World Journal of
Gastroenterology ®
Volume 18 Number 1
January 7, 2012

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Published by Baishideng Publishing Group Co., Limited
Room 1701, 17/F, Henan Building,
No. 90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-31158812
Telephone: +852-58042046
E-mail: bpg@wjgnet.com
http://www.wjgnet.com

ISSN 1007-9327 CN 14-1219/R Local Post Offices Code No. 82-261

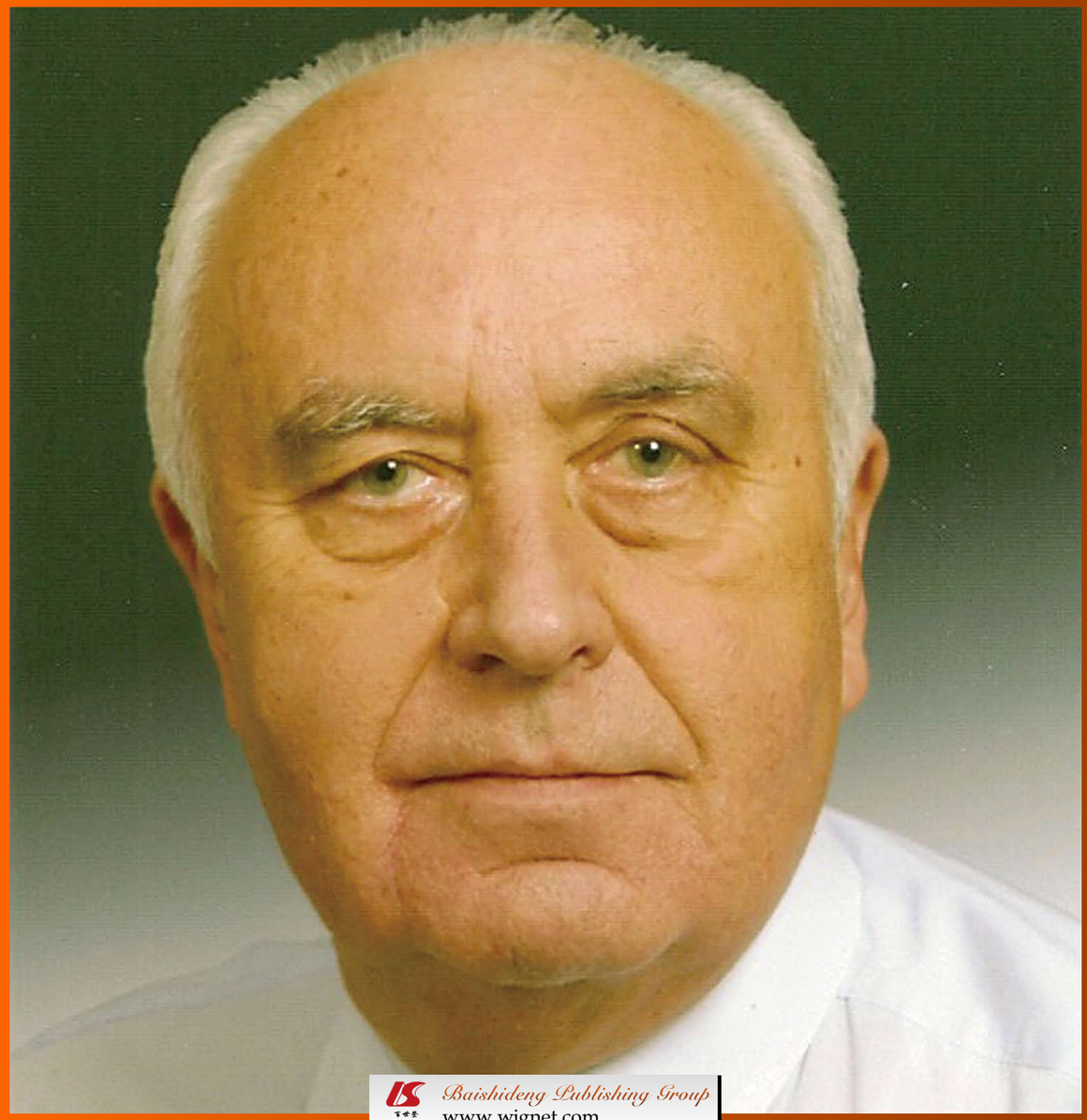
ISSN 1007-9327
0 1>

World Journal of Gastroenterology
www.wjgnet.com
Volume 18 Number 1 Jan 07 2012

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

World Journal of *Gastroenterology*

World J Gastroenterol 2012 January 7; 18(1): 1-98



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World Journal of Gastroenterology

Contents

Weekly Volume 18 Number 1 January 7, 2012

World Journal of Gastroenterology
Volume 18 Number 1 January 7, 2012

Contents

NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

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http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Co., Limited
Room 1701, 17/F, Henan Building,
No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-31158812
Telephone: +852-58042046
E-mail: bpg@baishideng.com
http://www.wjgnet.com

PRINT SUBSCRIPTION
RMB 300 Yuan for each issue, RMB 14400 Yuan for one year.

PUBLICATION DATE
January 7, 2012

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

ONLINE SUBMISSION
http://www.wjgnet.com/1007-9327office/

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i1.1

World J Gastroenterol 2012 January 7; 18(1): 1-15
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Recurrence and rejection in liver transplantation for primary sclerosing cholangitis

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Received: April 23, 2011 Revised: June 15, 2011
Accepted: June 22, 2011
Published online: January 7, 2012

Abstract

Primary sclerosing cholangitis (PSC) is a chronic progressive inflammatory disease affecting the bile ducts, leading to fibrosis and eventually cirrhosis in most patients. Its etiology is unknown and so far no effective medical therapy is available. Liver transplantation (LTX) is the only curative treatment and at present PSC is the main indication for LTX in the Scandinavian countries. Close to half of the PSC patients experience one or more episodes of acute cellular rejection (ACR) following transplantation and approximately 1/5 of the transplanted patients develop recurrent disease in the graft. In addition, some reports indicate that ACR early after LTX for PSC can influence the risk for recurrent disease. For these important post-transplantation entities affecting PSC patients, we have reviewed the current literature on epidemiology, pathogenesis, treatment and the possible influence of rejection on the risk of recurrent disease in the allograft.

Key words: Liver transplantation; Primary sclerosing cholangitis; Rejection; Recurrence

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Fosby B, Karlsen TH, Melum E. Recurrence and rejection in liver transplantation for primary sclerosing cholangitis. *World J Gastroenterol* 2012; 18(1): 1-15 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i1/1.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i1.1

INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease of unknown etiology. Histologically, it is characterized by the existence of intrahepatic and/or extrahepatic concentric, obliterative fibrosis of the biliary ducts. It is a progressive disorder that eventually leads to the development of cirrhosis and hepatic decompensation in the majority of the patients[1,2]. PSC is closely associated with inflammatory bowel disease (IBD), especially ulcerative colitis (UC), which is diagnosed in approximately two-thirds of northern European PSC patients[3-5]. Approximately 2/3 of the PSC patients are male[6] and most affected individuals are less than 40 years of age at the time of diagnosis. There is still no medical treatment with proven efficacy[7] and liver transplantation (LTX) is currently considered as the only curative option for end-stage liver disease due to PSC[8]. Although graft and patient survival following LTX has improved dramatically over the last two decades, and is now close to 80% at 5 years[9], it has become increasingly obvious that the disease can recur in the transplanted liver. In the earliest reports, recurrent primary sclerosing cholangitis (rPSC)

did not seem to influence graft and patient survival[10,11]. On the contrary, more recent data have indicated that a significant proportion of the affected patients will develop graft loss[12], thus influencing the long-term patient survival[13]. Therefore, rPSC represents an important condition in a steadily growing cohort of patients, for which little is known regarding its epidemiology, pathogenesis and treatment.

PSC patients are also believed to be at an increased risk of rejection, and probably up to 50% of the patients will experience one or more episodes of acute cellular rejection (ACR) during the first few weeks following transplantation. The need for increased immunosuppression represents a problem in the clinical management of these patients[14-16]. In addition, the immunological reactions related to rejection could influence the risk of developing rPSC in the graft as more reactive lymphocytes are likely to be present. To date, a possible effect of rejection on the risk of developing recurrent disease is supported by three published reports. This deserves increased attention and will be addressed in this review.

EPIDEMIOLOGY OF PSC AND rPSC

The incidence of PSC varies considerably among different parts of the world. Population-based studies of disease frequency from Norway, Great Britain and United States, indicate a comparable incidence (0.9-1.3/100 000 per year) and prevalence (8.5-13.6/100 000)[17-19] while the numbers from southern Europe and Asia have been reported to be 10-100 times lower[20-22]. PSC is today the number one indication for liver transplantation in the Scandinavian countries, while it in the United States is the fifth most common indication[23]. The first report of suspected PSC recurrence in a liver graft was published in 1988 by Lerut *et al*[24] describing a transplanted PSC patient developing intrahepatic biliary strictures in the absence of allograft rejection within the first year after the transplantation. Since then, numerous reports have corroborated rPSC as a clinical entity[25-28]. The incidence of recurrence varies widely in reports from different centers, probably reflecting variation in diagnostic criteria, study design, and length and type of follow-up. We have in this review identified, after exclusion of cohorts duplicated in previous reports[29-32], a total of 22 publications containing data from original studies on the outcome of LTX for PSC, which analyzed the incidence of recurrent disease (Table 1). For studies based on the same clinical material, the most recent and largest study was included. Of the total number of 1399 patients transplanted for PSC in these studies, 259 (18.5%) developed recurrence (range, 5.7%-59.1% in the individual studies). In the largest series reported so far (n = 230), focusing on the risk factors for rPSC, recurrence occurred in 23.5% of the patients at a median of 4.6 years after LTX[13]. This number is in accordance with the overall percentage for the studies we analyzed.

PATHOGENESIS OF PSC AND rPSC

The etiology and pathogenesis of both PSC and rPSC are currently unknown. Most studies have focused on pre-transplant ("primary") pathogenesis, and lessons from these studies may give insight and provide hypotheses for further research even on the pathogenesis of recurrent disease. The primary disease is characterized by chronic inflammation and progressive fibrotic strictures of the bile ducts[33,34]. By the time the patient is diagnosed with PSC, the changes in the liver architecture are already quite advanced. To determine at this stadium, at a cellular level, which observations that can be of primary importance in the pathogenesis of PSC or just a secondary phenomenon for the ongoing disease is difficult to judge. So far, there has been no unified pathogenetic mechanism for PSC development.

It is important to identify risk factors for recurrence, both in the search for mechanisms involved in the pathogenesis and in improving the management of these patients after transplantation. It might also shed light on the pathogenesis of the primary disease. The pathogenesis of rPSC can be considered multifactorial and influenced by pre- and/or post-operative factors in combination with a genetic predisposition. It is also likely that it is partly related to the pathogenesis of the primary disease. Although it is beyond the scope of this review to go into details regarding PSC pathogenesis, we will briefly mention the theories that have gained the most general acceptance in recent years[7], since these mechanisms may also be involved in recurrent disease. Four hypotheses have been put forward, each is potentially relevant at different stages of the disease process.

Strong evidence indicates that genetic variants play an important role in disease susceptibility and siblings of PSC patients are 9-39 times more likely to develop PSC compared with the general population[35]. Family members of PSC patients are also at increased risk of developing UC, indicating the existence of shared genetic risk factors between these two conditions[7]. Furthermore, unbiased genome-wide association studies have demonstrated shared susceptibility loci between UC and PSC[36,37]. PSC associated variants in the human leukocyte antigen (HLA)-region were first reported almost 30 years ago[38] and have since been verified numerous times. It has so far not been possible to pin-point the exact causative genes in the HLA-region, and it is likely that more than one susceptibility gene exists at this locus. A recent genome-wide association study has also provided strong evidence for involvement of two or more non-HLA genes; in particular *BCL2L11* involved in deletion of autoreactive lymphocytes and *MST1* involved in macrophage activation. Variants at the *MST1* locus are also associated with IBD[39,40]. The role of these genes in recurrent disease is currently unknown but it is plausible that some of these variants together with other factors determine the susceptibility to recurrent disease.

Table 1 Studies analyzing recurrence of primary sclerosing cholangitis after liver transplantation

Ref.	Yr	*n*	Median follow-up (range) in months	Recurrence *n* (%)	Median time to recurrence (range) in months	Diagnostic criteria for recurrence	Risk factor(s) for recurrence
Goss *et al*[11]	1997	127	36 (ND)	11 (8.6)	ND (ND)	Radiographic and histological evidence	ND
Jeyarajah *et al*[70]	1998	100	ND (12-108)	18 (18)	21 (mean) (ND)	Radiographic and/or histological evidence	ACR
Graziadei *et al*[87]	1999	120	55 (mean) (4-136)	24 (20)	8.5 (3-42)	Radiographic and/or histological evidence	None
Kubota *et al*[169]	1999	53	ND (12-156)	3 (5.7)	ND (16-48)	Radiographic and histological evidence	ND
Liden *et al*[170]	2001	61	ND (ND)	5 (8.2)	ND (ND)	Radiographic and histological evidence	ND
Renz *et al*[171]	2002	49	66 (ND)	7 (14)	ND (ND)	Radiographic or histological evidence	ND
Yusoff *et al*[172]	2002	12	58 (mean) (4-174)	2 (17)	ND	Radiographic and histological evidence	ND
Khettry *et al*[71]	2003	42	ND (24-168)	6 (14.3)	ND (ND)	Radiographic and/or histological evidence	Recipient-donor mismatch
Kugelmas *et al*[76]	2003	71	ND (14-91)	15 (21.1)	53 (mean) (12-110)	Radiographic or histological evidence	ND
Brandsaeter *et al*[75]	2005	49	77 (17-182)	9 (18)	ND	Radiographic evidence	Steroid-resistant ACR
Khuroo *et al*[173]	2005	5	90 (1-186)	2 (40)	ND	Radiographic evidence	ND
Oldakowska-Jedynak *et al*[174]	2005	17	32 (mean) (0.9-91)	2 (12)	29 (18-51)	Radiographic and/or histological evidence	ND
Cholongitas *et al*[77]	2007	53	110 (12-185)	7 (13.2)	60 (4-120)	Radiographic and histological evidence	Steroids for UC (> 3 mo post-LTX)
Campsen *et al*[78]	2007	130	66 (ND)	22 (16.9)	ND (ND)	Radiographic evidence	Pre-LTX CCA
Yamagiwa *et al*[175]	2007	44 (all LDLT)	ND (ND)	11 (25)	ND (ND)	Radiographic and histological evidence	ND
Tamura *et al*[176]	2007	8 (LDLT)	42 (ND)	4 (50)	40 (14-66)	Radiographic evidence	ND
Haga *et al*[74]	2007	22 (all LDLT)	31 (mean) (22-71)	13 (59.1)	31 (22-71)	Radiographic and histological evidence	ND
Alexander *et al*[69]	2008	69	50 (1-173)	7 (10)	68 (24-134)	Radiographic and/or histological evidence	ACR, steroid resistant ACR, HLA-DRB1*08 in donor or recipient
Alabraba *et al*[13]	2009	230	82.5 (0.0-239)	54 (23.5)	55.2 (6-155)	Radiographic and histological evidence	Intact colon at the time of LTX, use of EDC graft
Kashyap *et al*[177]	2009	58	41.5 (mean) (ND)	11 (19)	ND (ND)	Radiographic and/or histological evidence	ND
Egawa *et al*[73]	2009	20 (all LDLT)	63 (1-133)	11 (55)	ND (26-71)	Radiographic or histological evidence	Cytomegalovirus infection within 3 mo, related donor
Moncrief *et al*[178]	2009	59	68 (33-106)	15 (25)	40.2 (19.5-66.1)	Radiographic and/or histological evidence	ACR, cytomegalovirus mismatch

LDLT: Live donor liver transplantation; CCA: Cholangiocarcinoma; ND: Not determined; HLA: Human leukocyte antigen; ACR: Acute cellular rejection; LTX: Liver transplantation; EDC: Extended donor criteria; *n*: No. of patients.

In addition to the genetic associations at loci involved in the immune response, the fact that the majority of PSC patients have IBD, an increased frequency of other autoimmune diseases[41] and the presence of multiple autoantibodies[42] further support a role for autoimmune components in the pathogenesis. The most prevalent autoantibody, which is found in more than 90% of PSC patients, is a special type of perinuclear anti-neutrophil cytoplasmatic antibody (pANCA)[43,44]. The same antibody is observed in UC and in type 1 autoimmune hepatitis[44,45]. On the other hand, the male predominance, the lack of demonstration of a specific PSC autoantigen and the missing response to immunosuppressive treatment are atypical for an autoimmune disease[46,47]. The importance of autoantibodies in both PSC and rPSC is unknown, nevertheless mechanisms related to the immune response are likely candidates for overlapping mechanistic themes between the primary and recurrent disease.

Studies in murine models with intestinal bacterial overgrowth suggest that innate immune responses to the

bacterial products could initiate a PSC-like disease process[48-52], and despite the lack of convincing evidence to support a role of infectious agents in human studies[53-57], the presence of an infectious trigger or infectious modifier effects is still possible.

The strong connection to IBD has lead to the hypothesis that long-lived memory cells (lymphocytes) generated in the gut are responsible for the inflammation of the biliary tree in PSC[58]. This hypothesis is supported by the expression, in both liver and intestine in PSC patients, of vascular adhesion protein-1 (VAP-1)[59] and mucosal adressin cell adhesion molecule (MAdCAM)-1[60]. In contrast, MAdCAM-1 and VAP-1 expression in the physiological state is restricted to the gut and liver, respectively. In PSC and other inflammatory liver diseases, MAdCAM-1 may function to recruit intestinally activated T-lymphocytes to the liver[58,61]. It is an intriguing hypothesis that activated lymphocytes generated in this manner in the recipient can attack the new organ in a similar manner, and contribute to the occurrence of recurrent disease in the transplanted liver.

Several studies have focused on the composition of bile in PSC pathogenesis, this is partly based on the findings of a PSC-like disease in mice that lack proteins involved in the transportation of bile components. These changes closely resemble intrahepatic PSC in humans[62-65]. Interestingly, variants in the *ABCB4* gene (also called *MDR3*) influence the progression of PSC[66]. *ABCB4* variants are also of importance in the pathogenesis in some forms of intrahepatic cholestasis of pregnancy and type 3 progressive familiar intrahepatic cholestasis[67,68]. The influence of these variants, on disease progression, is necessarily dependent upon the genotype of the liver and it remains to be verified if variants in the donor liver potentially affect the progression of rPSC in a similar manner.

RISK FACTORS FOR rPSC

It is also important to identify the risk factors for rPSC because it can reveal essential clues in the pathogenesis of both PSC and rPSC, and potentially influence the management of PSC patients after transplantation. Since the first report on suspected recurrence in the liver graft appeared in 1988[24], potential risk factors have been sought. However, the data on specific risk factors are still limited and non-consistent, serving as an illustration of the complexity of the disease.

Several studies have shown one or more risk factors to be significantly associated with increased risk of rPSC as follows: the presence of HLA-DRB1*08 in either recipient or donor[69], absence of donor HLA DR52[70], recipient-donor gender mismatch[71], male recipient[32], older recipient age[72], younger recipient age[70], intact colon before transplantation[13,32], use of related donor[73,74], use of extended donor criteria (EDC) grafts[13], ACR[69,70], steroid-resistant ACR[69,75], use of OKT3[76], presence of UC after LTX[77], maintenance of steroid therapy for UC for more than 3 mo[77], presence of cholangiocarcinoma (CCA) before transplantation[78] and concurrent cytomegalovirus infection in the recipient[70,73]. Differences between various immunosuppressive regimes used after LTX have been hypothesized to be related to the risk of rPSC, however no such effect has been observed[13,77]. Also, no effect has been found according to post-transplant use of ursodeoxycholic acid (UDCA)[13].

Most of the studies on risk factors for rPSC have appropriately performed multivariate analysis and have found one or more factors to be significantly associated with rPSC. Nevertheless, almost none of the findings are reproduced by other groups. The discrepant results are not so surprising, considering that some of the studies included only a limited number of patients and were thus prone to both false positive and negative findings.

One of the risk factors that seem to be best documented is the link between IBD and recurrent disease in the liver allograft. This was first reported in a study by Vera *et al*[32] in 2002, which demonstrated a dramatic reduction in the risk of recurrence if the colon was removed before or at the time of the transplantation. This study evaluated 152 patients and found male gender and intact colon after transplantation to be the strongest predictors of rPSC. Fifty-six (37%) patients developed rPSC during follow-up, but only 1 (6%) of 17 patients who underwent colectomy before or at the time of LTX had recurrence. The importance of IBD in rPSC was considerably strengthened in a study by Cholongitas *et al*[77] in 2007 evaluating 69 patients receiving LTX for PSC. In this series, none of the PSC patients without UC or patients undergoing pre-LTX total colectomy developed rPSC. On the contrary, recurrence occurred in 7 (27%) of 26 patients with post-LTX UC. In their multivariate regression analysis, UC with the need for maintenance steroids for more than three months was the only risk factor significantly associated with rPSC. In 2009, Alabraba *et al*[13] published the largest study to date on risk factors for rPSC, of note, this study was a re-review and extension of the cohort studied by Vera *et al*[32]. A total of 230 consecutive adult patients who underwent liver transplantation for PSC were included. The protective effect of colectomy before or during LTX on the risk of developing rPSC was confirmed, while colectomy after LTX had no beneficial effect on the incidence of recurrent disease[13]. Taken together, these three studies give a relatively strong indication that absence of inflammation in the intestine, either due to absence of concurrent IBD or colectomy before or during LTX has a protective effect against rPSC[13,32,77]. These findings are consistent with the hypothesis of a common T-cell recruitment theme in UC and PSC, as reviewed by Grant *et al*[61], that may also be relevant in rPSC. Importantly, these data should not be interpreted as an advocation for pre-transplant colectomy that may in theory increase the risks for surgical complications during transplantation, but rather as input to understanding the mechanisms of rPSC development.

On the other side of arguments, Alexander *et al*[69] in a study on 69 patients transplanted for PSC, found no cor-

Table 2 Definition of recurrent primary sclerosing cholangitis following liver transplantation (according to Graziadei *et al*[87])

Diagnosis		
Confirmed diagnosis of primary sclerosing cholangitis prior to liver transplantation		
	And	
Cholangiography		**Histology**
Intrahepatic and/or extrahepatic biliary stricturing, beading, and irregularity > 90 d	**Or**	Fibrous cholangitis and/or fibro-obliterative lesions with or without ductopenia, biliary fibrosis, or biliary cirrhosis
Exclusion criteria		
Hepatic artery thrombosis/stenosis		
Established ductopenic rejection		
Anastomotic strictures alone		
Non Anastomotic strictures before post-transplantation day 90		
ABO incompatibility between donor and recipient		

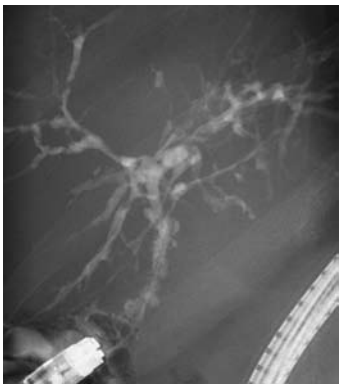

Figure 1 Endoscopic retrograde cholangiogram in a patient with recurrent primary sclerosing cholangitis, showing multifocal stenosis with intervening saccular dilatations affecting both intrahepatic and extrahepatic bile ducts.

relation of rPSC to IBD or the presence of an intact colon after transplantation. Neither did Gautam *et al*[28] in a systematic review from 2006 on rPSC, including only studies with available raw data. To investigate how different immunosuppressive regimens affected the natural course of PSC patients after transplantation, Kugelmas *et al*[76] reviewed a cohort of 71 patients with a 21% recurrence rate. No differences in the frequency of rPSC was observed in the patients with and without IBD, however they did find that OKT3 therapy for steroid-resistant ACR was associated with a higher risk of rPSC.

A few other groups have also independently reported an association between ACR and recurrent disease. In a study of 118 consecutive liver transplantations for PSC, Jeyarajah *et al*[70] found a significantly higher incidence of ACR in recipients that later developed rPSC or chronic rejection. Alexander *et al*[69] found ACR and steroid-resistant ACR to be predictive of an increased risk of rPSC and in a study of 49 patients transplanted for PSC evaluated by magnetic resonance cholangiography (MRC), steroid-resistant ACR was the only significant predictor for rPSC[75].

Up to date, the possible mechanism behind the association between ACR and an increased risk of recurrent disease is unknown. The biliary epithelium is one of the components that is attacked and injured in ACR, and it has been postulated that this can result in an increase in autoimmune epitopes, leading to ductal damage mediated by the immune system[70]. Others have suggested the existence of a common factor predisposing to both ACR and recurrent disease. Especially, the fact that PSC patients seem to be more prone to ACR than most other patient groups, has led to the speculation of a common link between the pathophysiology of rPSC and ACR[69]. One hypothesis is the existence of a hyperactive component of the immune system, and another theory is that the increased risk of both ACR and rPSC results from a defective mechanism in the immune regulation.

What is also worth mentioning is a reported higher rate of recurrence in recipients of grafts from living related donors, with rPSC occurring in 55% and 59% of the transplanted PSC patients[73,74]. Both studies involved a relatively low number of patients, but should urgently inspire further investigations in larger cohorts to confirm important pathogenic mechanisms.

DIAGNOSTIC CHALLENGES IN rPSC

Since the report by Lerut *et al*[24], a number of studies have indicated that biliary complications, especially nonanastomotic biliary strictures occurred more frequently after liver transplantation for PSC than for other end-stage liver diseases[79-85].

As with the diagnosis of PSC in the native liver, rPSC is diagnosed based on the combination of radiological, histological and biochemical findings. The diagnosis is considered problematic because of the difficulty in distinguishing rPSC from other conditions with similar biliary changes. A variety of potential insults to the liver graft can all result in biliary injury and stricturing[28,86]. In 1999, Graziadei *et al*[87] established specific criteria for diagnosing rPSC. This is a useful definition that has been followed since (Table 2). Strict biochemical indices or typical clinical symptoms are frequently absent in patients with rPSC. These patients often present with a cholestatic biochemical profile, and further examinations with cholangiography with either endoscopic retrograde cholangiopancreatography (ERCP), MRC or percutaneous cholangiography (PTC) show characteristic changes of the bile ducts with multifocal strictures and segmental dilatations[88]. However, emphasis must be put on exclusion of other causes that can cause similar radiographic and histological changes. Accordingly, the diagnosis is made in patients with a confirmed diagnosis of PSC prior to transplantation, and by either typical histological features on biopsy or by radiological demonstration of multiple nonanastomotic strictures occurring more than 90 d after LTX (Table 2 and Figure 1).

Cholangiographic findings

As PSC affects both the intra- and extra-hepatic bile ducts, most recipients will have a Roux-en-Y loop after

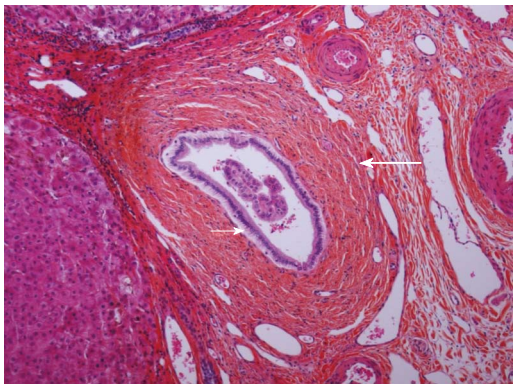

Figure 2 Histological changes demonstrated in a biopsy from a patient with recurrent primary sclerosing cholangitis. Bile duct (small arrow) surrounded by collar of connective tissue with concentric layers of collagen fibers (large arrow) illustrating the typical periductal lamellar fibrosis. (Original magnification, x 100).

LTX[89,90]. This renders access to the bile ducts *via* the endoscopic route technically challenging[91]. Some centers have addressed this challenge using single or double balloon enteroscopy and in this way are able to perform ERCP in patients with complex postsurgical gastrointestinal anatomy[92,93]. It is also worth mentioning that some centers have reported preliminary good results after bile duct reconstruction by direct choledochoduodenostomy[94,95], resulting in an anastomosis that have the advantage of easier access by conventional ERCP. Still, PTC or MRC is most widely used in the post-LTX population[92,96,97]. The typical findings on cholangiography in rPSC involve multifocal nonanastomotic strictures in both intra- and extrahepatic bile ducts, with intervening segments of normal or dilated ducts[86]. Sheng *et al*[85] compared the radiological signs of rPSC with other conditions after LTX. They included 32 patients transplanted for PSC who had developed biliary strictures in the graft and a control group of 32 non-PSC grafts who also had developed biliary strictures. This study demonstrated that intrahepatic strictures were much more common in patients with rPSC and the appearance of the strictures was different, mural irregularity and diverticular outpouchings being more common in rPSC. Even though these changes occurred significantly more frequently in patients with rPSC [15 (47%) patients and 6 (19%) patients, respectively], in the majority of the cases, it was impossible to distinguish rPSC from other conditions based solely on cholangiographic findings.

Several studies have evaluated the diagnostic accuracy of MRC in PSC patients[98-100]. A meta-analysis published last year found that MRC had a sensitivity and specificity of 86% and 94%, respectively in detecting PSC[100]. The latest guidelines by both the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASLD) recommend MRC rather than ERCP in patients with suspected PSC[101,102]. Nevertheless, some have raised concerns regarding the sensitivity of MRC when it comes to subtle intrahepatic changes[103] or in detailed characterization of extrahepatic biliary changes[104]. There has been no study so far comparing ERCP or PTC with MRC in rPSC and it is thus not clear whether the sensitivity/specificity figures are similar to those in the primary disease. Importantly, the quality of the MRC imaging is continuously and rapidly evolving and with the noninvasive nature and reduced cost, MRC has become the first choice for most clinicians to evaluate abnormalities of the extra- and intrahepatic bile ducts after LTX.

Histopathological findings

The use of liver biopsies in diagnosing both PSC and rPSC is usually regarded supplementary to cholangiography. This is partly due to the relative lack of specificity and the patchy involvement resulting in a certain degree of sample variability[27]. Histopathologically, the changes in rPSC (Figure 2) are identical to what is described in the native liver with PSC and can be extremely difficult to distinguish from biliary strictures of other causes in allografts, including recurrent biliary infections, ischemia due to arterial problems, chronic rejection, small-for-size syndrome, blood group incompatibility or reperfusion injury[86,105]. The early changes in rPSC are characterized by mild, nonspecific (peri) cholangitis and loss of small bile ducts. As the disease progresses, periductal lamellar edema, increased neutrophil and eosinophil inflammation in the portal tracts, periportal edema and an increased ductular reaction are becoming apparent. Marked deposits of copper with Mallory's hyaline in periportal hepatocytes can often be seen[26]. In medium and small bile ducts, the typical features of fibro-obliterative lesions are observed at this stage together with focal loss of medium and small bile ducts. Lobular changes in the early stages of rPSC include mild nodular regenerative hyperplasia. In the later stages, the characteristic changes of cholestasis, biliary cirrhosis, foam cell clusters and marked deposition of copper in the periportal hepatocytes are observed[105]. In the extrahepatic and large bile ducts of the hilum, ulceration, bile sludge, periductal fibrosis and foam cell accumulation are often seen[105].

The distinction between chronic rejection and rPSC is often challenging, as both conditions can cause loss of bile ducts and a cholestatic pattern of liver enzyme elevation[106]. The diagnosis of both rPSC and chronic rejection should therefore be based on a combination of histopathological, laboratory, radiological and clinical findings. In both conditions, it is important to review prior biopsies combined with the clinical course, and hence for the pathologist to take the clinical history into account. Often there is a history of failed compliance or episodes of severe rejection with unsatisfactory response to treatment in chronic rejection[105].

TREATMENT OF rPSC

Pharmacological therapy

There is currently no proven medical therapy to halt or slow the progression of PSC or rPSC. The major focus

regarding pharmacological treatment in PSC has been a possible effect of UDCA on the disease course. Some of the first studies could document an improvement in both hepatic, biochemical and histological parameters[107-109], but this effect did not significantly benefit transplant-free survival. In the three largest studies[110-112] using UDCA at different doses, no beneficial effect was found on the risk of liver transplantation or death. This conclusion is further supported by a recent meta-analysis by Shi *et al*[113]. In rPSC, no effect has been shown from any specific treatment. Some centers recommend discontinuation of corticosteroid therapy[26] and several centers prescribe UDCA[25]. Patients with coexisting UC may benefit from UDCA in reducing the risk of developing carcinoma in the colon[114], but regarding a possible effect on the progression of rPSC, documentation is so far lacking. The choice of immunosuppression seems to have no influence on the incidence or progression to rPSC[76]. This is, however, a complicated research field and difficult to review in retrospective studies, because detailed information for each patient is needed, including any potential changes in immunosuppressive medication and the disease stage where the change of medication took place. In this study, OKT3 was found associated with a higher risk of recurrence[76], but it is difficult to determine if it is the treatment or the reason for giving the treatment that had a deleterious effect.

Interventional therapy

Three retrospective studies on the effect of endoscopic treatment of dominant strictures in PSC patients have all suggested improved 3- and 5-year survival rates[115-117]. Whether endoscopic treatment influences the progression of rPSC is currently unknown. There have been no published studies with this specific focus in patients with recurrent disease, but based on the effect observed in PSC patients before LTX, further studies of endoscopic treatment modalities should be encouraged.

IMPACT OF rPSC ON GRAFT AND PATIENT SURVIVAL

Earlier studies reported no difference in graft or patient survival among recipients with or without rPSC[10,11]. Long-term data, however, indicate that recurrent disease has a significant impact on graft survival, rate of retransplantations and perhaps also patient survival[12,13]. A study from the United Network for Organ Sharing (UNOS) database on LTX in more than 3000 PSC patients compared with a similar number of patients with primary biliary cirrhosis (PBC), who have a very good outcome after liver transplantation found the retransplantation rate to be significantly higher in PSC patients than in PBC (12.4% *vs* 8.5%). PSC patients had significantly lower graft and patient survival than PBC patients after adjusting for other risk factors and a diagnosis of PSC was an independent predictor for retransplantation[118]. The reduced survival did not become evident until 7 years after the primary operation. In a retrospective study performed by Rowe *et al*[12] analyzing graft loss due to recurrence in 1840 patients undergoing primary LTX between 1982 and 2004, the risk of graft loss from recurrent disease was significantly higher in PSC patients than in PBC patients (hazard ratio 6.0; 95% CI 2.5-14.2). Cholongitas *et al*[77] reported in 2008 a recurrence rate of 13.5% after transplantation for PSC, and 43% of the patients with recurrence required retransplantation. In this study, no difference in survival was seen, but the number was relatively small with a total of 69 patients transplanted for PSC. In the largest series reported to date[13], rPSC developed in 61 grafts in 54 patients, of which, 23 grafts in 20 patients were lost and 13 retransplantations were carried out. After exclusion of all patients surviving less than 6 mo and adjustment for age, there was significantly better survival in patients without rPSC. This is not surprising, considering that retransplantation usually is a much more complicated procedure than the primary operation. A range of reports has shown that retransplantations, independent of the underlying disease, have a significantly worse outcome than the primary procedure[119-124]. In a review of the outcome after retransplantations (for any underlying cause) in 196 patients over a 25-year period at Queen Elisabeth Hospital, the 5- and 10-year survival after retransplantation were 57% and 47%, respectively[119]. The five-year survival after a second retransplantation was 40%. The risk of perioperative death increases significantly from less than 5% in the primary procedure to almost 20% in retransplantations[120,121].

EPIDEMIOLOGY OF REJECTION IN PSC PATIENTS

There is little doubt that the incidence of rejection after liver transplantation has been declining, yet still 20%-40% of the recipients will experience at least one episode of ACR requiring additional immunosuppressive treatment[125]. PSC patients have been suggested to have a higher risk of ACR compared with other liver recipients[10,126,127]. There are nevertheless relatively few published studies with specific focus on the incidence of rejection after LTX according to underlying disease, and the reported incidence varies substantially in different studies. In an attempt to clarify whether this patient population really is at an increased risk of rejection, we identified eleven publications containing specific data on the incidence of acute rejection in patients transplanted for PSC. These studies include a total of 656 patients transplanted for PSC, of whom, 373 (57%) recipients (range, 17%-100% in individual studies) had one or more episodes of acute rejection (Table 3) In the largest study including 150 consecutive transplantations for PSC, at least one episode of acute rejection occurred in 103 (69%) of the recipients, while the incidence in the control group was 59% (261/440)[10]. The reported incidence of ACR after LTX shows a large discrepancy between different centers and time periods. This is the case regarding ACR

Table 3 Studies containing data on the incidence of acute cellular rejection after liver transplantation for primary sclerosing cholangitis

Ref.	Yr	No. of PSC patients	Median follow-up (range) in months	ACR *n* (%)	Diagnostic criteria for rejection	Immunosuppression
Klintmalm *et al*[179]	1988	9	ND (ND)	4 (44)	Biopsy	CyA, CS and azathioprine
McEntee *et al*[180]	1991	44	ND (ND)	44 (100)	ND	ND
Shaked *et al*[181]	1992	36	ND (ND)	6 (17)	Biopsy	CyA/Tac, CS and azathioprine
Miki *et al*[3]	1994	55	ND (ND)	37 (67)	Biopsy	CyA/Tac, CS and azathioprine
Narumi *et al*[30]	1995	33	37.8 (6-73)	19(57)	Biopsy	ATG for 3-5 d, CS, azathioprine and delayed CyA
Farges *et al*[131]	1996	23	Minimal follow-up 18 mo	12 (52)	Biopsy	CyA/Tac, CS and azathioprine
Jeyarajah *et al*[70]	1998	115	Minimal follow-up 12 mo	45 (39)	Biopsy	CyA/Tac, CS and azathioprine
Wiesner *et al*[130]	1998	126	34	58 (46)	Biopsy	CyA/Tac, CS and azathioprine (1 group received induction with ATG)
Graziadei *et al*[10]	1999	150	55 (10-138)	103 (69.7)	Biopsy	CyA/Tac, CS and azathioprine
Bathgate *et al*[182]	2000	16	ND (ND)	10 (63)	Biopsy	ND
Brandsaeter *et al*[75]	2005	49	77 (17-182)	35 (71)	Biopsy	CyA/Tac, CS and azathioprine/ MMF

PSC: Primary sclerosing cholangitis; ACR: Acute cellular rejection; ND: Not determined; MMF: Mycophenolate mofetil; ATG: Anti-T-lymphocyte Globulin; CyA: Cyclosporine A; Tac: Tacrolimus; CS: Corticosteroid.

after LTX for PSC, as well as for other underlying diseases. Evolving immunosuppressive strategy clearly has a significant influence. No data regarding immunosuppression trends are currently available from Europe, but based on individual series and data from the UNOS registry, the majority of centers use triple drug therapy, including calcineurin inhibitors (CNIs), corticosteroids and antimetabolite drugs. However, the immunosuppressive regimens vary among centers and the use of induction therapy is inconsistent. Another important factor influencing the reported number of ACR is the use of protocol biopsies or not. An additional confounder is the fact that diagnostic criteria for rejection vary largely in earlier reports. This situation improved considerably with the introduction of the Banff classification in 1997[128]. Based on the current data, and the general difficulties in reporting rejection rates as discussed above, it is hard to state with certainty that transplanted PSC patients carry a higher risk of ACR than other patient groups. There are for instance several publications that show an equally high or even higher incidence of acute rejection in patients transplanted for PBC[84,127,129-131] and autoimmune hepatitis (AIH)[127,130,131]. What seems to be clear though is that there is a tendency that patients with an underlying "autoimmune" condition have an increased risk of ACR after LTX. Whether this is due to a generally more "active" immune system in these patients is unclear. Another interesting and relatively consistent finding in many reports supporting this hypothesis, is that patients treated for non-immunological conditions, like alcoholic liver disease or fulminant hepatic failure from paracetamol intoxication, seem to be at an especially low risk for acute rejection[127,130-133].

PATHOGENESIS AND RISK FACTORS FOR ACR IN PSC PATIENTS

In general, rejection of an allograft can be defined as "an immunologic reaction to the presence of a foreign tissue or organ that has the potential to result in graft dysfunction and failure"[134]. Since the start of experimental solid organ transplantation, it has been obvious that the liver has an immunological advantage over other organs. In several animal transplant models, spontaneous tolerance was achieved after LTX, while skin, heart or kidney transplants were aggressively rejected in the same models[135-139]. Tolerance following liver transplantation is rare in humans, however ACR is usually easily reversed.

Findings in studies on factors that increase the risk of acute rejection after LTX include: use of living donor[140], lack of induction treatment with anti-IL-2 receptor antibody[141,142], severe preservation injury[143], younger age of the recipient[130], lack of renal failure[130], fewer HLA-DR matches[130,144], cold ischemia time greater than 15 h[130] and being transplanted for PSC[10,30,70,87], PBC[129], autoimmune hepatitis[127] or HCV infection[127]. In addition, a meta-analysis of cytokine gene polymorphisms and acute liver graft rejection suggests a role for genetic variation at the *IL-10* locus[145].

For PSC, both disease recurrence and the presumed increased incidence of ACR have been speculated to be caused by a continuum of ongoing immunological attack against the bile ducts and the liver[10]. Whether different immune mechanisms are involved in the rejection process in PSC patients *vs* patients with other underlying disease is not known. To date, no study has focused on characterizing histopathological differences in the inflammatory infiltrates of ACR according to the underlying disease. The typical histological features described in ACR in the liver allograft (Figure 3) are similar to that in other organs, the dominant cell types being CD4+ and CD8+ T cells and macrophages[128,146]. B-cells, neutrophils and eosinophils have been observed in varying proportions, and scattered cells positive for the natural killer (NK) marker CD56+ have been described[147]. NK cells have emerged as a particular focus of interest in the transplant setting because of their ability to recognize allogenic major histo-compatibility complex (MHC) antigens and their potent cytolytic

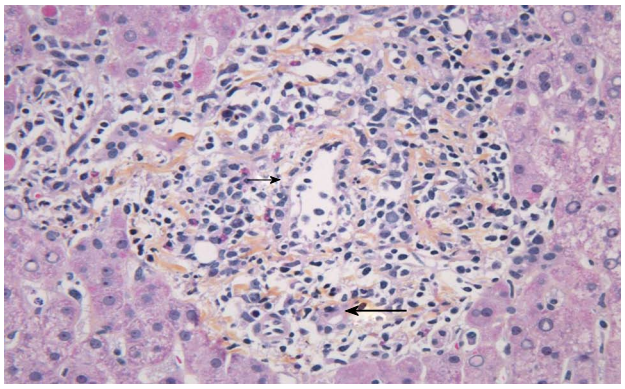

Figure 3 Histological changes seen in a biopsy with acute cellular rejection in a patient with primary sclerosing cholangitis. Portal tract with mixed inflammatory infiltrate containing blastic lymphocytes and eosinophils. Subendothelial localization of the inflammatory cells in a portal vein branch (small arrow). Inflammation of small bile duct (large arrow). (Original magnification, x 400).

activity[148]. They possess a variety of inhibitory and activating receptors, such as killer immunoglobulin-like receptors (KIRs), which recognize MHC class I molecules, and based on the "missing self" hypothesis, kill target cells that display reduced levels of MHC class I antigens. Interestingly, it has been shown that particular combinations of KIRs and HLA class I ligands that reduce NK cell inhibition increase the susceptibility to autoimmune diseases[149], and that certain genetic variants of ligands for NK cell receptors might contribute to the risk of PSC[150,151]. An increase in NK cells in the liver has been observed in PSC as compared with other liver diseases[152,153]. The effect of NK epitope mismatching on acute rejection after liver transplantation is uncertain. The few studies conducted so far have given conflicting results[154-156]. A recent paper by Hanvesakul *et al*[155] on 416 liver transplant recipients showed a striking influence of donor HLA-C genotype on graft survival and chronic rejection, but no effect on ACR. To what extent such phenomena may be restricted to patients with PSC has not been determined.

As previously addressed, there is conflicting data about whether the presence of IBD has an adverse impact on the risk of recurrence of PSC after the transplantation. Some reports also suggest that PSC patients with concomitant IBD may be at increased risk of ACR. In a study by Narumi *et al*[30], the incidence of moderate or severe rejection in patients with IBD was 70% *vs* 36% in PSC patients without IBD, and 37 % in a matched control group. This was further supported by a study by Miki *et al*[3] in which 87% of the patients with IBD developed ACR while only 41% of the patients without IBD developed ACR. Consistent with these studies, Gradziadei *et al*[10] also reported a significantly increased risk of ACR in patients with IBD in their large cohort of 150 transplanted PSC patients. On the other hand, in the study by Jeyarajah *et al*[70], including 118 transplantations for PSC, no association between concomitant IBD and ACR was found.

IMPORTANCE OF ACR AFTER LIVER TRANSPLANTATION FOR PSC

As opposed to kidney transplantation[157-159], there is no convincing data indicating that ACR in the early phase after liver transplantation (for any underlying condition) affects long-term graft or patient survival. After the introduction of more potent and specific immunosuppression, it is easier to prevent episodes of ACR and a major challenge in LTX today is the morbidity and mortality related to side effects of long- term use of immunosuppressive medication[157-160]. In animal models with spontaneous tolerance, the liver allograft undergoes an initial and transient acute rejection-like reaction[161-163], that could indicate that the histopathological diagnosis of ACR does not necessarily always require treatment, but as some argue that it might be a first step on the way to a degree of spontaneous tolerance.

Data regarding the incidence and significance of late acute rejection (LAR) are scarce, but an interpretation might indicate a risk of more serious consequences than with early ACR. LAR has been variably defined in different reports ranging from one, three, six or twelve months after the transplantation. The incidence of LAR after liver transplantation in general varies from 7% to 23% in the published reports[126,164-168]. In a study of more than 1600 LTX patients, where LAR was defined as ACR occurring later than six months after LTX, the incidence was 19%[126]. Interestingly, the study also showed that patients with a primary diagnosis of autoimmune hepatitis, PBC and PSC had the highest incidence of LAR and that patient and graft survival was significantly lower in the LAR group. Another study also reported an increased risk of LAR in PSC patients but did not find any negative effect on patient survival[168].

ACR represents an immunological insult towards the graft and could influence the risk of developing rPSC since more reactive lymphocytes are likely to be present. As previously mentioned, ACR or steroid resistant ACR were found to have a significant effect on the risk of developing recurrent disease[69,70,75,76]. In contrast, most studies evaluating risk factors for rPSC have not found an association with ACR. There is little data supporting the conclusion that mild or moderate ACR, affecting approximately 50% of the transplanted PSC patients, has any effect on the risk of recurrent disease. The question is if severe rejection is associated with recurrence or not. As of today, there is not enough data to answer this question with certainty.

CONCLUSION

Rejection and recurrence of the primary disease in the liver allograft remain as two major challenges in the clinical care of post-transplant PSC patients. Even as there are huge discrepancies in the reported incidence of

rPSC, it is at least present in approximately 1/5 of the transplanted patients. The pathogenesis of rPSC remains enigmatic and is believed to have similar features as the primary disease. There is so far an underutilized potential in studying the pathogenesis of the primary disease in parallel with recurrent disease, an approach that potentially can uncover shared mechanisms relevant to both conditions. Several studies have identified one or more risk factors for rPSC, but few have been confirmed from one study to another. The most convincing data seem to be the link with concurrent IBD and a protective effect on the development of rPSC of colectomy before or at the time of transplantation[13,32,77]. There is, importantly, no reason to advocate colectomy prior to liver transplantation for PSC on this basis. There seems also to be an increased incidence of acute rejection in PSC patients with a potential relevance to the development of recurrence. Rejection and recurrence might therefore represent a continuum of immunological affection of the graft in transplanted PSC patients.

ACKNOWLEDGMENTS

We are specially thankful to Grzyb Krzysztof for immunohistochemical images and Audun E Berstad for radiological images.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i1.16

World J Gastroenterol 2012 January 7; 18(1): 16-24
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

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Multi-modality treatment of colorectal liver metastases

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Received: March 17, 2011 Revised: June 9, 2011
Accepted: June 16, 2011
Published online: January 7, 2012

Abstract

Liver metastases synchronously or metachronously occur in approximately 50% of colorectal cancer patients. Multimodality comprehensive treatment is the best therapeutic strategy for these patients. However, the optimal pattern of multimodality therapy is still controversial, and it raises several significant concerns. Liver resection is the most important treatment for colorectal liver metastases. The definition of resectability has shifted to focus on the completion of R0 resection and normal liver function maintenance. The role of neoadjuvant and adjuvant chemotherapy still needs to be clarified. The management of either progression or complete remission during neoadjuvant chemotherapy is challenging. The optimal sequencing of surgery and chemotherapy in synchronous colorectal liver metastases patients is still unclear. Conversional chemotherapy, portal vein embolization, two-stage resection, and tumor ablation are effective approaches to improve resectability for initially unresectable patients. Several technical issues and concerns related to these methods need to be further explored. For patients with definitely unresectable liver disease, the necessity of resecting the primary tumor is still debatable, and evaluating and predicting the efficacy of targeted therapy deserve further investigation. This review discusses different patterns and important concerns of multidisciplinary treatment of colorectal liver metastases.

Key words: Colorectal cancer; Liver metastases; Multimodality therapy

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Cai GX, Cai SJ. Multi-modality treatment of colorectal liver metastases. *World J Gastroenterol* 2012; 18(1): 16-24 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i1/16.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i1.16

INTRODUCTION

Colorectal cancer is one of the most commonly diagnosed cancers, and it is ranked the third most common globally and fourth in China. Approximately 40% of colorectal cancer patients die of cancer recurrence and metastasis. The liver is the most frequent metastatic site of colorectal cancer. Approximately 15%-25% of colorectal cancer patients have synchronous liver metastases[1-3], and 20%-25% of patients with colorectal cancer develop metachronous hepatic metastases[4-7].

In recent decades, the 5-year overall survival (OS) after curative liver resection of colorectal liver metastases (CRLM) has increased to 35%-58%[8-10]. This improvement is largely due to advancements in CRLM multimodality treatment. Generally, CRLM can be categorized into three subsets: clearly resectable, potentially resectable, or definitely unresectable. This review discusses patterns and key issues of multidisciplinary treatment of these three different CRLM subsets with a focus on the

interactive influence of different therapeutic approaches.

