In the focal area, ectatic spaces appeared to dissect through the muscularis propria of the small intestine. Proteinaceous, fluid-containing lymphocytes seen in the cystic spaces revealed that the channel originated in the lymphatic system. The stroma was composed of a delicate meshwork of collagen punctated by lymphoid aggregates (Figure 1F). Immunohistochemical staining for CD34 showed positivity in the endothelial cells of the tumor (Figure 1G). The diagnosis of cavernous lymphangioma involving the jejunum and mesentery was established. The patient had an uneventful postoperative course and was discharged one week after surgery.

DISCUSSION

Lymphangioma or lymphangiomatosis affect the skin, the covering of various organs and areas except of the brain. About 90% are diagnosed within the first two years of their existence[1,2]. In adults, gastrointestinal tract involvement of mesenteric lymphangioma is very rare, the distal ileal mesentery is most frequently involved[3]. The etiology of lymphangiomas is still unclear. They are considered to be a congenital dysplasia of lymphatic tissue and abnormal development of the lymphatic vessels during fetal life[5,6]. The macroscopic appearance of lymphangioma is a cystic mass with partial septations and its histological characteristics are endothelial-lined, dilated, communicating lymphatic channels containing a variable amount of connective tissue and smooth muscle fiber[5,7]. Lymphangiomas are generally classified as simple capillary, cavernous, and cystic according to the size of lymphatic space and the nature of the lymphatic wall[8,9]. Cavernous lymphangioma is composed of dilated lymphatic vessels and lymphoid stroma and is connected with the adjacent normal lymphatics. Alternatively, cystic lymphangioma is composed of various-sized lymphatic spaces and has no connection with the adjacent normal lymphatics. However, as cystic lymphangiomas may have a cavernous area, clear differentiation between cystic and cavernous lymphangioma is not always possible[9,10].

Most intra-abdominal lymphangiomas are of cystic form and generally appear as a thin-walled, mutiseptated, cystic mass with or without intracystic debris. Although these lymphangioma characteristics may appear in typical, mutiseptated, cystic masses on images including ultrasound and CT, some lymphangiomas may appear to be solid masses because they contain intracystic debris or hemorrhage or due to the microcystic nature of cavernous lymphangiomas. MRI is advantageous for detecting fluid-filled cystic lesions as it may reveal the cystic nature of cavernous lymphangiomas that appear as solid masses on CT[11]. In our patient with rectal cancer, multiple nodular mesenteric masses infiltrating into the jejunum and adjacent mesentery were found. Because we did not suspect the microcystic nature of cavernous lymphangiomas, MRI was not performed to differentiate a microcystic tumor mimicking a solid mass.

As 18F-FDG is an analogue of glucose, its uptake

within viable tumor cells is in proportion to the rate of glycolysis. Therefore, as 18F-FDG PET/CT can detect hypermetabolic tumor cells, it can be widely used for the detection, staging, and management of various malignant tumors[4]. In our patient, as 18F-FDG PET/CT revealed no remarkable 18F-FDG uptake within the solid, mass-like lesions in the jejunal and adjacent mesentery, we were able to exclude the possibility of metastasis from the underlying rectal cancer.

The clinical symptoms of gastrointestinal and mesenteric lymphangiomas vary from being asymptomatic to acute abdominal symptoms such as obstruction or bleeding, according to the size and the localization of the tumor[10,12,13]. The treatment of choice is complete surgical resection. Because lymphangioma is benign in nature, the prognosis is usually good despite the possibility of tumor recurrence. As Goh *et al*[14] reported a 100% recurrence rate, complete lymphangioma resection is important in order to prevent tumor recurrence.

In conclusion, rarely occurring, cavernous mesenteric lymphangioma in adults occasionally appears on CT as a solid mass and it may be confused with metastasis in patients with malignancies. Therefore, 18F-FDG PET/CT may be helpful in excluding the presence of metastasis.

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CASE REPORT

Duodenal stenosis resulting from a preduodenal portal vein and an operation for scoliosis

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Abstract

A preduodenal portal vein (PDPV) is known to be a rare cause of duodenal stenosis. We treated a 22-year-old male patient with malnutrition as a result of PDPV and a previously performed operation for scoliosis, who showed an improvement in quality of life after being treated with a combination of nutritional support and surgery. The patient with PDPV had been admitted to our department with duodenal stenosis, ranging from the first to third portions. He had suffered from vomiting since 1 year of age, and he developed malnutrition during the last 6-mo period after orthopedic surgery for scoliosis. The stenosis was related to both the PDPV and the previously performed operation for scoliosis. After receiving nutritional support for 6 mo, a gastrojejunostomy with Braun's anastomosis for the first portion and a duodenojejunostomy for the second and third portions were performed. The postoperative course was almost uneventful. Three months later, he was discharged and able to attend university. In patients with widespread duodenal stenosis, there may be a complicated cause, such as PDPV and duodenal stretching induced by previous spinal surgery.

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Key words: Preduodenal portal vein; Duodenum; Malnutrition; Gastrojejunostomy; Nutritional support; Stenosis

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INTRODUCTION

Preduodenal portal vein (PDPV) is a rare congenital anomaly which was first described by Knight in 1921[1]. Clinically, 50% of the patients present with symptoms related to intestinal obstruction, but only in a few is the obstruction directly caused by the PDPV[2-4]. In the majority of cases, the obstruction is thought to be caused by associated anomalies such as duodenal diaphragm, intestinal malrotation, and annular pancreas. In the remaining 50%, PDPV is asymptomatic and diagnosed incidentally on preoperative investigation or during surgery for other diseases[2-4]. Patients with symptoms directly associated with PDPV or resulting from associated anomalies often require correction of the intestinal obstruction[2-5].

Scoliosis is a well known anomaly in the pediatric orthopedic field. In patients with severe scoliosis, a surgical correction is often needed. The hallmark of surgical treatment is thought to be early intervention before the development of large curvatures[6]. Recent orthopedic surgery for scoliosis has evolved to include the routine use of spinal instrumentation to achieve a good result.

No reports have been published regarding patients

with PDPV associated with severe scoliosis. We experienced one male patient with malnutrition resulting from PDPV and a previously performed operation for scoliosis. An improvement in the quality of life (QOL) was found after being treated with a combination of a gastrojejunostomy, with both Braun's anastomosis and a side-to-side anastomosis between the duodenal second portion and jejunum, and nutritional support. Therefore, we herein report and discuss the clinical course of this patient and his treatment.

CASE REPORT

A 22-year-old male patient with PDPV presented with duodenal stenosis, ranging from the first to the second portions. Frequent vomiting had occurred at 1 year of age. Therefore, fundoplication had been performed at 2 years of age in another hospital. However, he had occasionally suffered from vomiting since the fundoplication. In addition, he gradually developed severe scoliosis over time. Therefore, he underwent surgery twice for scoliosis at 21 and 22 years of age. After the orthopedic operations, he developed malnutrition because of severe vomiting during a 6-mo period. In another hospital, the cause of the vomiting was investigated, and found to be the result of widespread duodenal stenosis.

On admission to our department, an X-ray film showed a spine with a large curve to the right side although it was corrected by previous orthopedic surgery (Figure 1A). Abdominal computerized tomography (Figure 1B) and a gastrointestinal contrast study (Figure 1C) revealed that the stenosis was associated with both the PDPV and the previously performed operation for scoliosis. In addition, his nutritional condition at admission was extremely poor (body mass index: 11.4). Furthermore, he also had diabetes associated with dysfunction of Vater's sphincter. We therefore introduced nutritional support using both parenteral and enteral nutrition by jejunal feeding tube at first. Two months later, the blood sugar was well controlled by insulin therapy. During nutritional support with insulin therapy for 6 mo, his nutritional condition gradually improved (Figure 2). Six months later, his nutritional condition was observed to have returned to the normal level for his age (body mass index: 18.2, Figure 2).

Thereafter, an operation was performed. The operative findings showed that the PVPD had induced the severe stenosis of the duodenal first portion, and that the stenosis widely ranging from the second to third portions was a result of stretching by the corrected spine (Figure 3A). No intestinal malrotation was found. The liver was confirmed to be a symmetrical liver. The head of the pancreas was found to be attached to the stenosis of the first duodenal portion. However, the pancreas did not surround any portions of the duodenum. Although the second portion was mildly dilated after performing mobilization of the duodenal second portion, neither the first nor third duodenal portion was dilated (Figure 3B). Therefore, a gastrojejunostomy with Braun's anastomosis was selected and performed (Figure 3B). In addition, a side-to-side anastomosis between the duodenal second portion and jejunum was performed to allow smooth passage of the duodenal contents (Figure 3B).

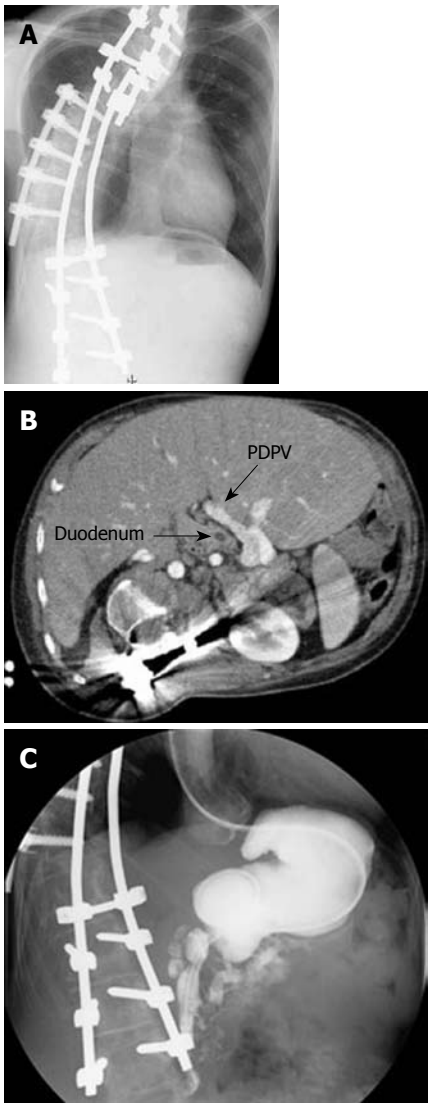

Figure 1 Images at admission. A: Plain X-ray film. Although the spine was corrected by a previous orthopedic operation, a severe scoliosis with right side protrusion was found at admission; B: Abdominal CT scan. The preduodenal portal vein (PDPV) was found to be located in the anterior side of the duodenum; C: Contrast study of the upper gastrointestinal tract. A contrast study of the duodenum showed a stenosis ranging from the second to third portions. In addition, the contents flowed to both the dilated main pancreatic duct and common bile duct.

The postoperative course was uneventful except for slight leakage at the site of anastomosis. Regarding his diabetes, he required insulin therapy for the control of blood sugar. The postoperative contrast study showed that duodenal passage in the second portion improved and that most of the contents in the stomach flowed smoothly into the connected jejunum through the gastrojejunostomy. Two months later, he could eat a normal volume of solid food by mouth (Figure 2). Three months later, he was discharged, using both feeding by mouth and home parenteral nutrition with insulin therapy, and was again able to attend university (Figure 2). Seven months after discharge, he no longer required home parenteral nutrition. Unfortunately, an improvement in his diabetes

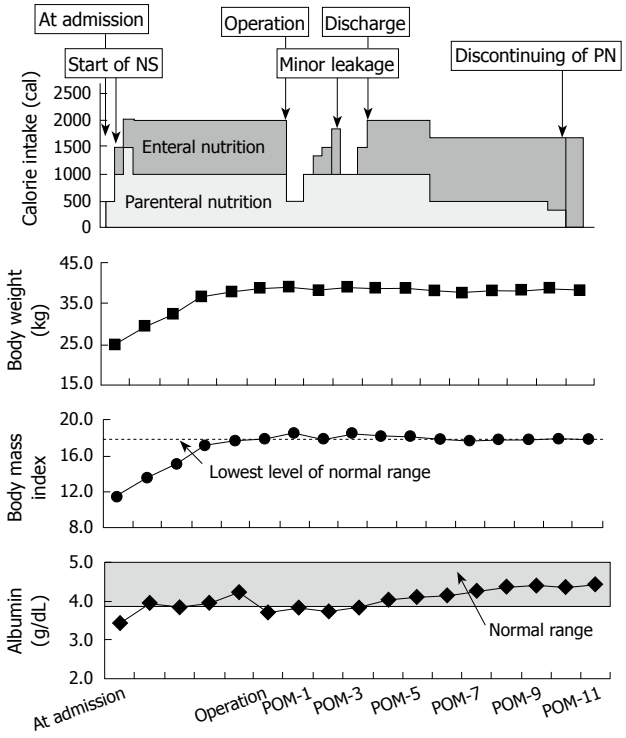

Figure 2 Clinical course of our patient. This figure shows the clinical course of our patient during the period from admission to the cessation of parenteral nutrition. After the preoperative nutritional support using both parenteral and enteral nutrition for 6 mo, an improvement in body weight and body mass index was seen. After the operation, the calorie intake from parenteral nutrition was gradually reduced. The postoperative anthropological markers were maintained. NS: Nutritional support; POM: Postoperative months.