CLEARLY RESECTABLE COLORECTAL LIVER METASTASES

Shifting definition of CRLM resectability

Liver lesions numbering more than three, an estimated resection margin < 1 cm, the presence of extrahepatic disease, or no expected sufficient remnant liver volume used to be deemed as contraindications for CRLM liver resection. According to this definition, only 10%-20% of CRLM patients were resectable. However, this definition has changed in recent years. The report by Malik *et al*[11] has indicated that patients with 4-7 or > 7 CRLMs still had a favorable outcome after liver resection (5-year OS 34.8% and 24.2%, respectively). In the past, it was widely accepted that at least a 1-cm resection margin must be achieved for CRLM resection. However, several studies have indicated that the actual clearance margin did not affect survival as long as R0 resection could be achieved[5,12]. The presence of extrahepatic metastases is also no longer considered an absolute contraindication for liver resection. Some cancer centers have reported that the 5-year OS after combined resection of lung and liver metastases is approximately 30%[13,14]. The CRLM resectability criteria have shifted to focus on whether R0 resection for all tumors can be achieved and if a sufficient volume of residual liver can be preserved. The requirement for residual liver volume can be different for patients receiving intensive chemotherapy. Although at least 20% of total liver volume should be preserved for a healthy liver, it is recommended that at least 30%-60% should be preserved for livers impaired by chemotherapy-associated steatosis or hepatitis[15].

Advantages and disadvantages of perioperative chemotherapy

The combination of surgery and chemotherapy is the most effective multidisciplinary therapeutic paradigm for CRLM with a curative intent. There are two patterns of perioperative chemotherapy for resectable CRLM: preoperative and postoperative chemotherapy. Postoperative chemotherapy is also known as adjuvant chemotherapy, although it is still debatable whether the alternative term adjuvant therapy should be used instead. Postoperative chemotherapy has become a common practice and is intended to reduce the high risk of recurrence after resection of metastases. Preoperative chemotherapy is also called neoadjuvant chemotherapy in the setting of resectable liver metastases. The role of preoperative chemotherapy is more controversial than postoperative chemotherapy because it can give rise to major concerns. Generally, the paradigm of preoperative chemotherapy plus liver resection plus postoperative chemotherapy has become the most prevalent treatment modality in real practice.

Survival benefit of perioperative chemotherapy: Adjuvant chemotherapy for stage Ⅲ colorectal cancer patients has been widely accepted based on solid evidence for survival benefit. Although postoperative chemotherapy after liver metastases resection is also accepted by many oncologists, there have been few prospective randomized clinical studies that have investigated the adjuvant chemotherapy survival benefit after liver resection, and the sample size of these studies has been limited due to difficult accrual. In the Fédération Francophone de la Cancérologie Digestive (FFCD) ACHBTH AURC 9002 clinical trial, CRLM patients receiving adjuvant chemotherapy of 5-fluorouracil (5-FU) and leucovorin (LV) after R0 liver resection had a significantly better 5-year disease-free survival (DFS) compared with the observation group (33.5% *vs* 26.7%, P = 0.028). There was also a trend toward better OS in the adjuvant chemotherapy group, although this was not statistically significant (51.1% *vs* 41.1%, P = 0.13)[16]. This study was prematurely stopped due to slow accrual. A pooled data analysis combined with another study (i.e., European Organisation for Research and Treatment of Cancer/ National Cancer Institute of Canada Clinical Trials Group/ Interdisciplinary Group for Cancer Care Evaluation trial), which had a similar design and stopped ahead of schedule for the same reason, was performed but also could not demonstrate a statistically significant improvement in OS (P = 0.095)[17].

Although the most fascinating benefit of preoperative chemotherapy is the conversion of unresectability to resectability, the role of neoadjuvant chemotherapy in patients with initially resectable CRLM is still controversial. The most important concern about neoadjuvant chemotherapy is whether it can bring about a survival benefit. The only published randomized prospective clinical trial to investigate the role of neoadjuvant chemotherapy in CRLM patients, EORTC 40983[18], indicated that patients with initially resectable CRLM undergoing liver resection plus six cycles of preoperative FOLFOX4 and six cycles of postoperative FOLFOX4 chemotherapy had a better 3-year progression-free survival (PFS) compared to those receiving liver resection alone. However, there was a significant defect in this study: patients in the control group did not undergo chemotherapy after hepatic resection. Therefore, it is difficult to determine whether the PFS improvement is brought about by preoperative chemotherapy, postoperative chemotherapy or both. To investigate the exact role of neoadjuvant chemotherapy, we still need to wait for the results of ongoing clinical studies to compare survival directly in patients undergoing postoperative chemotherapy alone with those undergoing both preoperative and postoperative chemotherapy.

Management of disappearing CRLM: A potential drawback of neoadjuvant chemotherapy in resectable CRLM patients is missing the optimal timing of liver resection because of complete response of liver tumors during chemotherapy. Approximately 4% of patients achieved a radiographic complete response (CR) to chemotherapy, and 9% had a pathological CR[19,20]. Radiographic CR does

not always mean true hepatic metastases remission. Viable cancer cells can be pathologically found in 80% (12/15) of patients with a radiographic CR and undergoing resection according to the prior sites[21]. If these radiographically disappearing liver metastases are kept in place without resection, 41%-75% will have recurrence *in situ*[21,22]. Nevertheless, it is not always easy to perform liver resection according to the previous site of disappearing liver metastases. To avoid such an intractable condition, it is recommended that the evaluation of liver lesions be repeated every 2 mo during preoperative chemotherapy[7,23-25].

Resection of CRLM progressing during neoadjuvant chemotherapy: A second potential risk of neoadjuvant chemotherapy is disease progression. Liver tumor progression may add to the difficulty of liver resection and even deprive patients of the opportunity for hepatic resection. The EORTC 40983 clinical study[18] reported that 7% of initially resectable CRLM patients had progressive disease (PD) during neoadjuvant chemotherapy, and 4% did not complete liver resection due to prior liver disease progression or the presence of new extrahepatic metastases. Another issue concerning liver metastases progression is whether they should be resected even if it is possible. Adam *et al*[26] have suggested that liver PD during chemotherapy indicates poor prognosis after resection and should be considered as a contraindication to liver resection. They reported a dismal 5-year OS (8%) and DFS (3%) after liver resection in patients with tumor progression during neoadjuvant chemotherapy. However, other studies have indicated that the response to neoadjuvant chemotherapy has no prognostic value. Reports from Neumann *et al*[27] and Gallagher *et al*[28] have indicated no difference in survival after liver resection among three groups of CRLM patients with PD, stable disease (SD) or objective response to neoadjuvant chemotherapy.

Impact of chemotherapy-induced hepatotoxicity on the outcome of hepatic resection: Another important concern related to neoadjuvant chemotherapy is whether the hepatotoxicity caused by preoperative chemotherapy increases the perioperative morbidity and mortality of liver surgery. There are two types of chemotherapy-associated hepatotoxicity: non-alcoholic fatty liver disease (i.e., macrovesicular steatosis/steatohepatitis) and vascular sinusoidal obstruction. All three commonly used chemotherapeutic agents for colorectal cancer, 5-FU, oxaliplatin and irinotecan, can induce steatosis with an incidence rate of 30%-40%[29,30]. Steatohepatitis is less common in patients with chemotherapy. Approximately 3.6%-8%[7,31] of patients have chemotherapy-associated steatohepatitis, which is relatively more common in patients receiving irinotecan as compared with those receiving 5-FU[32]. Vascular sinusoidal obstruction is associated with the use of oxaliplatin, and is present in 10%-52% of patients undergoing preoperative oxaliplatin therapy[7,25,33].

The impact of chemotherapy induced hepatic toxicity on the short-term outcome of patients receiving liver resection is still uncertain. A slightly increased morbidity was noted in patients undergoing six cycles of preoperative FOLFOX4 chemotherapy as compared with those without preoperative chemotherapy in the EORTC 40983 study[18]. Nevertheless, there was no difference in the perioperative mortality between these two groups. The study of Kooby *et al*[34] has demonstrated an increase in infection-related complications associated with moderate to severe steatosis in patients undergoing hepatic resection after chemotherapy, but no association with major surgical complications or mortality for preoperative chemotherapy was shown. However, Vauthey *et al*[25] have reported that, after the use of irinotecan, patients with steatohepatitis had a significantly higher 90-d postoperative mortality compared with those without steatohepatitis (15% *vs* 2%, $P = 0.001$). Therefore, it is recommended that irinotecan should be used cautiously in patients with known steatosis or steatohepatitis or those with a high risk for steatosis, such as those with obesity, hypertension or diabetes.

Management of resectable synchronous CRLM

Optimal sequencing of colorectal surgery, liver resection and perioperative chemotherapy: Approximately 15%-25% of patients have synchronous liver metastases at the diagnosis of colorectal cancer[1-3]. The optimal timing of primary tumor and liver metastases resection in synchronous resectable CRLM patients is still controversial. There are three approaches for the sequence of surgical treatment for primary tumor and liver disease: (1) simultaneous resection of primary cancer and liver metastases; (2) resection of primary colorectal tumor first followed by liver resection; and (3) hepatectomy first followed by primary cancer resection. The clinical decision usually depends on many factors, including surgical exposure, colectomy and hepatectomy complexity, surgeon expertise and patient comorbidity[35].

Based on the observation of the possible increased morbidity and mortality using a combination of hepatectomy and colectomy[36-39], a staged approach (i.e., liver resection following primary tumor resection and optional chemotherapy) was widely performed in the past. However, simultaneous resection of the primary cancer and the liver metastases has been increasingly adopted in recent years due to more recent reports that perioperative morbidity and mortality of simultaneous resection are comparable to that of staged resection[40-42]. No significant difference in 5-year survival was found between these two groups in a systemic analysis[43]. However, Reddy *et al*[35] have reported that patients undergoing simultaneous major hepatectomy (i.e., resection of three or more liver segments) had a significantly higher mortality (8.3% *vs* 1.4%) and severe morbidity (36.1% *vs* 17.6%) than those receiving staged resection. Therefore, simultaneous major hepatectomy is not highly recommended at present due to the potentially increased risk of severe complications. A new paradigm has been proposed more recently that is called the "liver-first" strategy[44], and includes first, liver resection, with or without preoperative

chemotherapy, followed by optional chemotherapy after hepatectomy, and finally, primary tumor resection. This approach may be suitable for borderline resectable liver metastases, which may lose the time frame of resection if delayed. Mentha *et al*[44] have reported 20 CRLM patients undergoing such a sequential resection with a resection rate of 80% and 4-year OS of 56%. However, there are some potential defects in the design of this approach. For patients with obstructive symptoms caused by the primary tumor, primary-tumor-directed treatment is more urgent and should be performed first. Another potential disadvantage of this approach is that the primary tumor may progress and require emergency surgery during this process. A decision-making analysis has demonstrated that it is least probable to complete all intended sequential treatment for the liver-first approach among the above three treatment sequences[45].

The role of minimally invasive surgery: It is difficult to perform a one-stage resection of primary and liver disease for rectal cancer liver metastases due to surgical exposure and lengthy incisions. In such a condition, laparoscopic surgery, particularly robot-assisted laparoscopic surgery, is advantageous to perform a simultaneous resection of liver metastases and rectal cancer. This type of surgery has been reported to be safe and feasible in a pilot study by Patriti *et al*[46]. An important concern about the laparoscopic hepatic resection is the oncologic outcome. It has been reported that the laparoscopic approach had a positive resection margin rate (5.6%) and 5-year OS (50%); comparable with open surgery for CRLMs. In a French study[47] comparing CRLM patients undergoing laparoscopic hepatic resection or open resection, the 5-year OS and DFS were similar in these two groups, whereas the laparoscopic surgery group even had a lower rate of positive resection margin than the open surgery group (13% *vs* 28%, $P = 0.04$).

UNRESECTABLE COLORECTAL LIVER METASTASES WITH POTENTIAL CONVERTIBILITY

Some CRLM patients are initially unresectable but have the potential to become resectable through conversion therapeutic strategies including chemotherapy, embolization, two-staged operation or the combination of ablation therapy.

Conversion chemotherapy

It is estimated that 80%-90% of CRLMs are considered unresectable at diagnosis. Due to the development of new chemotherapy agents and targeted therapeutic agents, chemotherapy can convert a considerable portion of initially unresectable CRLM into resectable disease, which is called conversion chemotherapy[7,48-50]. It was first reported in 1996 by Bismuth *et al*[49] that preoperative chemotherapy, using oxaliplatin plus 5-FU/LV, enabled 16% (53/330) of initially unresectable CRLM patients to gain the chance of undergoing liver resection with a 5-year OS of 40%. In 2001, Adam *et al*[23] reported that 13.6% (95/701) of initially unresectable CRLM patients underwent liver metastases resection after systemic chemotherapy and achieved a 5-year OS of 34%. Intensified chemotherapy such as FOLFOXIRI (i.e., oxaliplatin, 5-FU/LV and irinotecan) has been shown to have high response and conversion rates (19%)[50]; however, it has not been generally recommended thus far due to its considerable toxicity. In recent years, the addition of targeted agents such as cetuximab to chemotherapy has been shown to further improve the conversion rate to 30%-40%[51]. In the CELIM study[51], 106 patients with initially unresectable CRLM underwent cetuximab plus FOLFOX6 or cetuximab plus FOLFIRI and achieved an objective response rate of 68% and 57%, a liver resection rate of 40% and 38%, and a R0 liver resection rate of 38% and 30%, respectively.

Portal vein embolization and two-stage operation

Preserving at least 20% of future liver remnant is a major obstacle when performing an extended hemihepatectomy for extensive liver metastases. In this situation, Portal vein embolization (PVE) can be helpful to induce hypertrophy of the contralateral liver to fulfill the minimal liver volume requirement[52]. Generally, PVE is usually used before extended right hepatectomy and is seldom used for extended left hepatectomy because the right posterior sector generally provides > 30% of the liver volume. Even after preoperative chemotherapy or PVE, some patients cannot become eligible for complete CRLM resection through a single hepatectomy. PVE combined with a two-stage resection may be helpful in such circumstances. In 2000, Adam *et al*[53] first proposed the two-stage resection strategy when they reported the initial results from 13 patients undergoing two-stage hepatectomy with a 3-year survival rate of 35%. An updated result of a 5-year OS of 42% in 41 patients receiving two-staged resection was reported in 2008[54]. However, > 30% (18/59) of patients could not complete the second hepatectomy, mostly because of disease progression ($n = 17$). Additionally, the second hepatectomy has a significant higher postoperative mortality (7%) and morbidity (59%) than the first hepatectomy (0% and 20%, respectively).

Ablation therapy

The most commonly used approach for ablation therapy is radiofrequency ablation (RFA). Most previous studies have indicated that RFA is inferior to liver resection for CRLM with a high local recurrence rate[55]. However, for patients who cannot undergo liver resection because of extensive liver metastases and inadequate remnant liver volume, RFA can play an important role when combined with liver resection. RFA is generally recommended for CRLM less than 3 cm[56-64]. The local recurrence rate after RFA increases with tumor size in liver lesions > 3 cm[65]. Several studies have reported a significantly higher local

failure rate after ablation for tumors > 5 cm when compared to those 3cm-5 cm[64,66].

There are three approaches for RFA, including percutaneous, open and laparoscopic. Ablation through the open approach seems to be superior to the percutaneous or laparoscopic methods in terms of local failure rate[57,67,68]. However, the reported local recurrence rate of each approach has actually varied and overlapped each other in a range of 6%-40% in different studies[69-77].

DEFINITELY UNRESECTABLE CRLMs

Necessity of primary tumor resection

For patients with incurable metastatic colorectal cancer who have symptoms related to intestinal obstruction, perforation or intractable bleeding, palliative primary tumor resection is generally required and advocated. However, for asymptomatic patients with unresectable metastases, the value of primary tumor resection is still questionable. Early studies have indicated that primary tumor resection may have potential benefits in preventing tumor-related symptoms such as obstruction, which may require emergency operations with a high risk of surgical mortality[78-80]. However, this opinion may become outdated with the application of new efficient chemotherapy agents that have the ability to control intestinal symptoms well. Therefore, the US National Comprehensive Cancer Network guidelines recommend that colon resection should be considered only for impending obstruction risk or intractable bleeding. It is estimated that only 20%-30% of metastatic colorectal cancer patients are eligible for curative resection. Nevertheless, data from the US Surveillance, Epidemiology, and End Results (SEER) database have demonstrated that 66% of stage IV colorectal cancer patients received primary tumor resection[81]. In another study based on 9000 elderly metastatic colorectal cancer patients, 72% underwent primary tumor resection, whereas only 3.9% received metastasectomy and 20% had symptoms of bowel obstruction, perforation or bleeding[82]. It suggests that a considerable portion of incurable colorectal cancer patients receive intestinal resection without a clear and reasonable indication. The Memorial Sloan-Kettering Cancer Center reported 233 metastatic colorectal cancer patients receiving chemotherapy with the primary tumor left in place[83]. Only 7% of the patients required palliative primary tumor resection during the disease course. Thus, the authors recommended chemotherapy without prophylactic primary tumor resection as a standard management of metastatic colorectal cancer without obstruction or bleeding symptoms.

Targeted therapy in combination with chemotherapy

The survival benefit of adding targeted therapeutic agents such as bevacizumab, cetuximab and panitumumab to traditional chemotherapy in patients with unresectable metastatic colorectal cancer has been validated by several randomized clinical trials. The BEAT study[84] collected 1965 metastatic colorectal cancer patients undergoing bevacizumab combined with different types of chemotherapy as the first-line therapy, and demonstrated that the PFS in patients receiving bevacizumab plus FOLFIRI, FOLOFOX or Xelox was > 10 mo and the OS approached or exceeded 24 mo. This study indicated that bevacizumab-based combination chemotherapy is efficient in metastatic colorectal cancer. The PFS and OS were 8.6 and 18.0 mo, respectively, in patients receiving bevacizumab plus 5-FU, which is also comparable to a regimen including 5-FU plus oxaliplatin or 5-FU plus irinotecan.

The efficacy of cetuximab greatly depends on the status of the *KRAS* gene. The CRYSTAL study[85], which compared cetuximab plus FOLFIRI with FOLFIRI alone in the initial treatment of metastatic colorectal cancer patients, indicated that cetuximab improved the response rate (57.3% *vs* 39.7%, $P < 0.0001$), PFS (9.9 mo *vs* 8.4 mo, $P = 0.0012$) and OS (23.5 mo *vs* 20.0 mo, $P = 0.0094$) significantly in patients with wild-type KRAS. However, in a population subset with mutant KRAS, there was no significant difference in the response rate, PFS or OS between the two groups. The OPUS study[86] even exhibited a worse response rate and PFS for the cetuximab plus FOLFOX4 group as compared with FOLFOX4 group in patients with mutant *KRAS*. The status of the *BRAF* gene is another efficient predictor of cetuximab efficacy. Di Nicolantonio *et al*[87] have reported that the *BRAF* gene mutation rate in patients with wild-type *KRAS* was approximately 14% (11/79). None of the *BRAF* mutant patients responded to cetuximab or panitumumab or cetuximab plus chemotherapy. The PFS of patients with wild-type *BRAF* was better than their counterparts ($P < 0.001$). A considerable defect in the *BRAF* gene as a predictor of treatment response is that the mutation rate is low. The incidence rate of mutant *BRAF* is only 6.4%-14% in patients with wild-type *KRAS*, and no *BRAF* mutation has been reported in those with *KRAS* mutations.

In the second or third-line treatment settings, the addition of cetuximab or bevacizumab to chemotherapy, or a single treatment with cetuximab, has also been proven to be effective[88,89]. In a phase Ⅲ clinical trial[90], 463 metastatic colorectal cancer patients received either panitumumab plus best supportive care (BSC) or BSC alone after chemotherapy failure. Patients with panitumumab plus BSC had an objective response rate of 8% and a significantly better median PFS (96 d *vs* 60 d) than those who received BSC alone. In another clinical trial[91], the combination of panitumumab with FOLFIRI as a second treatment for metastatic colorectal cancer patients improved the PFS (5.9 mo *vs* 3.9 mo, $P = 0.004$) and objective response rate (35% *vs* 10%, $P < 0.001$) in patients with wild-type *KRAS* compared to the regimen of FOLFIRI alone.

CONCLUSION

Multidisciplinary treatment has become the standard practice for CRLM management. Nevertheless, the optimal paradigm of multimodality treatment still needs to be further investigated. As the most effective treatment

method, surgical CRLM resection has been rendered an expanded indication in recent years. CRLM patients can be categorized into three subtypes: clearly initially resectable, potentially resectable, or definitely unresectable. For patients with initially resectable CRLM, the survival benefit of neoadjuvant chemotherapy is still unclear. The management of CRLM disappearing or progressing during neoadjuvant chemotherapy is challenging and controversial. The influence of chemotherapy-related toxicity on the outcome of liver resection needs to be further clarified. The optimal sequencing of primary tumor resection and liver lesions and perioperative chemotherapy deserves further investigation in patients with resectable synchronous CRLM. For patients who are initially unresectable but potentially convertible, chemotherapy, PVE, two-staged operation and ablation therapy are effective methods to convert unresectability into resectability. How to utilize these methods in a reasonable and better way needs to be further explored. For definitely unresectable CRLMs, it is still being debated whether the primary tumor should be resected. Targeted therapy, in addition to traditional chemotherapy, has been shown to improve the survival of unresectable CRLM patients. How to accurately predict the tumor response to targeted therapy is an important issue that should be further investigated in consideration of its high cost. A better understanding of these issues will greatly improve the effect of multidisciplinary treatment of CRLM patients.

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S- Editor Sun H **L- Editor** Kerr C **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i1.25

World J Gastroenterol 2012 January 7; 18(1): 25-33
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

San-Jun Cai, MD, Professor, Director, *Series Editor*

An update on chemotherapy of colorectal liver metastases

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Received: March 31, 2011 Revised: June 24, 2011
Accepted: July 2, 2011
Published online: January 7, 2012

Abstract

Surgical resection of liver metastases of colorectal cancer greatly improves the clinical outcome of patients with advanced disease. Developments in chemotherapeutic agents and strategies bring hope of a cure to patients with initially unresectable colorectal liver metastases (CLM). Perioperative chemotherapy significantly improves the survival time of patients who receive curative-intent hepatectomy. Even for unresectable CLM, recent studies demonstrated that active preoperative chemotherapy could achieve shrinkage of liver metastasis and thus render some for resection. Furthermore, an increase in tumor resection rate and prolonged survival time among patients with CLM has been observed following the application of monoclonal antibodies in recent years. However, the value of chemotherapy *via* hepatic arterial infusion is still unclear. More trials should be conducted in patients with CLM in order to improve survival.

Key words: Chemotherapy; Colorectal cancer; Liver metastases; Resection rate; Targeted agents

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Wang CC, Li J. An update on chemotherapy of colorectal liver metastases. *World J Gastroenterol* 2012; 18(1): 25-33 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i1/25.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i1.25

INTRODUCTION

Colorectal carcinoma is one of the most common causes of cancer-related mortalities in both China and the Western world. Almost 50% of patients with colorectal cancer will eventually develop liver metastases during the natural course of the disease and 25% of patients present with liver metastases at the time of diagnosis. One third of patients with liver metastases have an isolated metastatic site limited to the liver, and the survival of this specific population is directly related to the progression of the hepatic lesions[1]. The management of patients with untreated colorectal liver metastases (CLM) remains a common clinical challenge as previous studies reported a median survival time of 4 mo[2].

The treatment goal for patients with limited CLM is to remove all evidence of disease for better long-term survival or even cure. Historically, only a minority of patients (10%-15%) are considered candidates for resection with overall 5-year survival rates ranging between 25% and 40%[3]. Significant advances have been achieved in the management of CLM in recent years, with improvement in the precision of cross-sectional imaging, surgical techniques, locoregional therapeutic options, and the availability of newer effective chemotherapeutic agents. A multimodality treatment approach for patients with resectable CLM results in more patients being considered for resection and better outcome has been noted, where resectability rate increased to 20%-30%, 5-year survival rate increased to 50%, and 25% of patients survived for not less than 10 years[4,5]. For the time being, although there are still many incurable cases in the most advanced stage, the course of progression can be greatly slowed by multimodality treatment encompassing surgery, chemo-

therapy and interventional locoregional therapy.

Even though surgical resection remains the mainstay of potentially curative therapy, the role of systemic preoperative chemotherapy has been gradually recognized. Standard chemotherapy regimens comprising 5-fluorouracil (5-FU) plus leucovorin (LV) in combination with irinotecan (FOLFIRI) or oxaliplatin (FOLFOX) have been reported to facilitate a resection rate of 9%-40% among patients with initially unresectable CLM[6,7]. Perioperative chemotherapy for those with resectable liver lesions confers a potential survival advantage. Moreover, mounting evidence suggests that the addition of targeted agents or a third cytotoxic agent might be even more effective[8].

In this review, we will focus mainly on updates in systemic chemotherapy for CLM, and a short discussion on regional interventional chemotherapy will also be presented.

SYSTEMIC POSTOPERATIVE CHEMOTHERAPY FOR RESECTABLE LIVER METASTASES

Currently, postoperative systemic chemotherapy for resectable CLM, a common practice, carries the same goal as that for stage Ⅲ colorectal cancer to effectively enhance the local disease control rate. However, in contrast to the well-established benefit noted for adjuvant chemotherapy for stage Ⅲ colorectal cancer, there has been few high quality randomized studies to formally evaluate the benefits of adjuvant chemotherapy for CLM, despite the fact that improved survival and reduced recurrence rates have been demonstrated in retrospective studies[9]. In a multicenter, phase Ⅲ Fédération Francophone de la Cancérologie Digestive Association Française de Chirurgie Hépato-Biliaire et de Transplantation Hépatique Association Universitaire de Recherche en Chirurgie (FFCD ACHBTH AURC) 9002 trial, 173 patients who had undergone R0 resection were randomized to surgery followed by bolus 5-FU/LV for 6 mo with interval days of 10-35 or surgery alone[10]. The 5-year disease-free survival (DFS) rates were 33.5% and 26.7% respectively (P = 0.028), suggesting a positive effect of chemotherapy after surgery. There was a trend toward increased 5-year overall survival (OS) in patients who received chemotherapy without statistical significance (51.1% *vs* 41.9%, P = 0.13). The study may have been statistically underpowered to detect a true difference in OS as a result of early termination of accrual due to low accrual rates. An another trial [European Organisation for Research and Treatment of Cancer (EORTC)/National Cancer Institute of Canada Clinical Trials Group (NCIC CTG)/Gruppo Interdisciplinare Valutazione Interventi in Oncologia (GIVIO) trial] with a similar design, also closed prematurely due to slow accrual, but showed a trend towards improved progression-free survival (PFS) and OS in the chemotherapy group. Multivariate analysis identified adjuvant chemotherapy as a significant independent prognostic factor even though between-group comparison was insignificant[11]. Some large United States and European retrospective analyses further suggested an urgent need for patients with recurrent disease to receive adjuvant chemotherapy and showed a better survival in resected CLM patients who received adjuvant therapy[12,13].

The choice of regimen is the key to the success of chemotherapy after tumor resection. The 5-FU/LV regimen is less commonly used nowadays, but the efficacy of combining 5-FU with oxaliplatin or irinotecan as postoperative chemotherapy for patients with resectable CLM remains to be elucidated. A randomized phase Ⅲ study comparing adjuvant 5-FU/LV with FOLFIRI in patients following complete resection of CLM reported a median DFS of 24.7 mo and 21.6 mo for FOLFIRI and 5-FU/LV, respectively, with no significant differences noted for DFS and OS, however, a trend in favor of improved DFS in patients treated with FOLFIRI could not be excluded[14]. At present, evidence to support significant additional benefit using combination therapies for resectable CLM has not been established. Thus, the use of postoperative therapy is individualized based on local practice as well-established data from clinical trials are not yet available. The expert panel of the European Colorectal Metastases Treatment Group recommends that systemic chemotherapy following liver resection should be considered as an option for patients with resected CLM, particularly for those patients who did not receive preoperative chemotherapy[8].

PREOPERATIVE CHEMOTHERAPY FOR RESECTABLE CLM

Rising enthusiasm for the role of perioperative chemotherapy in cases of operable carcinoma originating from the digestive system has been noted, including those with CLM. Convincing benefits of preoperative chemotherapy on long-term survival in patients with CLM is still not well-established, but it is gradually being accepted as the rationale to improve PFS and reduce recurrence rates[15]. A ten-year study on survival and recurrence after neoadjuvant chemotherapy followed by resection of liver metastases showed that the 1-, 3- and 5-year OS reached 90%, 59.2% and 46.1%, respectively and DFS at 1, 3 and 5 years was 68.1%, 34.8% and 27.9%, respectively. In addition, preoperative chemotherapy followed by liver metastases resection is associated with improved survival, low cancer involvement in resection margins and re-resection rates[16]. In 2008, Nordlinger *et al*[17] published the final results of the EORTC 40983 study, which compared perioperative chemotherapy with oxaliplatin, fluorouracil, and folinic acid (FOLFOX4) regimen to surgery alone in patients with resectable CLM. Patients were randomly assigned to six cycles of neoadjuvant FOLFOX4 before and after surgery (n = 182) or to surgery alone (n = 182). The 3-year PFS was improved from 28.1% for the surgery-alone group to 36.2% for the perioperative FOLFOX4 group, an increase of 8.1% [hazard ratio (HR) = 0.77; P = 0.041] for all eligible patients and 9.2% (HR = 0.73; P = 0.025) for all resected patients. Additional reports on the application of neoadjuvant chemotherapy came from a few prospective single-center clinical tri-

als[18,19]. In one trial, 50 patients with resectable liver metastases received neoadjuvant capecitabine plus oxaliplatin (XELOX) or FOLFOX4 for six cycles (3 mo) prior to surgical resection[20]. The results suggest that preoperative oxaliplatin-based chemotherapy provides high response rates (RRs) without increased risk of perioperative morbidity. The recurrence-free survival was significantly influenced by tumor response to neoadjuvant chemotherapy, which may identify the best candidates for a potentially curative treatment.

Perioperative chemotherapy with FOLFOX4 may be a possible treatment option for patients with resectable CLM, as prolonged PFS has been noted as mentioned above. Nevertheless, not all patients can be cured *via* surgery with perioperative chemotherapy. 7% of patients in the chemotherapy group in the EORTC 40 983 trial experienced disease progression after receiving 3-4 cycles of chemotherapy. More active regimens should be tried to provide better results[17]. A recent non-randomized trial revealed that the objective response following preoperative chemotherapy of XELOX plus bevacizumab was 73% for a cohort of 56 patients, the treatment being safely administered until 5 wk prior to surgery in patients with resectable CLM without increasing postoperative complications[19]. Liver regeneration was not affected by preoperative bevacizumab. A large randomized clinical trial to evaluate the efficacy of bevacizumab combined with preoperative chemotherapy would better assess the efficacy of this preoperative regimen in patients with resectable CLM. Another study is currently ongoing in Britain to determine if the combination of cetuximab with perioperative chemotherapy could contribute to better survival.

At present time, no mature data is available to support the combination of FOLFOX6, antibodies targeting both vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) in the perioperative setting. Therefore, a large multi-center EORTC 40051 BOS (Biologics, Oxaliplatin and Surgery) trial was launched to evaluate the combination of oxaliplatin-based chemotherapy plus cetuximab with or without bevacizumab in a preoperative (6 cycles) and postoperative (6 cycles) setting in patients with resectable CLM. Patients could have up to ten liver metastases, and up to two pulmonary metastases. The primary endpoints of the BOS trial are preoperative RR and safety. However, based on the disappointing results from the Panitumumab Advanced Colorectal Cancer Evaluation (PACCE) study where the combination of bevacizumab, panitumumab with chemotherapy was first-line therapy for advanced colorectal cancer[20], as well as the discouraging reports from combination chemotherapy with bevacizumab-cetuximab in the CApecitabine, IRinotecan, and Oxaliplatin (CAIRO) 2 study[21], this study is temporarily on hold.

CONVERSION CHEMOTHERAPY FOR INITIALLY UNRESECTABLE LIVER METASTASES

Approximately 80%-90% of patients with metastatic colorectal cancer (mCRC) referred to specialist centers have unresectable metastatic liver disease at presentation[22]. The role of chemotherapy in these patient populations is to downstage the liver lesions in an attempt to convert their disease from unresectable to resectable, while the goal of treatment for patients with little possibility of conversion to resectable disease is to prolong survival and improve quality of life.

Actually, it is more reasonable to use the term "conversion chemotherapy" than true neoadjuvant therapy[23]. The combination of 5-FU/LV with either irinotecan or oxaliplatin, or a triple cytotoxic drug combination such as fluorouracil, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI), with or without additional targeted agents has also been used as a preoperative strategy to achieve higher resection rates and a better clinical outcome. In a series of 44 patients with initially unresectable CLM, Alberts *et al*[24] reported their results with FOLFOX4 chemotherapy. Periodical reassessment for resectability with a high clinical RR of 60% was observed, consistent with other studies assessing the activity of FOLFOX4 as first-line therapy for colorectal cancer patients with isolated liver metastases, and 40% of these patients were able to undergo complete resection of their residual cancer. The efficacy of FOLFIRI as preoperative chemotherapy has also been demonstrated in terms of both high resection rate and favorable survival times[25-27]. In 2008, a major systematic review on irinotecan and oxaliplatin for the treatment of advanced colorectal cancer published by the United Kingdom Health Technology Assessment Agency evaluated all studies where irinotecan or oxaliplatin were combined with 5-FU to downstage patients with unresectable CLM[27]. The reported resection rates ranged from 9% to 35% for patients receiving irinotecan and 5-FU, while that for those receiving oxaliplatin and 5-FU ranged from 7% to 51%. There is no conclusive evidence that one is superior to the other as first-line therapy for the downstaging of CLM in terms of PFS and OS.

Current practice for patients whose metastases may be rendered resectable by conversion chemotherapy is to treat with the most effective regimen that offers a high RR in terms of resection rate and PFS, coupled with the recommendation that surgery should be conducted as early as possible to minimize chemical damage to the liver[6]. The effectiveness of FOLFIRI/FOLFOX draws the attention of adding a third cytotoxic drug to these regimens as initial chemotherapy in patients with CLM with good performance status for potential surgical intervention. Falcone *et al*[28] reported a phase Ⅲ randomized trial comparing FOLFOXIRI with a standard infusional fluorouracil, leucovorin, and irinotecan (FOLFIRI) regimen. Of a total of 244 patients with initially unresectable mCRC, an improved RR was achieved in the FOLFOXIRI arm (60% *vs* 34%, $P < 0.0001$). The R0 resection rate of metastases was greater in the FOLFOXIRI arm (15% *vs* 6%, $P = 0.033$, among all patients; and 36% *vs* 12%, $P = 0.017$ among patients with liver metastases only). PFS and OS were both significantly improved in the FOLFOXIRI arm (median PFS, 9.8 *vs* 6.9 mo, $P = 0.0006$;

median OS, 22.6 mo *vs* 16.7 mo, $P = 0.032$). A more recent long-term follow-up indicated that this regimen was associated with an increased RR (70.4%). The 5-year PFS for these patients was 16% and the 5-year and 8-year OS were 42% and 33%, respectively[29]. Another group demonstrated that chemotherapy with FOLFIRINOX (the same agents with FOLFOXIRI) shows a high RR (70.6%) in CLM, and further confirmed the high RR of this regimen. The rate of R0 hepatic resection in patients with initially unresectable liver metastases is attractive (26.5%)[30]. The randomized phase Ⅱ METHEP trial was conducted to compare standard double-agent chemotherapy with triple-agent intensified chemotherapy in patients with unresectable CLM. Various induction regimens including FOLFIRI, FOLFOX-4, high dose-FOLFIRI, FOLFOX-7, and FOLFIRINOX were evaluated. In the preliminary analysis after 4 cycles of treatment, the most promising regimens appeared to be FOLFIRINOX and high dose-FOLFIRI with an objective RR of 52% and 50%, respectively. Secondary resection rates of metastases were also highest in the high dose-FOLFIRI and FOLFIRINOX arms (37% and 36%, respectively). The safety profiles of FOLFIRINOX and FOLFOXIRI are generally acceptable[31].

For patients with initially unresectable CLM, disease progression during preoperative chemotherapy predicts poor prognosis, for which a change to other alternative chemotherapy regimens is usually recommended[32]. Liver resection could be reconsidered if the response evaluation shows response or stabilization after second-line chemotherapy. However, objective response rates (ORR) to second-line preoperative chemotherapy are only 4%-28%[33], and the safety of surgery in patients who received multiple lines of chemotherapy is still to be confirmed in larger series of patients. Hence, tumor progression during chemotherapy is considered a contraindication to CLM resection by most oncologists and surgery is seldom performed if patients fail first-line chemotherapy. In a recent prospective study which included the largest study population to evaluate the outcome of patients undergoing resection of CLM after a second-line chemotherapy regimen, a retrospective analysis by Brouquet *et al*[34] demonstrated that hepatectomy is safe and feasible, and associated with a modest survival benefit in these patients who present with advanced CLM who have a suboptimal response to systemic therapy, with 1-year, 3-year, and 5-year OS rates of 83%, 41%, and 22%, respectively. Although a 37% objective radiographic RR still illustrates the challenges in obtaining a tumor response with second-line chemotherapy, this rate remains acceptable compared to data reported previously[35]. This study indicated that liver resection could be considered an appropriate alternative for selected patients following second-line therapy. At the same time, due to the fact that only a few patients receiving second-line chemotherapy will benefit from resection, further investigations should be conducted to define the assessment criteria to identify potential surgical candidates in this challenging therapeutic setting.

TARGETED COMBINATION THERAPY FOR UNRESECTABLE MCRC

Abundant data are emerging from randomized trials of the added benefits conferred by the targeted agents on the prognosis of patients with unresectable mCRC, including those with CLM. Optimistic results from the randomized Cetuximab combined with iRinotecan in first line therapY for metaSTatic colorectAL cancer (CRYSTAL) trial[36] and OxaliPlatin and cetUximab in firSt-line treatment of mCRC (OPUS) trial[37] reinforce the role of cetuximab on the improvement of RRs and resection rates, combined with standard first-line chemotherapy in patients with advanced CRC. According to the published report of the CRYSTAL study, the addition of cetuximab to FOLFIRI significantly reduced the risk of progression (HR = 0.85; $P = 0.048$) and increased the ORR (HR = 1.4; $P = 0.004$), compared with FOLFIRI alone. The rate of R0 resection of secondary metastases was also slightly higher in the cetuximab-FOLFIRI arm ($P = 0.003$). Retrospective analysis suggested that the benefits of cetuximab were limited to patients with *KRAS* wild-type tumors[38]. The OPUS study which compared cetuximab plus FOLFOX to FOLFOX alone obtained similar results[37]. An update of a re-analysis of the CRYSTAL trial showed that greater benefits from the combination with cetuximab would be derived in patients with both wild-type *KRAS* and wild-type *BRAF*[39]. More patients with CLM would be rendered resectable with effective preoperative therapy, as the addition of cetuximab to chemotherapy is feasible in first-line therapy, which has been confirmed in a randomized phase Ⅱ multi-center study of cetuximab plus FOLFOX6 or cetuximab plus FOLFIRI in the preoperative setting for unresectable CLM (the CELIM study) published recently by Folprecht *et al*[40]. Partial or complete response was noted in 68% of 53 patients in the cetuximab plus FOLFOX6 arm, and 57% of 53 patients in the cetuximab plus FOLFIRI arm. In a combined analysis of both arms, 70% of patients with wild-type *KRAS* tumors achieved tumor response *versus* 42% of patients with mutated *KRAS* (OR = 3.42; $P = 0.008$). The R0 resection rates were as high as 34% in patients with wild-type *KRAS*. In retrospect, resectability rates increased from 32% at baseline to 60% after chemotherapy ($P < 0.0001$). It is concluded that cetuximab may increase the possibility of resection for patients with initially unresectable liver metastases and shows a high efficacy in the conversion treatment of CLM when combined with first-line chemotherapy. For patients with unresectable CLM refractory to conventional first-line chemotherapy, combination therapy with cetuximab could also increase resectability rates without increasing surgical mortality or liver injury[41]. A similar effective increase in RR was shown when cetuximab was added to either irinotecan- or oxaliplatin-based combinations[42-44]. However, the latest results from two randomized phase Ⅲ studies unexpectedly questioned the benefit of adding cetuximab to oxaliplatin-based combination chemotherapy[45-47]. In the MRC COIN study, 1394 patients received

oxaliplatin combination (CAPOX/FOLFOX) as standard chemotherapy with or without cetuximab. Analysis according to *KRAS* status did not result in any difference in either OS (median OS 17.9 mo *vs* 17.0 mo, $P = 0.68$) or PFS (median PFS 8.6 mo *vs* 8.6 mo, $P = 0.60$) between patients treated with CAPOX/FOLFOX and CAPOX/FOLFOX plus cetuximab, even in the *KRAS* wild-type group[45]. Only a small benefit was seen in the RR in the *KRAS* wild-type patients who received cetuximab combination therapy (59% *vs* 50%, $P = 0.02$). Further subgroup analysis reported at ASCO 2010 suggested that an interaction existed between the chemotherapy option, (CAPOX *vs* FOLFOX) and a positive effect on PFS was observed with cetuximab ($P = 0.07$), indicating a benefit from cetuximab in FOLFOX-treated patients, but not in CAPOX-treated patients. Currently, cetuximab is not recommended for combination therapies with capecitabine and oxaliplatin based on these data. Similarly, the NORDIC Ⅶ study, with 566 patients randomly assigned to receive 5-FU plus LV plus oxaliplatin (FLOX), FLOX plus cetuximab until disease progression, or FLOX intermittently plus continuous cetuximab, reported a negative result, demonstrating no added benefit when cetuximab was combined with oxaliplatin-based chemotherapy[47]. In the intent-to-treat population analysis, there were no statistically significant differences between the treatment groups in terms of RR, PFS or OS. Furthermore, the lack of benefit was also noted in both *KRAS* mutant and wild-type sub-groups, suggesting that *KRAS* status did not predict the effect of cetuximab in combination with FLOX in this study. The results from these two studies do not support the use of cetuximab in first-line therapy when given together with oxaliplatin-based regimens. Thus, caution should be taken when making decisions on combined chemotherapy regimens as the role of anti-EGFR agents in the first-line treatment of mCRC needs to be explored further.

In addition, cetuximab combined with triple cytotoxic drug therapy is also being evaluated, for potential extra efficacy on RR and clinical outcome[48,49]. Definitive results from the preoperative chemotherapy for hepatic resection (POCHER) study revealed an RR of 79% and a complete resection rate of 63% achieved by FOLFOXIRI plus cetuximab[48]. Preliminary results of another phase Ⅱ trial evaluating cetuximab in combination with FOLFIRINOX demonstrated an ORR as high as 82% and predicted the feasibility of this new therapeutic combination in first-line mCRC patients[49].

KRAS had been broadly accepted as a predictive factor of anti-EGFR antibody therapies prior to the negative results of the NORDIC Ⅶ study, and identification of additional predictors such as *BRAF* has attracted significant interest. A recent meta-analysis based on the CRYSTAL and OPUS trials reported the updated clinical efficacy of cetuximab combination therapy according to *KRAS* and *BRAF* mutation status[50]. This analysis confirmed that in *KRAS* wild-type patients, the addition of cetuximab in first-line treatment achieves a statistically significant improvement in RR, PFS, and OS compared with chemotherapy alone. However, it also concluded that *BRAF* mutation status does not appear to be a strong predictive biomarker for the addition of cetuximab. *BRAF* is more likely to be a prognostic factor as the clinical outcome in *BRAF*-mutant patients is worse than those with *BRAF* wild-type tumors in terms of RR, PFS and OS. Thus, *BRAF* testing is probably not essential when deciding whether cetuximab should be used. Larger clinical trials to further investigate the field of predictive molecular biomarkers are required since the present data are inconsistent.

The role of bevacizumab added to chemotherapy in the perioperative setting for initially unresectable metastases was evaluated in two large multi-center prospective trials (First BEAT and NO16966)[51]. The First BEAT trial reported a 6% R0 hepatic resection in an unselected population and 12.1% among patients with isolated liver metastases only. Resection rates were highest in patients who received oxaliplatin-based combination chemotherapy ($P = 0.002$). In NO16966[52], the addition of bevacizumab to XELOX/FOLFOX significantly improved PFS in the first-line therapy (9.4 mo *vs* 8.0 mo, $P = 0.0023$), but there were no statistically significant differences between resection rates or OS in patients treated with bevacizumab plus XELOX/FOLFOX *vs* placebo (6.3% *vs* 4.9%, $P = 0.24$). Bevacizumab improved RR when added to FOLFIRI regimen but did not improve RRs and resection rates when added to XELOX or FOLFOX. Recent data from a small phase Ⅱ trial by the GONO group revealed that FOLFOXIRI plus bevacizumab yielded an ORR of 76% and a disease control rate of 100%, with a secondary resection of metastases in 17% of patients[53]. It seems that the addition of bevacizumab to FOLFOXIRI regimen is effective with manageable toxicities, however, negative reports on its efficacy in heavily pretreated patients with advanced disease and its role as adjuvant therapy for stage Ⅲ colon cancer in the NSABPC-08 study remind us to be cautious of the optimal stage to start administration and to determine the best treatment sequence[54,55].

Results from the PACCE study[20] and CAIRO2 study[21] failed to demonstrate a biological synergistic effect in antibodies both against the EGFR (cetuximab or panitumumab) and VEGF (bevacizumab). Thus, specific combinations of targeted drugs are not recommended as first-line therapy for patients with mCRC, including CLM. The ongoing CALGB/SWOG 80404 trial which compared the addition of cetuximab, or bevacizumab or both to standard FOLFIRI/FOLFOX should help to define the preferred targeted partner primarily in terms of OS. The RR, PFS and the resection rate will be secondary end points[56].

LOCOREGIONAL CHEMOTHERAPY

Patients with multifocal CLM who are unfit for surgery or have tumor distribution technically unresectable with clear margins, are potential candidates for regional liver chemotherapy. Hepatic arterial infusion (HAI) with chemothera-

peutic agents can provide relatively high concentrations of drugs to micro- or macro-metastases remaining in the liver with less toxicity to extrahepatic organs. The most commonly used agent for HAI is Floxuridine (FUDR) which is an analogue of 5-FU, and has the advantage of having a first-pass extraction rate of over 94% within the liver[57]. For those whose liver metastases were initially unresectable, the use of HAI as pre-operative conversion therapy to downstage the disease for resection was recommended in some early studies, due to the efficacy results obtained[58-60]. A recent Cochrane meta-analysis of ten randomized trials which compared HAI with fluoropyrimidine chemotherapy to systemic chemotherapy or best supportive care in patients with initially unresectable liver metastases suggested that administration of fluoropyrimidines through HAI yielded higher tumor RRs as compared to the systematic chemotherapy regimens (42.9% *vs* 18.4%, $P < 0.0001$). However, this anticancer activity does not translate into a significant survival advantage for patients treated with HAI as compared to those given systemic chemotherapy (15.9 mo *vs* 12.4 mo)[61]. Only one out of ten studies indicated that HAI with 5-FU was superior to systemic bolus 5-FU/LV in terms of RR and survival[62]. Altogether, current data do not support the clinical or investigational use of fluoropyrimidine-based HAI alone in patients with initially unresectable liver metastases. The advantages of systemic oxaliplatin or irinotecan-based chemotherapy over the 5-FU/LV regimen also guided the use of these agents in HAI chemotherapy. Encouraging results were obtained in patients with initially unresectable metastases, with RRs as high as 55%-70% and resection rates of approximately 16%-18% in unresectable liver metastases[63,64].

HAI as post-operative chemotherapy was also investigated in some clinical trials for feasibility in CLM. A Cochrane meta-analysis performed on seven randomized trials with a total of 592 patients did not show improvement on OS in the HAI group even though fewer recurrences were noted in the remaining liver[65]. As early as 1999, Kemeny *et al*[66] reported the results from a single-institution study in which 156 patients were randomized to post-operative HAI with FUDR plus systemic 5-FU ± LV *vs* systemic therapy alone. An increase in two-year survival rate for the combination therapy group was observed (90% *vs* 60%, $P < 0.001$) as compared with the group receiving monotherapy. The liver relapse-free survival also significantly increased in the combination therapy group. Furthermore, an updated analysis with a median follow-up of 10.3 years reports a significantly greater PFS rate (31.3 mo *vs* 17.2 mo, $P = 0.02$) and a trend toward improved OS (68.8 mo *vs* 58.8 mo, $P = 0.10$) in the combined therapy group compared to the monotherapy group[67]. Other similar randomized studies also suggested an improved PFS of the liver in the HAI combination group compared to the control group, but none of these studies showed any advantage in OS and long-term DFS[68,69].

Negative outcomes in terms of OS and significant hepatobiliary toxicity related to HAI as well as the expertise required limit the implementation of this technique. Given the availability of an increasing number of active systemic chemotherapy regimens, especially the biologic agents, the value of HAI chemotherapy is less clear.

CONCLUSION

Surgical resection undoubtedly remains the gold standard for the treatment of resectable CLM. A well coordinated multidisciplinary approach is also necessary to achieve optimal outcomes for patients with CLM. The modality of perioperative chemotherapy over surgery alone has resulted in more patients with initially unresectable metastases receiving a complete resection and enjoying a prolonged survival after surgery. Emerging data has revealed that preoperative chemotherapy, as well as postoperative chemotherapy could be advantageous compared to surgery alone in terms of DFS for patients with resectable CLM. Newly emerging biologic targeted agents when added to the standard chemotherapy regimen have contributed to increased tumor RR, and to a larger extent, higher secondary resection rates. Insight into the molecular markers to predict the outcome of targeted therapy may define subgroups of patients within the same stage.

At present, there is insufficient evidence to demonstrate the efficacy of regional perioperative chemotherapy. Multi-center randomized prospective trials are needed to provide evidence of a survival advantage of regional perioperative chemotherapy with acceptable adverse effects.

Even though the intent of preoperative therapy followed by resection is probably curative, cure is rarely achieved, as the majority of patients who undergo hepatic resection will experience recurrence. More potent agents and strategies have to be developed to provide longer survival time and eventually cure this disease.

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S- Editor Sun H **L- Editor** Webster JR **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i1.34

World J Gastroenterol 2012 January 7; 18(1): 34-43
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Increased numbers of Foxp3-positive regulatory T cells in gastritis, peptic ulcer and gastric adenocarcinoma

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Supported by Grants from National Science Council, No. NSC98-2313-B-007-005-MY3, NSC98-3112-B-007-004 and NSC98-2627-B-007-013; partly from Boost Grant of National Tsing Hua University, Taiwan
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Received: March 4, 2011 Revised: June 20, 2011
Accepted: June 27, 2011
Published online: January 7, 2012

Abstract

AIM: To determine the number of regulatory T cells (Tregs) in gastric mucosa of patients with gastritis, peptic ulcers and gastric cancer.

METHODS: This study was a retrospective analysis of gastric antrum biopsy specimens from healthy controls (n = 22) and patients with gastritis (n = 30), peptic ulcer (n = 83), or gastric cancer (n = 32). Expression of CD4, CD25 and Foxp3 was determined by immunohistochemistry in three consecutive sections per sample.