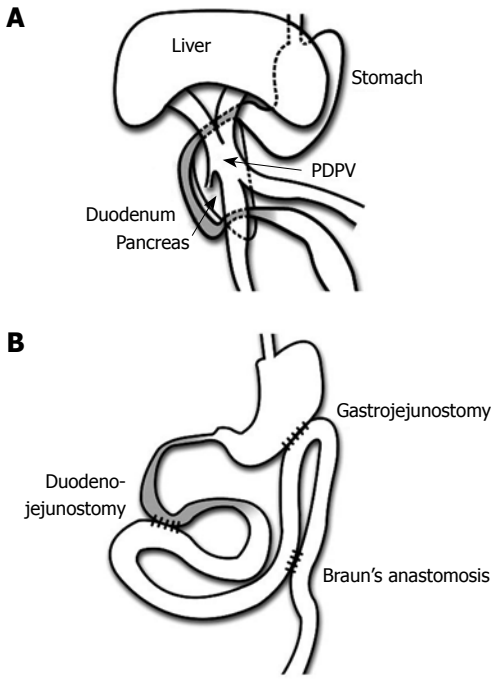

Figure 3 Operative findings and performed procedures in our patient. A: Operative findings. The PDPV was found to induce the severe stenosis of the duodenal first portion and the stenosis ranging from the second to third portions was the result of stretching by spinal correction. No intestinal malrotation was found. The liver was confirmed to be a symmetrical liver. The head of the pancreas was found to be attached to the stenosis of the first duodenal portion. The duodenum is colored gray in this figure; B: Performed procedures. A gastrojejunostomy with Braun's anastomosis was performed for the stenosis in the first portion. In addition, a duodenojejunostomy between the duodenal second portion and jejunum was performed for smooth passage of the duodenal contents. The duodenum is also colored gray in this figure.

was not observed after the operation and therefore he has needed to continue insulin therapy.

DISCUSSION

In general, a PDPV without associated anomalies may be the cause of duodenal stenosis in the first or second portion if symptoms related to PDPV occur[1-5,7,8]. Therefore, in such patients, an operation including duodenoduodenostomy, is thought to be required for the smooth passage of duodenal contents[1-5,7,8]. However, in this patient with PDPV, the stenosis ranged from the first to the third portions of the duodenum. This may be why duodenal stenosis in this patient was also associated with severe scoliosis. He had needed an operation for severe scoliosis but the surgery was not thought to be good for duodenal passage because the duodenum was stretched longitudinally to the spinal axis. After the orthopedic operation, his symptoms had further deteriorated. The first portion of the duodenum was thought to be anchored to both the thick pedicle of the PDPV and the head of the pancreas, and stretched to the hepatic side before the orthopedic operation. Therefore, the orthopedic operation may have induced excessive stretching of the duodenum, resulting in the duodenal stenosis, ranging from the second to third portions, in addition to the stenosis of first portion arising from the PDPV.

In this patient, the long-term symptoms related to PDPV, including frequent vomiting, induced malnutrition. In addition, the duodenal stretching after the orthopedic operation also contributed to the malnutrition. In general, if the severe symptoms are thought to be related to PDPV, duodenoduodenostomy is performed first[1,7,8]. However, in this patient, we preceded surgery with the recovery of his nutritional condition using both parenteral and enteral nutrition, since his nutritional condition had been very severe at admission. Although the time required for the nutritional recovery was long, the operation for the duodenal stenosis was performed smoothly, leading to a good postoperative clinical course. Therefore, in a specific case with severe malnutrition as in this report, nutritional support may precede the operation for severe duodenal stenosis.

In cases where duodenoduodenostomy is performed for duodenal stenosis resulting from PDPV, recent studies recommend the loose overbridging duodenoduodenostomy in order not to compress the blood flow of the portal vein[9]. In this patient, we could not perform the loose overbridging duodenoduodenostomy for stenosis of the duodenal first portion because the stenosis was widespread. Therefore, a gastrojejunostomy with Braun's anastomosis was performed. In addition, a side-to-side anastomosis between the duodenal second portion and the jejunum was performed to allow smooth passage of the duodenal contents, including the pancreatic juice and

bile, because Kocher's duodenal mobilization did not result in enough improvement of the stenosis ranging from the second to third portions. As no patient with a clinical course similar to our patient had been reported in the literature, we were uncertain which operations were the best choice for this patient. However, based on the postoperative clinical course in our patient, the operations we performed resulted in an improvement in the patient's QOL. Therefore, such a combination of operations is thought to be one choice for severe widespread duodenal stenosis.

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CASE REPORT

Jejunal small ectopic pancreas developing into jejunojejunal intussusception: A rare cause of ileus

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Abstract

Intussusception is rare in adults. We describe a 62-year-old man with jejunal ectopic pancreas that led to jejunojejunal intussusception and ileus. The patient was admitted to our hospital because of intermittent abdominal pain. Plain abdominal radiography showed some intestinal gas and fluid levels. Abdominal CT scan demonstrated a target sign suggesting bowel intussusception. Jejunography using a naso-jejunal tube showed an oval-shaped mass about 15 mm in diameter with a smooth surface in the jejunum, which suggested a submucosal tumor (SMT), and edematous mucosa around the mass. Partial jejunal resection was carried out and the resected oval-shaped tumor, 14 mm × 11 mm in size, was found to be covered with normal jejunal mucosa. The tumor was histologically diagnosed as type Ⅲ ectopic pancreas according to the classification proposed by Heinrich. Abdominal pain resolved postoperatively. This case reminds us that jejunal ectopic pancreas should be included in the differential diagnosis of intussusception caused by an SMT in the intestine.

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Key words: Jejunal submucosal tumor; Small intestine; Invagination; Aberrant pancreas; Surgery

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INTRODUCTION

Ectopic pancreas is not an extremely rare pathological condition[1-3]. It is defined as the presence of pancreatic tissue lacking anatomical and vascular continuity with the pancreas[1]. It occurs most commonly in the stomach, duodenum, and jejunum and has been reported in other locations, including the ileum, Meckel's diverticulum, colon, gall bladder, umbilicus, fallopian tube, mediastinum, spleen, and liver[2]. The majority of the cases have been found incidentally at laparotomy performed for other abdominal diseases. When an ectopic pancreas is found incidentally during surgery for other abdominal conditions, resection should be considered because of the risk of late clinical problems. This disease occasionally develops symptoms such as bleeding, vomiting or abdominal pain due to pancreatitis[2,3]. Even if symptoms are present, the preoperative diagnosis of ectopic pancreas in the small bowel appears to be difficult. Malignant transformation can occur in the ectopic pancreas tissue as well as in the tissue of a normally located pancreas. However, the incidence of malignant change in the ectopic pancreas has been estimated to be less than that of the normal pancreas itself[3]. Ectopic pancreas rarely occurs as intestinal invagination leading to ileus in adults[4]. Here, we describe a rare case of small jejunal ectopic pancreas that led to jejunojejunal intussusception and ileus.

CASE REPORT

The patient was a 62-year-old Japanese man. He was admitted to our hospital complaining of intermittent abdominal pain of several months' duration that was increasing in both magnitude and frequency. He had not had previous abdominal surgery and had been in good health. No specific family history was identified. His body temperature was 36.8℃, blood pressure was 132/76 mmHg, and radial pulse rate was 62 beats/min and regular. He had neither anemia nor jaundice. A neurological examination revealed no abnormal findings.

Figure 1 Jejunography using a naso-jejunal tube showed an oval-shaped mass in the jejunum (arrow) and edematous mucosa around the mass.

Figure 2 Macroscopic findings of the tumor. A 14 mm × 11 mm oval-shaped submucosal tumor covered with normal mucosa was observed.

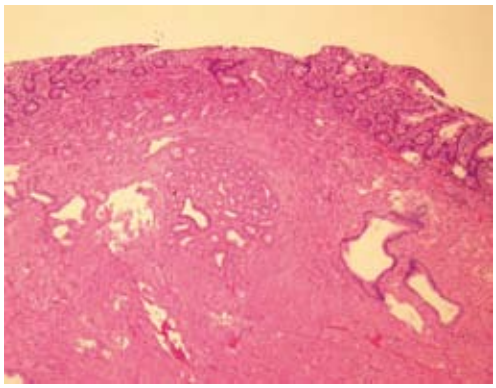

Figure 3 Histological findings of the tumor. The localized tumor was composed of proliferating ducts and proliferation of smooth muscle bundles without mitotic figures. However, both exocrine acini and endocrine elements were lacking (HE, × 100).

Laboratory tests showed hemoglobin concentration of 14.6 g/dL, a red blood cell count of 441 × 10^4/μL, a white blood cell count of 10300/μL (normal range: 4000-8000/μL), and a platelet count of 19.5 × 10^4/μL. Biochemical tests were within normal limits. Plain abdominal radiography showed some intestinal gas and fluid levels. Abdominal CT scan demonstrated a target sign suggesting bowel intussusception and dilated small bowel. Subsequent jejunography using a naso-jejunal tube showed an oval-shaped mass 15 mm in diameter with a smooth surface in the jejunum, which suggested a submucosal tumor (SMT), and edematous mucosa around the mass (Figure 1). Based on these findings, the patient was diagnosed with intussusception due to intestinal SMT. The patient underwent a laparotomy. At laparotomy, a small solid tumor that was 60 cm distal to the Treitz's ligament was palpable and the small bowel around the tumor was edematous. Liver and spleen were normal. There was no evidence of mesenteric or retroperitoneal lymphadenopathy, ascites or peritoneal disease. The resection of jejunum with 2 cm margins and an end-to-end anastomosis was performed. The resected oval shaped tumor, 14 mm × 11 mm in size, was covered with normal jejunal mucosa and no ulcer or erosion was seen on the mucosal surface (Figure 2). Opening of the rudimentary pancreatic duct was not detected. Histologically, the mass was distributed from submucosa to smooth muscle layer and was composed of proliferating ducts and proliferation of smooth muscle bundles without mitotic figures (Figure 3). However, both exocrine acini and endocrine elements were lacking. Based on the above findings, this tumor was diagnosed as jejunal ectopic pancreas (type Ⅲ ectopic pancreas according to the classification proposed by von Heinrich[5]). The postoperative course was uneventful. As a result of this treatment, the patient's abdominal pain resolved postoperatively.

DISCUSSION

Ectopic pancreas is defined as the presence of pancreatic tissue lacking anatomical and vascular continuity with the pancreas[1]. It occurs most commonly in the stomach, duodenum, and jejunum and has been reported in other locations, including the ileum, Meckel's diverticulum, colon, gall bladder, umbilicus, fallopian tube, mediastinum, spleen, and liver[2]. Ectopic pancreas occurs in 0.25%-13.7% of patients based on both autopsy and surgical series; approximately 70% of all such tissues are found in the stomach, duodenum, and jejunum[2]. When an ectopic pancreas is found coincidentally during surgery for other abdominal conditions, resection should be considered because of the risk of late clinical problems. Although this disease occasionally develops symptoms such as bleeding, vomiting or abdominal pain due to pancreatitis[2,3,6,7], intestinal obstruction or intussusception is rare[4,8,9]. No invagination case caused by ectopic pancreas had been observed in a series of 53 invaginations, reported by Ong *et al*[10]. Ectopic pancreas in the small intestine may rarely be fatal: however, Hitosugi *et al*[11] reported a sudden death case (11-year-old Japanese female) caused by intestinal obstruction due to jejunal ectopic pancreas (about 4 cm in diameter). Adult intussusception represents 5% of all cases of intussusception and accounts for only 1%-5% of intestinal obstructions in adults[12]. In contrast with intussusception in children, nearly all adult cases have primary causes such as a polyp, an SMT or a malignant tumor that needs to be resected[13,14]. In the present case, the small SMT (ectopic pancreas) in the jejunum caused ileus due to intussusception.

The preoperative diagnosis of ectopic pancreas in the small bowel appears to be difficult. However, there are some recent reports describing ectopic pancreas in the small bowel discovered by double-balloon enteroscopy or capsule endoscopy[15-17]. In spite of using double-balloon enteroscopy, endoscopic diagnosis is rarely

conclusive on endoscopic biopsies because the lesion locates mainly in the submucosal layer and the biopsy is often not deep enough to reach the submucosal tumor tissue. In the present case, the diagnosis could not be confirmed preoperatively although we knew that bowel intussusception due to small SMT might cause ileus. Because of the suspicion of ileus due to jejunal tumor, the patient underwent laparotomy; thus, double-balloon enteroscopy was not performed in the present case.

This disease is treated in principle by surgical resection if bowel intussusception or ileus occurs[4]. Moreover, physicians should be aware that ectopic pancreas in the small bowel may associate with endocrine tumor or carcinoma[18-21]. However, ectopic pancreas in the small intestine is usually benign. Ectopic pancreas in the small intestine is rarely fatal and patients remain asymptomatic in their daily lives, except for when bleeding, bowel intussusception, obstruction or pancreatitis occurs. Therefore, it is likely that there are patients with latent small bowel ectopic pancreas which may be incidentally discovered in the future, as a result of advances in diagnostic imaging, such as improved CT, MRI, capsule endoscopy and double-balloon enteroscopy.