RESULTS: Compared with healthy controls, there was an increased number of $CD25^+$ and $Foxp3^+$ cells in patients with gastritis (P = 0.004 and P = 0.008), peptic ulcer (P < 0.001 and P < 0.001), and gastric cancer (P < 0.001 and P < 0.001). The ratio of $CD25^+/CD4^+$ or $Foxp3^+/CD4^+$ cells was also significantly higher in all disease groups (P < 0.001, respectively). The number of $CD4^+$, $CD25^+$, and $Foxp3^+$ cells, and the ratio of $CD25^+/CD4^+$ and $Foxp3^+/CD4^+$ cells, were associated with the histological grade of the specimens, including acute inflammation, chronic inflammation, lymphoid follicle number, and *Helicobacter pylori* infection. The number of $CD4^+$, $CD25^+$ and $Foxp3^+$ cells, and the ratio of $CD25^+/CD4^+$ and $Foxp3^+/CD4^+$ cells, were negatively associated with intestinal metaplasia among gastritis (P < 0.001, P < 0.001, P < 0.001, P = 0.002 and P = 0.002) and peptic ulcer groups (P = 0.013, P = 0.004, P < 0.001, P = 0.040 and P = 0.003).

CONCLUSION: Tregs are positively associated with endoscopic findings of gastroduodenal diseases and histological grade but negatively associated with intestinal metaplasia in gastritis and peptic ulcer groups.

Key words: T regulatory cells; *Helicobacter pylori*; Gastroduodenal diseases; Intestinal metaplasia; Immunohistochemistry

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Cheng HH, Tseng GY, Yang HB, Wang HJ, Lin HJ, Wang WC. Increased numbers of Foxp3-positive regulatory T cells in gastritis, peptic ulcer and gastric adenocarcinoma. *World J Gastroenterol* 2012; 18(1): 34-43 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i1/34.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i1.34

INTRODUCTION

In 1988, Correa delineated a multistep pathway from active chronic gastritis to atrophic gastritis, intestinal metaplasia (IM), dysplasia and finally gastric adenocarcinoma[1]. The presence of severe gastric atrophy, corpus-predominant gastritis, or IM is suggested to have an increased risk of cancer development[2]. The progression from IM to gastric cancer is supported by studies from animal models[3-5]. Hence, finding markers associated with these early histological changes could improve the prognosis and treatment of the disease. A common pathogen, *Helicobacter pylori* (*H. pylori*), is recognized as a primary etiologic agent in chronic gastritis. Persistent infection by *H. pylori* is associated with peptic ulceration and/or gastric malignancy[6,7]. In 1994, *H. pylori* was categorized as a group I carcinogen by the World Health Organization's International Agency for Research on Cancer owing to its epidemiologic association with gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma.

Regulatory T cells are a small population of T lymphocytes that may induce and maintain immunologic self-tolerance to prevent the development of autoimmune diseases[8-10]. Naturally occurring $CD4^+CD25^+$ Tregs frequently co-express cytotoxic T lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), transforming growth factor β (TGF-β), and forkhead box p3 (Foxp3)[11]. Using a mouse model and other methods, Foxp3 has been identified as a crucial transcription factor that regulates the development of $CD4^+CD25^+$ Tregs function[12,13] and thus may serve as a reliable marker for $CD4^+CD25^+$ Tregs.

The roles of $CD4^+CD25^+$ Tregs in suppressing the immune response to *H. pylori* have been reported recently in several studies[14-17]. $CD4^+CD25^+$ Tregs reduce proinflammatory cytokine production and *H. pylori*-induced gastritis in mice[17-19]. Depletion of Tregs in infected mice results in increased gastric inflammation and reduced colonization by *H. pylori*[16]. Furthermore, there is a higher proportion of $Foxp3^+$ Tregs in tumor-infiltrating lymphocytes in the gastric mucosa of cancers as compared with normal gastric mucosa[15,20,21]. Apart from its positive correlation with tumor specimens, the number of $Foxp3^+$ Tregs is also elevated in *H. pylori*-associated gastritis[21].

These results suggest that Tregs play a pivotal role in persistent *H. pylori* colonization in gastric mucosa, which may then lead to development of gastric cancers. However, the relationship between $CD4^+CD25^+$ Tregs and precancerous lesions of the stomach remain unclear. In the present study, we investigated the association between Tregs, histological grade and clinical sequelae in the context of the multistep progression to gastric cancer by examining histological profiles in healthy controls and patients with chronic gastritis, peptic ulcers or adenocarcinoma. We retrospectively analyzed the number of $CD4^+$, $CD25^+$, or $Foxp3^+$ T cells by immunohistochemistry in the antrum mucosa and examined the relationship between marker expression and precancerous lesions.

MATERIALS AND METHODS

Study subjects

Between January 2008 and June 2009, a total of 4563 patients were examined by upper gastroduodenal endoscopy at Ton-Yen General Hospital, Hsinchu, Taiwan. Among them, 256 patients who had gastric antral biopsy for histological diagnosis were considered for this retrospective study. After medical chart review for these cases, we excluded patients with chronic heart, lung, liver or kidney diseases, patients with a history of prior gastric surgery or anti-*H. pylori* eradication therapy, and patients taking non-steroidal anti-inflammatory drugs within one week prior to endoscopy. This gave us a pool of 135 enrolled non-cancer subjects, including 113 symptomatic patients from our out-patient department (30 patients with gastritis and 83 patients with peptic ulcers) and 22 healthy controls (asymptomatic cases undergoing physical check up). In addition, 32 patients with histologically proven gastric adenocarcinoma diagnosed between 2004 and 2008 were enrolled as the gastric cancer group. The Institutional Review Board of Ton-Yen General Hospital approved this study.

Demographic factors reported for the 167 patients included age and gender. Endoscopic and histological data were reviewed exclusively by Guan-Ying Tseng and Hsiao-Bai Yang, respectively. We defined an ulcer as a circumscribed mucosal break (> 5 mm in diameter, with apparent depth) in the stomach or duodenum, covered with exudates. *H. pylori* status was assessed by histology with hematoxylin and eosin stain and by rapid urease test on biopsies using Pronto Dry (Medical Instruments Corp., Solothurn, Switzerland). Patient was considered *H. pylori* positive if results by one or both diagnostic methods were positive and *H. pylori* negative if results by both methods were negative.

Histological assessment

For diagnosis, the gastric antrum biopsy specimens were obtained from the non-ulcer region adjacent to the ulcer site of the non-cancer subjects, or taken from the tumor region of the cancer patients, fixed in 10% formalin buffer, and stained with hematoxylin-eosin. The histological findings were graded by Yang HB according to the updated Sydney System[22,23]. The parameters included: (1) chronic inflammatory score (CIS; range 1-3 for mild, moderate, or severe lymphocytic and plasma cell infiltration); (2) acute inflammatory score (AIS; range 0-3 for absence or degree of neutrophil infiltration in lamina propia, epithelia, crypt or gland lumens); (3) lymphoid follicle number (LF; range 0-7 for total LF numbers in one slide); (4) *H. pylori* density (HPD; range 0-5); (5) IM (0, absence or < 5% of the upper third of gastric mucosa; 1-2, goblet cells found in the upper third or upper half of gastric mucosa; 3, goblet cells and paneth cells found, also considered complete IM); and (6) atrophic change (AT; 0-3 for absence, mild, moderate or severe).

Table 1 Clinical characteristics of study subjects classified on endoscopy (n = 167)

	Healthy controls (n = 22)	Gastritis (n = 30)	Peptic ulcer (n = 83)	Gastric cancer (n = 32)
Age (yr, mean ± SD)	46.4 ± 14.3	55.4 ± 15.0[a]	61.7 ± 14.9[a]	67.9 ± 13.9[a]
Gender				
Male	13	17	53	30
Female	9	13	30	2
Helicobacter pylori infection				
Negative	22	20	33	21
Positive	0	10	50[b]	11

[a]P < 0.05, each disease *vs* healthy controls; [b]P = 0.02, peptic ulcer *vs* gastritis. Data were analyzed using the Mann-Whitney U test, χ^2 test and Fisher's exact test.

Table 2 Histological grading of non-cancer subjects (n = 135)

	Healthy controls	Gastritis	Peptic ulcer
Chronic inflammatory score (1/2/3)[a]	21/1/0	5/5/20	1/3/79
Acute inflammatory score (0/1/2/3)[a]	22/0/0/0	5/6/12/7	3/1/21/58
Lymphoid follicle number (0/1-2/≥ 3)[a]	21/1/0	17/9/4	39/28/16
Helicobacter pylori density (0/1/2/3/4/5)[a]	22/0/0/0/0/0	20/2/1/3/3/1	33/2/2/8/24/14
Intestinal metaplasia score (0/1/2/3)[a]	22/0/0/0	10/2/4/14	22/9/20/32
Atrophy score (0/1/2/3)[a]	19/1/1/1	8/9/11/2	14/40/20/9

[a]P < 0.001. Data were analyzed using Kruskal-Wallis H test.

Immunohistochemical staining

Consecutive paraffin-embedded serial sections of gastric biopsies (4 μm) were deparaffinized and rehydrated with xylene and ethanol for single staining of CD25, CD4, and Foxp3. Antigen retrieval was performed in a 95 ℃ water bath using Tris-ethylene diamine tetraacetic acid (EDTA) buffer (10 mmol/L Tris, pH 9.0, 1 mmol/L EDTA, 0.05% w/v Tween 20) for 10 min in CD4, or sodium citrate buffer (10 mmol/L sodium citrate, pH 6.0, 0.05% Tween 20) for 20 min in Foxp3 or 10 min in CD25. Sections were cooled for 20 min, and then endogenous peroxidase was blocked using 3% H_2O_2 for 10 min. Sections were incubated with blocking buffer (1% bovine serum albumin in phosphate-buffered solution) for 1 h at room temperature and then incubated with primary antibody for 2 h at room temperature. The working dilution of the primary antibody was 1:200 for mouse anti-human CD4 (clone 4B12; Novocastra, Newcastle, United Kingdom), 1:400 for mouse anti-human CD25 (clone 4C9; Novocastra), and 1:50 for mouse anti-human Foxp3 (clone 236A/E7; Santa Cruz Biotechnology, Santa Cruz, CA). Sections were stained using NovoLink Polymer Detection Systems (Novocastra) followed by 3,3'-diaminobenzidine (Sigma) for 5 min, and counterstained with hematoxylin (Sigma). Lymph node tissue was used as a positive control. Additional sections were processed without primary antibody as a negative control.

Quantification of the number of immunostained cells was conducted in three consecutive single-stained sections in order of CD25, CD4 and Foxp3. For enumeration of $CD4^+$ T cells, lymphocytes infiltrating the non-IM region were counted at least ten high-powered fields (HPF; 400 ×) from each CD4-stained section. Selected regions of each CD4-stained section were then retraced in the corresponding CD25- and Foxp3-stained sections to enumerate $CD25^+$ and $Foxp3^+$ cells. For each sample, the mean ratio of $CD25^+/CD4^+$ and $Foxp3^+/CD4^+$ cells was also calculated. Results were expressed as the median value and inter-quartile range of all tested patients in each group.

Statistical analysis

χ^2 test or Fisher's exact test was performed to compare groups with categorical variables. Continuous variables were compared between groups using a Mann-Whitney U test or Kruskal-Wallis H test. Correlations between the number of $CD25^+$ cells and $Foxp3^+$ cells were established based on Spearman's rank correlation analysis, and the ratio of $CD25^+/CD4^+$ cells and of $Foxp3^+/CD4^+$ cells was calculated. Statistical tests were two-sided, with $P < 0.05$ considered statistically significant. Statistical analysis was performed using SPSS version 12.0 (SPSS Inc., Chicago, IL).

RESULTS

Patients

The demographic and clinical characteristics of the 167 study subjects are shown in Table 1. On endoscopy, there were 22 normal, 30 gastritis, 83 peptic ulcer and 32 gastric cancer cases. Subjects ranged in age from 27 years to 95 years (59.7 ± 15.9 years). The mean age of the healthy controls (46.4 ± 14.3 years) was substantially lower than that of disease groups. A much higher male ratio was found in the gastric cancer group (93.8%) than in the healthy control group (59.1%, P = 0.004). The prevalence of *H. pylori* infection was significantly higher in the peptic ulcer group (60.2%) than in the gastritis group (33.3%, P = 0.020).

The histological grading of non-cancer subjects (n = 135) is shown in Table 2. The scores of the six parameters, including AIS, CIS, LF, HPD, IM and AT, were significantly associated with the severity of gastroduodenal diseases as determined endoscopically (AIS, $P < 0.001$; CIS, $P < 0.001$; LF: $P < 0.001$; HPD: $P < 0.001$; IM: $P < 0.001$; AT: $P < 0.001$). The number of $CD25^+$, $CD4^+$, or $Foxp3^+$ cells and the ratio of $CD25^+/CD4^+$ and of $Foxp3^+/CD4^+$ had no correlation with respect to age or sex in non-cancer patients.

Number of $Foxp3^+$ Tregs and ratio of $Foxp3^+/CD4^+$ Tregs are higher in gastroduodenal diseases

To enumerate Tregs present in the gastric mucosa of gastroduodenal diseases, we determined the number of

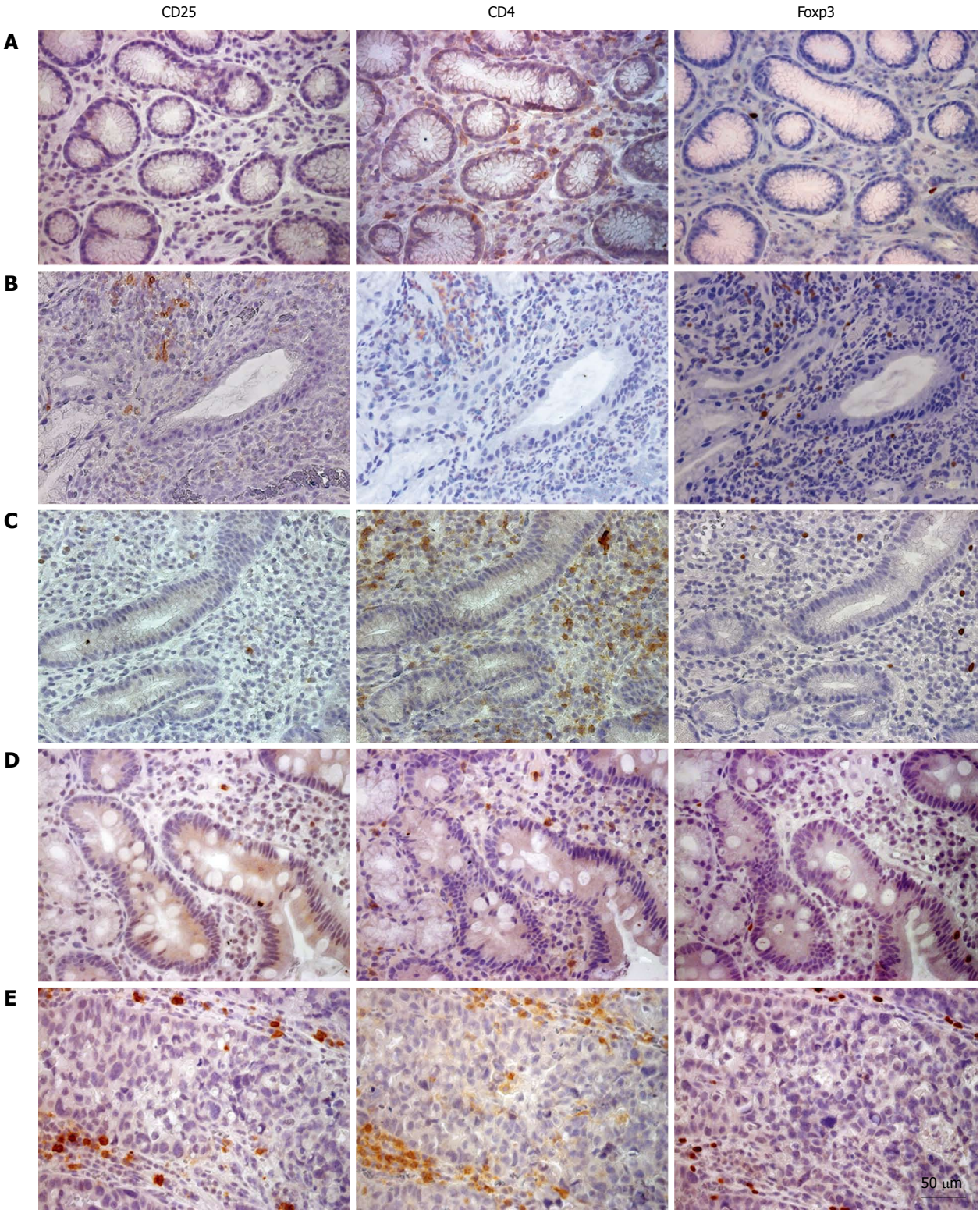

Figure 1 Immunohistochemistry of CD25, CD4, and Foxp3 in healthy controls, acute gastritis, chronic gastritis, intestinal metaplasia, and gastric cancer (original magnification, 400 ×). Immunohistochemistry of CD25 (left), CD4 (middle), and Foxp3 (right) in gastric mucosa from A: Healthy controls; B: Acute gastritis; C: Chronic gastritis; D: Intestinal metaplasia; E: Gastric cancer. CD4 and CD25 staining (brown) were found on the surface of T lymphocytes, and Foxp3 staining (brown) was located in the nucleus of T lymphocytes in lamina propria around glands.

$CD25^+$ cells, $CD4^+$ cells and $Foxp3^+$ cells in three consecutive sections. The expression of CD4 and CD25 was found on the surface of lymphocytes, whereas expression of Foxp3 was seen in the nucleus of lymphocytes (Figure 1).

The number of $CD25^+$ cells was significantly correlated with that of $Foxp3^+$ cells (Figure 2A; $r = 0.876$, $P < 0.001$). Furthermore, the ratio of $CD25^+/CD4^+$ was also significantly correlated with that of $Foxp3^+/CD4^+$ (Figure 2B; r

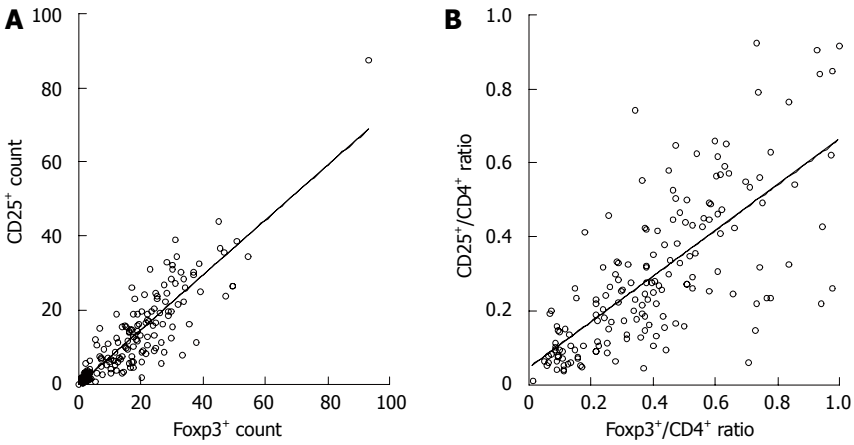

Figure 2 CD25 expression correlates with Foxp3 expression in human T lymphocytes. A: Correlation between $CD25^+$ and $Foxp3^+$ cells from 22 healthy controls and 30 gastritis, 83 peptic ulcer, and 32 gastric cancer patients; B: Correlation between the ratio of $CD25^+/CD4^+$ and of $Foxp3^+/CD4^+$ cells. Data were analyzed by Spearman's rank correlation.

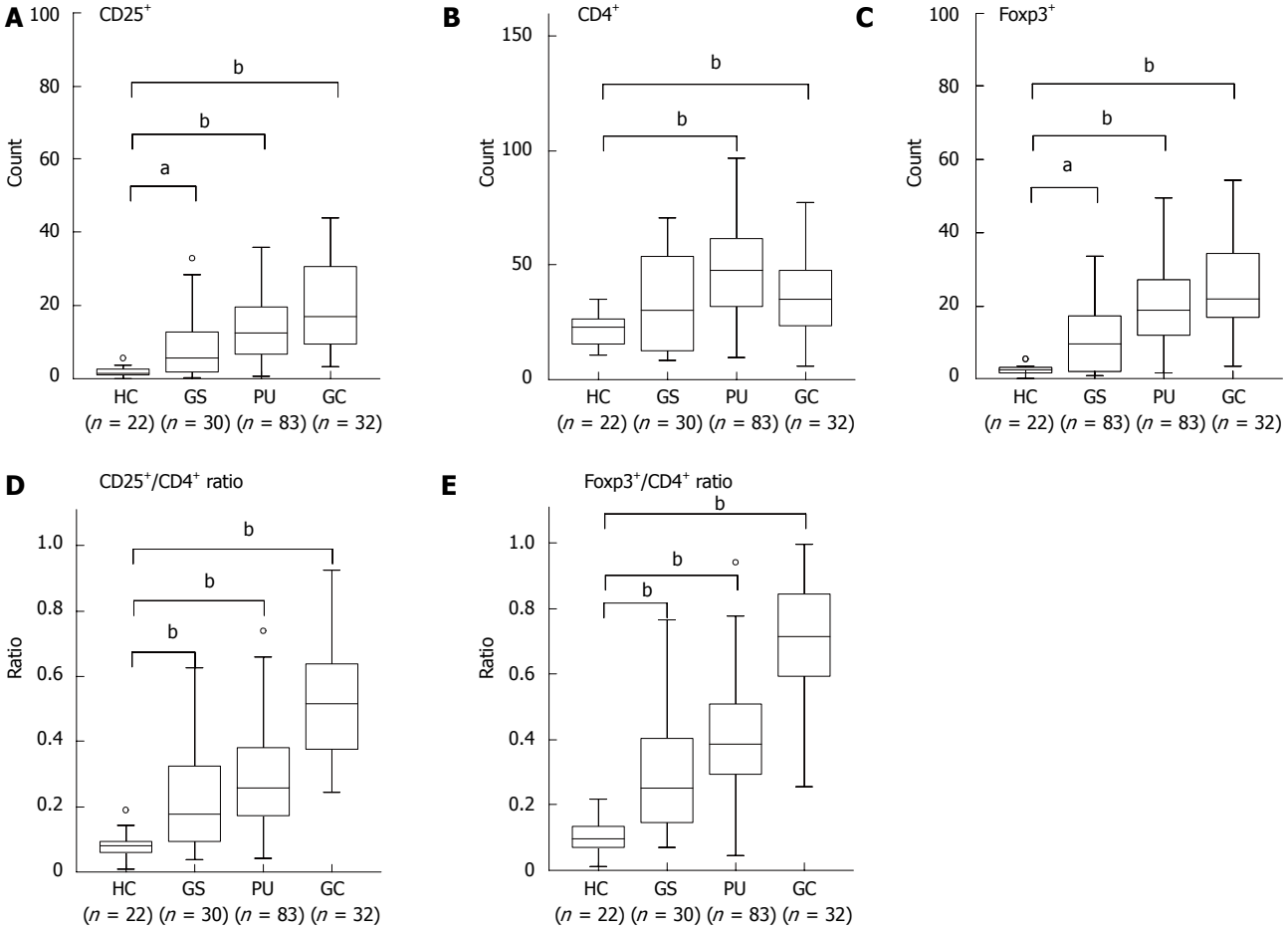

Figure 3 Box plots for the number of $CD25^+$, $CD4^+$, and $Foxp3^+$ cells, and the ratio of $CD25^+/CD4^+$ and $Foxp3^+/CD4^+$ in healthy controls, gastritis, peptic ulcer, and gastric cancer. Box plots for A: The number of $CD25^+$ cells; B: The number of $CD4^+$ cells; C: The number of $Foxp3^+$ cells; D: The ratio of $CD25^+/CD4^+$; E: The ratio of $Foxp3^+/CD4^+$ in non-intestinal metaplasia areas of antral gastric mucosa from healthy controls (HC) and patients with gastritis (GS), peptic ulcer (PU), and gastric cancer (GC). Data were analyzed by the Mann-Whitney *U* test. $^aP < 0.05$ and $^bP < 0.001$.

= 0.717, $P < 0.001$), revealing a strong positive correlation between CD25 and Foxp3.

Peptic ulcer and gastric cancer patients had a significantly higher number of $CD4^+$ cells than the healthy controls (Figure 3B; $P = 0.001$). The number of $CD25^+$ and $Foxp3^+$ cells was lowest in the healthy controls and increased progressively in the more severe disease groups (Figure 3A and 3C; gastritis *vs* healthy control: $CD25^+$, $P = 0.004$ and $Foxp3^+$, $P = 0.008$; peptic ulcer *vs* healthy control: $CD25^+$, $P < 0.001$ and $Foxp3^+$, $P < 0.001$; gastric

cancer *vs* healthy control: $CD25^+$, $P < 0.001$ and $Foxp3^+$, $P < 0.001$; peptic ulcer *vs* gastritis: $CD25^+$, $P = 0.001$ and $Foxp3^+$, $P < 0.001$; gastric cancer *vs* gastritis: $CD25^+$, $P < 0.001$ and $Foxp3^+$, $P < 0.001$; gastric cancer *vs* peptic ulcer: $CD25^+$, $P = 0.018$ and $Foxp3^+$, $P = 0.044$). In addition, the ratio of $CD25^+/CD4^+$ and of $Foxp3^+/CD4^+$ was increased progressively in the more severe disease groups (Figure 3D and 3E; gastritis *vs* healthy control: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$; peptic ulcer *vs* healthy control: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$; gastric cancer *vs* healthy control: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$; peptic ulcer *vs* gastritis: $CD25^+/CD4^+$, $P = 0.012$ and $Foxp3^+/CD4^+$, $P = 0.002$; gastric cancer *vs* gastritis: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$; gastric cancer *vs* peptic ulcer: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$). Together, these results suggested that Tregs were associated with gastritis, peptic ulcer and gastric cancer.

Foxp3$^+$/CD4$^+$ ratio is positively associated with degree of inflammation, number of LFs and H. pylori infection

We next analyzed the relationship between Tregs and histological findings in non-cancer subjects ($n = 135$; Figure 4). The ratios $CD25^+/CD4^+$ and $Foxp3^+/CD4^+$ were significantly higher with a higher degree of chronic and acute inflammation (CIS 3, AIS 3-4) than with a lower degree of chronic and acute inflammation (CIS 1-2, AIS 0-1) (Figure 4A and B; CIS 3 *vs* 1: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$; AIS 2 *vs* 0: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$; AIS 3 *vs* 0: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$). The ratios were also significantly higher in patients with higher numbers of LFs (Figure 4C; LF 1-2 *vs* 0: $CD25^+/CD4^+$, $P = 0.005$ and $Foxp3^+/CD4^+$, $P < 0.001$; LF $\geqslant$ 3 *vs* 0: $CD25^+/CD4^+$, $P = 0.013$ and $Foxp3^+/CD4^+$, $P < 0.001$). Moreover, the ratios were significantly higher in gastric mucosa of patients with *H. pylori* infection than in patients without infection (Figure 4D; Hp+ *vs* Hp-: $CD25^+/CD4^+$, $P = 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$). No significant association was found between Treg number and IM or AT. Together, the presence of Tregs was positively associated with inflammation, LFs, and *H. pylori* infection.

CD25$^+$/CD4$^+$ and Foxp3$^+$/CD4$^+$ ratios are lower in gastritis and peptic ulcer patients with IM than in those without IM

We compared Tregs in gastric mucosa of gastritis and peptic ulcer patients with and without IM (Figure 5). There were significantly fewer $CD4^+$, $CD25^+$, or $Foxp3^+$ cells in gastritis patients with IM than in those without (Figure 5A-C; IM+ *vs* IM-: $CD25^+$, $P < 0.001$; $CD4^+$, $P < 0.001$; $Foxp3^+$, $P < 0.001$). The ratios $CD25^+/CD4^+$ and $Foxp3^+/CD4^+$ were also significantly lower in gastritis patients with IM (Figure 5D and E; IM+ *vs* IM-: $CD25^+/CD4^+$, $P = 0.002$ and $Foxp3^+/CD4^+$, $P = 0.002$). A similar profile was observed in the peptic ulcer patients (Figure 5A-E; IM+ *vs* IM-: $CD25^+$, $P = 0.004$; $CD4^+$, $P = 0.013$; $Foxp3^+$, $P < 0.001$; $CD25^+/CD4^+$, $P = 0.040$; $Foxp3^+/CD4^+$, $P = 0.003$). Thus, IM was associated a reduced number of Tregs in patients with gastroduodenal diseases.

DISCUSSION

Most studies investigating the role of Tregs in gastroduodenal diseases have used fluorescence-activated cell sorting (FACS) to directly identify $CD4^+CD25^+$ Tregs in lymphocytes from peripheral blood or lamina propria[15,24-27]. Recently, Perrone *et al*[28] investigated tumor-infiltrating $Foxp3^+$ Tregs in radically resected (R0) gastric cancer by immunohistochemistry, and suggested that $Foxp3^+$ Tregs may be a novel in situ marker for identifying high-risk gastric cancer patients. In the present study, we investigated the expression of two markers for Tregs, namely Foxp3 and CD25, in relation to CD4 expression by immunohistocytochemistry in three consecutive sections of a panel of normal and diseased specimens. We confirmed a significantly high correlation between the numbers of $CD25^+$ and $Foxp3^+$ T lymphocytes, and between the $CD25^+/CD4^+$ and $Foxp3^+/CD4^+$ ratios.

The retrospective study of 135 non-cancer subjects in a single clinical institute demonstrates a positive correlation between the number of $Foxp3^+$ Tregs and histological grade, including AIS, CIS, and LFs. Furthermore, the presence of *H. pylori* was positively associated with the number of Tregs. These results suggest that gastritis can be induced by *H. pylori* infection, which contributes to the occurrence of lymphoid tissue hyperplasia and recruits Tregs. This result agrees with previous evidences for a positive link between *H. pylori*-induced gastritis and recruitment of $Foxp3^+$ Tregs[21,29-31]. It is interesting that Tregs isolated from gastric adenocarcinoma patients are able to suppress *H. pylori*-induced T cell responses *in vitro*, supporting the role of Tregs in facilitating persistent *H. pylori* colonization and hence gastric carcinogenesis[15].

The number of Tregs in our gastric cancer group was significantly higher than that of the peptic ulcer group or the gastritis group. Additionally, the number of $Foxp3^+$ Tregs and the $Foxp3^+/CD4^+$ ratio in antral mucosa were increased progressively from healthy controls to gastritis patients to gastric cancer patients. Interestingly, this sequential change corresponds to the sequential clinical sequelae of gastric carcinogenesis, or Correa's cascade[32,33]. Increasing evidence has indeed demonstrated the presence of significantly elevated numbers of $CD4^+$ Tregs in various types of cancers[15,24,34-39]. It has been hypothesized that Tregs suppress anti-tumor immunity, which leads to immune tolerance of tumor cells[40]. Recently, advanced tumor/node/metastasis (TNM) stage in gastric cancer patients was found associated with elevated expression of Foxp3 in tumor-infiltrating Treg cells[25]. Of note, cyclooxygenase-2/prostaglandin E_2 production might be involved in Treg-based immune suppression, which may have new implications for gastric cancer therapy.

We further examined the distribution of Tregs in patients with IM, a precancerous lesion of the intestinal-type gastric adenocarcinoma[32,33,41-46]. Notably, gastritis and peptic ulcer patients with IM had significantly fewer

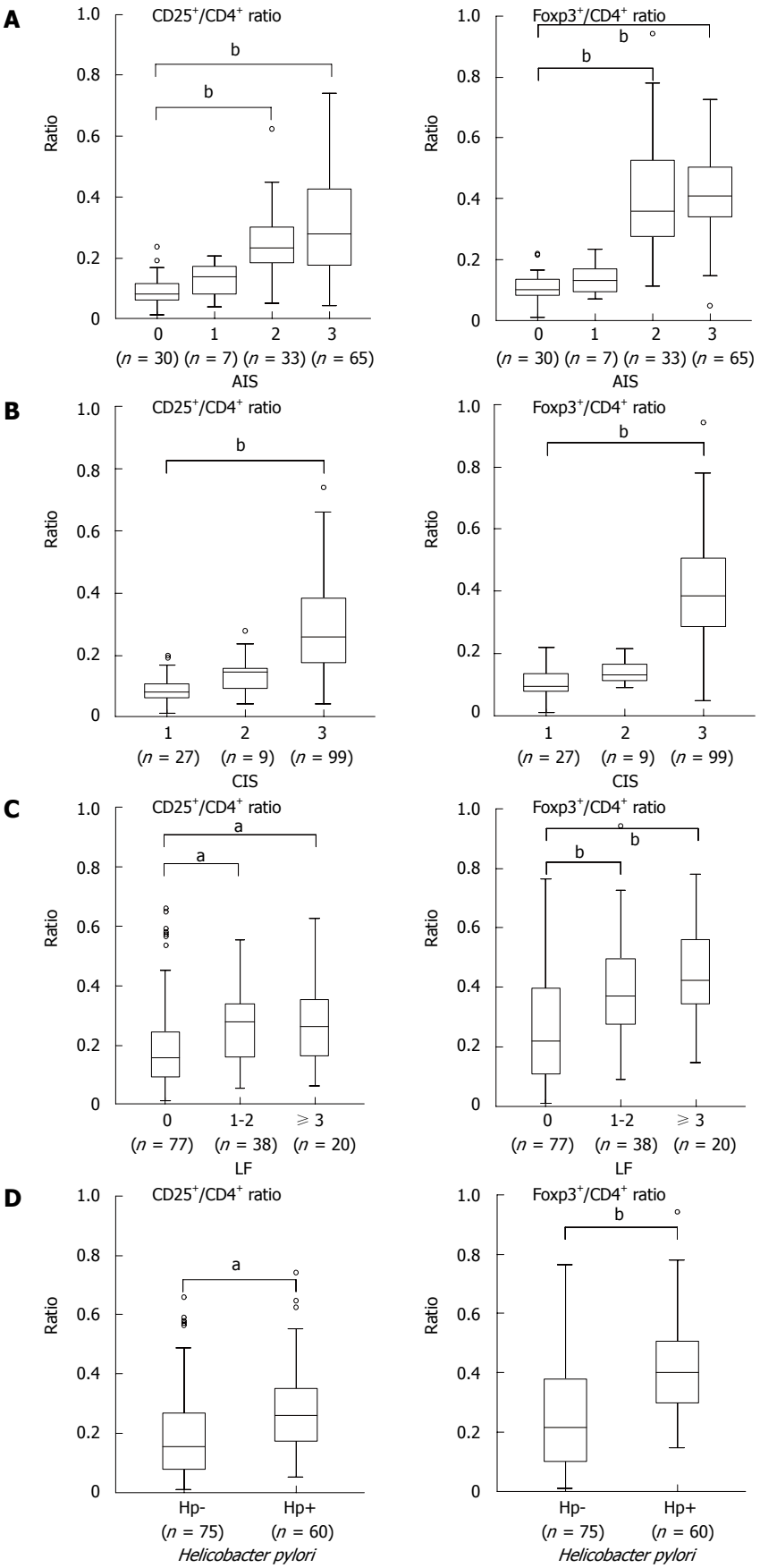

Figure 4 Box plots for the ratio of $CD25^+/CD4^+$ and of $Foxp3^+/CD4^+$ according to acute inflammatory score, chronic inflammatory score, lymphoid follicle number, and *Helicobacter pylori* infection. Box plots for the ratio of $CD25^+/CD4^+$ and of $Foxp3^+/CD4^+$ according to A: Acute inflammatory score (AIS); B: Chronic inflammatory score (CIS); C: Lymphoid follicle number (LF); D: *Helicobacter pylori* infection. Data were analyzed using the Mann-Whitney *U* test. $^aP < 0.05$ and $^bP < 0.001$. HP: *Helicobacter pylori*.

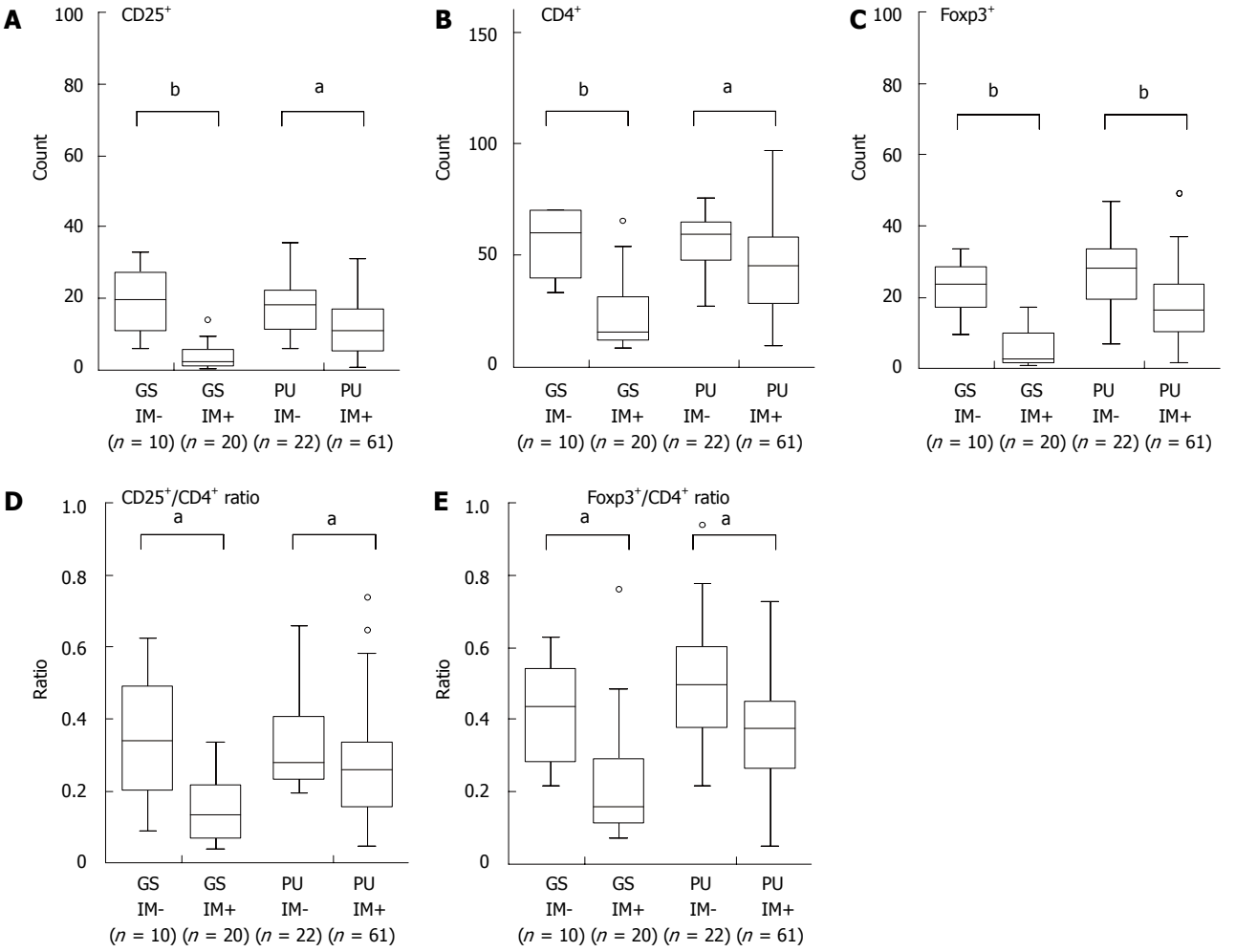

Figure 5 Box plots for the number of CD25$^+$, CD4$^+$, and Foxp3$^+$ cells, and the ratio of CD25$^+$/CD4$^+$ and of Foxp3$^+$/CD4$^+$ in gastritis and peptic ulcer patients with and without intestinal metaplasia. Box plots for A: The number of CD25$^+$ cells; B: The number of CD4$^+$ cells; C: The number of Foxp3$^+$ cells; D: The ratio of CD25$^+$/CD4$^+$; E: The ratio of Foxp3$^+$/CD4$^+$ in non-intestinal metaplasia (IM) areas of antral gastric mucosa from gastritis (GS) and peptic ulcer (PU) patients with and without IM. Data were analyzed using the Mann-Whitney *U* test. $^aP < 0.05$ and $^bP < 0.001$.

Tregs than patients without IM. Previous studies showed an increased level of TGF-β as well as Foxp3$^+$ Tregs in patients with *H. pylori*-induced gastritis[30], which supports the notion that TGF-β is a critical differentiation factor for Treg cells within a local microenvironment[47,48]. It is interesting that Mutoh *et al*[49] recently reported a lower level of TGF-β expression in the IM subjects, which was comparable to that in the normal tissues as opposed to a 6.5-fold increase in gastric carcinoma. Such a lower level of TGF-β expression might contribute to the lesser numbers of Foxp3$^+$ Tregs in GS/PU patients with IM. However, further investigations will be needed to explore this possibility.

There are limitations in our study, all stemming from its retrospective design. First, there is a possible selection bias, even though at least 30 cases were included in each disease group. Second, it is unlikely to rule out the remote NSAIDs used in our enrolled subjects based on reviewing their medical records. The potential effect of NSAIDs on Tregs in our patients thus might be undetermined. Third, it was not possible to use a functional assay for Treg expression to address detailed mechanisms.

In conclusion, the number of Tregs is elevated and positively correlated with histological grade of chronic gastritis, atrophic gastritis and adenocarcinoma, but is decreased and negatively correlated with histological grade of IM.

ACKNOWLEDGMENTS

We are grateful to Sheng-De Chen for his technical assistance.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) are associated with chronic gastroduodenal inflammation, atrophy, intestinal metaplasia, and gastric cancer. The role of CD4$^+$CD25$^+$ Tregs in suppressing the immune response to *H. pylori*, and increased populations of CD4$^+$CD25$^+$ T regulatory cells (Tregs) link to the *H. pylori*-infected pathologies have been reported.

Research frontiers

CD4$^+$CD25$^+$Foxp3$^+$ Tregs are expressed in various types of cancers including gastric cancer. Tregs is also elevated in *H. pylori*-associated gastritis. However, the relationship between Tregs with precancerous lesions of the stomach remains unclear. In this study, the authors analyzed the number of CD4$^+$, CD25$^+$,

or Foxp3$^+$ T cells by immunohistochemistry in the antrum mucosa and examined the relationship between marker expression and precancerous lesions.

Innovations and breakthroughs

Recent reports have highlighted the association between Tregs and precancerous lesions of the stomach. In this study, the authors report that the number of Tregs is positively correlated with histological grade of chronic gastritis, atrophic gastritis and adenocarcinoma, and negatively correlated with histological grade of IM, suggesting that Tregs may play a role in the progression of gastric cancer.

Applications

Foxp3$^+$ Tregs may be a novel *in situ* marker for identifying gastric cancer patients.

Terminology

Tregs are a small population of T lymphocytes that may induce and maintain immunologic self-tolerance to prevent the development of autoimmune diseases. Forkhead box p3 (Foxp3) has been identified as a crucial transcription factor that regulates the development of CD4$^+$CD25$^+$ Tregs function and thus may serve as a reliable marker for CD4$^+$CD25$^+$ Tregs.

Peer review

This manuscript is a well written contribution and the data merit publication. Significant patient material has been collected and relevant data have been retrieved. The immuno-based analysis is well carried out and justifies the conclusions reached by the authors.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i1.44

World J Gastroenterol 2012 January 7; 18(1): 44-48
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Influence of proton pump inhibitor treatment on *Helicobacter pylori* stool antigen test

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Received: February 25, 2011 Revised: July 6, 2011
Accepted: July 11, 2011
Published online: January 7, 2012

Abstract

AIM: To investigate the effects of proton pump inhibitor (PPI) treatment on stool antigen test using the TestMate pylori enzyme immunoassay.

METHODS: This study assessed 28 patients [16 men and 12 women; mean age (63.1 ± 5.9) years; range, 25-84 years] who underwent stool antigen test and urea breath test (UBT) before and after PPI administration.

RESULTS: Using the UBT as the standard, the sensitivity, specificity and agreement of the stool antigen test in all 28 patients were 95.2%, 71.4%, and 89.3%, respectively, before PPI administration, and 88.9%, 90.9%, and 89.3%, respectively, after PPI treatment. Mean UBT values were 23.98% ± 5.33% before and 16.19% ± 4.75% after PPI treatment and, in 15 patients treated for ⩾ 4 wk, were significantly lower after than before 4 wk of PPI treatment (12.58% ± 4.49% *vs* 24.53% ± 8.53%, P = 0.048). The mean optical density ($A_{450/630}$) ratios on the stool antigen test were 1.16 ± 0.20 before and 1.17 ± 0.24 after PPI treatment (P = 0.989), and were 1.02 ± 0.26 and 0.69 ± 0.28, respectively, in the group treated for > 4 wk (P = 0.099).

CONCLUSION: The stool antigen test was equally sensitive to the UBT, making it a useful and reliable diagnostic method, even during PPI administration.

Key words: *Helicobacter pylori*; Stool antigen test; Urea breath test; Proton pump inhibitor

Peer reviewers: Julio H Carri, Professor, Internal Medicine-Gastroenterology, National University of Córdoba, Av. Estrada 160-P 5-Department D, Córdoba 5000, Argentina; Mario M D' Elios, Professor, University of Florence, viale Morgagni 85, Florence 50134, Italy

Kodama M, Murakami K, Okimoto T, Fukuda Y, Shimoyama T, Okuda M, Kato C, Kobayashi I, Fujioka T. Influence of proton pump inhibitor treatment on *Helicobacter pylori* stool antigen test. *World J Gastroenterol* 2012; 18(1): 44-48 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i1/44.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i1.44

INTRODUCTION

The urea breath test (UBT) has shown high sensitivity

and specificity in the non-invasive diagnosis of *Helicobacter pylori* (*H. pylori*) infection, making the UBT one of the most valuable non-invasive tests for diagnosing *H. pylori* infection and eradication throughout the world[1]. This test has been recommended in the 2005 revision of the guidelines for *H. pylori* diagnosis and treatment of the European *Helicobacter* study group (Maastricht Ⅲ)[2].

The stool antigen test, using polyclonal and monoclonal antibodies, is another non-invasive method for detecting *H. pylori*[3], with comparable sensitivity and specificity to the UBT. Maastricht Ⅲ has also recommended the stool antigen test as a simple, easy and useful method for detecting the presence or eradication of *H. pylori*[2]. Native catalase was recently identified as an antigen produced by *H. pylori*[4]. Catalase is characterized by its stability, and the stool antigen test using a monoclonal antibody to catalase is both rapid, taking only about 70 min, and considered more specific than methods using polyclonal antibodies[4]. Although the UBT is non-invasive and the laboratory procedure is recognized as simple and easy, it requires a longer period of time and trained medical staff. Moreover, the UBT is difficult for some patients, including children, handicapped individuals with deteriorated activities of daily life (ADL) and elderly individuals. The stool antigen test is a simpler analytical process, does not require as many medical staff members and can be more readily applied for patients with reduced ADL, children, and the elderly.

Proton pump inhibitors (PPIs) have strong antigastric secretion effects, as well as being bacteriostatic against *H. pylori*. Since UBT may yield false-negative results in patients being treated with PPIs[5,6], it has been recommended that PPI treatment be suspended for at least 2 wk prior to UBT for *H. pylori* infection. One of the stool antigen tests available is the Testmate pylori antigen enzyme immunoassay (EIA)[7], which uses native catalase as an antigen. Thus, the precision of this test in diagnosing *H. pylori* infection should remain stable even during PPI treatment. To determine their comparative accuracy in patients being treated with PPIs, we compared this stool antigen test and the UBT in patients before and after PPI administration.

MATERIALS AND METHODS

We assessed 28 patients [16 men and 12 women; mean age, (63.1 ± 5.9) years; range, 25-84 years] who had been referred to the Department of Gastroenterology at Oita University Hospital and diagnosed with peptic ulcer, reflux esophagitis, or other diseases requiring PPI treatment.

The UBT and stool antigen test were performed before and after PPI administration. Patients were treated for > 2 wk with the 3 types of PPI commercially available in Japan: omeprazole (10 mg/d or 20 mg/d); lansoprazole (15 mg/d or 30 mg/d); or rabeprazole (10 mg/d or 20 mg/d).

Breath samples for UBT were collected before and 20 min after each subject took a 100 mg UBIT tablet® (Otsuka Pharmaceutical, Tokyo, Japan). $^{13}CO_2/^{12}CO_2$ ratios were analyzed by INIS (UBiT-IR300; Otsuka Electronics, Tokyo, Japan), with a cut-off point of 2.5‰.

Stool samples were collected from each patient before and after PPI treatment, and stored at -80 ℃ until assayed. The stool antigen test was performed using the Testmate pylori antigen EIA (Wakamoto Pharmaceutical, Tokyo, Japan; Kyowa Medex, Tokyo, Japan). An aliquot of 100 mg stool was diluted into 0.4 mL of diluted buffer, and 50 μL, together with peroxidase-conjugated anti-catalase monoclonal antibody, were added to a well and incubated for 1 h at 25 ℃. Absorbance was measured at wavelengths of 450 nm and 630 nm, with a cut-off value of 0.120.

Table 1 Results of urea breath test and stool antigen tests before proton pump inhibitor administration

	Urea breath test		
	Positive	Negative	Total
Stool antigen test			
Positive	20	2	22
Negative	1	5	6
Total	21	7	28

Sensitivity 95.2% (20/21), specificity 71.4% (5/7), agreement 89.3% (25/28).

Statistical analysis

Statistical comparisons were performed using the χ^2 test, the Wilcoxon signed-rank test, and the paired *t*-test.

RESULTS

Prior to PPI administration, both the UBT and stool antigen tests yielded positive results in 20 of 28 patients and negative results in 5. One patient was positive on the UBT and negative on the stool antigen test, and 2 patients were negative on the UBT and positive on the stool antigen test. Using UBT as the standard, the sensitivity, specificity and agreement of the stool antigen test before PPI treatment were 95.2%, 71.4%, and 89.3%, respectively (Table 1).

Following PPI administration, both the UBT and stool antigen test showed positive results in 16 patients and negative results in 9. Two patients were positive on the UBT and negative on the stool antigen test, and one was showed on the UBT and positive on the stool antigen test. Using UBT as the standard, the sensitivity, specificity and agreement of the stool antigen test after PPI treatment were 88.9%, 90.9%, and 89.3%, respectively (Table 2).