In conclusion, we have reported a very rare case of jejunal ectopic pancreas that led to jejunojejunal intussusception. Thus, it is necessary to be aware that a jejunal ectopic pancreas may cause jejunojejunal intussusception even though the likelihood is small. This etiology should be suspected in a patient with chronic atypical abdominal pain.

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CASE REPORT

Giant vesical diverticulum: A rare cause of defecation disturbance

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Abstract

Vesical diverticula frequently result from bladder outlet obstructions. However, giant vesical diverticula which cause acute abdomen or intestinal obstruction are very rare. Our review of the English medical literature found 3 cases of bladder diverticula which caused gastrointestinal symptoms. Here, we present a 57-year-old man with a giant diverticulum of the urinary bladder who complained of abdominal pain, nausea and vomiting, constipation, no passage of gas or feces, and abdominal distension for 3 d. A 20 cm × 15 cm diverticulum was observed upon laparotomy. The colonic obstruction was secondary to external compression of the rectum against the sacrum by a distended vesical diverticulum. We performed a diverticulectomy and primary closure. Twelve months postoperatively, the patient had no difficulty with voiding or defecation.

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Key words: Colonic obstruction; Defecation; Diverticulectomy; Urinary bladder; Vesical diverticulum

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INTRODUCTION

Vesical diverticula are herniations of the bladder mucosa and submucosa through the muscular wall of the bladder[1]. They are often asymptomatic and are discovered incidentally during an examination for other reasons[1,2]. Some patients present with urinary tract infections, obstruction, gallstones, or frequent voiding caused by diverticula, especially when they are large and empty poorly. The common causes of large bowel mechanical obstruction in adults are malignant tumors, diverticular disease, and volvulus. Extrinsic obstruction secondary to distension of a giant vesical diverticulum is rare. To our knowledge, this is the fourth report of a vesical diverticulum causing disrupted defecation or intestinal obstruction in the English medical literature since 1957[3-5]. We report an unusual case of large bowel obstruction caused by a giant bladder diverticulum. In addition, this is the largest vesical diverticulum reported in the literature.

CASE REPORT

A 57-year-old man was admitted to the general surgery department with abdominal pain, nausea and vomiting, constipation, no passage of gas or feces, and abdominal distension for 3 d. The relevant physical examination revealed a distended abdomen, decreased bowel sounds, diffuse sensitivity on palpation of the abdomen, and empty rectal ampulla. Laboratory investigations showed a blood urea nitrogen level of 31 mg/dL, a creatinine level of 1.1 mg/dL, and a C-reactive protein level of 35 mg/L. The blood cell count revealed leukocytosis at 16 500/μL, a hemoglobin level of 13.5 g/dL, and a platelet count of 423 000/μL. Other serum parameters, including prostate specific antigen (PSA, 1.9 ng/mL), were within normal limits. Computed tomography (CT) showed a 15 cm × 10 cm low density cystic lesion with smooth contours located in the presacral region, pushing the rectum to the right and the sigmoid colon and bladder superiorly (Figure 1A and B). The patient had a history of trauma because of a traffic accident 4 years previously, and

Table 1 A summary of 12 cases of giant vesical diverticula reported in the English medical literature from 1957 to 2009 and description of the studies

Ref.	Yr	Age	Sex	Medical history	Initial symptom	Diagnosis	Management	Complication
Kauffman *et al*[3]	1957	70	M	Not available	Constipation	X-ray films, intravenous urography	Diverticulectomy	Not found
Mirow *et al*[4]	2007	84	M	Sigmoid carcinoma	Abdominal pain, intestinal obstruction	Intraoperative	Diverticulectomy	Not found
Shaked *et al*[5]	2009	76	M	Hypertension, diabetes mellitus	Abdominal pain, constipation	CT		
Shukla *et al*[6]	2004	11	F	EDS	Infection, incomplete voiding	Cystogram	Diverticulectomy	Not found
		4 mo-3 yr	M M M	No medical history of voiding dysfunction	Decreasing urinary stream and urinary retention			
Burrows *et al*[7]	1998	16	M	EDS type 1	Outflow obstruction	Cystogram	Diverticulectomy	Not found
Suzuki *et al*[8]	2002	84	M	Bladder injury with bullet	Abdominal distension	CT, cystogram	Diverticulectomy	Not found
Farhi *et al*[9]	1991	31	F	Recurrent urinary infection	Ovarian cyst	USG, cystogram	Not available	Not available
Taha *et al*[10]	1987	65	M	Not available	Abdominal distension, slow stream of urine	Intravenous urography, CT	Reduction cystoplasty	Not found
Siddiqui *et al*[11]	2003	77	M	TUR-P was performed twice because of urinary retention	Acute urinary retention	Intravenous urography	Diverticulectomy	Not found

The first three references present cases of bladder diverticula which caused gastrointestinal symptoms. CT: Computed tomography; EDS: Ehlers-Danlos syndrome; TUR-P: Transurethral prostatectomy; USG: Ultrasonography.

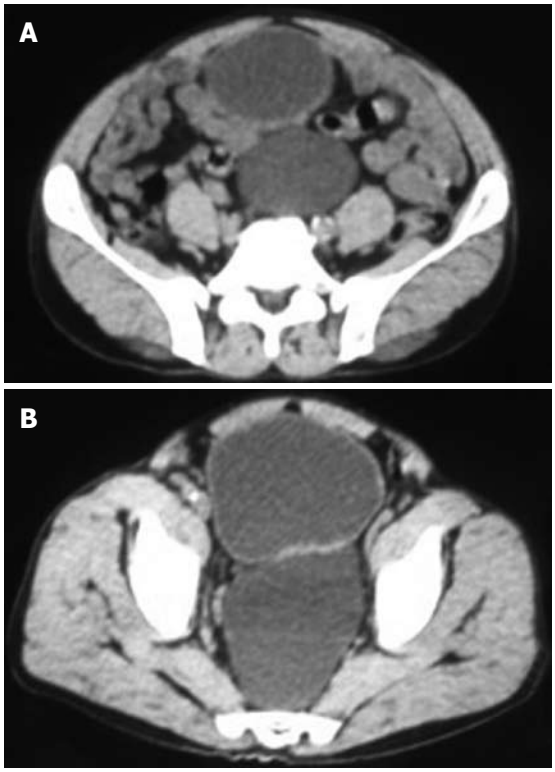

Figure 1 Computed tomography showed a 15 cm × 10 cm low density cystic lesion with smooth contours located in the presacral region, pushing the rectum to the right and the sigmoid colon and bladder superiorly (A and B).

because of this diagnostic laparotomy was performed and a few sutures were placed in the bladder. As the patient had symptoms of acute abdomen, laparotomy was performed.

During laparotomy, the mass was found to be a diverticulum originating from the posterosuperior region of the bladder. The colonic obstruction was secondary to external compression of the rectum against the sacrum by a distended vesical diverticulum. The diverticulum measured approximately 20 cm × 15 cm and was difficult to mobilize by dissecting its delicate attachments to the anterior rectal wall. The diverticular orifice was approximately 1-1.5 cm in diameter. A diverticulectomy and primary closure were performed. Postoperatively, the bladder was catheterized for 10 d. Three months postoperatively, the patient's urinary frequency and constipation had disappeared. An intravenous pyelography (IVP) showed no significant pathologic findings (Figure 2). The patient had no difficulty in voiding nor had constipation 12 mo postoperatively.

Figure 2 No significant pathology was observed on intravenous pyelography 3 mo postoperatively.

DISCUSSION

We described a rare cause of a giant vesical diverticulum causing mechanical bowel obstruction in an adult patient. We summarized the characteristics of the 12 cases of "giant bladder diverticulum" which we found in the literature in the Table 1[3-11]. Three of the cases

caused intestinal obstruction[3-5]. The bladder is a hollow muscular organ that stores and evacuates urine. The normal bladder can store approximately 350-400 mL of urine. A diverticulum is an abnormal sac or pouch protruding from the wall of a hollow organ. Most bladder diverticula are primary, congenital, or secondary to outflow obstruction or neurogenic bladders[12-14]. Congenital diverticula usually occur in areas where there is insufficient muscle, typically at the ureterovesical junction, or between bundles of hypertrophied muscle. They are usually asymptomatic and are discovered incidentally. Occasionally, a diverticulum may produce urinary obstruction as a result of compression of the urethra, or urinary tract infection arising from retention of urine within the diverticulum[8,15]. In this case, only minimal obstruction caused by the pressure on the ureters was observed. No obstruction of the urethra developed. Although diverticula generally develop from the ureteral orifices in the bladder, in this case the diverticulum was located in the posterosuperior region of the bladder distant from the orifices.

In this patient, the diverticulum was in the same location as previous trauma. Because of this and since trauma is known to be one of the etiologic factors for acquired diverticula, we believed that this diverticula had occurred as a result of the previous trauma.

The indications for surgery are persistent or recurrent urinary infection, the presence of a stone or tumor in the diverticulum[16,17], a vesicocutaneous fistula[18], lower urinary tract symptoms, and voiding symptoms or vesicoureteral reflux[19] resulting from the diverticulum or ureteral obstruction[6,12]. In this case, laparotomy was indicated by the normal IVP results and CT scan showing a mass exerting pressure on the ureters and rectum. Vesical diverticula are a common pathology of the urinary bladder, generally secondary to cervico-urethral obstruction. Shaked *et al*[5] presented a case of bladder distention and diverticulum arising from obstruction caused by a prostate adenoma. In their case, they reported that colonic obstruction occurred because the colon was packed between the sacrum and bladder. In our case, although the bladder diverticulum was large, no obstruction was observed because the diverticulum developed from the posterosuperior part of the bladder. This was reflected in the PSA and transrectal ultrasonography results.

In conclusion, there are several reported cases of bladder distention and diverticula causing colonic obstruction. For this reason, although rare, bladder pathology should be considered in patients with obstruction of the rectosigmoid region.

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CASE REPORT

Therapy of central pontine myelinolysis following living donor liver transplantation: Report of three cases

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Abstract

We analyzed the clinical manifestations and experiences of diagnosing and treating central pontine myelinolysis following living donor liver transplantation. The clinical data of three patients with central pontine myelinolysis following living donor liver transplantation from January 2005 to November 2007 were retrospectively analyzed at the West China Hospital, Sichuan University, China. The three patients developed hyponatremia prior to surgery. Case 1 suffered locked-in syndrome following surgery, and received a large dose of gamma globulin, and subsequently recovered. Case 2 was in a coma for three days, and received hyperbaric chamber treatment. This patient remained in a mild coma for six months following surgery. Case 3 developed consciousness disturbances, gradually went into a coma following surgery, and died due to pulmonary infection. Central pontine myelinolysis is a severe complication in patients following living donor liver transplantation. Large-dose gamma globulin treatment, as well as hyperbaric oxygen, might be effective therapeutic methods.

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Key words: Cyclosporine; Hyponatremia; Liver transplantation; Myelinolysis; Pontine

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INTRODUCTION

With the increased number of living donor liver transplantation (LDLT) patients, some special complications have become apparent. Central pontine myelinolysis (CPM) is a severe nervous system complication following liver transplantation[1-3]. To date, no studies have been performed addressing CPM occurrence and treatment following LDLT. This study aimed to report the clinical manifestations and treatment of three patients diagnosed with CPM following LDLT from January 2005 to November 2007. The three donors in this study were lineal relatives of these patients. No organs were used from prisoners and no subjects were prisoners at the time of data collection.

CASE REPORT

Case one

This patient was a 51-year-old female diagnosed with chronic, type B hepatitis, hepatic decompensation, and hepatic encephalopathy. Her blood type was B/Rh (-). Pre-operative examination revealed the following parameters: total bilirubin (107.9 μmol/L), direct bilirubin (53.6 μmol/L), aspartate aminotransferase (595 IU/L), glutamate-pyruvate transaminase (600 IU/L), and serum cholesterol (0.18 mmol/L). Blood electrolytes, Child-Turcotte-Pugh score, and MELD score are shown in Table 1. In November 2005, the patient underwent adult-to-adult LDLT with a right lobe graft. The donor was the patient's son, whose blood type was B/Rh (+). Electrolytes were re-examined in the intensive care unit (Table 1). Post-operative immunosuppressive regimen was administered. The patient was treated with cyclosporine A (250 mg, twice daily), 0.75 g mycophenolic acid (oral)

Table 1 Blood electrolytes, Child-Turcotte-Pugh (CTP) score, and "Model for end-stage liver disease" (MELD) score