Positivity of the UBT and stool antigen test before and after PPI treatment

The UBT positivity rates were 75.0% (21/28) before and 64.3% (18/28) after PPI treatment (P = 0.55; Table 3). The stool antigen test positivity rates were 78.6% (22/28) before and 60.7% (17/28 cases) after PPI treatment (P = 0.15). No significant differences in the positivity ratios of

Table 2 Results of urea breath test and stool antigen tests after proton pump inhibitor administration

	Urea breath test		
	Positive	Negative	Total
Stool antigen test			
Positive	16	1	17
Negative	2	9	11
Total	18	10	28

Sensitivity 88.9% (16/18), specificity 90.9% (9/10), agreement 89.3% (25/28).

Table 3 Differences in positivity rates from before to after proton pump inhibitor treatment

	Before PPI treatment	After PPI treatment	*P* value
Urea breath test	75.0% (21/28)	64.3% (18/28)	0.55
Stool antigen test	78.6% (22/28)	60.7% (17/28)	0.15

PPI: Proton pump inhibitor.

the two assays were observed before ($P = 0.75$) or after ($P = 0.58$) PPI treatment.

Measured values for UBT ($^{13}CO_2/^{12}CO_2$ ratios) and stool antigen test (A values) before and after PPI treatment

The mean UBT values were 23.98% ± 5.33% before and 16.19% ± 4.75% after PPI treatment ($P = 0.068$), and were 24.53% ± 8.53% and 12.58% ± 4.49%, respectively ($P = 0.048$), in the 15 patients treated with PPIs for ≥ 4 wk (Figure 1).

The mean *A* ratios on the stool antigen test were 1.16 ± 0.20 before and 1.17 ± 0.24 after PPI treatment ($P = 0.989$), and were 1.02 ± 0.26 and 0.69 ± 0.28, respectively ($P = 0.099$), in the 15 patients treated with PPI for ≥ 4 wk (Figure 2).

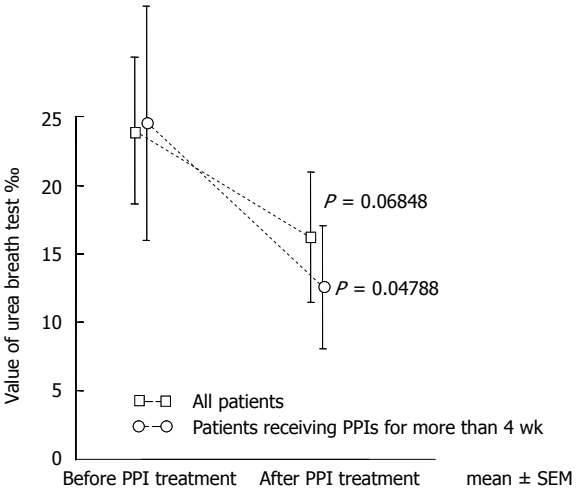

Figure 1 Urea breath test results ($^{13}CO_2/^{12}CO_2$ ratios) before and after proton pump inhibitor treatment. UBT results in all 28 patients were not altered by PPI treatment ($P = 0.068$). However, among patients treated for ≥ 4 wk, UBT results decreased significantly after PPI treatment ($P = 0.048$). UBT: Urea breath test; PPI: Proton pump inhibitor.

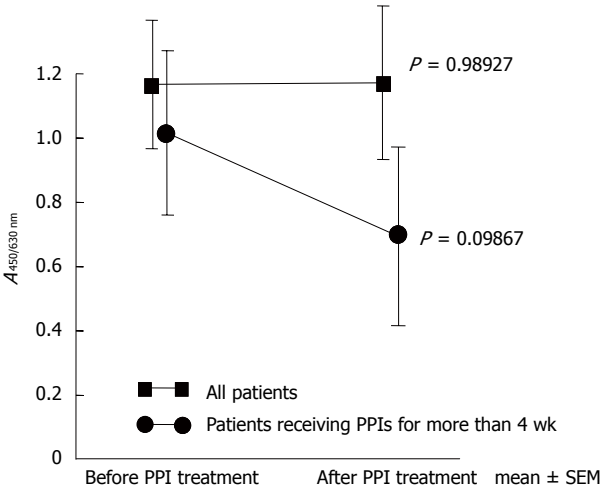

Figure 2 Stool antigen test results (A_{450}/A_{630} ratios) before and after proton pump inhibitor treatment. PPI treatment did not significantly alter stool antigen test results, both in all patients ($P = 0.989$) and in those treated for ≥ 4 wk ($P = 0.099$). PPI: Proton pump inhibitor.

DISCUSSION

The stool antigen test is a non-invasive test for *H. pylori*, similar to the UBT. The test is simple and easy to perform, with good sensitivity and specificity[8]. An analysis of 89 reports found that the mean sensitivity, specificity, positive predictive value and negative predictive value were 91%, 93%, 92%, and 87%, respectively, among 10 858 subjects who were not treated with *H. pylori* eradication therapy[3]. In addition, 8 reports of tests with monoclonal antibody showed significantly better results ($P < 0.001$), with mean sensitivity, specificity, positive predictive value and negative predictive value of 96%, 97%, 96%, and 97%, respectively. The Maastricht Ⅲ guidelines have found that the reliability of the stool antigen test is comparable to that of the UBT[2].

Despite its non-invasive nature, and high sensitivity and specificity, the UBT can lead to false-negative results in patients treated with drugs showing bacteriostatic activity against *H. pylori*, such as PPIs, or inhibiting urease activity[5,6,9-12]. In those studies, the false-negative rates of the UBT in patients treated with omeprazole or lansoprazole for 2 wk or 4 wk were ≥ 50%. We previously observed false-negative UBT results in 1 of 16 patients (6.3%) treated with 30 mg/d lansoprazole for 2 wk[6]. Several studies have also reported that the rate of false-negative results on the stool antigen test also increase in patients treated with PPI[13,14]. A comparison of stool antigen test and UBT results for 9 *H. pylori*-positive patients receiving PPI for 2 wk found a smaller degree of change on the stool antigen test than on the UBT[15].

The authors found that, before PPI treatment, the stool antigen test showed high sensitivity, but lower specificity (71.4%). This was likely due to the small number of patients showing negative results on the UBT ($n = 7$).

Similarly, although many reports have shown high sensitivity and specificity for stool antigen tests, others have reported lower specificity (54%-78%)[3].

The sensitivity of the stool antigen test fell slightly after PPI administration, but its specificity remained high level (90.9%). The concordance rate of UBT and stool antigen test results was high (89.3%) before and after PPI administration. Using the UBT as standard, the stool antigen test showed good sensitivity and specificity both before and after PPI administration. Although stool antigens have generally shown high sensitivity and specificity, divergent sensitivity and specificity between the UBT and stool antigen test have been reported[16]. However, increasing the cut-off for the stool antigen test reduced the percentage of conflicting results[16]. This discrepancy was attributed to the urease-based UBT not detecting the coccoid form of *H. pylori*, as well as the low cut-off index for the stool antigen test. The discrepancies we observed, with positive results on the stool antigen test and negative results on the UBT, were likely due to the same mechanism.

There were no significant differences in positive rates on the UBT and stool antigen test before and after PPI therapy, suggesting that the stool antigen test is a useful and reliable diagnostic test for *H. pylori*, similar to the UBT. The positivity rate for UBT decreased from 75.0% before to 64.3% after PPI administration, and the positivity rate for the stool antigen test decreased from 78.6% to 60.7%. These reductions indicate that the stool antigen test should be performed after limiting PPI administration as much as possible, but that, when PPI treatment cannot be stopped, the stool antigen test shows comparable utility to the UBT. Although both the stool antigen test and UBT results did not change significantly from before to after PPI treatment, the reductions were much less pronounced on the stool antigen test than on the UBT. Moreover, in patients treated for $\geqslant$ 4 wk, UBT, but not stool antigen test, results decreased significantly after PPI treatment. These findings indicate that, while the results of both assays were influenced by the bacteriostatic actions of PPIs, the stool antigen test was less influenced than the UBT.

Although several studies have reported increased false-negative results for the stool antigen test during PPI treatment[13,14], we found that stool antigen test results were more stable than UBT results in patients being treated with PPIs.

Despite the benefits of the UBT as a non-invasive test for *H. pylori*[17] and the slightly lower accuracy of the stool antigen test, the latter has several advantages, including ease, rapidity and lower cost. Others have also reported that the stool antigen test is highly sensitive and specific, with high utility due to the speed and simplicity of testing[18].

The 2005 Maastricht Ⅲ consensus report recommended that the stool antigen test be used for diagnosis when the UBT is unavailable[2]. The numbers of patients receiving PPI therapy have been increasing around the world, and many patients have difficulty undergoing the UBT, such as elderly patients and patients with reduced ADL. In these patients, the stool antigen test should be considered reliable and useful.

In conclusion, the stool antigen test showed stable results even during PPI treatment as well as sensitivity comparable to the UBT. In patients treated with PPI for $\geqslant$ 4 wk, the stool antigen test showed more stable results than the UBT. The stool antigen test is therefore a useful and reliable diagnostic method for *H. pylori* infection.

COMMENTS

Background

Although the urea breath test (UBT) is a reliable, non-invasive diagnostic test for *Helicobacter pylori* (*H. pylori*), it has a potential for false-negative results in patients treated with proton pump inhibitor (PPI). The stool antigen test is another useful, easy to perform, non-invasive assay for *H. pylori*, with high sensitivity and specificity. However, the effects of PPI treatment on stool antigen test results are unclear.

Research frontiers

Due to the increase in patients with gastroesophageal reflux disease and those treated with nonsteroidal anti-inflammatory drugs, the numbers treated with PPIs has increased throughout the world. Thus, reliable diagnostic tests for *H. pylori* are needed for patients treated with PPIs. The study describe the utility of a stool antigen test, using the TestMate pylori enzyme immunoassay, in patients treated with PPIs.

Innovations and breakthroughs

Although many studies have reported results of stool antigen tests in patients treated with PPIs, none has assessed the relationship between measured value of stool antigen test and UBT results. To our knowledge, this study is the first to clarify the stability of the stool antigen test, relative to the UBT, in patients treated with PPIs.

Applications

Elderly patients and those with reduced daily activities who are treated with PPIs have increased. The UBT is somewhat difficult to perform in these patients. The authors found that the stool antigen test may be a useful alternative to the UBT in these patients.

Terminology

The stool antigen test is a diagnostic assay for *H. pylori*. which identifies *H. pylori* antigens in feces using antigen-antibody reactions. This non-invasive assay has equal sensitivity and specificity to the UBT, and may be especially useful for children or elderly patients.

Peer review

The manuscript is well written. The methods are adequate. The results justify the conclusions drawn.

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S- Editor Zhang SJ **L- Editor** O'Neill M **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i1.49

World J Gastroenterol 2012 January 7; 18(1): 49-54
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Outcome after gastrectomy in gastric cancer patients with type 2 diabetes

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Received: March 17, 2011 Revised: April 15, 2011
Accepted: April 22, 2011
Published online: January 7, 2012

Abstract

AIM: To evaluate the prognosis of type Ⅱ diabetes mellitus (T2DM) after gastrectomy and related factors in gastric cancer patients.

METHODS: 403 gastric cancer patients with T2DM were studied, who underwent gastrectomy between May 2003 and September 2009. A review of medical records and telephone interviews was performed in this cross-sectional study. The factors included in the statistical analysis were as follows: gender, age, type of surgery, preoperative body mass index (BMI), current BMI, BMI reduction ratio, preoperative insulin or oral diabetic medicine requirement, follow-up duration, and current state of diabetes. Assessment of diabetes status after surgery was classified into four categories according to the change in hypoglycemic agents after surgery and present status of T2DM: resolution, improvement, same, and worse.

RESULTS: The mean follow-up duration was 33.7 mo (± 20.6 mo), preoperative BMI was 24.7 kg/m^2 (± 3.0 kg/m^2), and BMI reduction ratio was 9.8% (± 8.6%). After surgery, T2DM was cured in 58 patients (15.1%) and was improved in 117 patients (30.4%). According to the type of surgery, the BMI reduction ratio was significantly higher in the total gastrectomy and Roux-en-Y reconstruction group [14.2% ± 9.2% *vs* 9.2% ± 7.7% (Billroth Ⅱ group), $P < 0.001$] and significantly lower in the subtotal gastrectomy and Billroth Ⅰ reconstruction group [7.6% ± 8.0%, 9.2% ± 7.7% (Billroth Ⅱ group), $P < 0.001$]. The BMI reduction ratio, follow-up duration after surgery, type of surgery, extent of gastrectomy, and performance of duodenal bypass were significantly correlated to the course of T2DM ($P < 0.05$). The BMI reduction ratio was the most influential factor on T2DM status. In a subgroup analysis of patients with a BMI reduction ratio of 10% or less ($n = 206$), T2DM was cured in 15 (7.6%) patients and was improved in 57 (28.8%) patients after surgery, and only the duration of surgery was significantly correlated to T2DM status ($P = 0.022$).

CONCLUSION: The course of T2DM was significantly correlated to the BMI reduction ratio but not to the type of surgery without a significant change in BMI.

Key words: Gastric cancer; Diabetes mellitus; Metabolic surgery; Bariatric surgery

Peer reviewer: Kazuma Fujimoto, Professor, Department of Internal Medicine, Saga Medical School, Nabeshima, Saga 849-8501, Japan

Kim JW, Cheong JH, Hyung WJ, Choi SH, Noh SH. Outcome after gastrectomy in gastric cancer patients with type 2 diabetes. *World J Gastroenterol* 2012; 18(1): 49-54 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i1/49.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i1.49

INTRODUCTION

Gastrointestinal surgery has been established as the treatment of choice for patients with inadequately controlled type 2 diabetes mellitus (T2DM) and a body mass index (BMI) greater than or equal to 35 kg/m^2[1]. However, indications for bariatric surgery in the treatment of T2DM are not clear. T2DM is improved by calorie restriction and weight loss, which can be aided by bariatric surgery.

Recently, it was reported that bariatric surgery to treat morbid obesity along with T2DM resulted in amelioration of T2DM, including weight loss[2-6]. In addition, a non-randomized, comparative Swedish Obesity Study showed that regaining weight led to recurrence of diabetes through long-term follow-up[7]. However, bariatric surgery may improve glycemic control in T2DM within days, even before considerable weight loss occurs. The exact mechanism for the dramatic effect of obesity surgery on T2DM remains unknown. T2DM has been approached from a new angle as an intestinal disease, which is increasingly viewed as operable[8-11].

These findings prompted clinicians to extend indications for bariatric surgery to non-obese patients with T2DM. In the 1950s and 60s, amelioration of diabetes following subtotal gastrectomy was reported[12,13]. Since then, there have been few reports with inconsistent results in this area[14,15].

In this study, we compared the effects of gastrointestinal, anatomical rearrangements on diabetes after surgery for gastric cancer.

MATERIALS AND METHODS

Between May 2003 and September 2009, 6848 patients with gastric cancer underwent gastrectomy at Severance Hospital and Gangnam Severance Hospital, the Yonsei University College of Medicine. Of these patients, 576 had diabetes; the diagnosis of diabetes was based on the criteria of the American Diabetes Association and the World Health Organization. We excluded 81 patients who expired or had cancer recurrence and 92 patients who could not be surveyed, leaving 403 patients to be enrolled for this study. To exclude type 1 diabetes, we reviewed patient medical records and interviewed these individuals about their type and history of diabetes, but no patients were diagnosed with or suspected to have type 1 diabetes. Data were collected by a review of medical records and interviews *via* telephone. The following factors were statistically analyzed: gender, age, type of surgery, preoperative BMI, current BMI, BMI reduction ratio, preoperative insulin or oral diabetic medicine requirement, follow-up duration, and current state of diabetes. "Resolution" was defined as a case where the patient became euglycemic or an HbA1c level was maintained below 6% without the use of diabetes medication after surgery. "Improvement" was defined as a case where the patient showed a better fasting glucose level and either used a lower dose of hypoglycemic agents or converted to oral hypoglycemic agents from insulin, compared with preoperative diabetic control. "Same" or "worse" was defined as a case in which no change occurred ("same") or an aggravation in fasting glucose and medication after surgery ("worse") was observed. Patients were divided into three groups by type of surgery: gastroduodenostomy after subtotal gastrectomy [Billroth Ⅰ (B-Ⅰ) group], gastrojejunostomy after subtotal gastrectomy [Billroth Ⅱ (B-Ⅱ) group], and Roux-en-Y esophagojejunostomy after total gastrectomy (R-Y group).

Table 1 Clinical findings [*n* = 403, *n* (%) or mean ± SD]

Gender	Female	96 (23.8)
	Male	307 (76.2)
Age at OP (yr)		63.8 ± 7.6
OP type	B-Ⅰ	165 (40.9)
	B-Ⅱ	134 (33.3)
	R-Y	104 (25.8)
Extent of gastrectomy	Subtotal (B-Ⅰ, B-Ⅱ)	299 (74.2)
	Total (R-Y)	104 (25.8)
Duodenal bypass	Bypass – (B-Ⅰ)	165 (40.9)
	Bypass + (B-Ⅱ, R-Y)	238 (59.1)
Pre-OP BMI (kg/m^2)		24.7 ± 3.0 (18.4-39.9)
Current BMI (kg/m^2)		22.2 ± 2.9 (13.5-35.3)
BMI reduction ratio[1] (%)		9.8 ± 8.6
Pre-OP	Oral diabetic agent	368 (91.3)
diabetic therapy	Insulin	35 (8.7)
F/U duration (mo)		33.7 ± 20.6 (5.5-81.8)
Diabetes course	Worse	38 (9.9)
	Same	172 (44.7)
	Improvement	117 (30.4)
	Resolution	58 (15.1)

[1]BMI reduction ratio (%) = -[(Current BMI - Pre-OP BMI)/Pre-OP BMI] × 100. OP: Operation; BMI: Body mass index; F/U: Follow up; B-Ⅰ: Billroth Ⅰ; B-Ⅱ: Billroth Ⅱ; R-Y: Roux-en-Y.

SPSS version 12.0 for Windows (SPSS Inc., Chicago, IL, United States) was used for all statistical analyses. All data were analyzed using the paired sample *t* test, analysis of variance, and ordinal logistic regression analysis. A *P* value of less than 0.05 was considered significant.

This study was approved by the Institutional Review Board of Gangnam Severance Hospital (approval number 3-2020-0259).

RESULTS

The characteristics of the subjects are summarized in Table 1. Of 403 patients, 58 patients (15.1%) were euglycemic without medication, and 117 patients (30.4%) showed improved metabolic conditions but were still taking medication. In contrast, 172 patients (44.7%) did not change their medication for diabetes, and 38 patients (9.9%) had aggravated conditions (Table 1).

Univariate analyses were carried out to determine the impact of factors contributing to the amelioration of diabetes. The course of diabetes after surgery was significantly different between the R-Y and B-Ⅰ groups (P = 0.001) and between the R-Y and B-Ⅱ groups (P = 0.006), but not between the B-Ⅰ and B-Ⅱ groups (P = 0.161).

Table 2 Univariate analysis [*n* = 403, *n* (%) or mean ± SD]

		Diabetes course				*P* value
		Worse	Same	Improvement	Resolution	
Gender	Female	8 (8.6)	36 (38.7)	34 (36.6)	15 (16.1)	0.180
	Male	30 (10.3)	136 (46.6)	83 (28.4)	43 (14.7)	
Age at OP (yr)		62.6 ± 7.6	64.2 ± 7.4	63.5 ± 7.4	62.5 ± 8.4	0.453
OP type	B-Ⅰ[a]	20 (12.4)	82 (50.9)	42 (26.1)	17 (10.6)	0.001
	B-Ⅱ[a]	10 (8.0)	59 (47.2)	42 (33.6)	14 (11.2)	0.006
	R-Y	8 (8.1)	31 (31.3)	33 (33.3)	27 (27.3)	
Extent of gastrectomy	B-Ⅰ and B-Ⅱ (subtotal)	30 (10.5)	141 (49.3)	84 (29.4)	31 (10.8)	< 0.001
	R-Y (total)	8 (8.1)	31 (31.3)	33 (33.3)	27 (27.3)	
Duodenal bypass	B-Ⅰ (bypass -)	20 (12.4)	82 (50.9)	42 (26.1)	17 (10.6)	0.002
	B-Ⅱ and R-Y (bypass +)	18 (8.0)	90 (40.2)	75 (33.5)	41 (18.3)	
Pre-OP BMI (kg/m^2)		23.4 ± 2.5	24.9 ± 2.6	24.8 ± 3.5	24.9 ± 3.5	0.127
Current BMI (kg/m^2)		21.7 ± 2.7	22.7 ± 2.6	22.2 ± 3.2	21.2 ± 2.9	0.038
BMI reduction ratio[1] (%)		6.8 ± 10.2	8.5 ± 7.3	10.4 ± 8.7	14.4 ± 8.5	< 0.001
Pre-OP diabetic regimen	Oral diabetic agent	38 (10.8)	159 (45.3)	99 (28.2)	55 (15.7)	0.147
	Insulin	0 (0.0)	13 (38.2)	18 (52.9)	3 (8.8)	
F/U duration (mo)		36.4 ± 18.2	37.2 ± 21.6	30.0 ± 20.1	28.3 ± 17.8	0.001

[a]*P* = 0.161, B-Ⅰ *vs* B-Ⅱ. [1]BMI reduction ratio (%) = -[(Current BMI - Pre-OP BMI)/Pre-OP BMI] × 100. OP: Operation; BMI: Body mass index; F/U: Follow up; B-Ⅰ: Billroth Ⅰ; B-Ⅱ: Billroth Ⅱ; R-Y: Roux-en-Y.

We categorized patients into two groups by the extent of gastrectomy. Diabetes was significantly improved in the total gastrectomy group compared with the subtotal gastrectomy group ($P < 0.001$) All patients were then categorized into two groups by the presence of duodenal bypass. Patients who received duodenal bypass had significantly improved diabetes compared to those who did not ($P = 0.002$, Table 2). In addition, the course of diabetes was significantly correlated with current BMI and BMI reduction ratio ($P = 0.038$ and $P < 0.001$, respectively). Diabetes was improved immediately after surgery, gradually worsening over a longer term ($P = 0.001$, Table 2).

Ordinal logistic regression analysis was performed to determine significant factors affecting the course of diabetes. Because surgery type, extent of gastrectomy, and duodenal bypass were similar factors, when the three factors were entered simultaneously in multivariate analysis, all were insignificant. Therefore, we performed multivariate analysis three times according to the three types of surgery. In all three multivariate analyses, the BMI reduction ratio and follow-up duration were significantly related to amelioration of diabetes, but current BMI was not. Moreover, the absolute value of contribution degree of the BMI reduction ratio was greatest among significant factors, so the BMI reduction ratio was found to have the biggest impact on diabetic status after three multivariate analyses (Table 3). Performance of B-Ⅰ versus R-Y was a significant factor, but performance of B-Ⅱ versus R-Y was not. We used the extent of gastrectomy or duodenal bypass as a covariate, and these factors were also significant.

Based on these results, we investigated the association between weight change and type of surgery. There was a significant decrease in BMI in the R-Y group, compared with the B-Ⅰ and B-Ⅱ groups (Figure 1A). There was also a significant difference in the BMI reduction ratio between the B-Ⅰ and B-Ⅱ groups. The total gastrectomy group showed significant weight loss compared with the subtotal gastrectomy group ($P < 0.001$, Figure 1B), and duodenal bypass lost significantly more weight than the non-duodenal bypass group ($P < 0.001$, Figure 1C).

Table 3 Multivariate analysis (*n* = 403)

	P value	B-estimation	SE	Contribution degree
Current BMI (kg/m^2)	0.454	0.028	0.037	
BMI reduction ratio[1] (%)	< 0.001	0.051	0.013	3.948
F/U duration (mo)	0.007	-0.013	0.005	-2.703
OP type				
B-Ⅰ	0.006	-0.714	0.258	-2.770
B-Ⅱ	0.070	-0.473	0.262	
R-Y				
Current BMI (kg/m^2)	0.478	0.026	0.037	
BMI reduction ratio[1] (%)	< 0.001	0.052	0.013	4.031
F/U duration (mo)	0.006	-0.013	0.005	-2.727
Extent of gastrectomy				
Subtotal (B-Ⅰ, B-Ⅱ)	0.011	-0.597	0.234	-2.553
Total (R-Y)				
Current BMI (kg/m^2)	0.657	0.016	0.037	
BMI reduction ratio[1](%)	< 0.001	0.054	0.013	4.181
F/U duration (mo)	0.005	-0.013	0.005	-2.811
Duodenal bypass				
Bypass – (B-Ⅰ)	0.039	-0.416	0.202	-2.061
Bypass + (B-Ⅱ, R-Y)				

OP: Operation; BMI: Body mass index; F/U: Follow up; B-Ⅰ: Billroth Ⅰ; B-Ⅱ: Billroth Ⅱ; R-Y: Roux-en-Y; [1]BMI reduction ratio (%) = -[(Current BMI - Pre-OP BMI)/Pre-OP BMI] × 100.

These results suggest that the main factor influencing diabetes improvement might be weight loss instead of type of surgery. Therefore, we performed subgroup analysis of the patients whose BMI reduction ratio was less than 10% of the preoperative level. The mean BMI reduction ratio was 3.4% (± 5.4%; Table 4). In univariate analysis, type of surgery was not a significant factor for

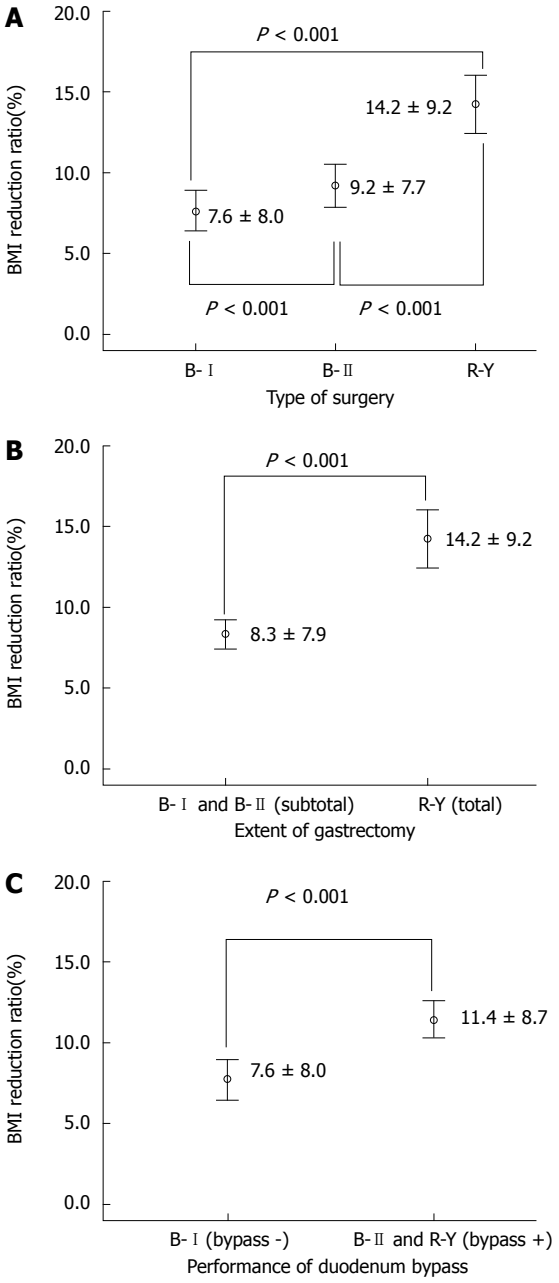

Figure 1 Body mass index reduction ratio according to A: Type of surgery; B: Extent of gastrectomy; C: Performance of duodenum bypass (*n* = 403, mean ± SD). BMI: Body mass index; B- Ⅰ: Billroth Ⅰ; B- Ⅱ: Billroth Ⅱ; R-Y: Roux-en-Y.

the amelioration of diabetes (Table 5). However, in this subgroup of 203 patients, diabetes was resolved without medication in 15 patients (7.6%), and 57 patients (36.4%) improved without significant weight loss.

DISCUSSION

This study was conducted in a retrospective manner. Therefore, the type of diabetes was not evaluated by laboratory testing but by reviewing medical records and interviewing. We interviewed all of the enrolled patients and asked about the type and history of diabetes to exclude type 1 diabetes. There were no patients who were diagnosed with or suspected to have type 1 diabetes. Furthermore, in Korea, the incidence of type 1 diabetes is very low, reported as approximately 0.6-2.2/100 000[16-18]. Considering that type 1 diabetes is extremely rare in Korea and the mean age of the study population was 63.8 years, the results of this study showing that there were no cases of type 1 diabetes in 6848 patients of the study population can be thought acceptable.

Table 4 Clinical findings in subgroup analysis [body mass index reduction ratio < 10%, *n* = 206, *n* (%) or mean ± SD]

Gender	Female	38 (18.4)
	Male	168 (81.6)
Age at OP (yr)		
OP type	B- Ⅰ	105 (51.0)
	B- Ⅱ	68 (33.0)
	R-Y	33 (16.0)
Extent of gastrectomy	Subtotal (B- Ⅰ, B- Ⅱ)	173 (84.0)
	Total (R-Y)	33 (16.0)
Duodenal bypass	Bypass – (B- Ⅰ)	105 (51.0)
	Bypass + (B- Ⅱ, R-Y)	101 (49.0)
Pre-OP BMI (kg/m^2)		23.8 ± 2.8 (18.4-32.6)
Current BMI (kg/m^2)		23.0 ± 2.7 (21.8-32.5)
BMI reduction ratio[1] (%)		3.4 ± 5.4
Pre-OP diabetic regimen	Oral diabetic agent	186 (90.3)
	Insulin	20 (9.7)
F/U duration (mo)		35.7 ± 20.7 (5.5-81.1)
Diabetes course	Worse	24 (12.1)
	Same	102 (51.5)
	Improvement	57 (28.8)
	Resolution	15 (7.6)

OP: Operation; BMI: Body mass index; F/U: Follow up; B- Ⅰ: Billroth Ⅰ; B- Ⅱ: Billroth Ⅱ; R-Y: Roux-en-Y; [1]BMI reduction ratio (%) = -[(Current BMI - Pre-OP BMI)/Pre-OP BMI] × 100.

Possible mechanisms through which metabolic surgery has led to the resolution of T2DM are a decrease in calorie intake, weight loss, and a rearranged gastrointestinal track, which could modulate hormones that affect glucose metabolism, insulin sensitivity, and beta pancreatic cell proliferation[19]. Several reports support a positive role for duodenojejunal exclusion and/or duodenal bypass for T2DM resolution after bariatric surgery in obese patients[20].

However, it is still unclear whether these effects extend to non-obese patients. One report stressed that gastrectomy and short Roux-en Y reconstruction in non-obese T2DM patients correlate with remission of diabetes in 65% of patients[14]. In that study, the preoperative BMI was 29.1 and the postoperative BMI was 24.6 kg/m^2; such significant weight loss might explain the relatively high rate of T2DM resolution after gastrectomy. Another study reported a resolution rate of 38.1% after gastrectomy with Roux-en Y gastrojejunostomy, which is similar to our results[15]. Early delivery of partially digested foods to the ileum and discrete enteroendocrine cells in the mucosa are suggested to release hormones that contribute to the ileal brake[21]. However, we could not evaluate this point due to procedural limitations.

Table 5 Subgroup analysis [body mass index reduction ratio < 10%, *n* = 206, *n* (%) or mean ± SD]

		Diabetes course				*P* value
		Worse	Same	Improvement	Remission	
Gender	Female	3 (8.1)	17 (45.9)	14 (37.8)	3 (8.1)	0.190
	Male	21 (13.0)	85 (52.8)	43 (26.7)	12 (7.5)	
Age at OP (yr)		61.9 ± 8.1	63.9 ± 8.0	63.8 ± 7.5	61.1 ± 9.3	0.976
OP type	B-Ⅰ[a]	14 (13.5)	56 (53.8)	27 (26.0)	7 (6.7)	0.076
	B-Ⅱ[a]	7 (11.3)	33 (53.2)	18 (29.0)	4 (6.5)	0.181
	R-Y	3 (9.4)	13 (40.6)	12 (37.5)	4 (12.5)	
Extent of gastrectomy	Subtotal (B-Ⅰ, B-Ⅱ)	21 (12.7)	89 (53.6)	45 (27.1)	11 (6.6)	0.084
	Total (R-Y)	3 (9.4)	13 (40.6)	12 (37.5)	4 (12.5)	
Duodenal bypass	Bypass – (B-Ⅰ)	14 (13.5)	56 (53.8)	27 (26.0)	7 (6.7)	0.250
	Bypass + (B-Ⅱ, R-Y)	10 (10.6)	46 (48.9)	30 (31.9)	8 (8.5)	
Pre-OP BMI (kg/m^2)		22.7 ± 2.3	24.3 ± 2.7	23.8 ± 3.0	22.2 ± 2.8	0.615
Current BMI (kg/m^2)		22.3 ± 2.2	23.4 ± 2.6	23.0 ± 3.1	21.3 ± 2.6	0.310
BMI reduction ratio[1] (%)		1.5 ± 6.6	3.7 ± 4.5	3.6 ± 5.9	4.2 ± 4.6	0.183
Pre-OP diabetic regimen	Oral diabetic agent	24 (13.5)	93 (52.2)	47 (26.4)	14 (7.9)	0.080
	Insulin	0 (0.0)	9 (45.0)	10 (50.0)	1 (5.0)	
F/U duration (mo)		37.7 ± 17.6	38.8 ± 21.7	30.7 ± 20.7	28.7 ± 14.1	0.022

[a]*P* = 0.674, B-Ⅰ *vs* B-Ⅱ; OP: Operation; BMI: Body mass index; F/U: Follow up; B-Ⅰ: Billroth Ⅰ; B-Ⅱ: Billroth Ⅱ; R-Y: Roux-en-Y; [1]BMI reduction ratio (%) = -[(Current BMI - Pre-OP BMI)/Pre-OP BMI] × 100.

One of the mainstream T2DM treatments is reduction of calorie intake; restrictive bariatric surgery effectively controls T2DM in obese patients[2]. In this study, univariate and multivariate analyses showed that duodenal bypass and/or duodenojejunal exclusion groups demonstrated a high rate of T2DM resolution compared to other procedures. However, for patients who lost less than 10% of their preoperative weight, the type of surgery was not significantly important for T2DM resolution. These results suggest that the usual procedures after gastrectomy for gastric cancer are ineffective to control T2DM directly. In addition, most gastrectomized patients for gastric cancer are not pathologically obese, and reconstruction should not induce severe weight loss.

Interestingly, diabetes worsened after improvements were observed for a short time after surgery. The limitation of this study, which is retrospective and cross-sectional analysis, should be considered. Moreover, most gastric cancer patients are cautious about maintaining their weight for good nutrition. Therefore, it is possible that weight loss immediately after surgery improves T2DM, and as the patient regains weight, the condition deteriorates. In meta-analysis, the proportion of patients with resolution or improvement was not significantly different at time points less than 2 years or 2 years or more after surgery, although it slightly decreased over time[2].

Several studies demonstrated that T2DM could be resolved after metabolic surgery, irrespective of weight loss, *via* a modification in the enteroinsular axis[2,20,21]. Based on these findings, we tried to establish a new procedure for the resolution of T2DM in non-obese patients. Unfortunately, routine reconstruction surgery after gastrectomy could not control diabetes in non-obese patients, and the main mechanism for gastrectomy-induced resolution of T2DM is possibly weight loss, instead of the type of surgery itself. Thus, a more sophisticated procedure should be developed and prospectively evaluated for its effectiveness in controlling T2DM in gastric cancer patients after gastrectomy.

COMMENTS

Background

It has been frequently reported that bariatric surgery for morbid obesity along with type Ⅱ diabetes mellitus (T2DM) results in amelioration of T2DM, including weight loss. Furthermore, in some reports, bariatric surgery may improve glycemic control in T2DM within days, even before considerable weight loss occurs. These findings prompted clinicians to consider T2DM as an operable gastrointestinal disease and extend indications for bariatric surgery to non-obese patients with T2DM. However, the application of surgery for non-obese T2DM patients has considerable risks. In Korea, many cases of gastric cancer have been performed by gastric cancer surgery. In this study, the effects of gastrointestinal, anatomical rearrangements on diabetes were compared after surgery for gastric cancer.

Research frontiers

This is a retrospective but very large-scale study. The authors interviewed all of the enrolled subjects *via* telephone.

Innovations and breakthroughs

In the 1950s and 60s, amelioration of diabetes following subtotal gastrectomy was reported. Since then, there have been few reports in this area with inconsistent results. In those reports, the numbers of cases were very small. In this study, 403 patients were enrolled, and we evaluated the factors affecting the course of diabetes. The authors demonstrated that 15% of T2DM was cured after gastrectomy with conventional reconstruction. The extent of gastrectomy, type of reconstruction, and change in body mass index (BMI) were related to the course of diabetes. Among these factors, the change in BMI was the strongest. In the subgroup analysis of small BMI changes, the extent of gastrectomy and the type of reconstruction were not statistically related to the course of diabetes, which did not support the foregut hypothesis.

Applications

The results of this study can be used as reference data for further study. In other words, in some study of innovative treatment for diabetes in gastric cancer patients, the results could be used for the calculation of the number of subjects required and provide reference data for comparison. In addition, duodenal bypass itself is not a critical factor in metabolic surgery.

Terminology

The term duodenal bypass means a condition in which ingested food material

does not traverse the duodenum. In both Billroth Ⅱ reconstruction after subtotal gastrectomy and Roux-en-Y reconstruction after total gastrectomy, ingested food materials go directly to the jejunum. Metabolic surgery is a field of surgery that attempts to treat metabolic syndrome, including diabetes. Hormonal changes after metabolic surgery are thought to be important. Both the hindgut hypothesis (the quick transit of nutrients to the distal bowel is important) and the foregut hypothesis (the exclusion of the proximal small intestine induces resolution of T2DM) have been proposed.

Peer review

This paper summarized the effect of gastrectomy on T2DM in a large number of patients.

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S- Editor Tian L **L- Editor** Rutherford A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i1.55

World J Gastroenterol 2012 January 7; 18(1): 55-63
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Efficacy and safety of treatment of hepatitis C virus infection in renal transplant recipients

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Supported by King Abdullah International Medical Research Center, National Guard Health Affairs, Riyadh, Saudi Arabia
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Received: February 22, 2011 Revised: April 20, 2011
Accepted: April 27, 2011
Published online: January 7, 2012

Abstract

AIM: To assess the efficacy and safety of combined pegylated interferon and ribavirin therapy in hepatitis C virus (HCV) infection in renal transplant recipients.

METHODS: This is a retrospective chart review of post renal transplant patients who were positive for anti-HCV and HCV-RNA, and who have received treatment with combination of pegylated interferon and ribavirin between October 2003 and December 2008. Only patients with stable graft function and absence of evidence of cirrhosis and who received the therapy for continuous 48 wk were included. Nineteen patients (13 male and 6 female) were identified and included. The patient's complete blood count, liver and kidney profile, and calculated glomerular filtration rate (GFR) were monitored every 6-8 wk while on treatment. HCV-RNA was tested at 12 wk for early virological response, at 48 wk for end of treatment response (ETR), and then retested at 24, and 48 wk after completion of therapy for sustained virological response (SVR). Liver biopsies were obtained before treatment from all patients and graft kidney biopsies were performed as required.

RESULTS: Of the entire cohort, 9 patients (47.4%) showed an ETR and 8 had SVR (42.1%). Of the 8 patients with abnormal alanine aminotransferase (ALT) levels at baseline, 78.9% had their ALT normalized (including the virological non responders). ALT was normal in all responders at the end of therapy and at 24 wk post therapy (100%). Only one patient (5.3%) developed an increase in creatinine and decline in GFR from baseline towards the end of treatment. This patient's kidney biopsy revealed borderline rejection. There was no impact on response by HCV-genotype, initial HCV RNA load, age or sex of the patient or duration post transplant before commencement of therapy. All patients tolerated treatment in the same way as non-transplant with no unusual or increased occurrence of side effects.

CONCLUSION: The combination of pegylated interferon and ribavirin is effective in suppressing HCV-RNA,

with a low risk of graft rejection or failure in HCV infected renal transplant recipients.

Key words: Allograft rejection; Hepatitis C; Pegylated interferon; Ribavirin; Renal transplant

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INTRODUCTION

Hepatitis C virus (HCV) is the major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma and is the leading indication for liver transplantation worldwide[1]. There is a marked geographic variation in seroprevalence and genotypes of HCV[2-4]. HCV infection is common among patients with end-stage renal disease. The prevalence of HCV infections in patients that undergo hemodialysis has been described in literature worldwide, reaching as high as 63%[5-8]. After implementation of regulations and routine screening in dialysis centers to prevent spread of infection, the incidence of HCV infection has declined in several countries[9-11]. A considerable number of dialysis patients will eventually undergo renal transplantation, which will ultimately increase the prevalence of HCV infection post renal transplantation. HCV has been recognized as one of the major causes of morbidity and mortality and indicates a poor prognosis of patient and graft survival in renal transplantation[12-15]. Reports on the prevalence of HCV in renal allograft recipients were variable. It has been reported to be from 10% to 49% in some centers, but may reach up to 64% in others[12,15-21]. Renal transplantation in HCV positive patients is associated with an aggressive course of liver disease[5,12,22,23]. Treatment of hepatitis C post renal transplant has been a debatable and controversial issue for a long time. Although there were some case reports of successful therapy with interferon with no serious side effects[24,25] many studies have suggested that interferon is contraindicated in such patients due to high incidence of allograft rejection, severe graft dysfunction and/or intolerance of patients to such therapy[26-33]. Most of the reported studies however, have included a small number of patients. Furthermore conventional interferon has been utilized in these studies and only limited experience with pegylated interferon (PEG-IFN) has been reported. In this retrospective study we report our experience with the largest cohort group of patients with hepatitis C post renal transplant treated with a combination of pegylated interferon and ribavirin, focusing on treatment response and allograft rejection.

MATERIALS AND METHODS

This is a retrospective, chart review study of post renal transplant recipients who were positive for anti-HCV and HCV-RNA, and who have received treatment with a combination of PEG-IFN and ribavirin, between October 2003-the time when pegylated interferon had become available as a formulary drug in our institution-and December 2008. Only patients with stable graft function, and absence of cirrhosis were included. Out of 230 renal transplant recipients followed at our institution, 40 consecutive recipients with positive hepatitis C serology were referred from nephrology to the hepatology clinic. Nineteen such patients (13 male and 6 female) were identified and included. Twenty one patients were excluded, as follows; one patient was anti-HCV positive but his HCV-RNA was negative, 4 patients refused to take therapy after they were told that they may lose their kidneys secondary to treatment, 1 patient had multiple medical problems, 3 patients had developed well established liver cirrhosis with portal hypertension, 4 patients had established rejection and had either considered or already started hemodialysis just before starting treatment, and 8 patients were recipients of double organ (liver and kidney) transplantation.

The patient's white cell count (WBC), hemoglobin, platelets, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, bilirubin, prothrombin time, blood urea nitrogen (BUN), creatinine, and glomerular filtration rate (GFR) (calculated using Cockcroft-Gault Formula)[34] were measured before treatment and repeated every 6-8 wk while on treatment and every 12 wk after completing therapy.

All patients were screened for hepatitis B virus (HBV), human immune deficiency virus (HIV) and autoimmune markers pre and post transplantation. Anti-HCV was tested using either the 3rd generation enzyme immunoassay (AxSYM HCV Version 3.0, Abbott laboratories, Diagnostics Division, Abbott Park, IL 60064, United States) or more recently with ARCHITECT Anti-HCV (Abbott GmbH and Co. KG, Max-Planck-Ring 2, 65205 Wiesbaden, Germany). Quantitative HCV-RNA was performed using Roche COBAS Ampliprep/ COBAS TaqMan System (Roche Molecular Systems, Pleasanton, United States). Qualitative HCV-RNA was performed using Roche Automated COBAS Amplicor Analyzer (Roche Molecular Systems, Pleasanton, United States). The HCV genotype detection assay was performed using m2000 real-time system (Abbott Molecular Diagnostics, Abbott Park, IL, United States). HCV genotype was determined in 14 of the patients. Quantitative HCV-RNA test was carried out in all patients prior to treatment, at 12 wk after starting treatment for early virological response (EVR), at 48 wk for end of treatment response (ETR), and then qualitative

and quantitative assays for HCV-RNA were performed at 24 and 48 wk after completion of therapy for sustained virological response (SVR). All patients had under gone liver biopsy prior to treatment. We used modified Ishak histological grading and staging of chronic hepatitis using the histological activity index (HAI) scoring system for the degree of necroinflammatory activity and a staging system for degree of fibrosis[35]. For simplification purposes, we classified necroinflammatory grading on liver histology into; minimal/mild chronic hepatitis if score is 0-6, moderate chronic hepatitis if score is 7-9, and marked chronic hepatitis, with or without bridging necrosis, if score is 10-18. Fibrosis staging was classified into minimal/mild fibrosis for score 0-3, and moderate/marked fibrosis for score 4-6.

Kidney biopsies were performed on patients who developed abnormal renal function or reduction of GFR, either before or during therapy according to the normal unit indications.

HCV treatment protocol used

This was based on the standard international guidelines for therapy of hepatitis C. However because these are a special group of patients and since there is no agreed-on defined protocol to treat such patients and as our study is a retrospective, the individual treatment protocol was left to the discretion of the treating hepatologist. Pegylated interferon α-2a (Pegasys, F. Hoffmann-la Roche Ltd., Basel, Switzerland) at a dosage of 135-180 μg (135 μg for 2 patients and 180 μg for 13 patients) every week in combination with ribavirin 400 mg (2 patients), 800 mg (11 patients), 1000 mg (1 patient), 1200 mg (1 patient) daily in two divided doses were given to 15 patients. Pegylated interferon α-2b (Peg-intron, Schering-Plough Corporation, Kenilworth, NJ, United States) at a dosage of 80-100 μg every week, in combination with ribavirin 400 mg (1 patients), 800 mg (3 patients) daily in divided doses were given to the remaining 4 patients.

All patients were treated for 48 wk. Patients whose hemoglobin dropped to below 100 mg/dL were given erythropoietin subcutaneously, and those whose absolute neutrophil count dropped to below 800/mm^3 were given granulocyte colony stimulating factor (G-CSF) subcutaneously. The dose and frequency of erythropoietin and G-CSF were given according to our center local guidelines, which are similar to the international protocols. Patients were followed up at the hepatology clinic every 2 wk for the first 6 wk and every 6-8 wk thereafter. Patients were also seen in the nephrology clinic every four to eight weeks.

Immunosuppression

All patients were on maintenance steroid therapy in the form of prednisone. Fifteen patients were on mycophenolate mofetil (CellCept), eight on cyclosporine and nine on tacrolimus. One patient received sirolimus. None of our patients received azathioprine.

Ethical and safety issues

Since there was no available international treatment protocol, the risks and benefits of therapy, including potential graft rejection were explained carefully to all patients by the nephrologists and further reinforced by the hepatologist before commencing therapy as normal precautions which were usually performed in similar conditions outside study protocols. Only those patients who consented received the therapy. The study was passed by the hospital's research committee and approved by the institutional review board.

Statistical analysis

The data was analyzed using SPSS version 17. Descriptive data was obtained for all the parameters tested (mean, median, SD). The change over time in liver profile, renal profile, and viral load were compared using ANOVA and changes between baseline and end of treatment parameters were examined using paired *t*-test. The effect of age, post transplant duration and gender on the response was tested using two-tailed independent *t* test and Pearson Chi square.

RESULTS

Of the nineteen recipients included in the study there were 13 males and 6 females. Age ranged from 20 years to 66 years, with a mean age of 39.9 (± 12.6) years. The time from transplant to initiation of treatment ranged from 14 mo to 156 mo with a mean of 66.3 (± 45.7) mo. All patients had undergone hemodialysis in more than one dialysis unit before renal transplant. Our patients tolerated the treatment fairly well. There were no unusual side effects to therapy. None of our patients were intolerant to therapy requiring discontinuation. None of our patients experienced a serious infection or sepsis during the course of therapy.

Liver biochemical profile

ALT was high in 9 patients at baseline, 15 patients (78.9%) had normal ALT at the end of therapy (including non responders). Nine patients had high AST at baseline, 13 (68.4%) of them had normal AST at end of therapy. ALT and AST were normal in all responders at the end of therapy and at 24 wk follow up post therapy (100%). There was a drop in AST between baseline and 48 wk of therapy but this was not statistically significant. The drop in ALT however was significant ($P = 0.01$) (Tables 1 and 2).

Liver histology profile

The histological activity index (HAI) scoring system revealed minimal/mild hepatitis in 14 patients (73.7%), and moderate hepatitis in 5 patients (26.3%). None of our patients was in the marked grade. The staging system for degree of fibrosis revealed 16 patients (84%) had minimal/mild fibrosis, and 3 patients (16%) had moderate/marked fibrosis.

Virological profile and genotype

All 19 patients had a high HCV-RNA load before treatment with values ranging from 5.1 log10 IU/mL to 7.4

Table 1 Mean ± SD of parameters measured at different stages of therapy (n = 19)

Parameters	Baseline	12 wk	24 wk	48 wk	*P* value (baseline *vs* 48 wk)
AST (U/L)	43.7 (30.4)	32.4 (29.6)	36.8 (35.4)	32.9 (34.3)	0.18
ALT (U/L)	65.4 (56.3)	34.0 (33.7)	34.9 (25.9)	30.8 (21.3)	0.01
HCV-RNA (log10 IU/mL)	6.3 (0.69)	3.0 (2.9)	2.6 (3.0)	1.1 (2.1)	0.0001
WBC (10^9/L)	5.5 (2.7)	4.5 (2.7)	5.1 (4.8)	6.6 (1.0)	0.698
Hemoglobin (G/L)	116.7 (43.6)	100.1 (33.8)	101.2 (24.7)	107.7 (25.8)	0.238
Platelets (10^9/L)	195 (101)	154 (76)	175 (83)	188 (98)	0.752
Creatinine (Umol/L)	122 (41)	122 (41)	138 (75)	130 (52)	0.183
GFR (mL/min)	70.4 (24.5)	69.0 (22.7)	68.2 (27.2)	69.7 (25.2)	0.772

AST: Aspartate aminotransferase; ALT: Abnormal alanine aminotransferase; HCV: Hepatitis C virus; WBC: White cell count; GFR: Glomerular filtration rate.