	Case 1	Case 2	Case 3
Pre-operative serum sodium (mmol/L)	124	119	119
Post-operative serum sodium (mmol/L)	151	153	141
Pre-operative serum magnesium (mmol/L)	0.52	0.47	0.64
Post-operative serum magnesium (mmol/L)	0.58	0.62	0.68
Pre-operative plasma osmotic pressure (mOsm/kg H_2O)	238.71	232.47	258.45
Post-operative plasma osmotic pressure (mOsm/kg H_2O)	295.36	288.93	291.92
Plasma cholesterol (mmol/L)	0.18	1.24	1.07
CTP score	B	C	C
MELD score	30	36	35
Cyclosporine A or Fk506 concentration	Cyclosporine A 180-250 mg/L	FK506 6.5-8 ng/mL	No
Clinical manifestations	Locked-in syndrome	Coma	Coma
Special therapy	Gamma globulin (i.v.)	Hyperbaric oxygen treatment	No

twice daily, and methylprednisolone (iv). Subsequent, cyclosporine A blood concentration was shown to be 180-250 mg/L. At 6 h after surgery, the patient was conscious, and the tracheal cannula was removed. On day one after surgery, the patient maintained clarity, answered questions with short sentences, exhibited locomotor activities, and drank water. On day three after surgery, the patient presented with decreased spontaneous speech, slowed actions, could not answer a simple question correctly, had indistinct pronunciation, and bucked while drinking water. On day five after surgery, the patient had stable vital body signs, clear consciousness, spontaneous eye opening, ocular movement, but did not exhibit spontaneous speech, spontaneous limb activities, or reaction to pain stimulation. Physical examination showed increased tension of limb muscles, muscle force 0°, decreased deep and superficial sensations, bilateral Babinski sign (+), and an Expanded Disability Status Scale score of 9.5. Skull nuclear magnetic resonance was subsequently performed and the findings are displayed in Figure 1. The patient was diagnosed with "locked-in syndrome" induced by CPM, and was administered citicoline (0.5 g daily *via* intravenous drip) and a large dose of gamma globulin (0.5 g/kg daily for ten days, iv). Oral cyclosporine A was not administered. On day twelve after surgery, the patient pronounced monosyllables, performed light limb activities with grade Ⅲ muscle force, but was still not able to swallow. On day fourteen after surgery, the patient was conscious, producing longer utterances, pronounced indistinctly and slowly, demonstrated increased autonomic activities, bucked during swallowing, and presented with an Expanded Disability Status Scale score of 6. Three months after surgery, the patient presented with normal liver function, clear consciousness, normal diet, self-care, slow speech rate, and slow motor reactions.

Figure 1 Magnetic resonance imaging displayed high-signal intensity on T2-weighted images of the central pons 7 d after surgery (arrow).

Case two

This patient was a 54-year-old male diagnosed with chronic severe hepatitis (type B), hepatic encephalopathy, and hepatorenal syndrome. His blood type was O/Rh (+). Pre-operative examination revealed the following parameters: total bilirubin (280 μmol/L), direct bilirubin (158.6 μmol/L), aspartate aminotransferase (269 IU/L), glutamate-pyruvate transaminase (188 IU/L), serum cholesterol (1.24 mmol/L), and serum creatinine (263.7 μmol/L). Blood electrolytes, Child-Turcotte-Pugh score, and MELD score are presented in Table 1. In March 2006, the patient received adult-to-adult LDLT with a right lobe graft. The donor was the patient's brother, whose blood type was O/Rh (+). Electrolytes were re-tested in the intensive care unit (Table 1).

Post-operative immunosuppressive regimen was administered. The patient received tacrolimus (FK506, 2 mg twice daily, oral), 0.75 g mycophenolic acid (oral, twice daily), and methylprednisolone (iv). Subsequent tacrolimus blood concentration was found to be 6.5-8 ng/mL. At 11 h after surgery, the patient was conscious, and the tracheal cannula was removed. The patient had stable vital body signs, poor mental state, and little spontaneous speech. On day two after surgery, the patient presented with lethargy, eye-opening reaction to powerful stimulation, and indistinct pronunciation. On day three after surgery, the bilateral pupils reacted poorly to light reflex and were equal in size and shape. The patient suffered from moderate coma, with decreased muscular tension, Babinski sign of the right side (+), Babinski sign of the left side (-), and a Glasgow-Pittsburgh score of 20. On day 6 after surgery, the patient received a tracheotomy, and respiration was supported by a respirator due to pulmonary infection. CPM was diagnosed with skull magnetic resonance imaging (Figure 2A). On day ten after surgery, the patient developed moderate coma, with good recovery of liver function. Following hyperbaric chamber treatment (2 h, once per day, for 14 d), the patient presented with good light reflex in both pupils, mild coma, increased autonomic activities, improved tension of limb muscles, and a Glasgow-Pittsburgh score of 26. At six months, the patient suffered from mild coma, but exhibited good liver function.

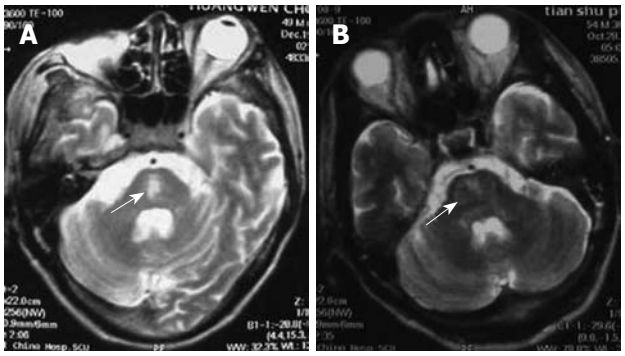

Figure 2 Magnetic resonance imaging displayed high-signal intensity on T2-weighted images of the central pons 9 d (A) and 8 d (B) after surgery (arrows).

Case three

This patient was a 47-year-old male diagnosed with chronic severe hepatitis (type B), upper gastrointestinal bleeding, and hepatorenal syndrome. His blood type was B/Rh (+). Pre-operative examination revealed the following parameters: total bilirubin (197.4 μmol/L), direct bilirubin (116.5 μmol/L), aspartate aminotransferase (141 IU/L), glutamate-pyruvate transaminase (107 IU/L), serum cholesterol (1.07 mmol/L), and serum creatinine (207.7 μmol/L). Blood electrolytes, Child-Turcotte-Pugh score, and MELD score are presented in Table 1. In August 2007, the patient received adult-to-adult LDLT with a right lobe graft. The donor was the patient's wife, whose blood type was B/Rh (+). Electrolytes were re-measured in the intensive care unit (Table 1). Post-operative immunosuppressive regimen was performed. The patient was treated with zenapax (containing daclizumab), 0.75 g mycophenolic acid (oral, twice daily), and methylprednisolone (iv). At 9 h following surgery, the patient was conscious, and the tracheal cannula was removed. The patient received hemofiltration due to worsened renal function. At 1 wk, the patient was conscious, displayed autonomic activities, reduced speech, low speech sound, and could provide correct answers to simple questions. Both pupils exhibited good light reflexes and normal ocular fundus, and were equal in size and shape. Tendon reflexes were symmetric and active, with pathological sign (-). Muscular tension of the upper limbs was increased, with flexion deformity and grade Ⅱ muscle force, but muscular tension of the lower limbs was decreased. On day 8 after surgery, the patient went into a coma, opened eyes when powerfully stimulated, could not pronounce or perform autonomic limb activities, and presented with normal liver and kidney function. CPM was diagnosed using skull magnetic resonance imaging (Figure 2B). On day 14 after surgery, the patient suffered from mild coma and received a tracheotomy, his respiration was supported by a respirator due to pulmonary infection. Subsequently, the patient developed severe sepsis and multiple organ failure, and died at twenty-three days following surgery.

DISCUSSION

CPM is a severe complication of the nervous system following liver transplantation, with high mortality and disability rates[4-6]. CPM is difficult to diagnose, due to the various clinical manifestations, and precise epidemiological data are lacking. From January 2005 to December 2007, 3/184 patients at the Center for Liver Transplantation, West China Hospital, Sichuan University, China undergoing living donor liver transplantation were diagnosed with CPM, with an occurrence rate of 1.63%.

CPM following liver transplantation is due to multiple factors. (1) Severe hyponatremia, especially during rapid correction of chronic hyponatremia: patients who develop CPM following liver transplantation suffer from severe hyponatremia prior to transplantation. However, not all hyponatremia patients develop CPM[7]. Rapid correction of hyponatremia, as well as larger alterations in plasma osmotic pressure, influences CPM occurrence[1]. Increased serum sodium (> 15 mmol/L) within 24 h has also been closely associated with CPM occurrence[1]. (2) Neurotoxicity of cyclosporine A and tacrolimus can induce or aggravate CPM. Cyclosporine A-related motor aphasia patients were shown to develop CPM, as diagnosed by skull magnetic resonance imaging[8,9]. (3) Severe damage to liver function prior to surgery is associated with CPM occurrence, especially in patients with hepatic encephalopathy and hypocholesterolemia. (4) Hemorrhage, infection, vascular complications, and impaired graft function following liver transplantation can result in CPM occurrence. Immune factors might participate in CPM pathogenesis, such as myelin sheath antibody formation[10,11]. In the present study, these three cases developed severe hyponatremia prior to surgery. Serum sodium ion concentration increased by > 20 mmol/L, and plasma osmotic pressure was greatly altered 6-8 h after surgery. These factors were responsible for CPM occurrence. Living donor liver transplantation is an elective procedure. Thus, there should be ample opportunity to correct serum sodium before surgery. A large number of blood products and synthetic colloids should be infused into patients during the peri-operative period, such as erythrocyte suspension, fresh frozen plasma, platelets, cryoprecipitate, hetastarch, and succinylated gelatin. These liquids contain many sodium salts, which increase serum sodium within a short period of time. More attention should be paid to regulating serum sodium during the peri-operative period to avoid adverse outcomes due to rapid correction of hyponatremia. It is also important for anesthesiologists to correct serum sodium very slowly during surgery. In case 1, the post-operative immunosuppressive agent was cyclosporine A. The blood drug concentration ranged between 180-250 mg/L. In case 2, the post-operative immunosuppressive agent was tacrolimus, and the blood drug concentration ranged between 6.5-8 ng/mL. Although blood drug levels were not significantly increased, severe liver disease-induced metabolic disturbances could lead to brain glial cell damage. It seems that critically ill patients with end-stage liver disease may have an organic mental syndrome. Any insult such as calcinerin inhibitors under these circumstances may cause further damage resulting in CPM.

The clinical manifestations of CPM are multi-faceted following liver transplantation, and include

mental disorder, cortical blindness, pseudobulbar palsy, aphasia, ataxia, visual hallucination, epileptic seizure, locked-in syndrome, and coma[12-14]. Case 1 was diagnosed with locked-in syndrome, meaning that the bilateral corticospinal tract and corticobulbar tract were blocked surrounding the pontine abducent nucleus. The characteristics of this syndrome include quadriplegia and anarthria, with preservation of consciousness. Patients retain vertical eye movement, and can facilitate non-verbal communication. Few patients with this syndrome have been reported, especially in LDLT. Cases 2 and 3 suffered from coma. Magnetic resonance imaging has proven to be a good diagnostic method for CPM. Positive diagnosis has not been detected in many patients using magnetic resonance imaging following the onset of clinical symptoms. This was associated with abnormal imaging manifestations following clinical symptoms.

To date, there remains a lack of effective therapeutic methods for CPM. The present methods primarily attempt to nourish brain cells, improve cerebral metabolism, and reduce immunosuppressive concentrations. In addition, gamma globulin has been injected intravenously into CPM patients, resulting in improved scores on the Expanded Disability Status Scale[10,11]. In the present study, case 1 was treated with a large dose of gamma globulin and was tested according to the Expanded Disability Status Scale. Case 2 received hyperbaric oxygen treatment and underwent Glasgow-Pittsburgh Score testing. Case 1 presented with improved (> 36%) scores on the Expanded Disability Status Scale following treatment. This might be correlated to treatment with large-dose gamma globulin which destroys myelin sheath-related antibodies by decreasing toxic substances in the myelin sheath and by accelerating remyelination. Case 2 presented with improved (30%) scores on the Glasgow-Pittsburgh Score following treatment. This might be correlated to hyperbaric oxygen, which reduced myelin sheath destruction, accelerated cell regeneration, and decreased edema in the injured regions. However, we cannot thoroughly assess the outcomes of these two methods, due to the limited number of cases.

CPM, a life-threatening illness, is difficult to diagnose and treat, and presents with a poor prognosis following living donor liver transplantation. More attention should be directed to the risk factors for CPM to avoid clinical complications, and to continue the search for an effective therapeutic method.

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Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
Marriott Wardman Park Hotel Washington, DC
13th International Symposium on Viral Hepatitis and Liver Disease

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April 8-9, 2009
Silver Spring, Maryland
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
http://www.easl.ch/

May 17-20, 2009
Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009
Orange County Convention Center Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
McCormick Place, Chicago, IL
DDW 2009
http://www.ddw.org

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North Bethesda, MD
Accelerating Anticancer Agent Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27 2009
Barcelona, Spain
ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

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Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
Aspen, CO, United States
Molecular Biology in Clinical Oncology

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
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Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
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Versailles, France
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October 30-November 3, 2009
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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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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/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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