Table 2 Hepatitis C virus-RNA, alanine aminotransferase, and aspartate aminotransferase at different times of treatment and follow up

	0 (before treatment)	24 wk	48 wk	24 wk post treatment
No. of patients with 2 log drop in HCV-RNA	0	1 (0.05%)	2 (0.1%)	NA
No. of patients with undetected HCV-RNA	0	9 (47.4%)	9 (47.4%)	8 (42.1%)
Normal ALT	10	14 (73.6%)	Out of all patients 15/19 (78.9%) Out of responders 9/9 (100%)	Out of all patients15/19 (78.9%) Out of responders 8/8 (100%)
Normal AST	10	13 (68.4%)	Out of all patients13/19 (68.4%) Out of responders 9/9 (100%)	Out of all patients13/19 (68.4%) Out of responders 9/9 (100%)

AST: Aspartate aminotransferase; ALT: Abnormal alanine aminotransferase; HCV: Hepatitis C virus; NA: Not applicable.

log10 IU/mL and a mean of 6.25 log10 IU/mL. Seven patients developed EVR (36.8%) at 12 wk of therapy, while two patients had a low target level at 12 wk of therapy. Nine patients had negative HCV-RNA at 24 wk and at the end of 48 wk of therapy (ETR) (47.4%). HCV-RNA utilizing qualitative and quantitative assays was performed at 24 wk and 48 wk after completion of therapy for SVR. This revealed negative HCV-RNA (SVR) in 8 of the 9 responders (42.1% of total treated patients and 88.9% of responders) and only one patient had relapsed at 24 wk. There was no impact of response by the initial HCV-RNA load. Several genotypes were identified: genotype 1 is the most common and found either alone or in combination with other genotypes (genotype 1 alone in 6 patients; 1 and 2 together in 3 patients; 1 and 3 together in 1 patient; 3 alone in 2 patients; 3 and 4 together in 1 patient; 1, 4, and 5 together in 1 patient). The multiple genotypes in one patient could have been acquired from using multiple dialysis units before transplant. Five patients did not have their genotype tested. Using ANOVA, we found that the genotype did not influence the 24 wk or the end of treatment HCV-RNA load (P = 0.084 and 0.059 respectively). There was a significant fall in the load from 6.3 log10 IU/mL (± 0.69) at baseline to 2.6 log10 IU/mL (± 3.05) at 24 wk, and a further fall at 48 wk to 1.1 log10 IU/mL (± 2.1), (P = 0.0001) (Tables 1 and 2).

Hematological profile

All patients had normal leukocyte count before treatment. One patient developed leucopenia, with an absolute neutrophil count of less than 800/mm³. This patient was given G-CSF and maintained a normal count throughout the course of treatment. Hemoglobin (Hb) level was above 100 mg/dL in all patients except for three who had their Hb between 92-95 mg/dL before treatment. Those patients were given erythropoietin injections to maintain their Hb above 100 mg/dL throughout the treatment course. Platelet count ranged from 108 000/m³ to 403 000/m³. There was no significant drop in platelet count in any of the patients. None of the changes in WBC, hemoglobin or platelets during the treatment period was statistically significant (Table 1).

Renal profile

Six patients had high serum creatinine before treatment. These patients underwent renal biopsies prior to treatment which revealed chronic allograft nephropathy (CAN) in 2 patients, membrano-proliferatve glomurulonephritis (MPGN) probably related to HCV in 2 patients, and calcineurin inhibitors toxicity in 2 patients. Three patients developed renal dysfunction while on treatment; one patient developed an increase in serum creatinine and decline in GFR from baseline towards 24 wk of treatment. This patient's serum creatinine rose from 109 μmol/L before treatment to 153 μmol/L after 24 wk of treatment. Kidney biopsy showed a borderline acute cellular rejection, probably related to interferon. However since he was showing an excellent response and only a mild change in

Table 3 Impact of patient gender on the parameters at the end of therapy

Parameters	Males (n = 13)	Females (n = 6)	P value
AST (U/L)	33.69	27.67	0.56
ALT (U/L)	32.23	28.33	0.62
HCV-RNA (log10 IU/mL)	2.3	3.27	0.54
WBC (10^9/L)	4.08	7.05	0.43
Hemoglobin (G/L)	99.77	102.33	0.88
Creatinine (Umol/L)	145.62	123.33	0.53
GFR (mL/min)	66.15	72.67	0.71

AST: Aspartate aminotransferase; ALT: Abnormal alanine aminotransferase; HCV: Hepatitis C virus; WBC: White cell count; GFR: Glomerular filtration rate.

Table 4 Impact of duration post transplant on the parameters at the end of therapy

Parameters	Duration post transplant		P value
	> 49 mo (n = 10)	< 49 mo (n = 9)	
AST (U/L)	27.6	36.44	0.63
ALT (U/L)	33.4	28.33	0.59
HCV-RNA (log10 IU/mL)	2.38	2.94	0.72
WBC (10^9/L)	4.38	5.73	0.59
Hemoglobin (G/L)	107	93.44	0.28
Creatinine (Umol/L)	161.80	112.78	0.15
GFR (mL/min)	61.90	75.22	0.37

AST: Aspartate aminotransferase; ALT: Abnormal alanine aminotransferase; HCV: Hepatitis C virus; WBC: White cell count; GFR: Glomerular filtration rate.

Table 5 Impact of age on the parameters at the end of therapy

Parameters	Age > 39 yr (n = 11)	Age < 39 yr (n = 8)	P value
AST (U/L)	32.91	30.25	0.82
ALT (U/L)	30.09	32.25	0.81
HCV-RNA (log10 IU/mL)	2.92	2.33	0.7
WBC (10^9/L)	4.28	6.04	0.53
Hemoglobin (G/L)	109.18	88.75	0.11
Creatinine (Umol/L)	148.73	124.63	0.46
GFR (mL/min)	67.73	68.88	0.92

AST: Aspartate aminotransferase; ALT: Abnormal alanine aminotransferase; HCV: Hepatitis C virus; WBC: White cell count; GFR: Glomerular filtration rate.

his creatinine, and after careful discussion between nephrologist, hepatologist and the patient, it was decided to continue treatment for a further 24 wk. Serum creatinine was 175 μmol/L at the end of treatment. Twelve weeks after completing the treatment, his creatinine went up to 195 μmol/L and then dropped to 172 μmol/L at 24 wk follow up. Although rejection was very mild, we consider this a serious issue and it was taken as a side effect of antiviral therapy. We consider this as a case of therapy related rejection giving a rejection rate of 5.3% in our series. The second patient had attempted treatment twice; initially for 12 wk when the serum creatinine showed a mild but gradual increase. Treatment was held and a kidney biopsy was performed and showed CAN with no features of acute rejection. Her creatinine continued to rise very gradually. The patient and the nephrologist agreed with the hepatologist on retreatment. She was then retreated when her creatinine was 196 μmol/L before treatment. Creatinine went up to 215 μmol/L, 12 wk after starting therapy. The patient showed a virological response and since the nephropathy was not related to interferon it was decided to continue a full course of treatment. The third patient had only a mild elevation of serum creatinine from 168 μmol/L before treatment to 199 μmol/L at the end of treatment; however, he also underwent a kidney biopsy before starting treatment and has shown MPGN, which has no contraindication to treatment, and indeed if HCV related, may respond well to antiviral therapy. These three patients who developed abnormal renal function and underwent kidney biopsies had an excellent ETR and 2 of them had SVR at 24 wk post therapy. There was no significant change in GFR during or after therapy from baseline except in the only patient who had rejection who had a decrease in GFR from 61 mL/min to 38 mL/min at the end of therapy. Serum creatinine and GFR remained stable during the treatment period (Table 1).

Impact of type of immunosuppression

There was no relation observed between response rate and the type of immunosuppression regimen used during therapy. There was no relation between the increase of creatinine or decrease in GFR rate and the type of immunosuppression regimen used.

Impact of gender, duration post transplant and age on the parameters at the end of therapy period

Stratification by gender, duration post transplant and age had no impact on the final post transplant levels of the parameters measured (Tables 3-5).

Table 6 summarises recent reports on the efficacy and rejection rate following the use of interferon in post renal transplant HCV infection. It shows that there is a high rate of response to PEG-IFN especially when it is combined with ribavirin, compared to conventional interferon. Furthermore, the rate of rejection and graft failure with this type of therapy is much lower than that with conventional interferon (Table 6).

DISCUSSION

Several studies have shown a negative impact of HCV on patient and graft survival post renal transplantation[12-15,19,25,26]. Immunosuppressive therapy after renal transplantation usually leads to a flare up of HCV viremia[28]. In the setting of renal and other organ transplantation, HCV infected post transplant patients have an aggressive and rapidly progressive liver disease with cirrhosis, liver failure and death[12,22,23,36,37]. Therefore every effort to suppress HCV and prevent liver and renal

Table 6 Summary of some reports on the efficacy and rejection rate following the use of interferon alone or in combination with ribavirin in post renal transplant hepatitis C virus infection

Ref.	Yr	No. of patients treated	Anti HCV therapy used (No. of patients)	Immunosuppression used (% of patients)	ETR (%)	SVR (%)	Rate of allograft rejection/failure (%)
Ozgur *et al*[32]	1995	5	CI alone (5)	CNI (100) Aza (100) S (100)	NR	NR	40
Rosting *et al*[31]	1995[1]	14	CI alone (14)	CNI (85.7) Aza (50) S (85.7)	28.60	0	35.70
Hanafusa *et al*[14]	1998	10	CI alone (10)	CNI (NS) Aza (100) S (100)	10	0	40
Baid *et al*[27]	2003[2]	12	CI + R (11) CI alone (1)	CNI (100) Aza (17) C (67) S (100)	NS	33	17
Sharma *et al*[26]	2006[3]	6	CI + R (5) PEG-INF + R (1)	CNI (100) Aza (50) C (14) S (100)	66.60	33.30	66.60
Pageaux *et al*[67]	2009	8	PEG-INF + R (4) PEG-INF alone (4)	CNI (75) Aza (37.5) C (25) S (100)	75	50	0
Aljumah *et al*	Current study	19	PEG-INF + R (19)	CNI (89.5) C (78.9) S (100)	47.40	42.10	5.30

HCV: Hepatitis C virus; CI: Conventional interferon; PEG-IFN: Pegylated interferon; R: Ribavirin; CNI: Calcineurin inhibitors; C: Cellcept; S: Steroids; Aza: azathioprine; ETR: End of treatment response; SVR: Sustained virological response; NR: Not reported; NS: Not specified. [1]Five patients developed acute renal failure, but biopsy did not confirm rejection. [2]CI changed to PEG-IFN in 3 patients but study did not specify these patients. [3]In this study 8 patients received ribavirin alone without interferon, so they were not included. [3]Four patients developed severe renal dysfunction, only one patient biopsied and showed chronic allograft nephropathy.

diseases progression is of paramount clinical importance.

The currently accepted therapy for hepatitis C in immunocompetent patients includes a combination of conventional interferon and ribavirin, and more recently PEG-IFN and ribavirin. Several local and international studies have shown that a combination of PEG-IFN and ribavirin is superior to conventional interferon and ribavirin with a sustained virological response ranging from 41% to 82% depending on several factors including viral load, genotype, liver histology, patient age and weight[38-42].

Ribavirin alone is not recommended in dialysis patients as it is generally not tolerated due to severe hemolysis and aggravation of anemia[43]. Conventional interferon or PEG-IFN alone or in combination with ribavirin were used with varying results in hemodialysis patients with some studies suggesting prolonged durability of response after renal transplantation[44-51].

Treatment of HCV post renal transplant is even more difficult and challenging. Ribavirin alone has been used in recurrence of HCV post liver and kidney transplant but this was not associated with virological response[52-56]. Several studies and case reports have shown that the response rate to a combination of conventional interferon and ribavirin is very low. Furthermore this is associated with severe side effects including allograft rejection[26-33]. Very few studies have reported good efficacy and safety of conventional interferon in renal transplant patients[24,25]. In general, there is a major reluctance to use interferon out of fear of rejection. Fabrizi *et al*[57] have reported the meta-analysis of renal transplant patients treated with conventional interferon and ribavirin between 1994 and 2004 and have concluded that the treatment of HCV in the setting of renal transplant with interferon is contraindicated due to poor safety and efficacy. In a study one patient who was treated with PEG-IFN and ribavirin failed to achieve SVR and has developed graft dysfunction[26]. On the other hand, reports of two cases of HCV in combined liver and kidney transplant recipients treated with a combination of PEG-IFN and ribavirin revealed excellent results[58,59]. Recently eight patients were treated with PEG-IFN either alone or in combination with ribavirin and the results were encouraging, with no episodes of rejection and a SVR of 50%. However in this study there was a high incidence of side effects and intolerance to treatment[60].

The mechanism of rejection induced by interferon in renal transplant recipients is unclear. Interferon is a known strong immune modulator; hence at least theoretically it is highly possible that rejection in this setting involves an immunological reaction. Interferon may produce cell-surface expression of HLA antigens with induction of cytokine gene expression and subsequent stimulation of antibody production[61]. Baid *et al*[27] have found de novo donor-specific human leukocyte antigen

(HLA) antibodies (DSA) in serum and C4d deposits with neutrophils in peritubular capillaries in 2 renal transplanted patients with acute humoral rejection treated with interferon and ribavirin.

The reason why we have less rejection than the other previously published studies is unclear. However, most published studies have a small number of patients and many did not differentiate between biopsy proven kidney rejection and renal dysfunction, unlike our study which has followed these patients carefully and performed kidney biopsies before and during treatment whenever there is decline of renal function. In addition, there are several hypotheses which may have played a role; firstly the fact that we have not used azathioprine in any patient and instead most of our patients were on mycophenolate mofetil, which has been reported to improve graft survival and decrease the incidence of rejection[62-64]; secondly, the average time frame between transplant and initiation of therapy in our patients is relatively long (66 mo). None of our patients had sepsis or severe infection during treatment course or follow up, as sepsis is a well known independent cause for morbidity and mortality in HCV positive renal transplantation[12,13]. All of our patients were on PEG-IFN and ribavirin, with 90% of them being on PEG-IFN α-2a (Pegasys, F.Hoffmann-la Roche Ltd., Basel, Switzerland) which is known for its safety in renal patients including renal failure compared with conventional interferon as its clearance is primarily by the liver[65-68]. Ribavirin has been suggested to have a protective effect against rejection in liver transplantation[69], and this could well be the case in renal transplant. We suggest that it is worth treating such patients with PEG-IFN and ribavirin. However close monitoring is essential. A weakness of our study is that it involves a small number and is retrospective in nature. Nevertheless it has shown that PEG-IFN in combination with ribavirin has a high safety profile and a very good sustained virological response. A prospective protocol involving a larger number of patients is advisable. We think that it would be much better and safer if HCV was treated before the transplant, and indeed this is the current practice at our institution; however even with this protocol there is a chance of recurrence of HCV in the post transplant period.

In conclusion, the combination of pegylated interferon and ribavirin in HCV-RNA positive renal transplant recipients was effective, with ALT normalized in 78% of patients in whom the levels were abnormal before therapy including non responders. ALT was normal in all responders at the end of therapy and at 24 wk post therapy (100%). Virological response was observed in 47.4% of all treated patients at the end of therapy and 42.1% (88.9% of responders) have a sustained virological response at 24 wk of follow up. Rejection occurred in only one patient (5.3%) during therapy. Our study, although retrospective and of small size, has shown that the combination of pegylated interferon and ribavirin has a significant role in suppressing HCV-RNA, without subjecting patients to a high risk of graft rejection or failure in HCV infected patients post renal transplantation. These findings need to be evaluated in large clinical studies. To our knowledge this is the largest reported series of HCV positive renal transplant recipients treated with pegylated interferon and ribavirin.

ACKNOWLEDGMENTS

Part of this work was presented in brief abstract form at the 60th annual meeting of the American Association for the Study of Liver Diseases, 2009, Boston, United States.

COMMENTS

Background

Treatment of hepatitis C post renal transplant has been a debatable and controversial issue for a long time. Many studies have suggested that interferon is contraindicated in such patients due to high incidence of rejection, kidney failure and/or intolerance of patients to such therapy. Limited experience with pegylated interferon has been reported.

Research frontiers

This study reports on the largest group of patients with hepatitis C post renal transplant treated with a combination of pegylated interferon and ribavirin in the world, focusing on treatment response and kidney rejection.

Innovations and breakthroughs

The authors have treated 19 patients, who have hepatitis C and had kidney transplant with pegylated interferon and ribavirin for 48 wk. The virus was cleared in 42.1% and only one patient (5.3%) developed impairment of his kidney function towards the end of treatment period.

Applications

This research has clearly demonstrated the efficacy and safety of pegylated interferon and ribavirin in treating patients with hepatitis C virus (HCV) after renal transplant. This shall open doors for treating such patients, which were in the past left untreated because of a potential risk of kidney failure.

Terminology

Pegylated interferon is an antiviral therapy used for treatment of hepatitis C. Ribavirin is oral antiviral therapy that is used in combination with interferon for treatment of hepatitis C. Rejection is the process by which the human body refuses to accept the transplanted organ, and eventually leads to the organ failure.

Peer review

This is the largest reported series of HCV infected renal transplant recipients treated with pegylated interferon and ribavirin. The paper is generally well written and adds to the current experience regarding hepatitis C treatment in renal transplant patients.

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S- Editor Zhang SJ **L- Editor** O'Neill M **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i1.64

World J Gastroenterol 2012 January 7; 18(1): 64-69
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Risk factors for adverse outcome in low rectal cancer

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Received: April 8, 2011 Revised: July 7, 2011
Accepted: July 14, 2011
Published online: January 7, 2012

Abstract

AIM: To demonstrate the oncologic outcomes of low rectal cancer and to clarify the risk factors for survival, focusing particularly on the type of surgery performed.

METHODS: Data from patients with low rectal carcinomas who underwent surgery, either sphincter-preserving surgery (SPS) or abdominoperineal resection (APR), at The First Affiliated Hospital of Sun Yat-sen University in China from August 1994 to December 2005 were retrospectively analyzed.

RESULTS: Of 331 patients with low rectal cancer, 159 (48.0%) were treated with SPS. A higher incidence of positive resection margins and a higher 5-year cumulative local recurrence rate (14.7% *vs* 6.8%, $P = 0.041$) were observed in patients after APR compared to SPS. The five-year overall survival (OS) was 54.6% after APR and 66.8% after SPS ($P = 0.018$), and the 5-year disease-free survival (DFS) was 52.9% after APR and 65.5% after SPS ($P = 0.013$). In multivariate analysis, poor OS and DFS were significantly related to positive resection margins, pT3-4, and pTNM Ⅲ-Ⅳ but not to the type of surgery.

CONCLUSION: Despite a higher rate of positive resection margins after APR, the type of surgery was not identified as an independent risk factor for survival.

Key words: Abdominoperineal resection; Prognosis; Rectal neoplasms; Sphincter-preserving surgery; Surgery

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Chen ZH, Song XM, Chen SC, Li MZ, Li XX, Zhan WH, He YL. Risk factors for adverse outcome in low rectal cancer. *World J Gastroenterol* 2012; 18(1): 64-69 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i1/64.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i1.64

INTRODUCTION

Colorectal cancer is the fourth most prevalent malignancy in the world. In 2010, it was estimated that there were approximately 39 670 new rectal cancers in the United States[1]. In China, approximately 70% of rectal cancers are located below the peritoneal reflection[2], an obvious difference from the typical location of rectal cancers in patients in Western countries.

Treatment outcomes for rectal cancer have been dramatically improved by applying the total mesorectum excision (TME) principle[3], the double-stapling technique[4], and the concept of shorter distal margins over the past

few decades[5]. More patients with rectal tumors are being managed with various types of sphincter-preserving surgery (SPS). However, for low rectal cancer, challenges remain for some patients who require abdominoperineal resection (APR) to achieve a safe distal margin. Improvements in survival and control of local tumor recurrence for patients with mid and upper rectal cancers have not been as difficult to achieve as in those patients with lower-third rectal tumors. One of the possible reasons for this discrepancy may be the higher rate of circumferential margin (CRM) and inadvertent bowel perforations in APR[6]. However, whether the type of surgery is a risk factor for oncologic outcomes in patients with low rectal cancers is still controversial[7-10].

Herein, we collected data from a single institute in China and performed a retrospective, consecutive cohort study attempting to demonstrate the oncologic outcomes of low rectal cancer and to clarify risk factors for survival, especially focusing on the type of surgery.

MATERIALS AND METHODS

From August 1994 to December 2005, a total of 353 patients with primary low rectal carcinomas (0 to 7 cm from the anal verge) underwent open surgery at The First Affiliated Hospital of Sun Yat-sen University in China. Patients who underwent local excision (n = 3), the Hartmann procedure (n = 2), palliative colostomy (n = 7) or who were lost follow-up (n = 10) were excluded, resulting in 331 patients enrolled into the final study.

The clinicopathologic features, including gender, age, tumor size (maximum tumor diameter), distance of the tumor from the anal verge, operative procedure, resection margins, histopathologic grade, pathologic stage, mucin production[11], adjuvant therapy, and oncologic outcome, were fully reviewed.

Surgical procedures

All patients underwent surgery according to the principles of TME[3]. APR was performed in cases where the tumor was too close (usually ⩽ 2 cm) to the dentate line or where the differentiation of the tumor was poor (necessitating a longer distal resection margin). Low anterior resection (LAR) was adopted as often as possible, especially for tumors with a distance < 2 cm from the dentate line. To observe the effects of LAR with a colonic J-pouch, we performed a prospective clinical trial from 1998 to 2002 in which 16 out of 331 patients had a J-pouch created. In this study, LAR (with or without the creation of a J-pouch) and Bacon and Parks procedure were combined as SPS.

The Bacon pull-through procedure was performed for some patients with ultra low rectal cancers. In brief, in this procedure the mobilization of the sigmoid colon and the rectum is identical to that in LAR and APR. The anal sphincter is dilated to four to six finger breadths, and then the submucosa is infiltrated with 1:300 000 epinephrine. A circular incision is made in the mucosa at the anoderm at a point 5 mm from the dentate line. Isolation is performed upward beneath the rectal mucosa until reaching the upper limit of the internal sphincter; then the rectum is cut off and moved away from the abdomen. A 2-cm soft plastic pipe is inserted into the proximal colon. The colon is pulled through from the anus, and several sutures are made between the sigmoid colon and the anal canal. The second stage of the procedure is performed 14 to 21 days later. Excess bowel is amputated, and the anastomosis is completed. The Parks procedure is similar to the Bacon pull-through procedure, the difference being that the anastomosis between the colon and the anal canal is accomplished directly in the Parks procedure without the need to pull through the colon.

The CRM was not used at our institute during the study period. Instead, "resection margins" were adopted to record the status of the proximal, distal, and circumferential resection margins. Neoadjuvant chemoradiation therapy (CRT) was not extensively performed at our institute during the study period. Only 5 patients in the SPS group and 12 in the APR group received neoadjuvant therapy.

Follow-up

All of the patients were followed up every three to six months for the first two years, then every six months for the next three years, and then once a year thereafter. Digital palpation, abdominal and pelvic computed tomography (CT) scan, chest X-ray, total colonoscopy and carcinoembryonic antigen (CEA) were routinely measured to exclude local recurrence or metastasis.

Statistical analysis

Statistic analysis was performed using the SPSS 17.0 statistical package (SPSS, Inc., Chicago, IL, United States). The Student t-test and Chi-square test were used to analyze continuous and categorical variables, respectively. Overall survival, cancer-related survival and local recurrence rates were calculated using the Kaplan-Meier method. Potential prognostic factors were investigated using the log-rank test first, and then covariates with P-values < 0.05 were selected for backward multivariate Cox regression analysis. All P-values less than 0.05 with two sides were considered significant.

RESULTS

Clinicopathologic features

Patient characteristics and treatment details are summarized in Table 1. There were 211 male and 120 female patients with a median age of 56 years (range, 17 years-91 years). The median tumor size was 4.0 cm (range, 0.5 cm-15.0 cm). The median distance between the anal verge and the tumor was 4.0 cm (range, 1.0 cm-7.0 cm), and tumors in the APR group were significantly closer to the anus than those in the SPS group (median, 3.0 cm *vs* 5.0 cm, P < 0.001).

Of the 331 patients with low rectal cancer, 172 patients

Table 1 Characteristics of 331 patients with low rectal cancer

Characteristic	APR (%) *n* = 172	SPS (%) *n* = 159	*P* value
Median age (yr)	56.0	58.0	0.334
Median tumor size (cm)	4.0	4.0	0.301
Median distance of tumor from the anal verge (cm)	3.0	5.0	< 0.001
Gender (male)	115 (66.9)	96 (60.4)	0.220
Positive resection margins	23 (13.4)	10 (6.3)	0.032
Histopathologic grade			0.238
Well	34 (19.8)	27 (17.0)	
Moderate	102 (59.3)	108 (67.9)	
Poor	36 (20.9)	24 (15.1)	
pTNM stage			0.445
Ⅰ	20 (11.6)	22 (13.8)	
Ⅱ	63 (36.6)	69 (43.4)	
Ⅲ	73 (42.4)	56 (35.2)	
Ⅳ	16 (9.4)	12 (7.6)	
Mucin production	14 (8.1)	10 (6.3)	0.517
Adjuvant chemotherapy	34 (19.8)	29 (18.2)	
Year of surgery			0.001
1994-2001	101 (58.7)	65 (40.9)	
2002-2005	71 (41.3)	94 (59.1)	

APR: Abdominoperineal resection; SPS: Sphincter-preserving surgery.

received the APR procedure and 159 underwent SPS, including 130 APR, 13 Bacon or Parks procedures, and 16 APR with a colonic J-pouch. Before 2001, only 40.9% (65 out of 166) of patients underwent SPS, while after 2001 more (59.1%, 94 out of 165) patients were able to undergo anus-sparing surgery (65/166 *vs* 94/165, $P = 0.001$). Combined resection of organs was performed for 35 patients, including 10 patients who underwent resection of solitary liver metastasis. Only 4 (2.5%) patients had a protective stoma after SPS.

Resection margins were microscopically positive in 33 (10%) patients. More patients in the APR group were diagnosed with "positive resection margins" than in the SPS group (13.4% *vs* 6.3%, $P = 0.032$). Data on tumor stage and tumor differentiation are presented in Table 1.

There was no mortality. Anastomotic leakage occurred in 6 (3.8%, 6 out of 159 SPS) patients, all of whom did not have a protective stoma before, and these patients were treated conservatively. In total, 63 (19.0%) patients received adjuvant chemotherapy with 5-fluorouracil plus leucovorin or FOLFOX (oxaliplatin, 5-fluorouracil, and folinic acid).

Recurrence

Patients were followed up with a median follow-up time of 61 mo (range, 1 mo-194 mo). At the last follow-up in November 2010, 177 patients (53.5%) were alive, and 154 (46.7%) were dead.

A total of 97 (27.3%) patients experienced recurrence, including 34 (10.3%) local, 58 (17.5%) distant, and 5 (1.5%) combined recurrence. Seventy-five patients died of cancer recurrence. The median disease-free interval for the 35 patients with local recurrence was 15.5 mo (range, 4 mo-104 mo). For the 51 patients with distant metastasis, the median time to metastasis was 25 mo (range, 4 mo-123 mo). The overall 5-year cumulative local recurrence rate was 10.9%. The estimated local recurrence rate at 5 years was 14.7% for APR and 6.8% for SPS ($P = 0.041$, Figure 1A).

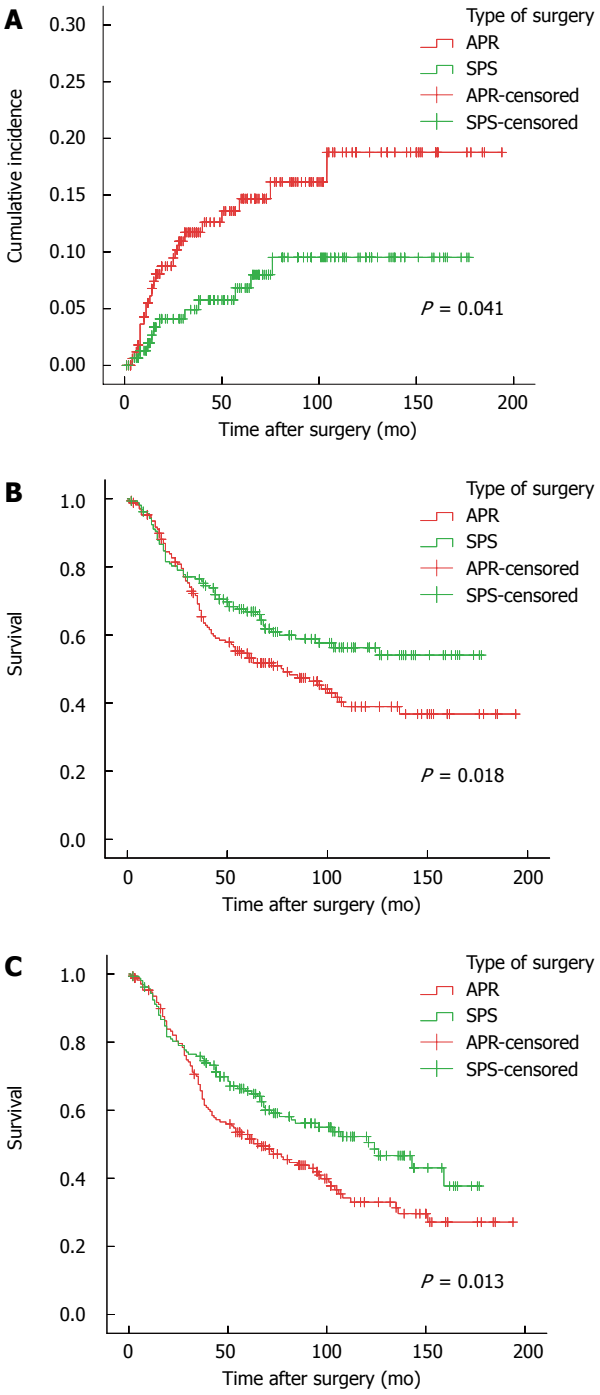

Figure 1 Surgical outcomes among 331 patients who underwent abdominoperineal resection and sphincter-preserving surgery. A: Cumulative local recurrence rate; B: Overall survival rate; C: Disease-free survival rate. APR: Abdominoperineal resection; SPS: Sphincter-preserving surgery.

Survival

The five-year overall (OS) and disease-free survival (DFS) were 60.6% and 59.1%, respectively. The five-year OS was 54.6% after APR and 66.8% after SPS ($P = 0.018$,

Table 2 Univariate analysis of 331 patients with low rectal cancer

Characteristic	No. of patients	5-year OS		5-year DFS	
		%	*P* value	%	*P* value
Gender			0.987		0.768
Male	211	61.2		59.2	
Female	120	59.6		58.9	
Age (yr)			0.707		0.987
< 60	182	58.8		56.4	
≥ 60	149	62.7		62.2	
Tumor size (cm)			0.011		0.003
< 4	103	74.8		74.0	
≥ 4	228	54.1		52.3	
Distance of tumor from the anal verge (cm)			0.189		0.197
< 3.5	117	60.1		58.1	
≥ 3.5	214	60.9		59.6	
Type of surgery			0.018		0.013
APR	172	54.6		52.9	
SPS	159	66.8		65.6	
Resection margins			< 0.001		< 0.001
Negative	298	63.8		62.1	
Positive	33	32.5		32.5	
Histopathologic grade			0.006		0.002
Well	61	66.5		66.5	
Moderate	210	63.6		61.9	
Poor	60	43.6		41.6	
pT stage			< 0.001		< 0.001
1-2	125	74.0		71.9	
3-4	206	52.4		51.2	
pTNM stage			< 0.001		< 0.001
Ⅰ-Ⅱ	174	75.4		73.3	
Ⅲ-Ⅳ	157	44.0		43.1	
Mucin production			0.212		0.121
No	307	61.7		60.3	
Yes	24	44.8		42.6	
Anastomotic leakage[1]			0.310		0.386
No	153	67.6		66.3	
Yes	6	50.0		50.0	
Adjuvant chemotherapy			0.950		0.573
No	268	60.2		58.4	
Yes	63	62.2		62.2	

OS: Overall survival; DFS: Disease-free survival; APR: Abdominoperineal resection; SPS: Sphincter-preserving surgery. [1]Analysis of 159 patients who underwent sphincter-preserving surgery.

Figure 1B). The 5-year DFS was 52.9% after APR and 65.5% after SPS ($P = 0.013$, Figure 1C).

In univariate analysis, OS and DFS were both significantly influenced by tumor size, type of surgery, resection margins, tumor histopathologic grade, pT stage, and pTNM stage (Table 2).

Multivariate analysis was performed for those factors which were statistically significantly associated with OS and DFS in the univariate analysis. In backward multivariate Cox regression analysis, poor OS was significantly related to positive resection margins [hazard ratio (HR) = 1.644, $P = 0.031$], pT3-4 (HR = 1.781, $P = 0.003$), and pTNM Ⅲ-Ⅳ (HR = 2.153, $P < 0.001$). Positive resection margins (HR = 1.728, $P = 0.012$), pT3-4 (HR = 1.669, $P = 0.006$), pTNM Ⅲ-Ⅳ (HR = 1.839, $P < 0.001$), and poor tumor differentiation (HR = 1.665, $P = 0.034$) were significantly associated with poor DFS (Table 3).

Table 3 Multivariate analysis of 331 patients with low rectal cancer

Characteristic	OS			DFS		
	HR	95% CI for HR	*P* value	HR	95% CI for HR	*P* value
Tumor size (cm)			0.131			0.067
< 4	1.000			1.000		
≥ 4	1.327	0.920-1.915		1.388	0.977-1.971	
Type of surgery			0.091			0.112
APR	1.000			1.000		
SPS	0.754	0.544-1.047		0.778	0.572-1.060	
Resection margins			0.031			0.012
Negative	1.000			1.000		
Positive	1.644	1.047-2.582		1.728	1.129-2.644	
Histopathologic grade			0.150			0.047
Well	1.000			1.000		
Moderate	1.061	0.688-1.637	0.788	1.108	0.735-1.670	0.624
Poor	1.515	0.917-2.504	0.105	1.665	1.039-2.666	0.034
pT stage			0.003			0.006
1-2	1.000			1.000		
3-4	1.781	1.212-2.616		1.669	1.162-2.396	
pTNM stage			< 0.001			< 0.001
Ⅰ-Ⅱ	1.000			1.000		
Ⅲ-Ⅳ	2.153	1.515	3.058	1.839	1.332-2.540	

OS: Overall survival; DFS: Disease-free survival; HR: Hazard ratio; CI: Confidence interval; APR: Abdominoperineal resection; SPS: Sphincter-preserving surgery.

DISCUSSION

Curative resection of local rectal cancer consists of complete removal of the primary tumor and its lymphatic drainage by sharp mesorectal excision with or without sphincter preservation. The choice of APR or SPS may be affected by different opinions regarding safe distal margin, views on the functional aspects associated with intersphincteric dissection, a variety of institution-specific cultural features and the availability of staple anastomosis[12-15]. Our study demonstrated that the rate of sphincter-preserving surgery increased from 40.9% before 2001 to 59.1% after 2001. This may be attributed to the acceptance of a shorter distal margin (1-2 cm) and the availability of the double stapling technique.

This study shows that more patients had positive resection margins after APR than after SPS, which is in line with other studies[6,10,16-18]. Nevertheless, a positive margin should lead to a worse prognosis, and this was confirmed by the multivariate analysis in this study. Inadequate resection at the level of the pelvic floor with TME in APR may result in an increased risk for positive resection margins, contributing to the observed higher risk for positive circumferential resection margins and local recurrence after APR than after SPS. The five-year OS and DFS after APR were inferior to those after SPS, and the OS and DFS were both significantly influenced by the type of surgery in the univariate analysis. However, after adjustment for other potential risk factors, we failed to find a significant association between the type of surgery and

survival.

Low rectal cancers lie at the pelvic floor and close to the anal sphincter, which makes completion of a radical operation challenging. To reduce the incidence of circumferential resection margin involvement and intraoperative tumor perforation, some surgeons have made a change in their approach to APR. West *et al*[14] applied cylindrical abdominoperineal excision of the rectum and anus, entailing resection of the levator muscles en bloc with the lower rectum and anal canal, for patients with low rectal cancer, leading to a decrease in circumferential resection margin involvement from 40.6% after traditional APR to 14.8%. They also reduced the rate of intra-operative perforations from 22.8% to 3.7%. The cylindrical technique may possess the potential to improve patient outcomes, and close attention should be paid to the perineal part of the APR surgery.

It has been reported that preoperative CRT has a strong influence on the prognosis in rectal cancer[18,19]. However, only 17 patients in this study received preoperative CRT due to economic considerations and lack of acceptance of it as a major part of the management of rectal cancer. It was not until recently that we began to include preoperative CRT widely for advanced rectal cancers. We also investigated other variables for poor survival outcome and determined that the pathologic TNM stage remained the strongest risk factors for OS and DFS, which is consisted with previous studies[20-21].

In conclusion, this consecutive cohort study from a single institution in China demonstrates that a higher risk of positive resection margins and local failure was observed in patients after APR. The survival after APR is inferior to that after SPS. However, after adjustment for other covariates, we failed to confirm that the type of surgery is an independent risk factor for survival outcome. To reduce positive resection margins and local recurrence, there has been a call to change the approach to APR.

COMMENTS

Background

Risk factors for survival in low rectal cancer patients still need to be clarified. There is evidence that the type of surgery, sphincter-preserving surgery (SPS) or abdominoperineal resection (APR), may influence the prognosis in low rectal cancer.

Research frontiers

A retrospective, consecutive cohort study was conducted, attempting to demonstrate the oncologic outcomes of low rectal cancer and to clarify the risk factors for survival. Univariate and multivariate Cox regression analyses were performed to determine whether gender, age, tumor size, distance of the tumor from the anal verge, operative procedure, resection margins, histopathologic grade, pathologic stage, mucin production, and adjuvant therapy were associated with the prognosis of low rectal cancer.

Innovations and breakthroughs

A higher risk for positive resection margins and local failures were observed in APR. The survival outcome after APR is inferior to that after SPS. However, after adjustment for other covariates, the type of surgery was not identified as an independent risk factor for survival.

Applications

To reduce positive resection margins and local recurrence, there has been a call for a change in the approach to APR.

Terminology

Total mesorectum excision (TME) is the standard technique for the treatment of rectal cancer. It was devised some 20 years ago by Professor Bill Heald at the Basingstoke District Hospital in the United Kingdom. TME is accomplished by precise sharp dissection under direct visualization with the true pelvis around the integral mesentery enveloping the entire mid-rectum and with preservation of the hypogastric plexus.

Peer review

This is an important paper, comparing the outcome of low mesorectal anterior resections for carcinoma of the rectum with abdominoperineal resections.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i1.70

World J Gastroenterol 2012 January 7; 18(1): 70-78
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Germline promoter hypermethylation of tumor suppressor genes in gastric cancer

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Supported by The National Natural Science Foundation of China, No. 30972535; the Natural Science Foundation of Jiangsu, China, No. BK2008269; the Fundamental Research Funds for the Central Universities of China, No. 1112021402
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Received: April 15, 2011 Revised: July 11, 2011
Accepted: July 18, 2011
Published online: January 7, 2012

Abstract

AIM: To explore germline hypermethylation of the tumor suppressor genes *MLH1*, *CDH1* and *P16*^{INK4a} in suspected cases of hereditary gastric cancer (GC).

METHODS: A group of 140 Chinese GC patients in whom the primary cancer had developed before the age of 60 or who had a familial history of cancer were screened for germline hypermethylation of the *MLH1*, *CDH1* and *P16*^{INK4a} tumor suppressor genes. Genomic DNA was extracted from peripheral blood leukocytes and modified by sodium bisulfite. The treated DNA was then subjected to bisulfite DNA sequencing for a specific region of the *MLH1* promoter. The methylation status of *CDH1* or *P16*^{INK4a} was assayed using methylation-specific PCR. Clonal bisulfite allelic sequencing in positive samples was performed to obtain a comprehensive analysis of the CpG island methylation status of these promoter regions.

RESULTS: Methylation of the *MLH1* gene promoter was detected in the peripheral blood DNA of only 1/140 (0.7%) of the GC patient group. However, this methylation pattern was mosaic rather than the allelic pattern which has previously been reported for *MLH1* in hereditary non-polyposis colorectal cancer (HNPCC) patients. We found that 10% of the *MLH1* alleles in the peripheral blood DNA of this patient were methylated, consistent with 20% of cells having one methylated allele. No germline promoter methylation of the *CDH1* or *P16*^{INK4a} genes was detected.

CONCLUSION: Mosaic germline epimutation of the *MLH1* gene is present in suspected hereditary GC patients in China but at a very low level. Germline epimutation of the *CDH1* or *P16*^{INK4a} gene is not a frequent event.

Key words: Gastric cancer; Germline promoter methylation; *MLH1*; *CDH1*; *P16*^{INK4a}

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Wu PY, Zhang Z, Wang JM, Guo WW, Xiao N, He Q, Wang YP, Fan YM. Germline promoter hypermethylation of tumor suppressor genes in gastric cancer. *World J Gastroenterol* 2012; 18(1): 70-78 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i1/70.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i1.70

INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies worldwide and is the most frequent form of cancer in East Asian countries. Point mutations or deletions within large genomic segments of tumor suppressor genes have previously been detected in about 30% of individuals with a genetic predisposition to GC[1,2]. However, the underlying genetic abnormalities in more than 70% of GC patients thus remain unknown. The methylation of cytosines in CpG dinucleotides within the promoter regions of tumor suppressor genes can result in transcriptional silencing and this occurs frequently in GC tumors. As one of the major genes in the mismatch repair (MMR) system, *MLH1* is considered to be one of the key causative genes for GC. Hypermethylation of the *MLH1* gene promoter is extremely frequent and often correlates well with loss of the MLH1 protein in tumor cells[3-6]. The epithelial cadherin gene *CDH1* is also associated with GC and shows decreased expression in GC tumors. However, germline mutations in *CDH1* have been found in only some patients with hereditary diffuse gastric cancer (HDGC). A high rate of promoter methylation of *CDH1* is observed in GC tumors and is associated with the inactivation of this gene[7-11]. As an important protein in the cell cycle regulatory pathways, the inactivation of $P16^{INK4a}$ is one of the most commonly observed abnormalities in human cancer. This can occur *via* the hypermethylation of CpG islands within its promoter in many tumor types including gastric cancers and germline mutations in this gene in GC are relatively rare[10,11].

Recent reports have shown that promoter hypermethylation of the *MLH1* gene is not limited to tumor cells but might also occur in the peripheral blood and other tissues of patients with early-onset colorectal cancer (CRC), suggesting a germline origin[12-18]. Importantly however, although the promoters of *MLH1*, *CDH1* and $P16^{INK4a}$ are hypermethylated in GC tumors, it is currently not known whether these epigenetic events exist in the germline and cause a GC predisposition in affected individuals.

In our current study, we characterize the germline promoter methylation of the *MLH1*, *CDH1* and $P16^{INK4a}$ tumor suppressor genes in GC. We screened a selected cohort of Chinese GC cases in whom the cancer had developed before the age of 60 years or in which there was familial history of *MLH1*, *CDH1* and $P16^{INK4a}$ germline epimutations.

MATERIALS AND METHODS

Subjects

A cohort of 140 GC patients from the Jiangsu, Anhui and Zhejiang provinces of China was assembled for this study. The selection of patients was based on the GC family history and onset ages i.e., (1) individuals with GC and two or more first-degree relatives with GC, denoted as high familial cancer history (HF) cases; (2) individuals with GC and one first- or second-degree relative with GC, designated low familial cancer history (LF) cases; and (3) individuals diagnosed with GC prior to 60 years of age, but no family history of this disease, referred to as young onset (Y) patients. Of the 140 GC patients in our selected cohort, we identified 18 HF, 43 LF and 79 Y cases. Thirty age-matched normal controls were also included in the analysis. Informed consent was obtained from each subject who underwent genetic testing, in accordance with the guidelines of the Ethics Committee of the Medical School of Nanjing University.

Methylation screening and bisulfite sequencing

Peripheral blood leukocyte DNA was bisulfite modified using the CpGenome™ DNA Modification Kit (Chemicon International, Temecula, CA, United States) in accordance with the manufacturer's instructions. A specific CpG-rich sequence in the *MLH1* promoter region (from -427 to -53 bp relative to the translation start site for human *MLH1*, a 375 bp fragment containing 20 CpG sites; Figure 1A) was selected. This region is purported to be strongly associated with *MLH1* silencing[19-22]. Bisulfite DNA sequencing (BSP) of this region was performed to determine the comprehensive CpG island methylation status of the *MLH1* gene promoter using an ABI 3100-Avant automated sequencer (Applied Biosystems, Foster City, CA, United States). The primers used for BSP were MLH1-BF, 5'- TAAGGGGAGAGGAGGAGTTTGA-3' (sense) and MLH1-BR, 5'-CAACCAATCACCTCAATACCTC-3' (antisense). The obtained PCR products displaying methylated CpG sites were subcloned into the PMD18-T vector [TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China] and at least 10 clones were selected and sequenced for each sample using universal M13 primers to determine the level and extent of gene promoter methylation.

The methylation status of *CDH1* and *P16* was determined by methylation-specific PCR (MSP) after treatment of the DNA with sodium bisulfite. The primer sequences used have been reported previously for *CDH1*[8,11] and *P16*[7,23] and are as follows: CDH1-M-F, 5'-GGTGAATTTTTAGTTAATTAGCGGTAC-3' (sense) and CDH1-M-R, 5'-CATAACTAACCGAAAACGCCG-3' (antisense) for methylated *CDH1*; CDH1-U-F, 5'-GGTAGGTGAATTTTTAGTTAATTAGTGGTA-3' (sense); and CDH1-U-R, 5'-ACCCATAACTAACCAAAAACACCA-3' (antisense) for unmethylated *CDH1*; P16-M-F, 5'-TTATTAGAGGGTGGGGCGGATCGC-3' (sense) and P16-M-R, 5'-GACCCCGAACCGCGACCGTAA-3' (antisense) for methylated *P16*; P16-U-F, 5'-TTATTAGAGGGTGGGGTGGATTGT-3' (sense), and P16-U-R, 5'- CAACCCCAAACCACAACCATAA-3' (antisense) for unmethylated *P16*. The PCR-amplified regions for the methylated (from -78 to +127 bp relative to the transcription initiation site for human *CDH1*, 205 bp) and unmethylated (from -78 to +130 bp relative to the transcription initiation site, 208 bp) *CDH1* alleles contained 19 CpG dinucleotides, including five CpGs at the primer annealing sites (Figure 3A). The primer sets spanned the transcriptional start site and were designed to include methylation sites that best corresponded with the transcriptional

silencing of *CDH1* in the published literature[24].

The PCR-amplified region for the methylated (from -80 to +70 bp relative to the translation initiation site for human *P16*, 150 bp) and unmethylated (from -80 to +71 bp relative to the translation initiation site, 151 bp) *p16* alleles contained 19 CpG dinucleotides, including eight CpGs at the primer annealing sites (Figure 6A).

The positive samples were further amplified using *CDH1* or *p16* gene-specific primers CDH1-BF-5'-TAGTAATTTTAGGTTAGAGGGTTA-3' (sense) and CDH1-BR-5'-AAATACCTACAACAACAACAACAACAAC-3' (anti-sense)[25]; P16-BF-5'-TTTTTAGAGGATTTGAGGGATAGG-3' (sense) and P16-BR-5'-CTACCTAATTCCAATTCCCCTACA-3' (anti-sense)[19]. The amplified fragments (363 bp, from -185 to +178 bp relative to the transcription initiation site of *CDH1*, Figure 3A; 392 bp, from -159 to +233 bp relative to the translation initiation site of *P16*, Figure 6A), were sequenced using an ABI 3100-Avant automated sequencer (Applied Biosystems, Foster City, CA, United States). CpGenome universal methylated and unmethylated DNA (Chemicon International, Temecula, CA, United States) served as a positive control for gene promoter hypermethylation and hypomethylation, respectively.

RESULTS

Methylation analysis of the MLH1 promoter

A stretch of 375 bases incorporating 20 CpG sites in the *MLH1* proximal promoter was analyzed (Figure 1A). In one of the 140 suspected hereditary GC patients in our cohort (patient G46), partial methylation was detected by BSP through the 6th to 20th CpG sites of the *MLH1* promoter from blood DNA (Figure 1B). This individual was a 60-year-old female with no family history of GC. For the remaining samples, partial methylation was evident at the 9th and 10th CpG sites (-269 and -262 bp from the translation initiation site, respectively), but no other CpGs in this region were found to be methylated. The peripheral blood leukocyte DNA of 30 age-matched normal controls was examined for *MLH1* methylation in comparison with the GC patients. None of the control samples showed detectable *MLH1* promoter methylation by bisulfite DNA sequencing apart from the 9th and 10th CpG sites which seemed to have methylated alleles more than 50% for all the patients. However, because this alteration was also found in unmethylated controls and in healthy blood donor samples, it is far less likely to be linked to GC (Figure 1B).

To determine the extent of *MLH1* allele methylation in patient G46, clonal bisulfite allelic sequencing of the *MLH1* promoter in peripheral blood from this individual was performed. Upon analysis of the peripheral blood DNA from patient G46, one of ten clones had a cytosine present through the 6th to 20th CpG sites (Figure 2). All other non-CpG cytosines were converted, indicating that this corresponded to a methylated allele. For one other clone, only one CpG (the 12th) was found to be methylated and this may have been due to an incomplete conversion. Four of these ten clones had a cytosine present at the 9th and 10th CpGs, confirming the methylation pattern evidenced by bisulfite DNA sequencing. This result supported the possibility that mosaic germline *MLH1* methylation could be occurring in this patient. Indeed, 10% of the *MLH1* allele was methylated in this individual, equating to 20% of the cells found with one methylated *MLH1* allele in the peripheral blood. Unfortunately, tumor material was not available from patient G46 to evaluate the methylation profile of the *MLH1* gene in the GC cancer cells.

Methylation analysis of the CDH1 promoter in GC patients

None of the GC cases under study showed detectable *CDH1* promoter methylation in their peripheral blood leukocytes by MSP assay (Figure 3B). Direct sequencing of the *CDH1* promoter region from -185 to +178 bp relative to the transcription initiation site confirmed this result (Figure 4). Several C bases (including the 4th and 9th CpG sites, and at positions -120 and -65 bp relative to the transcription initiation site) were evident (more than 50%) in all patients. However, because this variation was also found in the healthy blood donor samples, it is unlikely to be linked to GC (Figure 5). This result also suggested that the incidence of germline hypermethylation of the *CDH1* promoter is low in Chinese GC patients.

Methylation analysis of the P16 promoter

None of the GC cases under study showed detectable *P16* promoter methylation of the DNA from their peripheral blood leukocytes by MSP assay (Figure 6B). Direct sequencing of the *P16* promoter region from -159 to +233 bp relative to the translation initiation site confirmed that all of the 35 CpG dinucleotides present in the fragment were unmethylated (Figure 7). This result suggested that germline hypermethylation of the *P16* promoter is also rare in Chinese GC patients.

DISCUSSION

In our current study, we demonstrated that a mosaic *MLH1* promoter methylation pattern existed in the peripheral blood of a Chinese GC patient. Deng *et al*[19] previously divided the *MLH1* promoter area into four regions, A–D, and proposed that methylation in region C plays an important role in silencing hMLH1 expression. In our current experiments, we carried out methylation analysis on region C of the *MLH1* promoter. We excluded the 9th and 10th CpG sites of the *MLH1* promoter region as they were found to be methylated in the control samples (Figure 1B). Our analyses also showed that rather than an allelic methylation pattern as has previously been reported for *MLH1* in CRC patients, a mosaic level of *MLH1* methylation was found from the 6th to 20th CpG sites in this promoter region in the germline genomic DNA of GC patient G46 in our study cohort. Clonal sequencing further revealed a

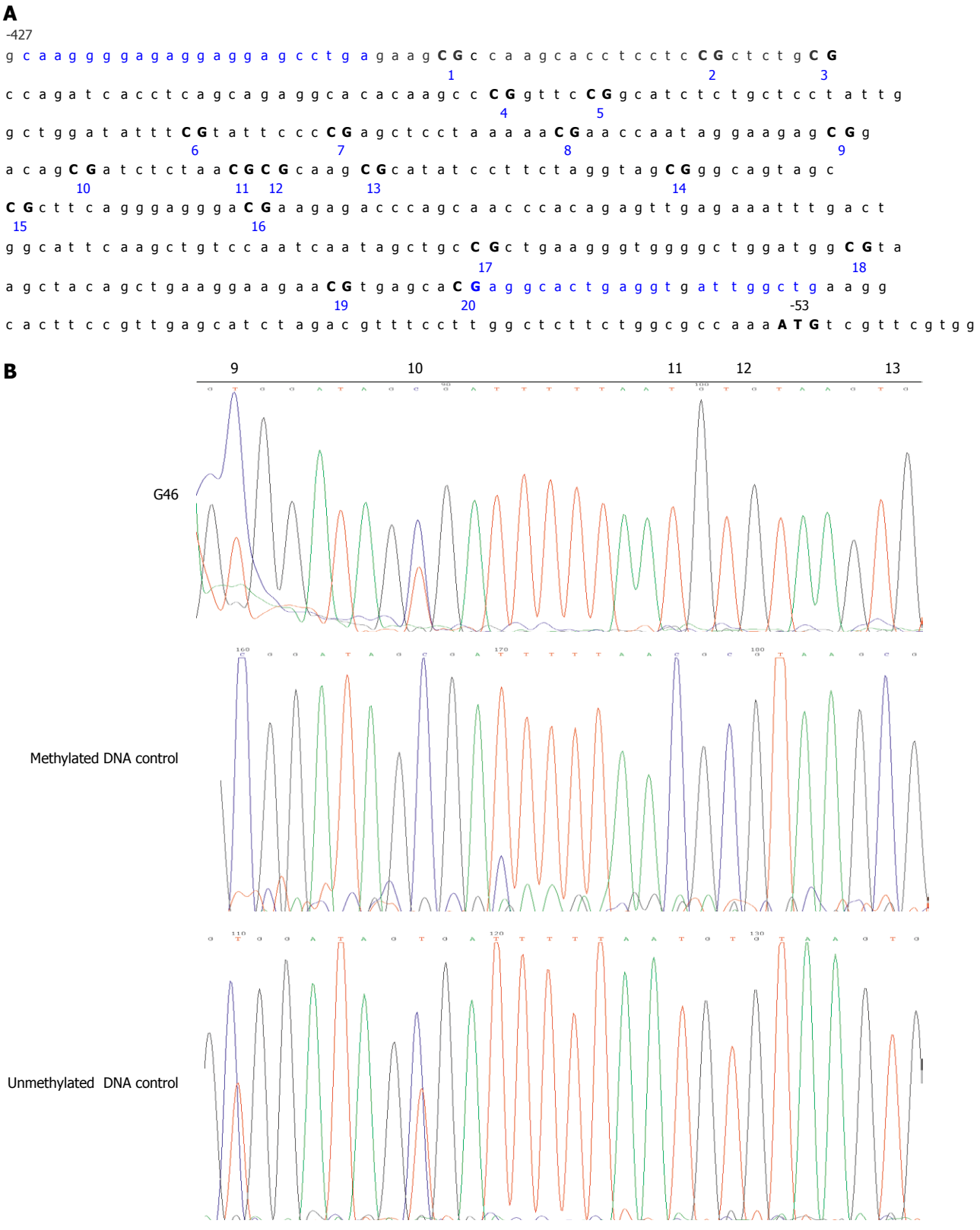

Figure 1 Germline *MLH1* promoter hypermethylation analysis in a gastric cancer patient cohort. A: Map of the CpG island structure in the *MLH1* promoter. The sequence is numbered relative to the translation start site for human *MLH1* (bolded "ATG"). Characters in blue indicate the primer binding sites for bisulfite sequencing. Individual CpG sites in the sequence are numbered consecutively; B: Bisulfite sequencing of *MLH1* promoter sequences. *G46,* DNA isolated from the blood of case G46 showing a mixture of C and T at the 6th to 20th CpG sites attributable to partial modification of the DNA due to partial methylation; *Methylated DNA control*, CpGenome Universal Methylated DNA in which *MLH1* is completely methylated showing a high C content at all CpGs attributable to reduced modification because of complete methylation of the DNA; Unmethylated DNA control, CpGenome Universal Unmethylated DNA showing T bases at all CpGs except the 9th and 10th CpG sites due to almost complete modification of the DNA. The 9th and 10th CpG sites show a high number of Cs in G46, but also in the unmethylated control, suggesting that these sites are uninformative in relation to gastric cancer.

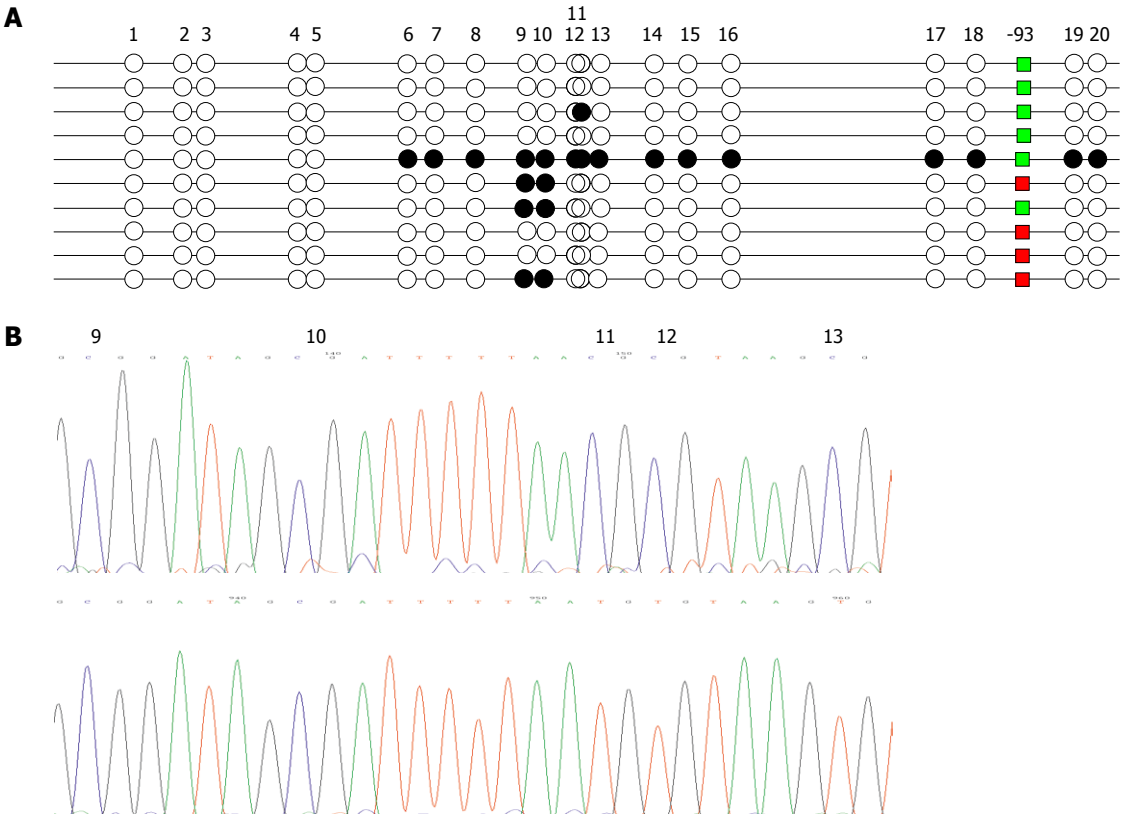

Figure 2 Clonal bisulfite allelic sequencing of the *MLH1* promoter in the peripheral blood from gastric cancer patient G46. A: Each horizontal line of circles represents an isolated allele. The numbering scheme is derived from the map shown in Figure 1A. White circles represent non-methylated CpG sites, and black circles indicate a methylated CpG. This subject displayed 10% methylated alleles at the 6th to 20th CpG sites, suggestive of mosaic allele-specific germline epimutation. Green, -93A; Red, -93G; B: Upper figure, sequencing of clone 5 from case G46 showing methylation at the 6th to 20th CpG sites. Lower figure, sequencing of G46 clone 7 revealing unmethylated CpGs except for the 9th and 10th CpG sites. The numbers above the sequences are derived from the map shown in Figure 1A, indicating the location of the CpG sites.

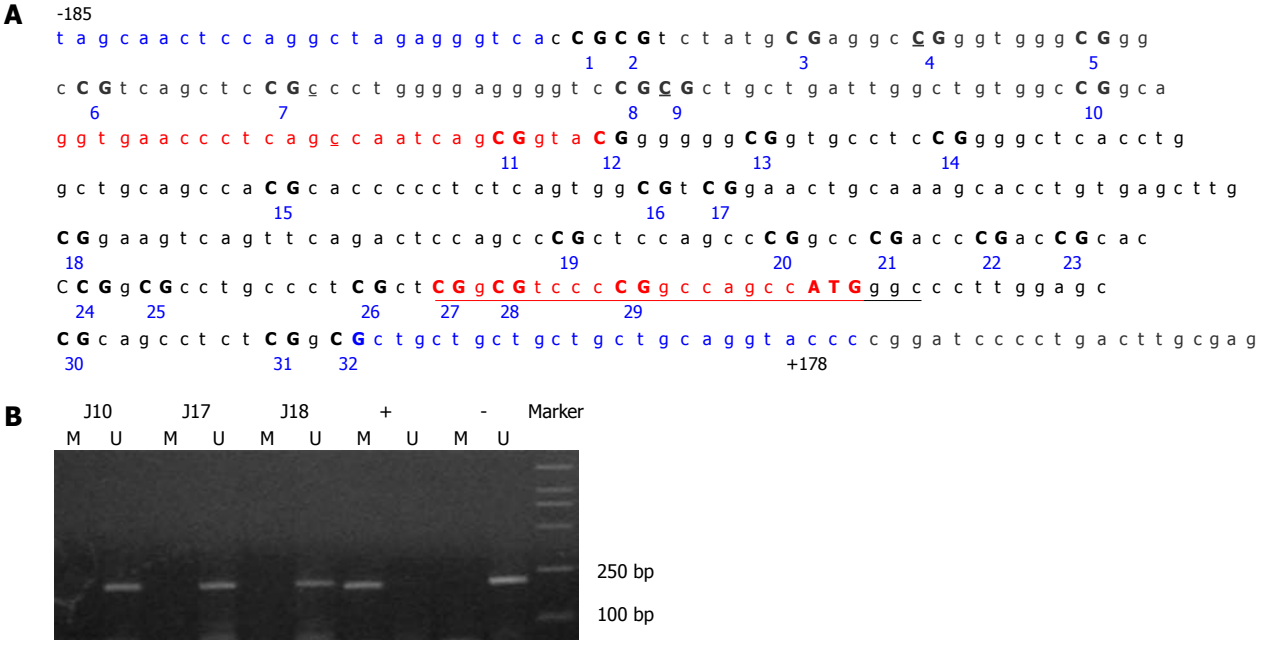

Figure 3 Germline *CDH1* promoter hypermethylation analysis in GC patients. A: Map of the *CDH1* promoter region and primer positions. The sequence is numbered relative to the transcription start site for human *CDH1*. Characters in red indicate the primer binding sites for methylation-specific PCR (MSP), those in blue for bisulfite sequencing. Individual CpG sites in the sequence are numbered consecutively; B: MSP of the *CDH1* promoter from the peripheral blood of GC patients. Marker, DL2000 DNA Markers (TaKaRa); +, CpGenome Universal Methylated DNA control; -, CpGenome Universal Unmethylated DNA control; M, methylated band; U, unmethylated band. As a control, the fragments corresponding to the *CDH1* promoter of fully methylated DNA showed a clear band when amplified with methylated-specific primers and did not display a band when treated with unmethylated primers. The unmethylated DNA control showed the reverse pattern.

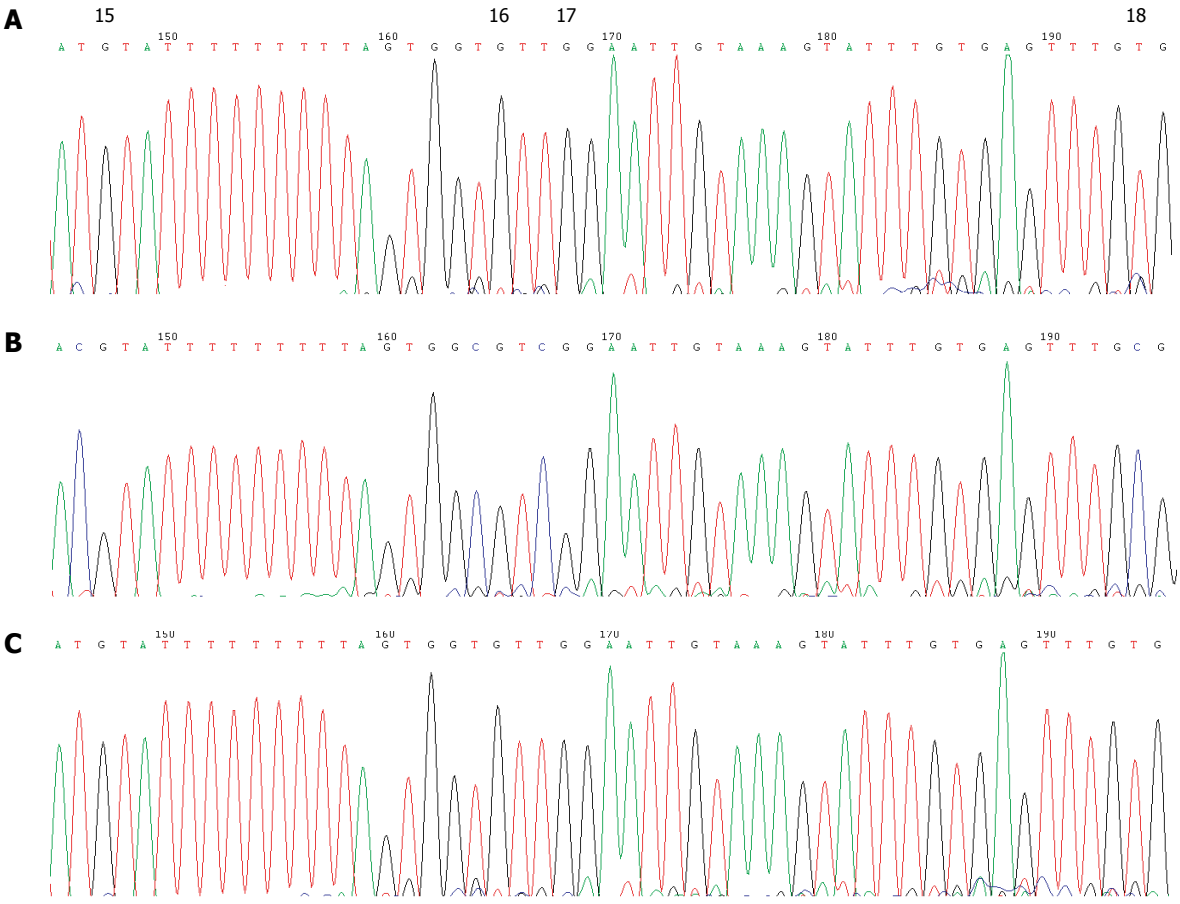

Figure 4 Bisulfite sequencing of *CDH1* promoter sequences from gastric cancer patients. A: DNA isolated from the blood of a gastric cancer patient showing T bases at the CpG sites around the transcription start site due to complete modification of the DNA; B: CpGenome Universal Methylated DNA control in which *CDH1* is completely methylated showing a high level of C at all CpGs due to reduced bisulfite modification; C: CpGenome Universal Unmethylated DNA control showing T bases at the CpG sites around the transcription start site attributable to complete bisulfite modification of the DNA.

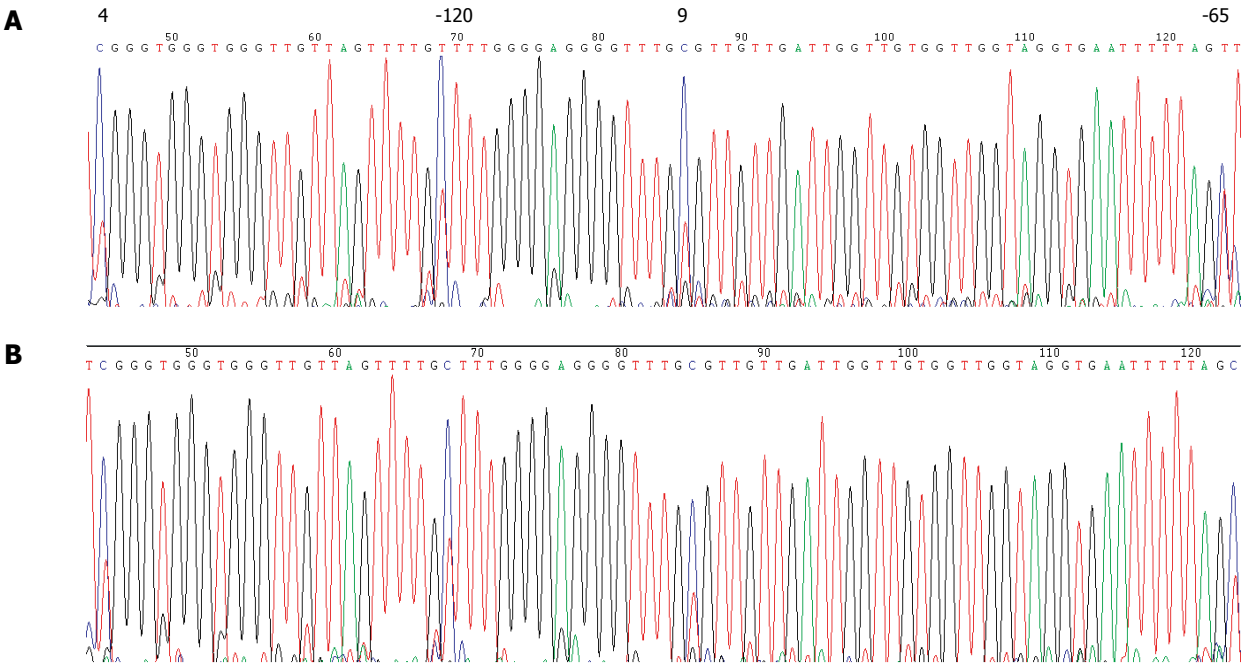

Figure 5 Bisulfite sequencing of the *CDH1* promoter sequences from gastric cancer patients showing uninformative CpG sites. A: DNA isolated from the blood of a gastric cancer (GC) patient; B: DNA isolated from the blood of a normal control. The 4th and 9th CpG sites showed a high C content in all patients, but also in each of the normal controls, suggesting that these sites are not associated with GC.

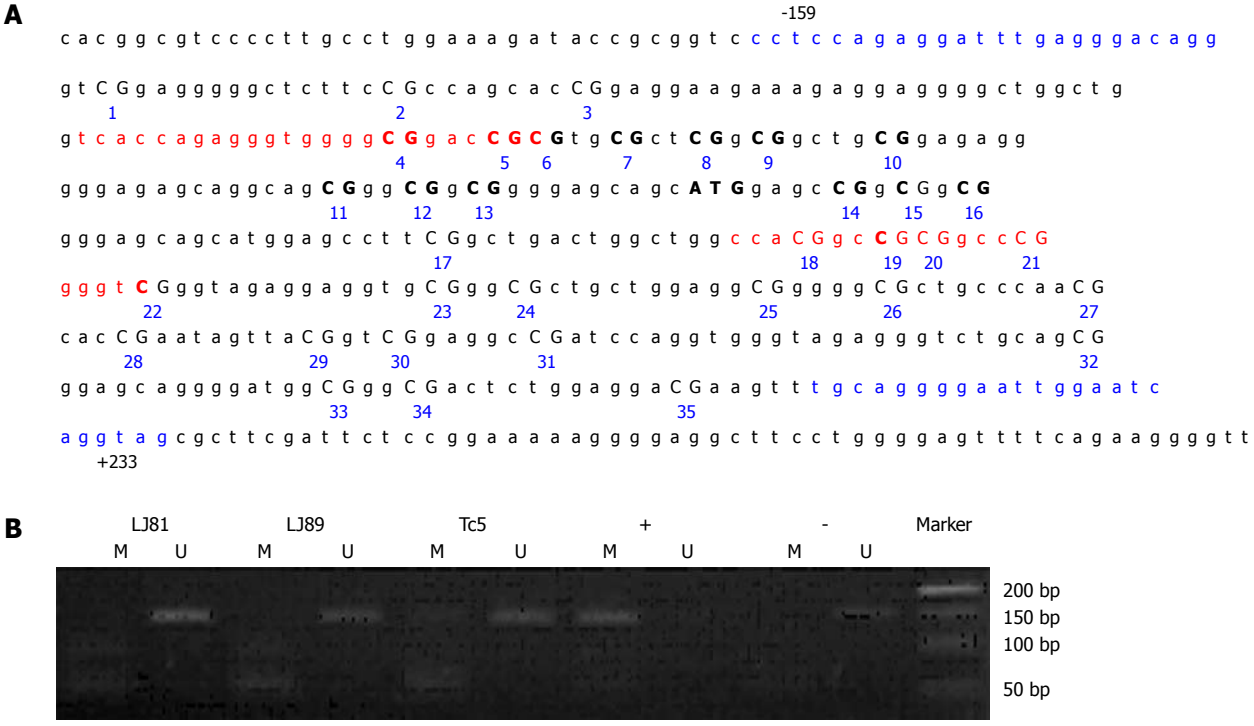

Figure 6 Analysis of the methylation pattern of the *P16* gene in blood cells from gastric cancer patients. A: Map of the *P16* promoter region and positions of the primers used in the analysis. The sequence is numbered relative to the translation start site for human *P16*. Characters in red indicate the primer binding sites for MSP and those in blue for bisulfite sequencing. Individual CpG sites are numbered consecutively; B: MSP of the *P16* promoter in peripheral blood from patients with gastric cancer. Marker, DL500 DNA Marker (TaKaRa); +, fully methylated DNA control; -, fully unmethylated DNA control; M, methylated band; U, unmethylated band. As a control, the fragments corresponding to the *P16* promoter of fully methylated DNA showed a clear band when amplified with methylated-specific primers and did not display a band when treated with unmethylated primers. The fully unmethylated DNA control showed the reverse pattern.

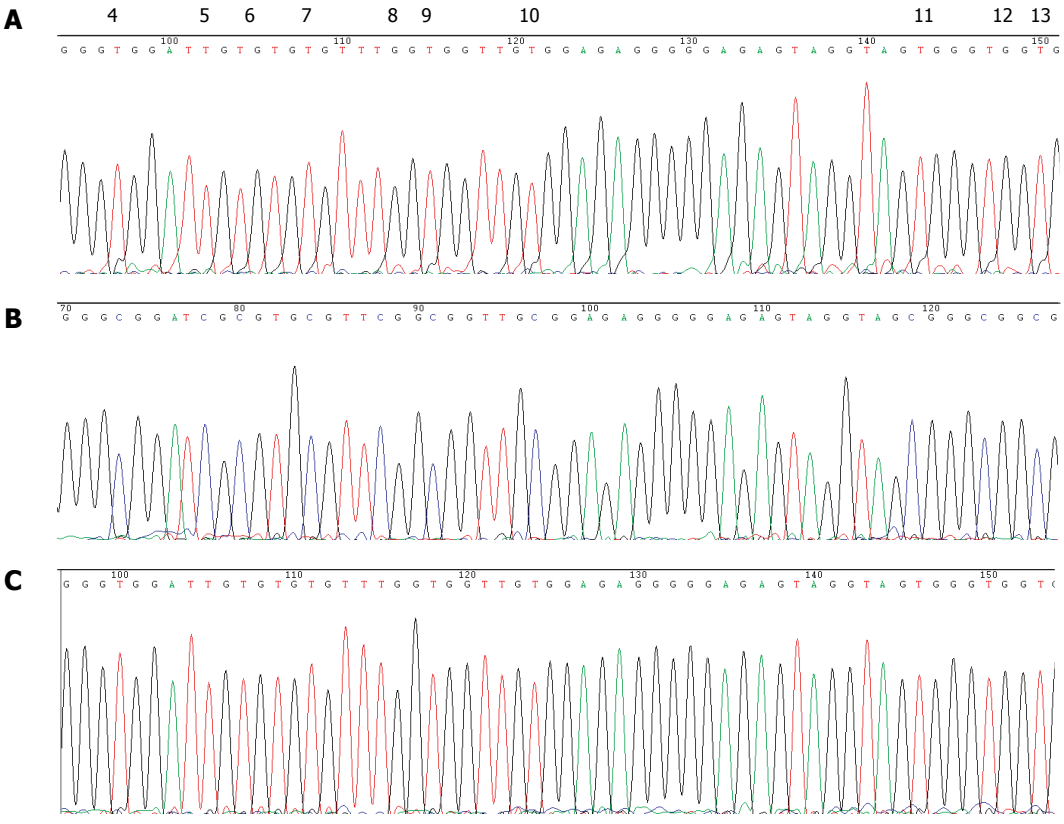

Figure 7 Bisulfite sequencing of *P16* promoter sequences. A: DNA isolated from the blood of a GC patient showing a T at all CpG sites of the *P16* gene promoter region due to complete modification of the DNA; B: CpGenome Universal Methylated DNA control in which a high C content can be found at all CpGs in the *P16* gene; C: CpGenome Universal Unmethylated DNA control showing a T at all CpG sites in the *P16* gene promoter.

10% methylation level at each of these CpG sites. Taken together, our data suggest that a mosaic germline *MLH1* epimutation might be present in some patients who develop gastric cancer. This mosaic methylation of *MLH1* might not be due to disseminated GC cells in the blood because these do not occur at sufficiently high levels to be detected by our assay. In patient G46, aberrant DNA methylation may have occurred after fertilization, when the maternal CpG methylation pattern is established[26]. It is also possible that CpG methylation is not always faithfully replicated during early embryogenesis, and the degree of mosaicism might be influenced by the genetic or epigenetic background. Our results raise the further possibility that silencing of *MLH1* by promoter methylation could occur as a germline or an early somatic event that generates a predisposition to GC.

We did not detect germline *CDH1* or *P16* promoter CpG methylation in any of the 140 GC patients investigated (Figures 3-7), suggesting that germline hypermethylation in the *CDH1* or *P16* promoter is not a common mechanism of *CDH1* or *P16* inactivation leading to GC. A detailed inspection of the bisulfate transformed sequence corresponding to the promoter region of *CDH1* revealed two methylated CpGs (the 4th and 9th), and also two methylated Cs at positions -120 and -65 bp relative to the transcription start site of the *CDH1* gene. However, given that this methylation was found in all of the patients and normal controls, it is unlikely to be associated with GC.

In summary, we report here that germline mosaic hypermethylation of the CpG islands in the *MLH1* promoter region may be the underlying cause of some gastric cancers, while germline hypermethylation of *CDH1* or *P16* gene is not a common mechanism responsible for GC in familial patients.

COMMENTS

Background

Gastric cancer is considered to be one of the leading cancers in East Asia but the underlying genetic abnormalities that lead to more than 70% of these cases remain unknown.

Research frontiers

Epigenetic methylation-associated inactivation of genes is not limited to tumor cells. Monoallelic promoter hypermethylation of the *MLH1* gene in the peripheral blood of patients with early-onset colorectal cancer has recently been reported in the literature. However, it is uncertain whether germline epimutations are responsible for gastric cancer onset in hereditary cases.

Innovations and breakthroughs

In this report, we provide a comprehensive description of germline epimutations of the tumor suppressor genes *MLH1*, *CDH1* and $P16^{INK4a}$ in Chinese gastric cancer cases. We report the rare occurrence of germline mosaic hypermethylation of the CpG islands in the *MLH1* promoter region in gastric cancer. We also demonstrate that germline epimutation of the *CDH1* or $P16^{INK4a}$ gene is not a frequent occurrence in this disease.

Applications

The finding of human *MLH1* gene germline epimutations is of some significance as it not only reveals a possible new mechanism in the tumorigenesis of gastric cancers, but also indicates that germline epimutations may be associated with a wide range of human diseases. On the other hand, germline epimutations show both mosaic and non-Mendelian characteristics, which may explain the phenotypic variability and changes in the genetic penetrance of some complex diseases, and thereby provide new avenues for studying the etiology of such disorders.

Terminology

Germline epimutations not only arise in tumor cells but in nontumor cells of a germline origin.

Peer review

The manuscript is well written and the methods are adequate. The results justify the conclusions drawn.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i1.79

World J Gastroenterol 2012 January 7; 18(1): 79-83
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Growth inhibitory effect of 4-phenyl butyric acid on human gastric cancer cells is associated with cell cycle arrest

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Supported by Natural Science Foundation of Ningbo, No. 2009A610134; Natural Sciences Foundation of Zhejiang, No. Y207244; College Students' Science-Technology Innovation Program of Zhejiang Province, No. 200959; the Excellent Dissertation Foundation of Ningbo University, No. 201014; KC Wong Magna Fund of Ningbo University; the Scientific Innovation Team Project of Ningbo, No.2011B82014

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Received: April 17, 2011 Revised: June 21, 2011
Accepted: June 28, 2011
Published online: January 7, 2012

Abstract

AIM: To investigate the growth effects of 4-phenyl butyric acid (PBA) on human gastric carcinoma cells and their mechanisms.

METHODS: Moderately-differentiated human gastric carcinoma SGC-7901 and lowly-differentiated MGC-803 cells were treated with 5, 10, 20, 40, and 60 μmol/L PBA for 1-4 d. Cell proliferation was detected using the MTT colorimetric assay. Cell cycle distributions were examined using flow cytometry.

RESULTS: The proliferation of gastric carcinoma cells was inhibited by PBA in a dose- and time-dependent fashion. Flow cytometry showed that SGC-7901 cells treated with low concentrations of PBA were arrested at the G_0/G_1 phase, whereas cells treated with high concentrations of PBA were arrested at the G_2/M phase. Although MGC-803 cells treated with low concentrations of PBA were also arrested at the G_0/G_1 phase, cells treated with high concentrations of PBA were arrested at the S phase.

CONCLUSION: The growth inhibitory effect of PBA on gastric cancer cells is associated with alteration of the cell cycle. For moderately-differentiated gastric cancer cells, the cell cycle was arrested at the G_0/G_1 and G_2/M phases. For lowly-differentiated gastric cancer cells, the cell cycle was arrested at the G_0/G_1 and S phases.

Key words: Histone deacetylase inhibitor; 4-phenyl butyric acid; Gastric carcinoma; Anticancer effect; Cell cycle; MGC-803; SGC-7901

Peer reviewer: Tomoyuki Shibata, Dr., Department of Gastroenterology, Fujita Health University, Toyoake 470-1192, Japan

Li LZ, Deng HX, Lou WZ, Sun XY, Song MW, Tao J, Xiao BX, Guo JM. Growth inhibitory effect of 4-phenyl butyric acid on human gastric cancer cells is associated with cell cycle arrest. *World J Gastroenterol* 2012; 18(1): 79-83 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i1/79.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i1.79

INTRODUCTION

Modification of the N-terminal of chromosomal histone

can alter chromatin structure by affecting the affinity between the histone and DNA[1,2]. Histone acetylation is a reversible dynamic process and can be regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC). Usually, HATs lead to the relaxation of chromatin structures and gene transcriptional activation, while HDACs are associated with chromatin condensation and transcriptional silence. Many studies have shown the close correlation of low histone acetylation or high expression of HDACs with the genesis and development of some tumors[3,4]. The activation of HATs and/or the suppression of HDACs were considered as a new approach to tumor therapy. The antitumor effect of histone deacetylase inhibitors (HDACIs) are attributed mainly to growth inhibition, apoptosis, or the induction of differentiation[5].

Gastric cancer is the second leading cause of cancer death in the world and will likely remain as one of the leading causes of all deaths in the near future[6,7]. In the past few years, evidence has accumulated showing that modification of acetylation status plays a central role in gastric carcinogenesis[8]. 4-phenyl butyric acid (PBA), a short-chain fatty acid, is a commonly used HDACI. However, the antitumor effect of PBA in gastric cancers has not yet been elucidated.

MATERIALS AND METHODS

Cell culture and reagents

Human gastric cancer cell lines, MGC-803 (lowly differentiated) and SGC-7901 (moderately differentiated), were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). MGC-803 and SGC-7901 cells were cultured in RPMI-1640 medium (Life Technologies, Grand Island, NY, United States) supplemented with 10% fetal bovine serum, glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified, 5% CO_2 in air atmosphere at 37 ℃. Exponentially growing cells were used for the experiments.

MTT assay

To observe the effect of PBA on the growth of gastric carcinoma cells, the MTT assay was used. MGC-803 and SGC-7901 cells (1.5×10^4/mL) were added to 96-well plates. Twenty-four hours later, PBA was added at final concentrations of 5, 10, 20, 40 and 60 μmol/L, respectively. Four wells were used for each dose. After the cells were incubated for 24, 48, 72, and 96 h, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution [2 g/L in phosphate buffered saline (PBS)] was added into each of the 96 wells. The cells were incubated at 37 ℃ for 4 h, the medium was removed, and 150 μL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan. The microplate was shaken on a rotary platform for 10 min. Then, the optical density (OD) values were measured at 490 nm using a Wellscan reader (Labsystems, Santa Fe, NM, United States). The inhibition rate was determined to indicate the suppressive effect of PBA on gastric carcinoma cells. The relative inhibition rate was calculated as a percentage, as follows: $(1-A_{experiment}/A_{control}) \times 100\%$. Three independent experiments were performed.

Cell cycle analysis

MGC-803 and SGC-7901 cells were treated with PBA at the above-mentioned concentrations for 24 h and 48 h. Then, the cells were collected and washed twice with PBS. Cold 70% ethanol was added and the cells were kept at 4 ℃ overnight. Subsequently, the cells were rinsed twice with PBS. Finally, the cells were incubated in propidium iodide/RNase Staining Buffer (BD, San Diego, CA, United States) according to the manufacturer's manual, and the cell cycle was analyzed with a fluorescence-activated cell sorting Calibur Flow Cytometer (BD, San Diego, CA, United States). The percentage of cells in the different phases of the cell cycle (G_0/G_1, S or G_2/M phase) was calculated by the BD FACStation™ Data Management System. Three wells of a 6-well plate were used for each dose. Three independent experiments were performed.

Statistical analysis

Statistical analysis was performed using the Statistical Program for Social Sciences (SPSS) software 13.0 (SPSS Inc, Chicago, IL, United States). Data were expressed as the mean ± SD. One-way analysis of variance (ANOVA) was used to analyze significant differences between control and treatment groups. A P value of less than 0.05 was considered statistically significant.

RESULTS

Effect of 4-phenyl butyric acid on the growth of gastric carcinoma cells

After exposure to PBA for 24, 48, 72, and 96 h, the growth of gastric carcinoma MGC-803 and SGC-7901 cells was inhibited significantly (5 and 10 μmol/L, $P < 0.05$; 20, 40, and 60 μmol/L, $P < 0.01$). The inhibitory effect was dose- and time-dependent (Figure 1).

Effects of cell cycle distribution of 4-phenyl butyric acid on gastric carcinoma cells

To decipher the suppressive mechanisms of PBA on gastric cancer cells, we monitored the changes in the cell cycle distribution by flow cytometry. The results of 48 h treatments showed that gastric carcinoma cells treated with various concentrations of PBA were arrested at different phases. MGC-803 cells treated with low concentrations (5 and 10 μmol/L) of PBA were arrested at the G_0/G_1 phase, while those treated with high concentrations (20-60 μmol/L) were arrested at G_2/M (Figure 2A). There was a big difference in the cell cycle interruption between MGC-803 and SGC-7901. SGC-7901 cells were arrested at the G_0/G_1 phase at low concentrations (5 and 10 μmol/L) and at the S phase with high concentrations (Figure 2B). Based on these observations, we investigated

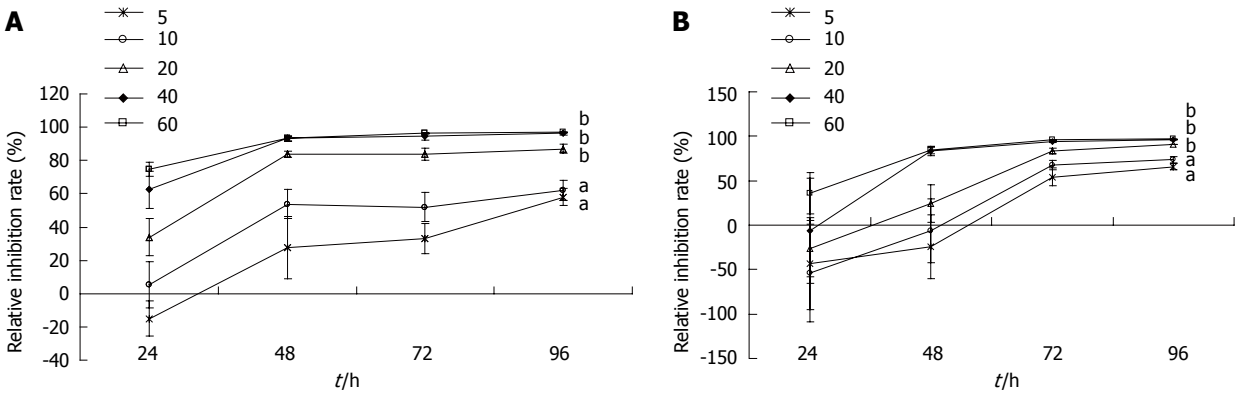

Figure 1 Effects of 4-phenyl butyric acid on the proliferation of gastric carcinoma MGC-803 cells (A) and SGC-7901 cells (B). Cells were incubated with 4-phenyl butyric acid at various concentrations for 24, 48, 72, and 96 h. The proliferation of cells was determined by the MTT assay. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control. The relative inhibition rate was calculated as a percentage, as follows: $(1\text{-}A_{experiment}/A_{control}) \times 100\%$.

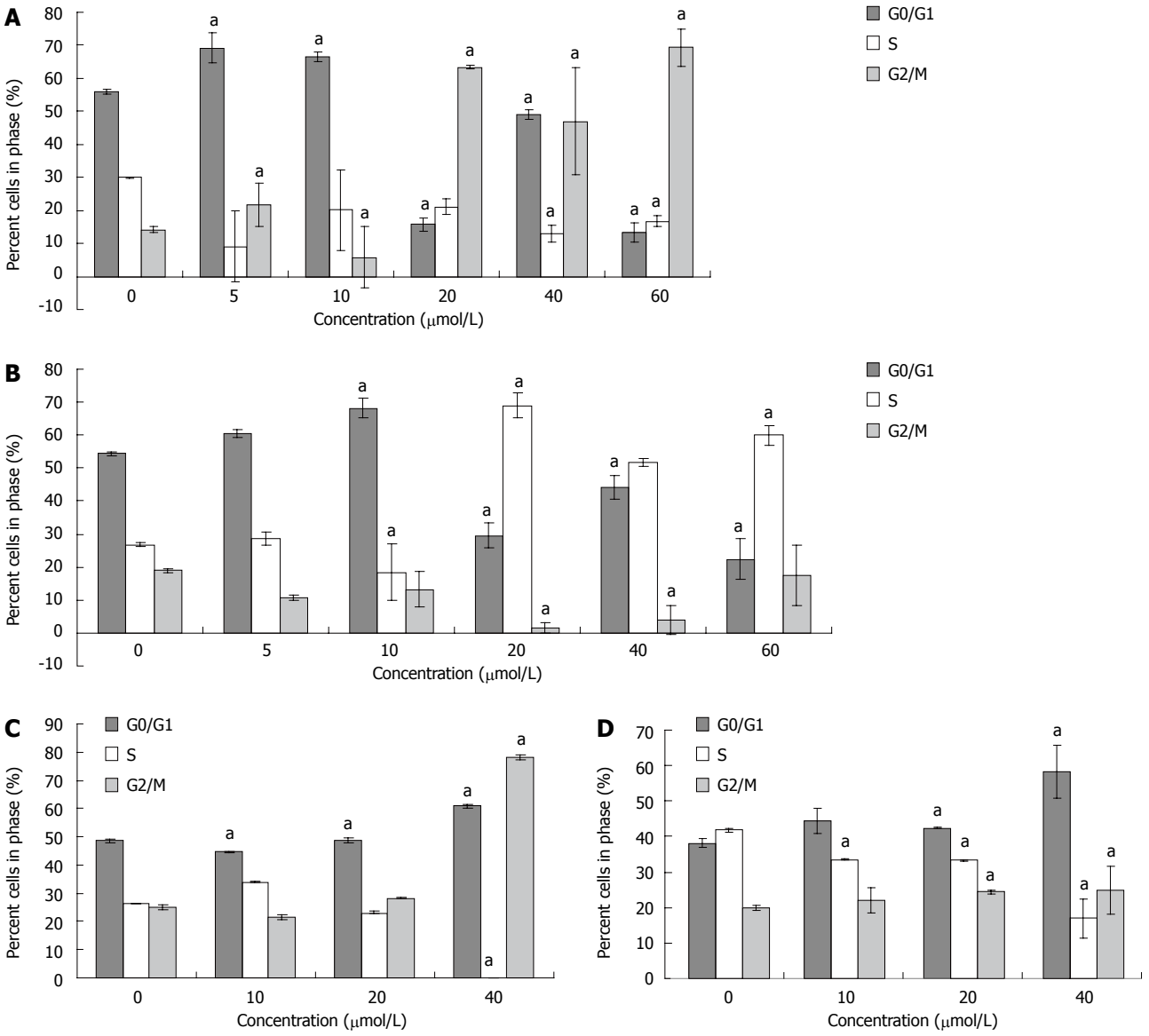

Figure 2 Effects of 4-phenyl butyric acid on cell cycle distribution of gastric carcinoma cells. The cell cycle was measured by propidium iodide staining and fluorescence-activated cell sorting analysis. $^{a}P < 0.05$ *vs* control. MGC-803 cells (A) and SGC-7901 cells (B) were treated for 48 h; and MGC-803 cells (C) and SGC-7901 cells (D) were treated for 24 h.

another time-course effect. Three doses, 10, 20 and 40 μmol/L, which were among the significant concentrations for both cells at the 48 h treatment, were chosen to treat cells for 24 h. As indicated in Figures 2C and D, similar effects were found.

DISCUSSION

PBA is one of the HDACIs already tested in clinical trials in the treatment of recurrent malignant gliomas and myelodysplastic syndrome[9,10]. In addition, it is a FDA-approved, and well-tolerated drug for urea cycle disorders[11]. It is converted into phenylacetate (PA) by β-oxidation in liver and kidney mitochondria[12]. It has recently been demonstrated that PBA has various cellular effects, such as induction of differentiation and apoptosis[13]. PBA has also been proved to be an effective chemical compound in preventing gene mutations and in preventing the aggregation of denatured α-lactalbumin and bovine serum albumin[14,15]. Interestingly, PBA treatment results in the induction of apoptosis in prostate cancer cells, medulloblastoma cells and colon cancer cells[12,13-17]. Furthermore, PBA was found to cause the regression of tumors derived from hepatocarcinoma cells in a rat model system[18].

Histone acetylation and DNA methylation represent epigenetic modifications that are essential for chromatin organization and the regulation of gene expression. Histone acetylation leads to an open chromatin structure favoring gene transcription, whereas deacetylation induces transcriptional repression through chromatin condensation[19]. In addition, the function of nonhistone proteins can be modified by acetylation and deacetylation[20]. Aberrant gene expression resulting from epigenetic alterations is critical for tumor development in many tumors, including gastric cancer, and it is also implicated in response to chemotherapy[21,22]. Modulation of chromatin structure has been suggested to influence the accessibility of DNA-targeting drugs such as short-chain fatty acid, and thus to affect the extent of the DNA damage[23-25]. Enzymes involved in these chromatin modifications with opposing activities are the histone acetyltransferases and HDACs. According to the structure, there are four types of HDACIs: hydroxamic acid and analogs, short-chain fatty acids, circum tetrapeptide, and benzamides[26]. PBA, a short-chain fatty acid, has not been used widely against gastric cancer, and its antineoplastic function has not been studied.

The present study has shown that PBA has a time- and dose-dependent effect on the proliferation of gastric carcinoma cells (Figure 1). These effects are similar to those of PBA on prostate cancer[27]. It is known that chemotherapeutic drugs act on the cell cycle of tumor cells. To decipher the growth suppressive mechanisms of PBA on gastric cancer cells, cell cycle distribution was measured by flow cytometry. The results indicated that the cell cycle distributions of two types of gastric carcinoma cells were interrupted by PBA (Figure 2). It was interesting to find that the modes of cell cycle arrests were different when high concentrations of PBA were used. The state of differentiation of MGC-803 and SGC-7901 cells is very different. MGC-803 cells are lowly differentiated, and SGC-7901 cells are moderately differentiated. These effects are similar to those of lycium barbarum polysaccharide (LBP) on MGC-803 and SGC-7901 cells. Miao *et al*[28] found that LBP treatment inhibited the growth of MGC-803 and SGC-7901 cells, with cell-cycle arrest at the G_0/G_1 and S phase, respectively. The cell cycles are regulated by the cell cycle-associated proteins, cyclin and cyclin-dependent kinase (CDK). Cyclin A binds and activates CDK2, and thus promotes both G_1/S and G_2/M transitions in the cell cycle[29]. For SGC-7901 cells, LBP increased the expression of cyclin A and decreased the expression of CDK2, and thus arrested cells at the S phase[28]. At the G_0/G_1 phase, the main cell cycle regulators are cyclin D, cyclin E, and CDK2[30]. For MGC-803 cells, LBP decreased the expressions of cyclin D, cyclin E, and CDK2[28]. These results are consistent with the effect of LBP on the arrest of the MGC-803 cell cycle at the G_0/G_1 phase.

In summary, our study identified PBA effects on gastric cancer cell growth. These observations may be correlated with the suppression of the cell cycle, and they suggest that an HDAC inhibitor such as PBA may be a potential anti-cancer drug.

COMMENTS

Background

Gastric cancer is the second leading cause of cancer death in the world and will likely remain as one of the leading causes of all deaths in the near future. Evidence has accumulated showing that modification of acetylation status plays a central role in gastric carcinogenesis.

Research frontiers

4-phenyl butyric acid (PBA), a short-chain fatty acid, is a commonly used histone deacetylase inhibitor (HDACI). However, the antitumor effect of PBA in gastric cancer has not yet been elucidated. In this study, the authors demonstrate that the proliferation of gastric carcinoma cells was inhibited by PBA in a dose- and time-dependent fashion.

Innovations and breakthroughs

Recent reports have highlighted the importance of PBA in the growth inhibitory effects on prostate cancer cells. This is the first study to report that PBA also suppresses the growth of gastric cancer cells. Furthermore, our studies show that the cell cycle arrest of PBA in gastric cancer cells is different between differentiated level of cancer cells.

Applications

By understanding how PBA inhibits the growth of gastric cancer cells, this study may represent a future strategy for therapeutic intervention in the treatment of patients with gastric adenocarcinoma.

Terminology

Histone acetylation is a reversible dynamic process which can be regulated by histone acetyltransferase and histone deacetylase (HDAC). Many studies have shown the close correlation of low histone acetylation or high expression of HDACs with the genesis and development of some tumors. Unsurprisingly, the use of PBA, a HDACI, will suppress the growth of gastric cancer cells.

Peer review

The authors assessed the inhibitory effect of PBA on human gastric cancer cells. They showed that PBA had growth inhibitory effects in a dose- and time-dependent manner. They also found cell cycle arrest was different between differentiated levels of gastric cancer cells. Advanced gastric cancer is a high mortality tumor as the result of repeated anti-cancer therapy. A further therapeutic strategy is needed to raise the remission rate of gastric cancer. The approach

of the authors in this study is one of the next generation therapies for gastric cancer. Therefore, the concept of this study is reasonable and important. It is interesting that the inhibitory effect was different between differentiated levels of gastric cancer cells. Also, methodology is interesting to investigate the mechanism of inhibitory effect of HDACIs.

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S- Editor Zhang SJ **L- Editor** Logan S **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i1.84

World J Gastroenterol 2012 January 7; 18(1): 84-89
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Unsuccessful treatment of four patients with acute graft-*vs*-host disease after liver transplantation

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Author contributions: Xu MQ and Yan LN contributed equally to this work; Xu MQ designed the research protocol; Yang J collected clinical data; Xu MQ, Wen TF and Yan LN contributed to the clinical work; Chen XB and Xu MQ wrote the paper.

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Received: May 4, 2011 Revised: June 23, 2011
Accepted: June 30, 2011
Published online: January 7, 2012

Abstract

AIM: To investigate appropriate therapeutic strategies for graft-*vs*-host disease (GVHD) following liver transplantation.

METHODS: Four patients who developed GVHD after liver transplantation in West China Hospital were included in this study. Therapeutic strategies with augmentation or withdrawal of immunosuppressants combined with supportive therapy were investigated in these patients. In addition, a literature review of patients who developed GVHD after liver transplantation was performed.

RESULTS: Although a transient response to initial treatment was detected, all four patients died of complications from GVHD: one from sepsis with multiple organ failure, one from gastrointestinal bleeding, and the other two from sepsis with gastrointestinal bleeding. Few consensuses for the treatment of GVHD after liver transplantation have been reached.

CONCLUSION: New and effective treatments are required for GVHD after liver transplantation to improve the prognosis of patients with this diagnosis.

Key words: Graft-*vs*-host disease; Immunosuppressant; Immunosuppression; Liver transplantation; Treatment

Peer reviewer: Rui Marinho, Professor, Department of Gastroenterology and Hepatology, Rua Prof. Aires de Sousa, 1 r/c A, Lisboa 1600-590, Portugal

Chen XB, Yang J, Xu MQ, Wen TF, Yan LN. Unsuccessful treatment of four patients with acute graft-*vs*-host disease after liver transplantation. *World J Gastroenterol* 2012; 18(1): 84-89 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i1/84.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i1.84

INTRODUCTION

Although the liver is recognized as an immunologically privileged organ, acute graft-*vs*-host disease (GVHD) may occur in 1%-2% of patients after liver transplantation (LT), and the mortality rate of patients with GVHD is very high (> 85%)[1-3]. The donor lymphocytes remaining in the portal tracts and the parenchyma of the donor liver graft after flushing with cold preservative solution[4,5] colonize the recipient, recognizing the host tissue antigens as foreign and react against the host tissue. The typical clinical presentations of GVHD include fever, skin rash (Figures 1-4), diarrhea, and pancytopenia beginning 2 to 6 wk after LT[1,2,6]. The diagnosis is usually made according to the typical clinical manifestations mentioned above, with the exclusion of other phenomena such as infection, drug allergies or rejection, which share the same clinical features as GVHD. A rapid diagnosis by the detection of lymphocyte macrochimerism through DNA-short tandem repeat (STR) has been recommended[7], while human leukocyte antigen (HLA)-

typing has also proven critical in confirming GVHD and elucidating its cause[8].

Treatment of GVHD typically consists of increasing immunosuppression using antibody preparations, such as OKT3 or antithymocyte globulin, to eliminate the donor lymphocytes and supporting myelopoiesis through the use of cytokines. However, this treatment has been unsuccessful; the majority of GVHD patients died of native bone marrow failure, resulting in fatal sepsis[1,9,10]. In one case, immunosuppressant withdrawal to control GVHD was suggested by Chinnakotla *et al*[11]. In their report, two of three patients exhibited the rapid loss of donor T-cell chimerism and resolution of their symptoms; however, the remaining patient continued to progress to severe GVHD and subsequently died.

In this study, we describe the unsuccessful results of different treatment regimens involving augmentation or withdrawal of immunosuppressant therapy in four patients with established GVHD after LT.

MATERIALS AND METHODS

From January 1999 to December 2010, 836 cases of LT were performed in West China Hospital. A total of four recipients developed postoperative GVHD (Table 1). Each of these patients was given a pathological diagnosis of GVHD following skin rash biopsy; HLA typing and polymerase chain reaction (PCR)-STR were not performed. The various treatments in these patients are shown in Table 2. One patient received increased immunosuppressive therapy (methylprednisolone 500 mg, iv, qd, for 3 d). This therapy was then augmented by consecutive use of cyclosporine A (CsA) and mycophenolate mofetil, while granulocyte colony-stimulating factor (G-CSF) was subsequently administered as a supportive treatment. The other three patients also initially received increased immunosuppressive therapy (methylprednisolone 500 mg, iv, qd, for 2 d); however, this was followed by the complete withdrawal of immunosuppressant therapy combined with the administration of supportive treatment including G-CSF (0.075 mg, bid), intravenous immunoglobulin (20 g, qd) or thymosin α1 (Zadaxin 1.6 mg, qd). Due to immune dysfunction, the patient who received increased immunosuppressive therapy eventually developed a severe infection. *Enterobacter cloacae* (a yeast-like organism) and *Candida albicans* were identified through repeat sputum and buccal swab culture in this patient. Subsequently, many different antibiotics (imipenem/cilastatin sodium, azithromycin, moxifloxacin, meropenem and teicoplanin) and an anti-fungal agent (fluconazole) were administered. Broad-spectrum antibiotics with or without anti-fungal medicines were also administered in the other three patients after GVHD onset to control infection or as a routine prophylaxis (Table 2). A flowchart of treatment is shown in Figure 5.

RESULTS

All patients with confirmed pathological diagnoses of GVHD developed fever, skin rash, diarrhea, severe bone marrow suppression and gastrointestinal bleeding. In response to the initial treatment, the rash faded, the skin underwent desquamation and pigmentation (Figure 2), and the temperature displayed a transient decrease. However, diarrhea persisted, bone marrow failure and gastrointestinal bleeding developed, and the levels of white blood cells (WBC) and platelets dropped. The most conspicuous changes in platelet number and white blood cell count were observed in cases 4 and 3, from a normal

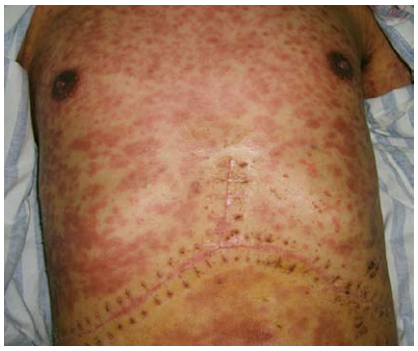

Figure 1 The maculopapular rash on the skin of the patient's chest wall and abdominal wall.

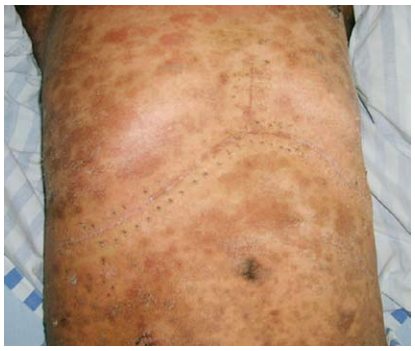

Figure 2 The skin underwent desquamation and pigmentation (the same patient as in Figure 1).

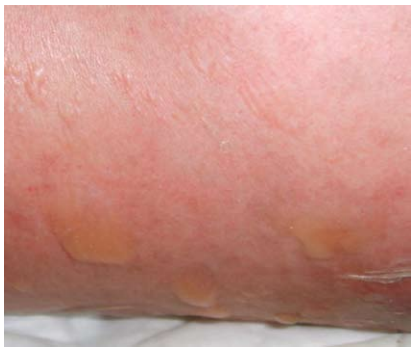

Figure 3 Skin blisters and rash on the flank and back of the patient.

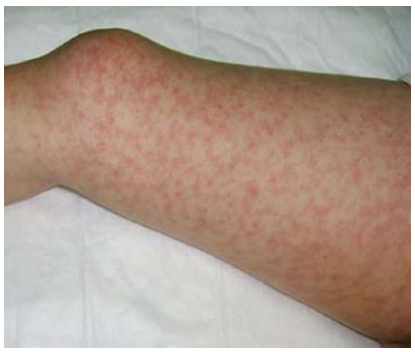

Figure 4 Maculopapular rash on the leg.

Table 1 Clinic data of recipients with graft-*vs*-host disease

Case	Age (yr)/sex	Etiology	HCC criteria	MELD score	Onset day	Presenting symptoms	Changes in PLT count	Changes in WBC count
1	48/M	HCC/LC/HBV	UCSF	28	POD 23	Fever, rash, diarrhea, BMS, gastrointestinal bleeding	111 × 10^9/L to 12 × 10^9/L	8.69 × 10^9/L to 0.28 × 10^9/L
2	43/M	HCC/LC/HBV	Milan	21	POD 21	Fever, rash, diarrhea, BMS, gastrointestinal bleeding	124 × 10^9/L to 45 × 10^9/L	7.59 × 10^9/L to 0.11 × 10^9/L
3	55/M	HCC/LC/HBV	Milan	23	POD 20	Fever, rash, diarrhea, BMS, gastrointestinal bleeding	273 × 10^9/L to 18 × 10^9/L	4.39 × 10^9/L to 0.03 × 10^9/L
4	56/F	LC/HBV	-	27	POD 21	Fever, rash, diarrhea, BMS, gastrointestinal bleeding	150 × 10^9/L to 1 × 10^9/L	5.12 × 10^9/L to 0.16 × 10^9/L

M: Male; F: Female; HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; HBV: Hepatitis B virus; UCSF: University of California, San Francisco criteria for liver transplantation; BMS: Bone marrow suppression; POD: Post-operation day; MELD: Model for End-Stage Liver Disease; PLT: Platelet; WBC: White blood cell. Normal value for PLT is (100-300) × 10^9/L; Normal value for WBC is (4-10) × 10^9/L.

Table 2 Treatment for graft-*vs*-host disease and outcome

Case	Immunosuppression and supportive therapy	Time of death	Cause of death
1	Increased immunosuppressant (methylprednisolone 500 mg, iv, qd, for 3 d), then CsA and mycophenolate mofetil + G-CSF (0.075 mg, bid, for 11 d) + antibiotics (imipenem/cilastatin sodium, azithromycin, moxifloxacin, meropenem and teicoplanin) + anti-fungal (fluconazole)	POD 34	MOF and sepsis
2	Increased immunosuppressant (methylprednisolone 500 mg, iv, qd, for 2 d), then withdrawal (POD 29) of immunosuppressant + G-CSF (0.075 mg, bid, for 9 d) + IVIG (20 g, qd, for 9 d) + broad spectrum antibiotics (cefuroxime, ceftriaxone and imipenem/cilastatin) + anti-fungal (fluconazole)	POD 38	Gastrointestinal bleeding
3	Increased immunosuppressant (methylprednisolone 500 mg, qd, for 2 d), then withdrawal (POD 29) of immunosuppressant + thymosin α1 (Zadaxin 1.6 mg, qd, for 7 d) + G-CSF (0.075 mg, bid, for 7 d) + broad spectrum antibiotics (cefminox)	POD 35	Gastrointestinal bleeding and sepsis
4	Increased immunosuppressant (methylprednisolone 500 mg, iv, qd, for 2 d), then withdrawal (POD 30) of immunosuppressant + IVIG (20 g, qd, for 7 d) + thymosin α1 (Zadaxin 1.6 mg, qd, for 7 d) + G-CSF (0.075 mg, bid, for 5 d) + broad spectrum antibiotics (cefminox, cefoperazone/sulbactam)	POD 36	Gastrointestinal bleeding and sepsis

CsA: Cyclosporine A; G-CSF: Granulocyte colony-stimulating factor; IVIG: Intravenous immunoglobulin therapy; MOF: Multiple organ failure; POD: Post-operation day.

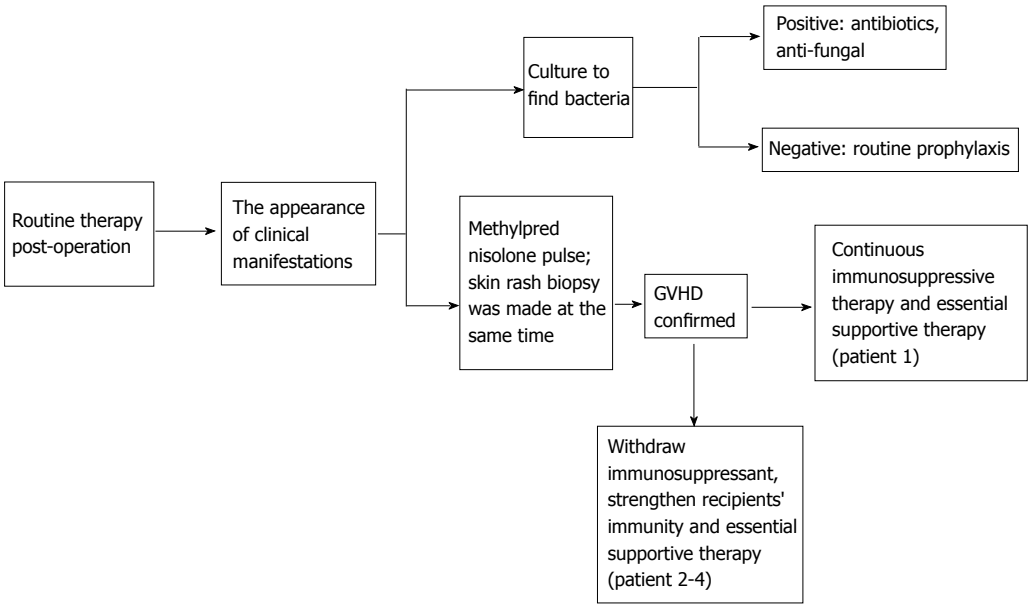

Figure 5 Flowchart of the management of graft-*vs*-host disease after liver transplantation in our center. GVHD: Graft-*vs*-host disease.

level to 1 × 10^9/L and from a normal level to 0.03 × 10^9/L, respectively (Table 1). All of these patients died of complications from GVHD: one from sepsis with multiple organ failure (MOF), one from gastrointestinal bleeding, and the other two from sepsis with gastrointestinal bleeding (Table 2). The average survival time from onset of GVHD was 14.25 d.

DISCUSSION

GVHD following LT is an uncommon but fatal complication that poses a major diagnostic and therapeutic challenge. Research has shown that GVHD involves activation of donor T lymphocytes by antigen-presenting cells (APCs), causing an alloreactive T-cell response to recipient tissues mediated by cytotoxic T-cells and inflammatory cytokines[12], such as tumor necrosis factor-alpha (TNF-α), interleukins 1, 2, 6 and 10, and interferon γ[13]. Of these cytokines, TNF-α is recognized as the key inflammatory cytokine involved in the pathogenesis of GVHD, which can activate APCs, recruit effector cells, and cause tissue damage[13,14]. Taking the role of TNF-α during GVHD into account, certain anti-TNF-α agents such as etanercept and infliximab have been used in steroid-refractory GVHD following hematopoietic stem cell transplantation (HSCT), and one case who developed GVHD after LT was reported to be successfully treated with infliximab[14,15]. Etanercept, a soluble recombinant human TNF-α receptor type Ⅱ fusion protein, which is usually used to treat rheumatoid arthritis and inflammatory bowel disease, was reported to be used in acute GVHD with steroids. A substantial majority of patients displayed a complete response (CR), which was very promising[15,16].

At present, most cases of GVHD after LT are diagnosed by typical clinical manifestations. However, the following laboratory examinations can help with the diagnosis: (1) biopsy of the skin rash; (2) detection of donor peripheral blood leukocytic chimerism; (3) microsatellite phenotype; (4) detection of donor HLA types in the peripheral blood, mucous membrane, or skin by PCR; and (5) detection of donor lymphocytes using immunohistochemistry on the skin rash[17]. Once diagnosis is confirmed, treatment should follow. Due to the low incidence of the disease and difficulty in evaluating the efficacy of treatment modalities, the therapeutic schedule for GVHD after LT has not yet been standardized. Most treatment methods are derived from those experiences of GVHD management following HSCT. Most treatments focus on increasing immunosuppression, usually in the form of antibody preparations such as antithymocyte globulin (ATG) or OKT3, which are used to treat conventional GVHD in a bone marrow transplant recipient. Unfortunately, these experiences did not demonstrate any survival benefit[9,10,18-21].

CsA is the mainstay of pharmacologic prevention of acute GVHD[22], while high-dose corticosteroid is considered an important part of first-line treatment for acute GVHD after LT[1,3]. The use of corticosteroids for the treatment of GVHD after LT is derived from the HSCT experience: corticosteroids resolve symptoms in many patients. The mechanism by which glucocorticoids ameliorate acute GVHD is not completely clear, but it is likely related primarily to the suppression of lymphocytic activity. However, GVHD after LT is less responsive to corticosteroids than GVHD after stem cell transplantation. The literature includes reports on 12 patients who were treated primarily with corticosteroids and/or increased immunosuppressive medications following the diagnosis of GVHD after LT[1,3,9,10,21,23-27]. Of these patients, all adult patients died of GVHD-related complications during the subsequent 11 d; only two children[28,29] were alive at the time of the report. These results suggest that, at least in the adult patients, treatment of GVHD after LT exclusively with corticosteroids or increasing immunosuppression is an inadequate approach to long-term therapy. Although temporary symptom relief may be available, the resolution of GVHD cannot be expected.

In the first case in our series, high-dose methylprednisolone followed by CsA and mycophenolate mofetil were administered to treat GVHD. Although the fever improved transiently in this patient, diarrhea and bone marrow suppression did not respond to this treatment regimen; continuous diarrhea and bone marrow failure developed, and WBC and platelet counts progressively decreased. Furthermore, hyperpyrexia developed again after two days' amelioration. This patient soon died of severe lung infection and MOF 11 d after GVHD onset. Due to the unsuccessful outcome of acute GVHD in a liver allograft recipient following increased immunosuppression, reduction and even complete withdrawal of immunosuppressant therapy has been proposed as a treatment for GVHD after LT. Theoretically, such an approach should allow the recipient immune system to reject alloreactive donor lymphocytes mediating the GVHD[29-31]. Attempts at implementing this approach were met with an initial worsening of symptoms[2,32] and involved a risk of the donor liver being rejected[30]. These limitations would explain why few patients were treated primarily with the reduction or discontinuation of immunosuppression. Antilymphocyte globulin, ATG and OKT3 were also used in these patients, but there was no evidence that these agents altered the eventual outcome[9,10,8-21].

Six adult patients were reported to have been treated for GVHD after LT primarily by the reduction or discontinuation of immunosuppression before 2004[9,26,33-35]. Two of these patients were reported to be alive at the time of the report, with their recovery attributed to a reduction in immunosuppression[26,30]. Five children have been treated with this approach[1,29,31,33,36]. Of these children, three were alive, one was alive with chronic GVHD, and one child died. Chinnakotla *et al*[11] reported the success of immunosuppression withdrawal in three patients diagnosed with GVHD in 2007. This approach

was successful in two of three cases, but the other patient died. Another successful approach to treatment involving improvement of the immunity of the patient was also reported by Lu *et al*[17].

In our series, three patients were treated first by increasing immunosuppression (high-dose methylprednisolone 500 mg once daily for 2 d + FK506 or CsA) upon the initial appearance of GVHD symptoms, such as hyperpyrexia and skin rash. Because the diagnosis of GVHD was confirmed by skin rash biopsy, the amelioration of fever, and progressive bone marrow cell proliferation suppression, complete withdrawal of the immunosuppressant was proposed. At the same time, supportive therapies such as immunoglobulin, thymosin α1 and G-CSF were administered. Unfortunately, these three patients did not respond effectively to this regimen of immunosuppression withdrawal combined with supportive therapy; one patient died of gastrointestinal bleeding, while two died of sepsis with gastrointestinal bleeding.

In sum, although two major strategies (augmentation or withdrawal of immunosuppressant therapy) have been proposed for the treatment of GVHD after LT, many novel strategies have been reported to be effective: anti-TNF-α with etanercept or infliximab, use of alefacept to elevate the blood cell count[36], pulse cyclophosphamide to treat the steroid-refractory hepatitis form of liver GVHD not associated with gut GVHD[37], interleukin 2-receptor antibody (basiliximab or daclizumab) therapy[7], and the preventive broad-spectrum chemokine-inhibitor NR58-3.14.3 (in animal experiments only)[38]. However, few consensuses have been reached. For non-typical manifestation at an early stage, which involve symptoms similar to those of a drug allergy or mimicking a more common clinical condition such as infection, GVHD can easily be misdiagnosed or even omitted. Moreover, as a rare post-transplant complication with no standardized treatment protocol hitherto, early diagnosis and effective treatment of GVHD would be complex, frustrating and laden with additional difficulties. Although a handful of successful cases have been reported, further research is necessary. It is therefore imperative to develop a *de novo* therapeutic schedule with definite effects.

COMMENTS

Background

Graft-*vs*-host disease (GVHD) following liver transplantation (LT) is a rare but fatal complication. The incidence is about 1%-2%, and the mortality rate is over 85%. People who develop GVHD after LT usually die of serious complications such as multiple organ failure, gastrointestinal bleeding and sepsis. So, a rapid diagnosis and treatment of GVHD is critical. At present, the consensuses of treatment methods for GVHD after LT have not been reached and the therapeutic experiences are derived from those of management following hematopoietic stem cell transplantation.Two major strategies (augmentation or withdrawal of immunosuppressant therapy) have been proposed as the treatment of GVHD after LT. Also, many novel strategies have been reported to be effective.

Research frontiers

A rapid diagnosis by the detection of lymphocyte macrochimerism through DNA-short tandem repeat has been recommended, while human leukocyte antigen-typing has also proven critical in confirming GVHD and elucidating its cause. Tumor necrosis factor-alpha (TNF-α) is recognized as the key inflammatory cytokine involved in the pathogenesis of GVHD, which can activate antigen presenting cells, recruit effector cells and cause tissue damage. So, certain anti-TNF-α agents such as etanercept and infliximab have been used in steroid-refractory GVHD.

Innovations and breakthroughs

The authors herein report four patients who developed GVHD after liver transplantation. Therapeutic strategies with augmentation or withdrawal of immunosuppressant combined with supportive therapy were investigated in these patients and a literature review of patients who developed GVHD after liver transplantation was performed in this article to investigate appropriate therapeutic strategies for GVHD following LT.

Applications

This article describes the clinical manifestations and diagnostic method of GVHD after LT and summarizes different therapeutic strategies, which would be helpful to the diagnosis or cure of this disease.

Terminology

GVHD: An immunological disorder that affects many organ systems, including the gastrointestinal tract, liver, skin and lungs, is a common and serious complication of transplantation where there is a reaction of donated organ/bone marrow against a patient's own tissue. It is an incompatibility reaction when donor lymphocytes or a graft containing lymphocytes that immunologically competent are given to a patient that has low immunological competence. Due to antibodies from the donor against antigens in the host, it can produce lymphocyte clones that will react by a variety of processes against the host and cause damage. Immunosuppressant: The agent administrated by patients who accept organ/bone marrow transplantation that decrease the immunity of the patients.

Peer review

The manuscript is well written and summarizes the treatment methods of GVHD after LT.

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S- Editor Sun H **L- Editor** Webster JR **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i1.90

World J Gastroenterol 2012 January 7; 18(1): 90-95
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Inflammatory pseudotumor of the liver and spleen diagnosed by percutaneous needle biopsy

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Received: February 12, 2011 Revised: June 3, 2011
Accepted: June 10, 2011
Published online: January 7, 2012

Abstract

An inflammatory pseudotumor (IPT) is a relatively rare lesion characterized by chronic infiltration of inflammatory cells and areas of fibrosis. IPTs are difficult to diagnose because of the absence of specific symptoms or of characteristic hematological or radiological findings. In this study, a case of a woman aged over 70 years was reported, who presented with a general malaise lasting more than two months. A computed tomography scan demonstrated a diffusely spread lesion of the liver with a portal vein occlusion and a splenic lesion surrounded by a soft density layer. Since the percutaneous liver biopsy showed findings that suggested an IPT, although the radiological findings did not exclude the possibility of a malignancy, we performed a percutaneous spleen biopsy to enable a more definitive diagnosis. The microscopic findings from the spleen specimen lead us to a diagnosis of IPT involving the liver and spleen. Subsequent steroid pulse therapy was effective, and rapid resolution of the disease was observed.

Key words: Inflammatory pseudotumor; Percutaneous liver biopsy; Percutaneous spleen biopsy; Steroid pulse therapy

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Kawaguchi T, Mochizuki K, Kizu T, Miyazaki M, Yakushijin T, Tsutsui S, Morii E, Takehara T. Inflammatory pseudotumor of the liver and spleen diagnosed by percutaneous needle biopsy. *World J Gastroenterol* 2012; 18(1): 90-95 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i1/90.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i1.90

INTRODUCTION

Inflammatory pseudotumors (IPT) are unusual lesions from a histological standpoint as they are characterized by nonspecific inflammatory cell infiltration and fibrosis. This tumor type was first described in the lung by Brunn[1] in 1939, and has been reported subsequently in various organs including the liver[2] and spleen[3].

No radiological findings have been found that are characteristics of IPT. This may be because the proportion or the distribution of inflammatory cells and fibrosis differs according to the cause and the period of inflammation[4]. Hence, it is difficult to establish a definite diagnosis by radiological imaging. Moreover, a proper differential

diagnosis of IPT from a malignant tumor could delay vital treatment. As a result of these issues, surgical removal is chosen only when the possibility of malignancy cannot be ruled out and when the treatment can be expected to be effective, particularly if growth of the lesion is observed during the evaluation period. There are some reports emphasizing the importance of undertaking a percutaneous needle biopsy in cases of suspected hepatic IPT to avoid unnecessary surgery, although misdiagnoses of adenocarcinoma[5] or an uncertain type of sarcoma[6] have been reported for some microscopic examinations. However, only one case of a needle biopsy for splenic IPT has been reported to date[7], and it is extremely rare to find IPT of the liver and spleen at the same time[8-10].

We report a rare case of IPT involving the liver and spleen which was difficult to diagnose by hematological examination or radiological study, but was confirmed following a percutaneous liver and spleen biopsy.

CASE REPORT

A woman aged over 70 with a past history of early-stage gastric cancer that had been treated by endoscopic mucosal resection at another hospital four years previously, was being followed annually by upper esophagogastroduodenoscopy. In one examination, compression from outside of the stomach at the fundus was observed, and she was referred to our hospital for further investigation.

At the time of the referral, this patient had been suffering from a general malaise lasting more than two months but did not have any other clinical symptoms such as fever, appetite loss, weight loss, or abdominal pain. The initial physical examination revealed nothing of note. Blood tests further showed a reduction in hemoglobin [8.0 g/dL (normal range 12.0-15.0 g/dL)] and an elevation of the white blood cell count [10 300/mm^3 (normal range 3300-9400/mm^3)], platelet count [549 000/mm^3 (normal range 130 000-320 000/mm^3)], and C-reactive protein level [10.98 mg/dL (normal range < 0.2 mg/dL)]. There was a slight increase in the total protein content [8.2 g/dL (normal range 6.4-8.1 g/dL)], whereas the albumin concentration was 1.9 g/dL (normal range 3.6-4.7 g/dL), indicating a substantial increase in globulin levels, which was consistent with an elevated IgG [3774 mg/dL (normal range 870-1700 mg/dL)]. The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were within the normal range, whereas the serum levels of alkaline phosphatase and γ-glutamyl transpeptidase were elevated [507 U/L (normal range 134-359 U/L) and 94 U/L (normal range 8-51 U/L), respectively]. The patient was negative for hepatitis B surface antigen (HBsAg) and hepatitis C virus antibody (anti-HCV). Antinuclear antibodies (ANA) were positive at a titer of 1:40 (normal range < 1:40) whereas other autoantibodies were negative, including antimitochondrial antibody (AMA), cytoplasmic anti-neutrophil cytoplasmic antibody (C-ANCA), and perinuclear anti-neutrophil cytoplasmic antibody (P-ANCA). Carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), α-fetoprotein (AFP), and des-γ-carboxy prothrombin (DCP) were all found to be within normal limits.

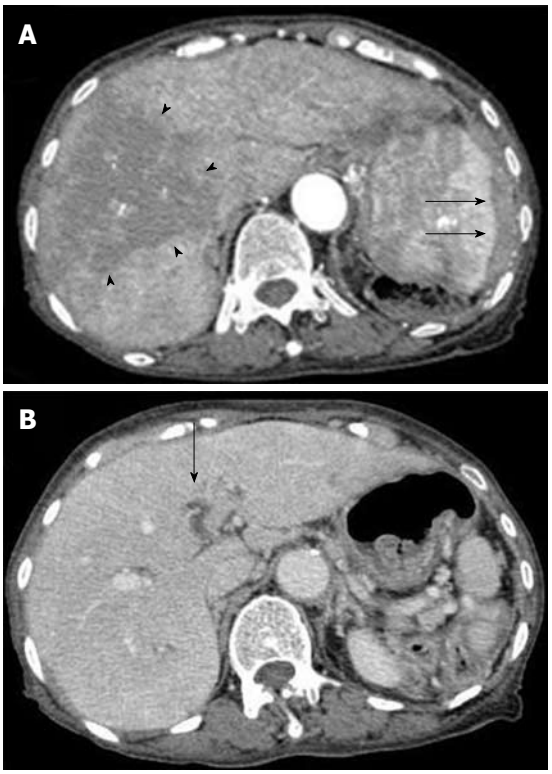

Figure 1 Contrast computed tomography scanning of the arterial phase revealed a diffusely and non-homogeneously enhanced liver, with the anterior compartment showing less enhancement (arrow-head). The spleen was found to be protruding inward, and also showed a diffuse and inhomogeneous enhancement. A: Computed tomography (CT) scans also revealed a soft density layer around the spleen (arrow). B: A CT of the portal phase showed an occlusion (arrow) of the left branch of the portal vein.

Contrast computed tomography (CT) during the arterial phase showed a diffusely and non-homogeneously enhanced liver, with a less enhanced anterior compartment (Figure 1A). The left branch of the portal vein was occluded in the portal phase (Figure 1B). The spleen was protruding inward, with diffuse and inhomogeneous enhancement of the arterial phase, as observed for the liver. CT also revealed a soft density layer around the spleen (Figure 1A). The results of fluorodeoxyglucose positron emission tomography/computed tomography (FDG-PET/CT) exhibited abnormal metabolic activity with a high standardized uptake value (SUV) of 7.1 around the spleen (Figure 2A). This was consistent with previous CT findings, and with a multiple abnormal uptake in segment six of the liver (Figure 2B). There was no abnormal uptake in other body sites. These radiological findings indicated the presence of a malignancy of the area surrounding the spleen and liver, with the possibility of a differential diagnosis of angiosarcoma, malignant lymphoma, hepatocellular carcinoma or metastatic cancer of the liver, with tumor thrombosis of the portal vein and the spleen.

A percutaneous needle liver biopsy was performed

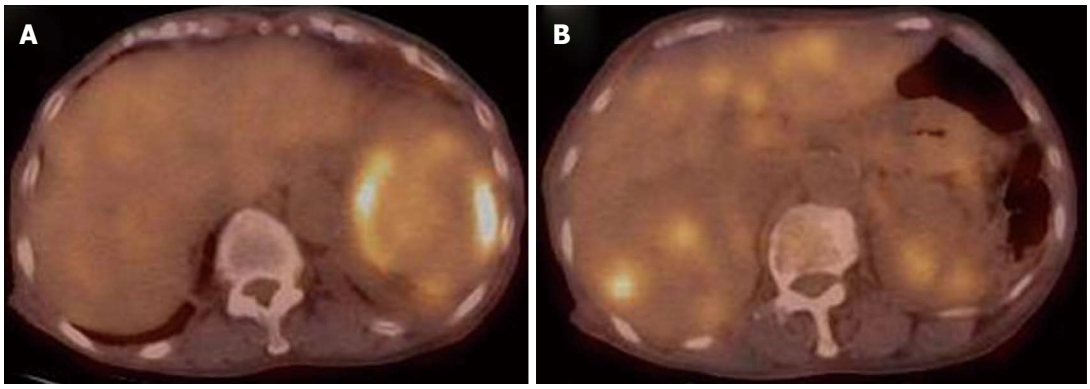

Figure 2 Fluorodeoxyglucose positron emission tomography/computed tomography analysis showing abnormal metabolic activity with a high standardized uptake value of 7.1 surrounding the spleen (A), and multiple abnormal uptakes in segment six of the liver (B).

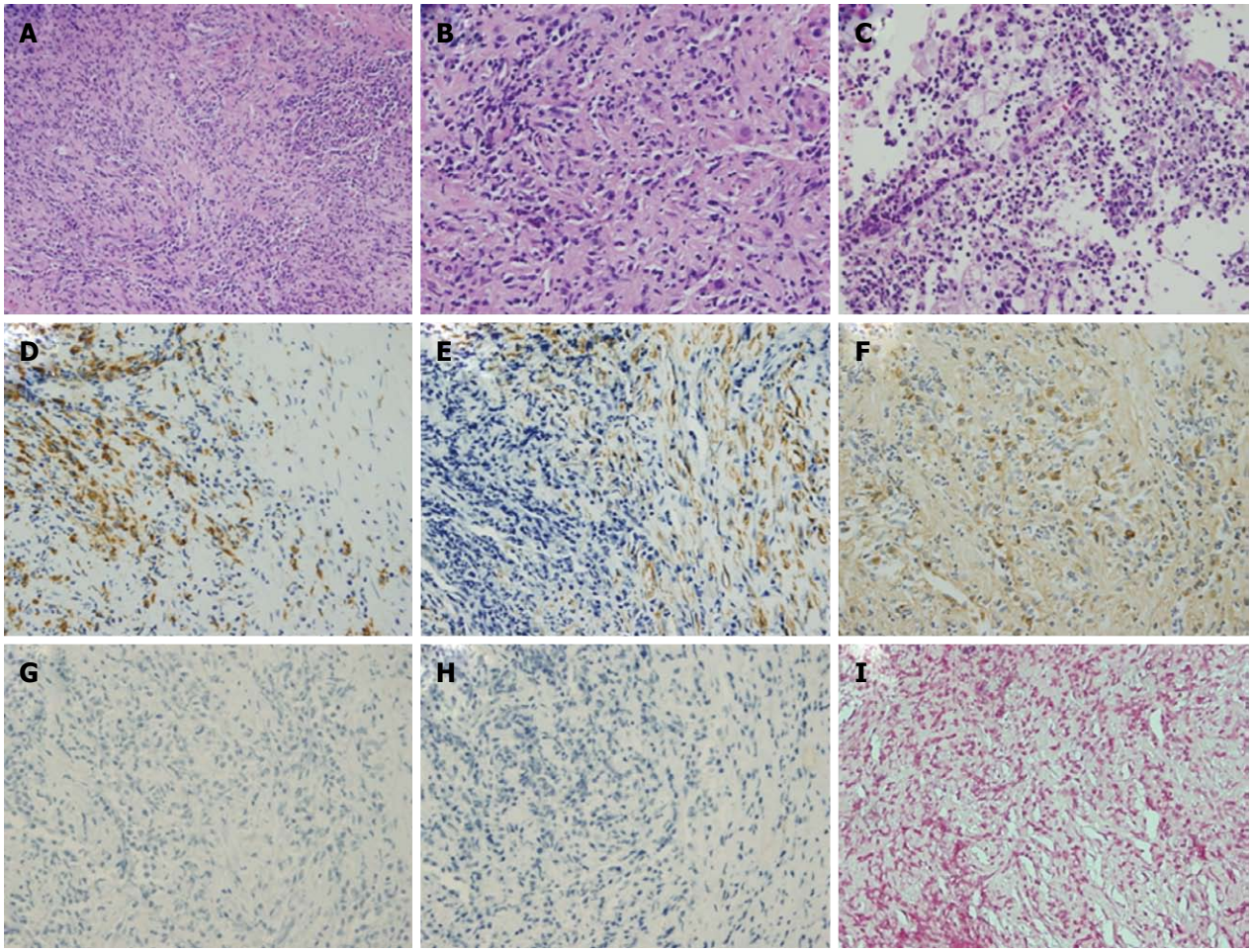

Figure 3 Histological findings for the liver *via* hematoxylin and eosin staining showing patchy fibroses and inflammatory cell infiltration (original magnification × 100). A: Mainly consisting of lymphocytes and plasma cells (original magnification × 200); B and C: Histological findings for the spleen following HE staining showed infiltration by plasma cells (original magnification × 200); D-I: Immunohistochemical analysis of the liver showed that the lesion was positive for CD68 (D), α-smooth muscle actin (SMA) (E), and IgG (F), but not for IgG4 (G), anaplastic lymphoma kinase (ALK) (H), or Epstein-Barr virus (EBV) encoded RNA (EBER) (I). HE: Hematoxylin and eosin.

under ultrasonic guidance to enable a more definite diagnosis. Sonographic examination revealed rough echogenicity of the right hepatic lobe, and a liver biopsy was subsequently performed but did not show a focal liver mass. The sonographic appearance of the spleen was similar to the liver biopsy region. The liver specimen showed a patchy fibrosis and inflammatory cell infiltration, mainly consisting of lymphocytes and plasma cells, but was free of malignant cells (Figure 3A and B). From this point of view, we considered a possible diagnosis of

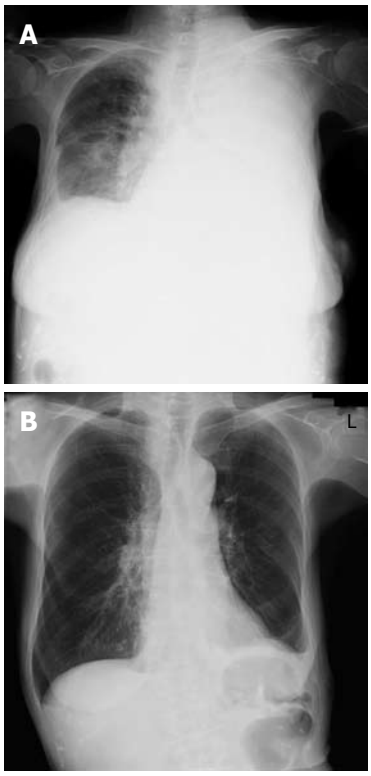

Figure 4 Chest X-ray showing massive pleural effusion of the left side before treatment (A), and full correction following steroid pulse therapy (B).

IPT, but the radiological findings so strongly indicated malignancy that we planned splenectomy for diagnostic confirmation. However, due to the patient's severe shortness of breath, caused by massive pleural effusion, a splenectomy was considered unsafe at that time (Figure 4A). Subsequent cytologic analysis showed that the pleural effusion was transudative and negative for malignancy. Hence, a percutaneous needle biopsy of the splenic lesion was performed as an alternative examination under ultrasonic guidance in the supine position *via* an intracostal approach. Microscopic examination of the spleen specimen revealed infiltration by lymphocytes and plasma cells, but no evidence of malignant cells (Figure 3C), as found in the liver specimen. Immunohistochemical examination of the liver specimen showed that the lesion was positive for CD68, α-smooth muscle actin (SMA) and IgG, but was negative for IgG4, anaplastic lymphoma kinase (ALK), and Epstein-Barr virus (EBV) encoded RNA (EBER) (Figure 3D-I). We were thus able to rule out an IgG4-related lesion, inflammatory myofibroblastic tumor (IMT), and an EBV-associated ITP-like follicular dendritic cell (FDC) tumor.

Finally, based on the results of our microscopic examination of both the liver and the spleen specimens, we diagnosed the pathological status of this patient as an IPT. However, despite an extensive clinical search for the potential cause of this IPT, mainly for infectious diseases including tuberculosis, its etiology could not be identified. As IgG levels were elevated in this patient and the ANA test was positive, we speculated that an autoimmune process could be possible. The patient did not respond for 19 d to intravenous antibiotics, including ampicillin hydrate (ABPC), sultamicillin tosilate hydrate (SBTPC), ciprofloxacin hydrochloride (CPFX), and meropenem hydrate (MEPN) and no underlying disease that could be a possible cause of IPT was detected. Because of the lack of any evidence for infection and her deteriorating clinical condition, we commenced steroid pulse therapy (three days of intravenous methylprednisolone; 1000 mg). C-reactive protein levels decreased immediately from 9.25 mg/dL to 0.93 mg/dL and there was no subsequent flare-up of inflammation during the gradual tapering of the steroid dose. The pleural effusion also decreased gradually (Figure 4B) accompanied by the restoration of normal breathing. Follow-up CT imaging after one month showed a nearly complete resolution of the hepatic and splenic lesions, except for the soft density layer remaining around the spleen (Figure 5). The occlusion of the portal vein remained unresolved however, and was subsequently found to be due to a thrombus which may have resulted from the severe inflammation. At 15 mo after the initial steroid pulse therapy, the patient was in good health and free of recurrence with continuing treatment of 5 mg of oral prednisone.

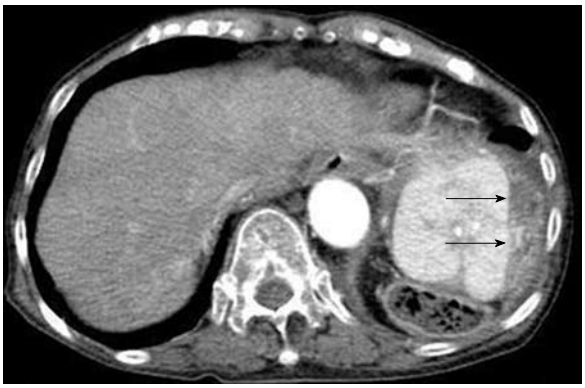

Figure 5 Follow-up computed tomography showing nearly complete resolution of the hepatic and splenic lesions other than the remaining soft density layer around the spleen (arrow).

DISCUSSION

The sites of predilection for IPT are the lungs and eyes followed by the liver, but involvement of the spleen is rare. The coexistence of IPT of the liver and spleen is therefore unusual.

It is very difficult for a physician to diagnose IPT because of the absence of specific symptoms, hematological abnormalities or anomalous radiological findings. Patients with IPT can present with fever, abdominal pain, abdominal discomfort, or leukocytosis, but these symptoms are not specific to IPT. Radiological results for IPT are also inconsistent, because fatty depositions, tissue inflammation and necrosis, fibrosis and bleeding can affect the imaging[11]. Moreover, the degree and distribution of proliferating capillaries influences the staining patterns

obtained by CT or MRI examination. A further problem is that IPT imaging findings are often similar to those of malignant tumors. For example, delayed enhancement on contrast CT, especially at the periphery of the lesions, is considered to be characteristic of IPT[12,13], but cholangiocellular carcinoma or metastatic tumor shows the same enhancement pattern. IPT sometimes shows an early enhancement pattern followed by a washout in the delayed phase on contrast CT[14], but this is a typical finding also for hepatocellular carcinoma. FDG uptake can be semiquantitatively measured using an SUV, which is generally higher in malignant tumors than in inflammatory disease. However, this method of differentiation is of limited value for IPT as the FDG uptake varies with the proportion of fibrosis and inflammatory cell infiltration. In fact, there have been some FDG-PET studies of IPT that have reported a high SUV[15,16] that is comparable to malignancy.

Due to the difficulty in diagnosing IPT through an assessment of clinical symptoms, blood examinations or radiological imaging, a definitive diagnosis often requires histopathological confirmation. Percutaneous needle liver biopsies are usually performed to diagnose IPT of the liver but may sometimes lead to a misdiagnosis[5,6]. Despite the absence of malignant findings by histological examination, there are also some IPT cases in which the possible existence of malignancy cannot be completely excluded even though various clinical factors are considered in tandem. Exploratory laparotomies or hepatectomies are often then performed[17] for confirmation. In cases of splenic IPT, almost all final diagnoses require surgery since a splenectomy is not only diagnostic but can also be curative. Recently, a less invasive laparoscopic splenectomy has been introduced in clinical settings[18], making it easier to perform. On the other hand, only one case of splenic IPT diagnosed by percutaneous spleen biopsy has been reported to date[7].

Only three cases of the coexistence of IPT of the liver and spleen have been previously reported[8-10]. Each was at first clinically or radiologically believed to be another disease such as lymphoma or metastatic cancer, but was eventually histologically diagnosed as IPT. One case was diagnosed by percutaneous liver biopsy without further radical study including spleen biopsy, as the patient was in the remission stage at the time of the biopsy. In the other two cases, a splenectomy was performed to enable the final diagnosis. In our current case, CT and FDG-PET findings strongly suggested the existence of a malignant tumor of the liver and the spleen with peritoneal dissemination, even though the histological appearance of the liver biopsy sample showed inflammatory cell infiltration and fibrosis indicating IPT. We therefore planned to conduct a splenectomy to confirm the diagnosis, but this became too risky as a massive pleural effusion had reduced the patient's respiratory function and her clinical status was worsening rapidly at that time. We therefore decided to conduct a spleen biopsy after obtaining informed consent from the patient. The results from the earlier liver biopsy were confirmed by the matching evidence obtained from the spleen sample.

IPT is clinically classified into several types according to their etiology, and the treatment options vary. Infections and autoimmune disorders are representative of originally-defined IPT, which is a benign and a reactive lesion. IPT of infectious origin can be caused by *Mycobacterium tuberculosis*[19], non-tuberculous mycobacteria[20], *Escherichia coli*[21], gram-positive cocci[5] and *Klebsiella pneumonia*[22]. Detection of the infecting organism by bacterial cultivation can confirm the existence of such instances of IPT, and the diagnosis can be further validated by the patient's response to administered antibacterial agents.

In our current patient, IMT[23], an EBV associated IPT-like FDC tumor[24], and IgG4-related diseases[25] were all excluded by immunohistochemical staining. In addition, although we obtained blood cultures and analyzed tissue from the lesions, no microorganisms were detected. Furthermore, the patient did not respond to a serial course of treatment with antibiotics, thus excluding the possibility of active infection. There are some reports of IgG4-related IPT[25] indicating the possibility that an autoimmune response contributes to the disease pathogenesis. Although the etiology of IPT remains unclear, based on the deteriorating clinical condition of our patient, we undertook steroid pulse therapy which proved to be very effective. The indication for steroid therapy has not been firmly established, but seems to be appropriate for IPT considering that this disorder was originally attributed to an inflammatory or a reactive process.

In conclusion, we report a rare case of the coexistence of hepatic and splenic IPT which was diagnosed by percutaneous liver and spleen biopsies. We conclude that a percutaneous biopsy can be an effective choice for suspected cases of IPT, even when a laparotomy or splenectomy is clinically difficult to perform.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i1.96

World J Gastroenterol 2012 January 7; 18(1): 96-98
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Laparoscopic resection of gastric gastrointestinal stromal tumors presenting as left adrenal tumors

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Received: March 16, 2011 Revised: April 5, 2011
Accepted: April 12, 2011
Published online: January 7, 2012

Abstract

Gastrointestinal stromal tumors (GISTs) are rare gastrointestinal malignancies. They are rarely seen near the urinary tract. In a literature review, only one case of GIST presenting as a left adrenal tumor was reported. We report two documented cases of gastric GISTs mimicking left adrenal tumors which were successfully treated with pure laparoscopic adrenalectomy and wedge resection of the stomach by excising the tumor from the stomach with serial firing of endoscopic gastrointestinal staplers. The surgical margins were clear, and the patients recovered smoothly. No adjuvant therapy with imatinib was prescribed. During the surveillance for 9 mo and 44 mo respectively, no tumor recurrence and metastasis were documented. Laparoscopic tumor excision, when adhering to the principles of surgical oncology, seems feasible and the prognosis is favorable for such tumors.

Key words: Gastrointestinal stromal tumor; Stomach; Laparoscopy

Peer reviewer: Marek Bebenek, Dr., MD, PhD, Department of Surgical Oncology, Regional Comprehensive Cancer Center, Plac Hirszfelda 12, 53-413 Wroclaw, Poland

Chung SD, Chueh JS, Yu HJ. Laparoscopic resection of gastric gastrointestinal stromal tumors presenting as left adrenal tumors. *World J Gastroenterol* 2012; 18(1): 96-98 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i1/96.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i1.96

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are rare neoplasms that account for less than 1% of all GI malignancies. The stomach is the most common site of origin for GISTs, accounting for 50%-60% of these tumors[1,2]. Of these, about 12% arise from the greater curvature[1]. It may mimic an adrenal tumor if no irregular gastric contour is noted. When the tumor is detected at this location incidentally, a patient without gastrointestinal (GI) symptoms may be referred to a urologist and adrenalectomy may be conducted. However, many urologists may be unfamiliar with this disease entity and may encounter difficulty in managing these tumors intra-operatively. Herein, we reported two such cases and detail our management.

CASE REPORT

Case 1

A 56-year-old female presented with microscopic hematuria found during a health examination. She did not

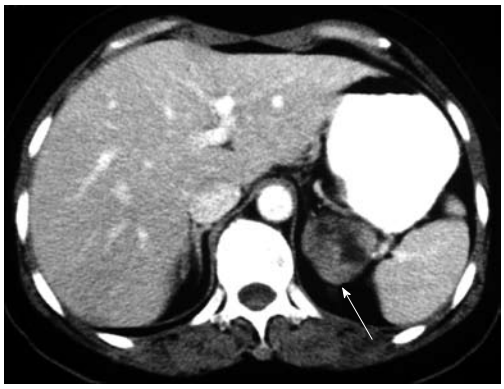

Figure 1 Computed tomography scan showing a soft tissue mass above the upper pole of the left kidney (arrow).

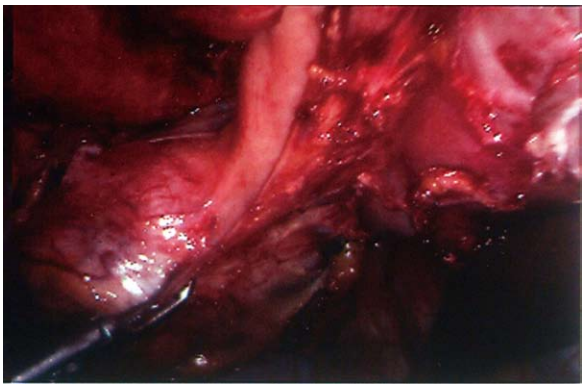

Figure 2 Intra-operative picture of a pedunculated tumor (T) originating from the greater curvature of the stomach (S).

suffer from flank pain, dysuria, epigastralgia, melena or hematemesis. Her medical history was unremarkable. Physical examination revealed no icteric sclera, flank knocking tenderness or palpable abdominal mass. Urine cytology disclosed a negative result for malignancy. Renal ultrasonography showed a left suprarenal mass. Abdominal computed tomography was performed, which showed a soft-tissue density mass 4.5 cm in diameter above the upper pole of the left kidney (Figure 1). This tumor was not enhanced with intravenous contrast and did not have calcification. Laboratory screening did not display anemia, or occult blood in stool. Endocrine examination showed normal adrenal hormonal profiles.

Case 2

A 76-year-old man presented with dyspepsia. A firm tumor in the left epigastrium could be palpated physically. Abdominal ultrasonography showed a large tumor located at the left retroperitoneum. He had a history of peptic ulcer, but no definite tumor had been documented in a panendoscopic examination of the upper gastrointestinal tract. Abdominal computed tomography showed a soft-tissue density mass 8 cm in diameter above the upper pole of the left kidney. Neither hepatic lesions nor lymphadenopathy was found. A bone scan and laboratory screening, including all endocrine survey, were all unremarkable.

Surgical technique: The surgical procedures for these two patients were quite similar and described together as follows. With a preoperative diagnosis of a non-functional adrenal tumor (case 1), and an adrenal mass ruling out adrenal carcinoma (case 2), transperitoneal laparoscopic tumor excision was planned. During surgery the tumors were noted to be undistinguishable from the left adrenal gland; hence no effort was made to dissect the tumor from the adrenal gland. Instead, the tumor along with the whole left adrenal gland was excised *en bloc,* in order to adhere to the principles of surgical oncology. After the main left adrenal vein, several small adrenal arteries and veins, and other surrounding connective tissues were divided, a pedunculated tumor strongly attached to the greater curvature of the stomach was noted (Figure 2). *En bloc* tumor excision was accomplished with wedge resection of the attached stomach by firing 2-3 endoscopic gastrointestinal anastomosis staplers (ENDO-GIA, Tyco HealthCare, Norwalk, CT, United States) consecutively to divide the gastric attachment of the tumor from the normal part of the stomach.

Pathology and follow-up: The pathological examination showed a 4 cm yellowish and elastic tumor beneath the smooth gastric mucosa in patient 1, and a 7 cm yellowish-gray and soft tumor in patient 2. The mitotic counts in both cases were less than 5 mitoses per 50 high power fields. Tumor cells were immunohistochemically positive for c-kit (CD117). Hence the diagnosis of GIST was confirmed for both tumors. The surgical margins in each specimen were free of tumor in either direction. Postoperatively, there was no gastric leakage; the nasogastric tube drainage was removed 2 d later and the patients resumed intake of a soft diet in 2-3 d. No adjuvant therapy with imatinib was prescribed for either patient. Serial follow-up computed tomography during surveillance revealed no local tumor recurrence or metastasis 9 (case 1) and 44 (case 2) mo after their respective operations.

DISCUSSION

The peak age of patients diagnosed with GISTs is around 60 years in most series. In a large series (1765 cases) of gastric GISTs, 54.4% of patients presented with symptoms related to GI bleeding such as anemia, melena or hematemesis[1]. Others had upper abdominal pain (16.8%), while acute abdomen resulted from tumor rupture was very rare (1.7%)[1]. GISTs are often discovered as incidental findings during radiological imaging for unrelated conditions, or as unexpected findings in surgical resections[1].

In the medical literature only one patient with GIST presenting as an adrenal tumor was reported before[3]. In both our patients, it was difficult to differentiate these two GISTs from usual cases of nonfunctional adrenal tumors since there were no clues from their symptoms

(no GI bleeding-related symptoms and normal endoscopy result) or image studies (no irregular intra-luminal contour of the stomach). Mild adhesion of the mass to the stomach might be a clue, but this phenomenon can be frequently observed if the adrenal tumor is big enough and/or the stomach is distended during the imaging studies. However, GISTs were suspected during the operation due to the severe adhesion of the mass to the greater curvature of the stomach wall, and surgery was adapted to achieve *en bloc* resection according to the principles of surgical oncology.

Surgical resection is the mainstay of treatment for localized gastric GISTs. It should be performed very carefully to avoid tumor rupture, which was associated with a high risk of intra-abdominal dissemination, subsequent recurrence and short overall survival[4]. In addition, complete resection of tumors is mandatory. The survival duration was similar between patients with tumor rupture and patients with incomplete surgical resections[4]. Unlike adenocarcinoma of the stomach, metastasis of GISTs to regional lymph nodes is very rare[1,5]. Routine lymphadenectomy is, hence, not indicated[6]. Several scholars had reported successful laparoscopic resection of gastric GISTs[7-9]. However, a large GIST of the stomach sometimes obscured the laparoscopic field. Yano *et al*[10] reported that two patients with large GISTs were successfully treated with hand-assisted laparoscopic surgery. They believed a hand-assisted procedure may offer adequate traction for the resection with sufficient surgical margins. In our patients, although the tumors were also quite large (4 cm and 7 cm in diameter) they had been completely dissected from all the other surrounding tissues except their final attachments from the greater curvature of the stomach, thus there was adequate space for manipulation and they were readily removed from the stomach with laparoscopic GIA staplers. Transperitoneally pure laparoscopic resection was hence feasible and successful in these two cases.

In conclusion, evaluation of patients with left adrenal tumors should include not only endocrine examinations but also GI workups if patients present with concurrent GI symptoms, or with hormonally inactive asymptomatic left adrenal tumors where there is no obvious demarcation between the tumor and the stomach wall in preoperative images[11]. If a GIST is suspected intra-operatively, we need to excise the tumors carefully with adequate safety margin and without causing tumor rupture. Postoperative surveillance is recommended especially in high risk patients (tumor size > 5 cm, mitoses > 5/50 high power fields).

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
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www.wjgnet.com

World J Gastroenterol 2012 January 7; 18(1): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2012 January 7; 18(1): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

November 9-13, 2012
American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel Diseases
Hollywood, FL 33028, United States

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2012 January 7; 18(1): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1361 experts in gastroenterology and hepatology from 64 countries.

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

Name of journal

World Journal of Gastroenterology

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

Editorial office
World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
Ocean International Center,
No. 62 Dongsihuan Zhonglu,
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-59080039
Fax: +86-10-85381893

Indexed and abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifer, and Directory of Open Access Journals. ISI, Thomson Reuters, 2010 Impact Factor: 2.240 (35/71 Gastroenterology and Hepatology).

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Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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Format

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1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunolog-

ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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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

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

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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Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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World Journal of
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Volume 18 Number 2
January 14, 2012

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中国精品科技期刊
OUTSTANDING S&T JOURNALS OF CHINA

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9 771007 932045 0 2>

ISSN 1007-9327 CN 14-1219/R Local Post Offices Code No. 82-261

World Journal of Gastroenterology

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Volume 18 Number 2 Jan 14 2012

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

World Journal of *Gastroenterology*

World J Gastroenterol 2012 January 14; 18(2): 99-196

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World Journal of Gastroenterology

Contents

Weekly Volume 18 Number 2 January 14, 2012

World Journal of Gastroenterology
Volume 18 Number 2 January 14, 2012

Contents

NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
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PUBLISHER
Baishideng Publishing Group Co., Limited
Room 1701, 17/F, Henan Building,
No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-31158812
Telephone: +852-58042046
E-mail: bpg@baishideng.com
http://www.wjgnet.com

PRINT SUBSCRIPTION
RMB 300 Yuan for each issue, RMB 14400 Yuan for one year.

PUBLICATION DATE
January 14, 2012

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

ONLINE SUBMISSION
http://www.wjgnet.com/1007-9327office/

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i2.99

World J Gastroenterol 2012 January 14; 18(2): 99-104
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Current treatment options and response rates in children with chronic hepatitis C

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Received: April 20, 2011 Revised: June 16, 2011
Accepted: June 23, 2011
Published online: January 14, 2012

Abstract

Vertical transmission has become the most common mode of transmission of hepatitis C virus (HCV) in children. The rate of perinatal transmission from an HCV-infected mother to her child ranges from 2% to 5% and the prevalence of HCV in children in developed countries ranges between 0.1% and 0.4%. Spontaneous viral clearance seems to be dependent on the genotype and has been reported between 2.4%-25%. For chronically infected patients, treatment with recombinant polyethylene glycol (PEG)-interferon α-2b and daily ribavirin has now been approved as standard treatment for children 2-17 years of age. In five large prospective studies, a total of 318 children and adolescents aged 3-17 years were treated either with subcutaneous PEG-interferon α-2b at a dose of 1-1.5 μg/kg or 60 μg/m² once a week in combination with oral ribavirin (15 mg/kg per day) or PEG-interferon α-2a with ribavirin. Subjects with genotype 1 and 4 received the medication for 48 wk and individuals with genotype 2 and 3 mainly for 24 wk. Overall sustained viral response (SVR) was achieved in 193/318 (60.7%) of treated patients. Stratified for genotype; 120/234 (51%) with genotype 1, 68/73 (93%) with genotype 2/3, and 6/11 (55%) with genotype 4 showed SVR. Relapse rate was between 7.7% and 17%. Overall, treatment was well tolerated; however, notable side effects were present in approximately 20%. According to recent experiences in the treatment of chronic hepatitis C in children and adolescents, a combination of PEG-interferon α with ribavirin has been found to be well tolerated and highly efficacious, particularly in individuals with genotype 2/3. Thus, this treatment can be recommended as standard of care until more effective treatment options will become available for genotype 1 patients.

Key words: Chronic hepatitis C; Treatment; Children; Polyethylene glycol-interferon and ribavirin; Response rate

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Wirth S. Current treatment options and response rates in children with chronic hepatitis C. *World J Gastroenterol* 2012; 18(2): 99-104 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i2/99.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i2.99

INTRODUCTION

Combination therapy of polyethylene glycol (PEG)-interferon α-2a or α-2b with ribavirin is standard of care for adults with chronic hepatitis C. Clear benefits in terms of sustained viral response (SVR) and side effect profile have been documented with PEG-interferon α compared with recombinant interferon α with and without ribavirin. An additional advantage of the pegylated form of interferon is the extended serum half-life, which allows a once-weekly administration regimen. Until recently, only recombinant interferon α-2b in combination with ribavirin had been approved by the Food and Drug Administration

(FDA) and European Medicines Agency (EMA) for use in children and adolescents. Since December 2008 and September 2009, respectively, the FDA and EMA approved PEG-interferon α-2b in combination with ribavirin in the United States and Europe for children aged 3 years and older[1]. Although most experts believe treatment is beneficial, due to several factors associated with treating young patients with chronic hepatitis C, this topic remains controversial[2-4]. However, there is no doubt that chronic hepatitis C remains an epidemiologically important health care issue in children and adolescents. Associated costs in the United States are estimated between $17 and $40 million annually[5]. Effective treatment of chronic hepatitis C virus (HCV) at an early age would help to prevent the long-term sequelae of chronic infection, improve the prognosis of patients, and reduce health care expenditure.

The prevalence of HCV in children in developed countries ranges between 0.1% and 0.4% but may even exceed 10% in some regions of Saudi Arabia and Africa[6-8]. The rate of perinatal HCV transmission from an infected mother to her child ranges from 2% to 5%. Clinically most relevant are genotypes 1, 2 and 3; considerably less spread is genotype 4[2]. It is estimated that there are 1 million individuals aged less than 18 years infected with chronic hepatitis C worldwide[9].

Since the early 1990s, transmission of HCV infection has occurred predominantly by parenteral transfusion of blood products or by non-use of disposable syringes. However, transfusion-associated hepatitis C has now become extremely rare in countries with adequate hygienic facilities. Subjects who were particularly at risk such as premature infants, hemophiliacs, patients with thalassemia, and children with malignant diseases or organ transplantations have now reached adulthood and vertical transmission from HCV-infected mothers to their offspring has become the most common cause of chronic hepatitis C in children. Importantly, in the case of vertical infection, the chronicity rate is very high[10].

Children chronically infected with HCV may be at risk for social disintegration and impaired quality of life. A possible psychological burden may be present and some physical impairment has been described. To date, only two rather small studies have been published reporting significantly lower physical and psychosocial scores and worse cognitive functioning compared with non-infected controls[11,12].

NATURAL COURSE

Spontaneous viral clearance in vertically infected children seems to be dependent on genotype and was found to range from 2.4%-25%[13,14]. It may be higher in parenterally infected individuals and was reported to reach 35%-45% by adolescence[15,16]. Children infected with genotype 3 have a higher spontaneous clearance rate than those infected with genotype 1. Beyond the age of 4 years, spontaneous viral clearance seems to become rather unlikely[13]. Patients who do not clear the virus within the first years of life will develop chronic hepatitis C. Overall, the cumulative probability of progression to chronicity is approximately 80%[17,18]. Most children are clinically asymptomatic or show only mild unspecific symptoms. In roughly 10% of patients, hepatomegaly may be present[17]. During the chronic course, alanine aminotransferase (ALT) levels may be normal or intermittently elevated. Only few patients show persistent markedly elevated ALT levels. Inflammatory activity in liver tissue is usually mild and the risk of severe complications is low. However, despite the favourable prognosis during the first and second decade of life, approximately 4%-6% of children will develop evidence of advanced liver fibrosis or cirrhosis[19,20]. A recently published study in pediatric patients with chronic hepatitis C cured of malignancy reported liver cirrhosis in 5% after three decades of observation[21]. Progression of fibrosis depends on age and additional risk factors such as obesity and alcohol consumption. Thus, progression usually starts beyond the second life decade and there is evidence that it seems to proceed more rapidly in patients with genotype 3[22]. Large liver transplantation units have reported on children who needed liver transplantation due to progressive HCV infection[23].

TREATMENT OPTIONS

Many years ago, treatment started in adults with the use of interferons, yielding SVR rates in the 10%-15% range. According to the use of different treatment regimens and small numbers of treated children, it was difficult to compare the response rates in children to those in adults. Overall, SVR seemed to be better in children. Nineteen studies using recombinant α-interferon were published between 1992 and 2003[24]. A meta-analysis of trials with interferon-α monotherapy revealed a wide range (0%-76%, mean 27%) of SVR. Subjects infected with genotype 2 and 3 clearly responded better than patients harbouring genotype 1. Based on an increasing number of randomized controlled trials in adults, ribavirin was added to interferon-α in treatment trials for children. Between 2000 and 2005, six studies were published all demonstrating an SVR from 27% to 64%[25]. The stratification according to genotypes showed a very good response (> 80%) in patients with genotype 2 and 3 and an SVR of approximately 36%-53% in those with genotype 1. Results of an extensive trial in children published by Gonzalez-Peralta led to the approval of recombinant interferon α-2b in combination with ribavirin[26].

However, when PEG-interferon in combination with ribavirin became the standard of care for adults with chronic hepatitis C, trials in children promptly started. Some advantages were present such as a reduced injection frequency to once per week, better SVR, and better interferon tolerance. Interestingly, the sole controlled randomized trial, comparing a pegylated interferon α (PEG-interferon α-2a) with and without additional ribavirin, was only published in 2011. It clearly demonstrated that in the pediatric age group, the addition of ribavirin was necessary to obtain significantly better treatment re-

Table 1 Sustained viral response in five representative prospective trials using polyethylene glycol-interferon alpha-2b and polyethylene glycol-interferon alpha-2a in combination with ribavirin and stratified for different clinical and laboratory parameters and genotypes, published between 2005 and 2011

	Wirth 2005[1]	Jara 2008[1]	Wirth 2010[1]	Total PEG-interferon a-2b trials	Schwarz 2011[2]	Sokal 2010[2]	Total all trials
Dosage	1.5 μg/kg per week	1.0 μg/kg per week	60 μg/m² per week		180 μg/1.73 m² per week	100 μg/m² per week	
Total (%)	36/61 (59)	15/30 (50)	70/107 (65.4)	121/198 (61.1)	29/55 (53)	43/65 (66.1)	193/318 (60.7)
Genotype (%)							
1	22/46 (48)	12/26 (46)	38/72 (53)	72/144 (50)	21/45 (47)	27/47 (59)	120/236 (51)
2/3	13/13 (100)	3/3 (100)	28/30 (93)	44/46 (96)	8/10 (80)	16/17 (94)	68/73 (93)
4	1/2	0/1	4/5 (80)	5/8 (62)		Included in G1	
ALT-levels (%)							
Elevated	12/25 (48)		27/44 (61)			19/33 (58)	58/102 (57)
Normal	24/36 (67)		42/63 (67)			24/30 (80)	90/129 (70)
Mode of infection (%)							
Parenteral	19/27 (70)	7/9 (78)	5/5 (100)	31/41(76)			
Genotype 1	13/21 (62)		1/1				
Vertical	12/25 (48)	8/21 (38)	46/75 (61)	66/121 (55)			
Genotype 1	7/20 (35)		26/52 (50)	33/72 (46)			
Break through	9.8%				6/41 (15)		
Relapse	7.7%		8%		6/35 (17)		

ALT: Alanine aminotransferase; PEG: Polyethylene glycol. [1]PEG-interferon α-2b; [2]PEG-interferon α-2a.

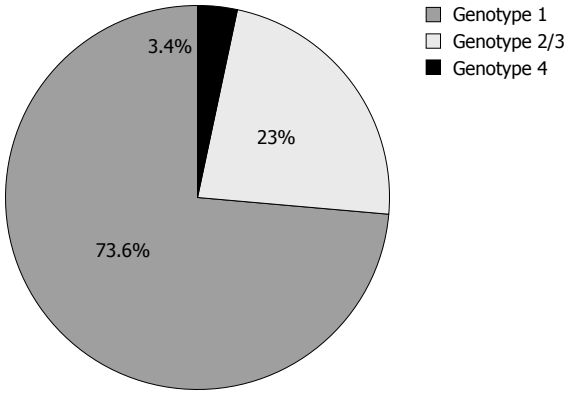

Figure 1 Distribution of genotypes in the five representative prospective trials using polyethylene glycol-interferon α-2b and polyethylene glycol-interferon α-2a in combination with ribavirin, published between 2005 and 2011.

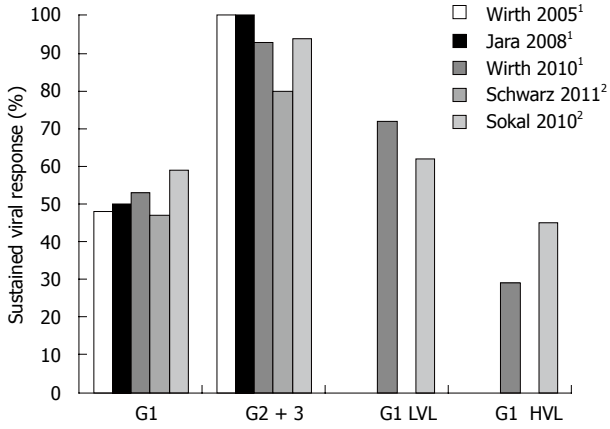

Figure 2 Sustained viral response in five large prospective trials with polyethylene glycol-interferon α-2b[1]/α-2a[2] and ribavirin stratified for genotype and viral load[27,28,30-32]. G: Genotype; HVL: High viral load, > 600 000 U/mL, (Wirth *et al*[32]), > 500 000 U/mL (Sokal *et al*[30]); LVL: Low viral load, < 600 000 U/mL (Wirth *et al*[32]), < 500 000 U/mL (Sokal *et al*[30]).

sults[27]. Specifically, in genotype 1 patients, SVR rate was 17% with PEG-interferon monotherapy compared with 47% in individuals with combination treatment. The difference was also striking in subjects infected with genotype 2 and 3 (36% *vs* 80%).

Up to now, results of seven trials using PEG-interferon α in combination with ribavirin have been reported[27-32]. SVR rates in patients with genotype 1 from 5 trials with more than 30 patients ranged from 44% to 59%. Achieving SVR in children with genotype 2 and 3 was very successful and yielded rates of more than 90%. The relapse rate was between 7.7% and 17%. Four trials used PEG-interferon α-2b and two used PEG-interferon α-2a in combination with ribavirin. An additional report presented the retrospective data in 33 treated Japanese children and young adults[33]. SVR rate in these patients was approximately 82%. Unfortunately, no information regarding genotypes was provided. Table 1 and Figure 1 summarize the characteristics of the five prospective studies. Peg-interferon α-2b and ribavirin were approved for patients aged 3 to 17 years of age by the FDA in December 2008 and the EMA in September 2009.

Baseline viral load

Two studies stratified the results in genotype 1 patients according to the viral load before treatment. In the first study, the cut-off level was 600 000 IU/mL: 32% of children with genotype 1 and high viral load (> 600 000 IU/mL) and 73% with low viral load (< 600 000 IU/mL) achieved SVR[32]. In the second trial, the cut-off value was 500 000 IU/mL: 45% of children with genotype 1 and > 500 000 IU/mL and 62% with < 500 000 IU/mL achieved SVR[30].

Figure 2 summarizes the SVR in relevant pediatric trials using PEG-interferon in combination with ribavirin.

Table 2 Most frequent adverse events during polyethylene glycol-interferon treatment in combination with ribavirin and its appraisal of clinical significance

Interferon α-treatment:
Leukopenia, thrombocytopenia: Frequent, not really significant; if necessary dose reduction
Flu-like symptoms: In all treated patients, not significant
Alopecia: Not significant
Autoimmune thyroiditis: At least 15 %, significant, mostly reversible
Acute psychosis, depression: Very seldom before puberty (< 1 %), rare in adolescents, significant in cases with manifestation; should be under investigation in future trials
Growth delay: Clinically not significant, catch-up growth, but under investigation with relative high priority
Anorexia, weight loss: Mostly not significant with exceptions, normalisation after therapy stop
Ribavirin:
Anemia: Mostly clinically not significant with exceptions, reversible

Most side effects' intensity is decreasing after some weeks of treatment.

Baseline aminotransferases

It is remarkable that the level of aminotransferases or histological findings by liver biopsy do not significantly correlate with SVR. However, interestingly, there was a trend towards a slightly better SVR in patients with normal aminotransferases.

Mode of infection

There is no significant correlation between SVR and the mode of infection. Nevertheless, it seems that individuals with parenteral infection may have a slightly higher probability to obtain SVR. However, the overall response rate in vertically infected subjects was 55% and in genotype 1 patients 46%, which is comparable to the SVR in adults who are mainly parenterally infected (Table 1).

Standard of care

According to approval, in principle, treatment with interferon α-2b and ribavirin administering injections thrice per week can be performed. However, the majority of experts will prefer once weekly dosing using PEG-interferon. To date in America and Europe, only PEG-interferon α-2b (60 μg/m² per week) in combination with ribavirin (15 mg/kg per day) is approved by the FDA and EMA[1]. Patients with genotypes 1 and 4 should be treated for 48 wk, with treatment discontinued at 4-6 mo if there has been no viral response. Patients with genotypes 2 and 3 should be treated for 24 wk irrespective of pre-treatment viral load. In routine clinical practice, there is no need to perform liver biopsy before initiating treatment. In addition, pre-treatment levels of aminotransferases and mode of infection are not predictive for SVR. A five-year follow-up study of children with SVR treated with interferon α and ribavirin showed permanent viral elimination in 98% (Kelly D, personal communication).

Re-treatment

Response rates in patients retreated with a standard of care protocol are dependent on the primary treatment regimen. Individuals with previous interferon α monotherapy or recombinant α-interferon in combination with ribavirin may achieve a higher response rate. There are no studies specifically addressing re-treatment except for the trial by Gerner *et al*[34], which has been performed with a natural interferon α in combination with ribavirin. Previously published reports have only included small numbers of children with failed response, demonstrating a re-treatment response rate of 40%-50% in those with previous interferon α monotherapy. Gerner *et al*[34] reported SVR in only 2/18 patients. Thus, re-treatment, particularly in individuals who have been primarily treated with PEG-interferon and ribavirin, remains prognostically difficult and cannot be recommended until new combination treatment options including directly acting antivirals such as protease inhibitors become available.

ADVERSE EVENTS

The majority of treated children and adolescents will tolerate PEG-interferon and ribavirin well. Nevertheless, almost all patients will experience at least one side effect. The clinical significance of adverse events is summarized in Table 2. Most adverse events are mild to moderate, such as flu-like symptoms including fever, anorexia, fatigue, dry skin and moderate hair loss. In some patients, dose reduction of PEG-interferon may be necessary due to decreased white blood cell counts. Severe anemia is very rare; hence, the need for dose reduction of ribavirin is extremely infrequent. The rates of discontinuation of treatment due to adverse events were low in all trials published. Severe psychiatric side effects were rare in prepubertal individuals, but may be of significance in affected individuals. Appearance of thyroid autoantibodies and thyroid dysfunction during long-term treatment (> 24 wk) has to be considered and carefully monitored. Up to 20% of treated patients, particularly with genotype 1, may have abnormal thyroid stimulating hormone levels or other signs of thyroid dysfunction[31,35]. Another notable side effect is transient growth impairment. Inhibited growth can be observed in 50%-70% with decrease of growth velocity below the 3rd percentile. Shortly after the end of treatment, catch-up growth usually starts with an increased growth velocity followed by achievement of previous growth velocity levels, which can be observed during the follow-up period. Nevertheless, if possible, treatment during pubertal growth spurt should be avoided[36]. In addition, weight loss is very common during the treatment phase; however, most patients experience compensatory weight gain after treatment ends[32]. Regarding quality of life, and behavioral, emotional and cognitive outcomes during and after treatment, no significant impairment has been detected in the PEDS-C trial[37]. More follow-up studies are in progress to evaluate long-term sequelae.

NEW DEVELOPMENTS

There is no doubt that treatment response in patients with genotype 1 is not entirely satisfactory and improved treat-

Table 3 Indication for hepatitis C virus treatment in children-pros and cons

In favour of treatment	Deferral might be considered
High response rate, sustained viral response means cure of the disease	Before 3-4 years of age because of possible spontaneous viral elimination
Prevention of disease progression and social burden	Psychiatric disorder
Better tolerability and less side effects in younger patients (particularly before puberty)	Low response rate in subjects with genotype 1 and high viral load
More favourable factors for response in children (e.g., low viral load)	Pubertal growth spurt
Parents facilitate compliance	More effective treatments in future in genotype 1 non-responders

ment regimens are desirable. A number of directly acting antiviral agents, designed to target viral encoded proteins essential to the HCV life cycle, are currently under development. Phase Ⅲ trials in adults have been completed for two protease inhibitors (telaprevir and boceprevir) and have shown a significantly increased viral elimination rate in combination with PEG-interferon and ribavirin[38-40]. Brand new data indicate that in a considerable number of patients with rapid response, exposure to PEG-interferon and ribavirin may be shortened and response-guided therapy will become the treatment of choice[41]. Approval of telaprevir and boceprevir has been sanctioned by the FDA and EMA in 2011, and pediatric trials will follow in the near future. In adults, genotype 1 non-responders have also demonstrated SVR rates ranging between 59% and 66%, depending on the duration of boceprevir treatment, compared to 20% with standard of care[42]. Given that efficacy data could be extrapolated from adults to children, an approved triple therapy regimen should be expected for non-responders. Nevertheless, they should definitely be included in future pediatric trials[36].

CONCLUSION

In children and adolescents, PEG-interferon treatment in combination with ribavirin for 48 wk produces a sustained viral response rate in approximately 50% of adequately treated individuals. Thus, this option can be offered to all patients irrespective of the level of aminotransferases or mode of infection. There is evidence that subjects with low viral load may respond better than patients with high viral load. In patients infected with genotype 2 or 3, a 90% or even better SVR rate can be achieved. Thus, treatment for 24 wk should be administered in all patients with genotype 2 and 3. According to the approval of the drugs, treatment start is possible beyond three years of age. However, because spontaneous viral elimination may occur within the first 4-5 years of life in vertically infected individuals, watchful waiting for up to five years of age is a justified alternative to an early treatment start. Additionally, different individual and family variables may influence the appropriate time to initiate therapy. An experienced pediatric gastroenterologist should supervise the management of treating these patients. Mid-childhood age before pubertal growth spurt is preferable. Table 3 summarizes pros and cons to indicate or possibly to defer treatment. Adverse events are usually well tolerated, but severe side effects may occur in a small number of patients making dose adjustment necessary. Overall, the encouraging results, particularly in patients with relatively low viral load and/or favourable genotypes and in line with an appropriate consideration of early stopping rules, endorse application of treatment in eligible patients. Re-treatment in non-responding genotype 1 patients should be deferred until a combination of standard care with direct acting antivirals has become available.

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S- Editor Zhang SJ **L- Editor** Logan S **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i2.105

World J Gastroenterol 2012 January 14; 18(2): 105-118
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

Selwyn Odes, MD, AGAF, Professor, *Series Editor*

Role of genetics in the diagnosis and prognosis of Crohn's disease

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Received: March 22, 2011 Revised: April 25, 2011
Accepted: May 2, 2011
Published online: January 14, 2012

Abstract

Considering epidemiological, genetic and immunological data, we can conclude that the inflammatory bowel diseases are heterogeneous disorders of multifactorial etiology in which hereditability and environment interact to produce the disease. It is probable that patients have a genetic predisposition for the development of the disease coupled with disturbances in immunoregulation. Several genes have been so far related to the diagnosis of Crohn's disease. Those genes are related to innate pattern recognition receptors, to epithelial barrier homeostasis and maintenance of epithelial barrier integrity, to autophagy and to lymphocyte differentiation. So far, the most strong and replicated associations with Crohn's disease have been done with *NOD2*, *IL23R* and *ATG16L1* genes. Many genes have so far been implicated in prognosis of Crohn's disease and many attempts have been made to classify genetic profiles in Crohn's disease. *CARD15* seems not only a susceptibility gene, but also a disease-modifier gene for Crohn's disease. Enriching our understanding on Crohn's disease genetics is important but when combining genetic data with functional data the outcome could be of major importance to clinicians.

Key words: Crohn's; Genetics; Polymorphism; Diagnosis; Prognosis; Genome wide scan; Genetic consortium

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Tsianos EV, Katsanos KH, Tsianos VE. Role of genetics in the diagnosis and prognosis of Crohn's disease. *World J Gastroenterol* 2012; 18(2): 105-118 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i2/105.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i2.105

EVOLVING ROLE OF GENETICS IN CROHN'S DISEASE

Despite decades of research the etiology of inflammatory bowel diseases (IBD) remains largely unexplained, but considering together epidemiological, genetic and immunological data, we can conclude that IBD are heterogeneous disorders of multifactorial etiology in which hereditability (genetic) and environment (microbial, behavior) interact to produce the immunological background of the disease. It is probable that patients have a genetic predisposition for the development of the disease coupled with disturbances in immunoregulation. The disease can then be triggered by any of a number of different unknown environmental factors and sustained by an abnormal immune response to these factors. Rather, the intensive interaction between intestinal epithelial cells and immune competent cells is critical to maintain and perpetuate the chronic inflammatory process characteristic

for IBD[1].

Early epidemiologic evidence for the role of genetic factors in the pathogenesis of Crohn's disease (CD) came from studies demonstrating higher rates of CD among individuals of Caucasian and Jewish ethnicity, familial aggregation of CD and higher concordance rates of both twins developing CD in monozygotic compared with dizygotic twins. The search for specific CD susceptibility genes, however, has been difficult due to complex genetics, including factors such as the lack of simple Mendelian inheritance patterns, involvement of several genes, and the influence of environmental factors and intestinal microflora on disease development. More than 30 distinct genomic loci encode genes involved in a number of homeostatic mechanisms and have been suggested to be involved in CD etiopathogenesis and prognosis[2].

Until very recently, two main approaches could be undertaken to identify genes in complex diseases: the positional cloning approach, based on linkage analysis, and the candidate gene approach, based on association studies. Linkage analysis studies the co-segregation of the disease with a marker within families. The candidate gene approach uses case-control cohorts or trios of affected offspring with both parents. Here, a specific gene with known or potential interest for the disease is studied. The allelic frequencies (in the case of case-control study) or the transmission of a single nucleotide polymorphism (SNP) towards affected offspring (in the case of trios) are analyzed, and differences between patients and controls, or distortion of transmission towards affected children, will point towards implication of the gene in the pathogenesis of the disease under investigation.

Despite the large numbers of genome-wide association studies (GWAS) established to date, most diseases have only managed to explain some additional percentage of the hereditability estimates. In an attempt to explain some of this missing hereditability, researchers have adopted several complementary strategies. Larger cohorts of cases are being collected, through either further patient recruitment or collaborations. The meta-analysis data generated to date has demonstrated how increasing the cohort sample size generates additional statistical power to detect smaller and smaller odds ratios[3]. Advances in technology and particularly bioinformatics have now made it possible to perform GWAS using common copy number variation probes. Many groups are looking to high-throughput sequencing technology, with the aim of sequencing candidate gene regions identified by GWAS, to hopefully identify either the causal or rare variants[4,5]. Several GWAS have been published in the last decade and have identified many genes associated with Crohn's disease (Table 1). Among these there are recognition-related genes such as *NOD1* and *TLRs*, other susceptibility genes including *DLG5*, *OCTN* and *HLA* and the newest susceptibility genes in CD resulting from GWAS: *IL23R* gene, *ATG16L1* gene and *IRGM* gene[6].

Table 1 Genetic polymorphisms related to Crohn's disease

Genes and the diagnosis of Crohn's disease
Genes related to innate pattern recognition receptors
NOD2/CARD15
OCTN
TLR
Genes related to epithelial barrier homeostasis
IBD5
DLG5
Genes related to molecular mimicry and autophagy
ATG16L1
IRGM
LRRK2
Genes related to lymphocyte differentiation
IL23R
STAT3
Genes related to secondary immune response and apoptosis
MHC
HLA
Genes and the prognosis of Crohn's disease
Genes related to age of Crohn's disease onset
TNFRSF6B, CXCL9, IL23R, NOD2 , ATG16L1, CNR1, IL-10, MDR1, DLG5, IRGM
Genes related to Crohn's disease behaviour
Stenotic/structuring behaviour: *NOD2, TLR4, IL-12B, CX3CR1, IL-10, IL-6*
Penetrating/fistulizing behaviour: *NOD2, IRGM, TNF, HLADRB1, CDKAL1*
Inflammatory behaviour: *HLA*
Granulomatous disease: *TLR4/CARD15*
Genes related to Crohn's disease location
Upper gastrointestinal: *NOD2, MIF*
Ileal: *IL-10, CRP, NOD2, ZNF365, STAT3*
Ileocolonic: *ATG16L1, TCF-4 (TCF7L2)*
Colonic: *HLA, TLR4, TLR1, -2, -6*
Genes related to Crohn's disease activity
HSP70-2, NOD2, PAI-1, CNR1
Genes related to surgery
NOD2 , HLA-G
Genes related to dysplasia and cancer
FHIT
Genes related to extraintestinal manifestations
*CARD15, FcRL3, HLADRB*103, HLAB*27 HLA-B*44, HLA-B*35, TNFa-308A, TNF-1031C, STAT3*
Pharmacogenetics in Crohn's disease
CARD15, NAT, TPMT, MDR1, MIF, DLG5, TNF, LTA

ROLE OF GENES IN THE DIAGNOSIS OF CROHN'S DISEASE

Several genes have been related to the diagnosis of Crohn's disease so far. Those genes are related to innate pattern recognition receptors, to epithelial barrier homeostasis and maintenance of epithelial barrier integrity, to autophagy and to lymphocyte differentiation. So far, the strongest and replicated associations with CD have been done with *NOD2*, *IL23R* and *ATG16L1* genes.

Genes related to innate pattern recognition receptors

***NOD2/CARD15* gene:** *NOD2*/Caspase Recruitment Domain Family member 15 (*CARD15*) acts as a pattern recognition receptor (PRR); this locus has been characterized as the IBD1 locus on 16q12-13[7].

Fine mapping of the IBD1 locus identified the underlying gene on chromosome 16 as the *CARD15* (previous *NOD2)* gene. *CARD15* represents homology with the R genes in plants, genes that confer resistance to infection[8]. Thirty nonconservative polymorphisms have been identified within the gene, which are associated with CD, but only three are common (Arg702Trp, Gly908Arg and Leuc1007insC). The three common variants account for approximately 82% of the mutated alleles. CARD15 is associated with CD only and not with UC. CARD15 codes for a protein expressed in monocytes, macrophages, dentritic cells, epithelial cells and Paneth cells. CARD15 is involved in the recognition of bacterial peptidoglycan-derived muramyl dipeptide through the leucine-rich repeat (LRR) region. Of importance, the frameshift mutation 1007fsinsC that leads to a truncated protein lacking the 33 distal amino acids was associated with impaired activation of the transcription factor NF-κB after stimulation.

It has been shown that Paneth cells play an important role in innate host defense via their ability to secrete antimicrobial peptides and proteins. Although NODs are expressed at low levels in absorptive and secretory intestinal epithelial cells, Paneth cells in the small intestine have been recognized as the predominant site of expression of NOD2 in the epithelium. Furthermore, NOD2 mutations have been associated with decreased expression of antimicrobial peptides, the α-defensins, by Paneth cells. In addition, a distinct gene polymorphism resulting in low β-defensin 2-gene copy number has been associated with a predisposition to colonic Crohn's disease. In addition, NOD2 plays important roles in the promotion of antibacterial T-helper-17 (Th-17) cells in the IL-23-IL-1-IL-17 axis.

CARD15 variants are found in 35% to 45% of white CD patients, with the exception of Scandinavian, Irish and Scottish patients[9,10], in whom the prevalence is much lower. Genotype relative risks of 3 (simple mutation) and 10-44 (double mutations) have been reported in European Caucasians[9,10]. However, CARD15 mutation is not frequent or even absent in African-American populations, in Indians, Chinese and Japanese[11-13]. Other CARD related genetic loci that have been associated with CD diagnosis are the CARD4 (NOD1), CARD8 and CARD9 loci[14,15].

Organic cation transporter genes: Organic cation transporters (*OCTNs, 5q31-33*) are membrane transporters for drugs and positively charged endogenous metabolites. The novel OCTN subfamily may also transport carnitine, which is essential for metabolism of lipids and is involved in transport of light chain fatty acids into mitochondria for b-oxidation. The first study reported on two functional mutations in the carnitine/OCTN cluster on 5q31 (the IBD5 locus) that were associated with Crohn's disease. As membrane transporters of organic cations, OCTNs are therefore important in the maintenance of intracellular homeostasis. In humans OCTN1 and OCTN2 map to IBD5 on 5q31. An OCTN3 has recently been described in humans[16].

Toll-like receptor genes: Host response to microbial pathogens includes self-defense mechanisms such as defensins, PRRs, pathogen-associated molecular patterns and toll-like receptors (TLRs). TLRs recognize conserved motifs on pathogens that are not found in higher eukaryotes and initiate an "innate" (rapid and non-specific) immune response[17]. Subsequently, specific receptors recognizing chemo-attractant molecules mobilize phagocytic leukocytes and induce their migration to inflammatory sites. There, leukocytes encounter the invading microorganisms and ingest them through the activation of phagocytic receptors that mediate the uptake process. Innate immune responses are linked to the generation of corresponding adaptive immune responses and studies of genetically engineered or cellularly manipulated animal models have generated a great deal of new information[18].

Leucocyte-epithelial interactions are of special interest as exposure of epithelial TLRs to microbial ligands has been shown to result in transcriptional upregulation of inflammatory mediators whereas ligation of leucocyte TLRs modulate specific antimicrobial responses[19]. It has been shown that Paneth cells play an important role in innate host defense *via* their ability to secrete antimicrobial peptides and proteins. In addition, it has been shown that NOD2 mutations lead to loss of negative regulatory effects on TLR signaling while activation of the CARD domain results in activation of NF-κB[20].

TLRs are the most important receptors of the innate immune system. They are expressed by immune cells and by intestinal epithelial cells in IBD patients. In humans, at least 10 different TLRs are described and each recognizes a specific pathogen-associated molecular pattern. A transmission disequilibrium test on Belgian IBD trios with CD demonstrated preferential transmission of the TLR4 Asp299Gly polymorphism from heterozygous parents to affected children[21]. TLR9 modulates CD susceptibility and there is interaction between other polymorphisms such as NOD2, IL23R and DLG5[22,23].

Genes related to epithelial barrier homeostasis

The gastrointestinal tract uses a system of tolerance and controlled inflammation to limit the response to dietary or bacteria-derived antigens in the gut[24]. When this complex system breaks down, either by a chemical or pathogenic insult in a genetically predisposed individual the resulting immune response may lead to IBD[25]. Genes or loci involved in the maintenance of epithelial barrier integrity and associated with Crohn's disease are the *IBD5* and the Discs Large Homolog 5 (*DLG5*)[26].

The *DLG5* gene is a 180-kb protein containing 1900 amino acids. DLG5 protein harbours a CARD domain, is a further CD susceptibility gene of the CARD family and contributes to CARD-mediated mechanisms of host defense. In fact, the *DLG5* gene associated protein is a member of Membrane Associated Guanylate Kinase family of scaffolding proteins. Scaffolding proteins

organize protein complexes at cellular junctions to integrate the tethering of adhesion molecules, receptors and intracellular signaling enzymes. Of interest is a population variation in DLG5 variants. For example, DLG5 R30Q variant was not confirmed in other European studies[27,28]. Other genes of potential importance in the same panel are the *PTGER4, ITLN1, DMBT1, BPI* and *XBP1* genes[29].

Genes related to molecular mimicry and autophagy

The innate immune system is the first line of defense against infection. Of interest, virulence factors from bacteria and viruses have been identified that manipulate host innate immune signaling pathways through molecular mimicry. These microbial proteins contain signaling domains that bear sequence and structural similarity to their host targets, and thereby potentially sabotage host immunity by hijacking crucial signaling pathways and uncouple receptor activation from effector induction. Several protein families have evolved to function as receptors or sensors of pathogen invasion. There are two types of signaling domains for the above receptors: the TIR domain for the TLRs and the Pyrin domain or CARD for the NOD-like receptors (NLRs) and retinoic acid-inducible gene 1-like receptors or helicases (RLRs or RLHs).

Molecular mimicry has been invoked as one of the mechanisms responsible for the activation of autoreactive cells by microbial peptides that have structural similarities to self peptides but there is also evidence that antigenically unrelated infections or specific inflammatory signals can result in autoaggressiveness and induction of organ-specific autoimmunity including the gut. The extent and severity of this loss of tolerance is still being defined, as it has demonstrated that loss of tolerance in IBD patients is not exclusive for bacterial antigens and occurs also to orally administered soluble proteins[30]. This subversion of innate immune signaling through molecular mimicry is closely related to the phenomenon of autophagy. Autophagy is the tightly orchestrated cellular 'housekeeping' process responsible for the degradation of damaged and dysfunctional cellular organelles and protein aggregates and is well recognized as playing an important role in maintaining cellular homeostasis under physiological and pathophysiological conditions. Regulated degradation and turnover of subcellular components is essential for normal cellular function, growth, and development. The major catabolic pathway responsible for the disposal of obsolete or damaged organelles and protein aggregates is autophagy (i.e., "self-digestion"). During this process organelles and proteins are encircled in a double-membrane vesicle (the autophagosome), delivered to lysosomes, and the substrates for ATP generation that can be recycled to synthesize new proteins, high-energy phosphates, and other cellular components. Autophagy has evolved as a conserved mechanism for cell survival under conditions of starvation and stress. In addition to (macro)autophagy, characterized by the sequestration of organelles and proteins within an autophagosome, there are two additional subtypes of self-digestion, microautophagy which is protrusion of the lysosomal membrane per se around a region of cytoplasm and chaperone-mediated autophagy in which degradation is restricted only to those proteins with a consensus peptide sequence recognized by specific chaperone complexes[31]. Autophagy is now considered to be important for host defense against intracellular microorganisms. The associations of these autophagy-associated genes with Crohn's disease strongly support the hypothesis that abnormal innate immune responses to intracellular pathogens contribute to the pathogenesis of Crohn's disease. In fact, the pathological characteristics of human Crohn's disease represent "granuloma" formation. The mechanisms of granuloma formation remain unclear. Recent studies have demonstrated functional roles for IL-23 in the differentiation and promotion of Th-17 cells. Autophagy genes that have been related to CD diagnosis are the *ATG16L1*[32,33], *IRGM* and the *LRRK2* gene[34]. Unraveling the mechanisms of such molecular mimicry is crucial to our understanding and clinical intervention of infectious diseases and inflammatory disorders of unkown aetiopathogenesis including Crohn's disease.

Genes related to lymphocyte differentiation

***IL23R* gene:** Dysregulated cytokine production by mucosal lymphocytes and macrophages has been implicated in the pathogenesis of CD. In fact, an exclusive increase of $CD4^+$ T cells in inflammatory bowel disease and their recruitment as intraepithelial lymphocytes has been demonstrated[35]. $CD4^+$ T cells secreting interleukin-17 (T helper type 17) cells have emerged as a key effector population driving colitis in animal models previously associated with exaggerated T helper type 1 responses.

Of the genes involved in the differentiation of Th-17 lymphocytes the *IL23R* gene has been proved of great importance and has been related to Crohn's disease[36,37].

The IL23R, consisting of an IL-12β1 and an IL23R chain, is highly expressed on memory T cells. IL23 is a novel cytokine formed *via* the binding of IL12p40 to a p19 protein. After binding to the IL23 receptor, IL23 preferentially activates memory T cells. IL23 does exhibit some similar biological activities to IL-12; however, IL-12 is more involved in the differentiation of naïve T-cells into Th1 lymphocytes and subsequent interferon-gamma production. IL23, on the other hand, mediates proinflammatory activities in part by the production of IL17 through activation of Th17 lymphocytes[38].

Signal transducer and activator of transcription 3 gene: Signal transducer and activator of transcription 3 (STAT3) play an important role in various autoimmune disorders including IBD[39,40]. STAT3 was initially identified as an acute phase response factor, an inducible DNA binding protein that binds to the IL-6 responsive element within the promoters of hepatic acute phase protein genes and is involved in IL-6 dependent T-cell proliferation through prevention of apoptosis. Subsequent studies indicate that STAT3 becomes activated in response to

a wide variety of cytokines and growth factors. Recent studies have revealed that STAT3 activation plays distinctly different roles between innate immune responses and acquired immune responses in colitis. STAT-3 mediated activation of acquired immune responses plays a pathogenic role in colitis by enhancing the survival of pathogenic T-cells. In contrast, STAT3-mediated activation of innate responses contributes to the suppression of colitis. Emerging data indicate that STAT3 is one of the crucial targets for the treatment of IBD. However, as the receptors of these cytokines and growth factors are present in both innate and acquired cells, activation of STAT3 is likely to occur in both cell types. Therefore as the function of STAT3 is a double-edged sword, careful attention should be directed toward the cell population that is being targeted when one contemplates STAT3 inhibition or activation in human IBD[41]. Within the same panel, other than *STAT3* genes, and with probable importance are the *TNFSF15, JAK2, CCR6* and *ICOSLG* genes[42-44].

Genes related to secondary immune response, apoptosis and other pathways

Chemokines play a central role in the pathogenesis of IBD as they are able to trigger multiple inflammatory actions including leukocyte activation and chemoattraction, granule exocytosis, production of metalloproteinases for matrix degradation and upregulation of the oxidative burst[45]. Therefore, further support is given for genes that relates to secondary immune response, apoptosis and other pathways. For example, in the IBD4 locus 4 several interesting candidate genes, which may be relevant in the pathogenesis of CD, lie within this region (e.g., genes regulating apoptosis, signal transduction proteins, chemokine receptors, T cell receptor, metalloproteinases).

Gene expression profiles from colon lamina propria fibroblasts have demonstrated several functional changes in some proteins coded from the corresponding genes: collagen types Ⅰ, Ⅳ, XIV, matrix metalloproteinase 1, cathepsin K, stroma cell-derived factor-1, chitinase3-like-1 and many others[46]. The major histocompatability complex (MHC) has been extensively investigated. Human leucocyte antigen (HLA) class Ⅱ molecules present partially digested antigen to the T-cell receptor and play a central role in the immune response. In CD MHC and HLA studies have yielded conflicting and heterogenous results. HLADR1 has been implicated with CD[47].

Many other genes, loci and chromosomes involved in CD have also been advocated in several studies that however still require wide replication and association with clinical practice. These include *CNR1, MCP-1*[48], *PTPN2* (protein tyrosine phosphatase)[49], *PTPN22, NKX-3, IL-18 RAP /IL-18R1, IL12/IL23* pathway[50], *PTGER4, MST1/BSN/MST1R*[50,51,52], *IL-2/IL-21*[53], *TYK2, JUN, NAT2*[54], *IL-10, NELL1, NKX2-3*[55], *Cyclin Y, Hect domain, 1q24, 10q21, 5p13, RCC1-like domain, ICOSLG, CDKAL1*[56], *13q13.3, 1p35.2, 3p29, 5p13.1*[57,58], *X chromosome*[59], *NLRP3*[60], *Vitamin D receptor polymorphisms*[61] and many others as well.

Genes in family and ethnic group studies

Linkage studies performed in complex genetic disorders such as CD frequently use model-free analytic methods, which are non-parametric analyses that do not assume Mendelian recessive or dominant models of inheritance.

The strongest risk factor for IBD is having a relative with the same disease. First-degree relatives of patients with CD have a 12-to-15 times greater risk of developing CD than do people of comparable age in the general population[61]. Familial clustering can also result from exposure to common environmental risk factors. Twin studies are very useful to determine the degree of genetic versus nongenetic etiologies for a trait. Today, there is no evidence of a separate entity of familial IBD[62,63]. Based on the current literature, phenotypic differences between familial and sporadic cases of IBD are weak. Available data are to be accepted with caution, however, as they are mostly retrospective and may be biased. CARD15 explains around 20% of the genetic predisposition to Crohn's disease[64]. The relative risk of developing CD in the presence of one mutation is 2-4, but increases dramatically in the case of two mutations (compound heterozygous or homozygous).

Although NOD2 provides no clear familial predisposition, unaffected relatives carry an increased rate of CARD15 variants (37.1%) compared to controls, and it would be interesting to see if they will eventually develop symptoms[65-67]. In addition, maternal transmission of CARD15 variants seems protective with a lower ratio of affected/unaffected children when compared to fathers[68,69]. In the light of the foregoing data, it seems that genetic counselling should be done with caution. In addition, families should not receive genetic counseling/information about age at onset and disease severity. Ethnic group studies and ethnic variation were first demonstrated in the Jewish population, and those studies are of major importance in this context[70].

ROLE OF GENES IN PROGNOSIS OF CROHN'S DISEASE

This is a major issue that greatly concerns patients. Many genes have so far been implicated in the prognosis of CD and numerous attempts have been made to classify the genetic profiles in CD. Of interest, CARD15 seems not only a susceptibility gene, but also a disease-modifier gene for CD. Of the many studies published on the clinical relevance of CARD15 mutations, there are several providing data on disease location, and the majority of them support a significant association of CARD15 mutations with ileal disease site, while some demonstrate a connection with the absence of colonic location. Some studies also provide data supporting the relevance to CARD15 variants with stricturing disease behavior, and also penetrating behaviour. Other pertinent studies revealed an association with early onset of the disease. These investigations also support the thesis that pediatric Crohn's is like a "more genetic disease" consistent with other polygenic disease

models. Other reports provide data on an increased risk or need of surgery related to CD[71].

Differences among studies are difficult to explain, and we could argue about the low number of patients in some of the studies, the disease variability among Caucasians and finally differences regarding disease assessment and interobserver agreement. Whether the described relationship between the CARD15 variants and both stenosing phenotype and increased need for surgery in CD patients is a true association or only reflects the high proportion of ileal CD developing bowel stenosis and, therefore, requiring surgery, is still a matter of controversy.

Genes related to age of Crohn's disease onset

With respect to age of CD onset and more specially to childhood or early-onset Crohn's disease, many genes/loci have been implicated: *TNFRSF6B, CXCL9*[72], *IL23R*[73,74], *NOD2*[75], *ATG16L1 rs2241880*[76], *CNR1*[77], *IL-10*[78], *MDR1*[79]. Of interest DLG5 seems protective for female children[80] while there are also studies not supporting the relation of genes and early onset of CD[81] or supporting the relation of IL-10 and IRGM with adult onset[82].

Genes related to crohn's disease behaviour

Genes related to stenotic/structuring behaviour in CD are: *NOD2/CARD15*[83], *TLR4*[84], *IL-12B*[85], and *CX-3CR1*[86,87]. Of importance NOD2/CARD15 has been also related to acute intestinal obstruction[88]. IL-10 and IL-6 are also potentially related to stenotic/structuring behaviour in CD while genetic variants of several metalloproteinases and their inhibitors would be excellent candidate genes, since these molecules are considered to play a key role in the abnormal fibrogenesis that underlies the development of bowel stenosis in CD patients. Genes related to penetrating/fistulizing behaviour in CD are as follows: *NOD2, IRGM, TNF*[89], *HLADRB1*[90]; the C-allele in CDKAL1 rs6908425 SNP is associated with NOD2 (-) perianal fistula, whereas OCTN and the near *IL-12B* gene rs12704036 T-allele have a relationship with non perianal fistula[91]. Inflammatory CD behaviour has been related to HLA variation[92] while granulomatous disease has been related with TLR4/CARD15 variants[93].

Genes related to Crohn's disease location

Upper gastrointestinal Crohn's disease has been related to NOD2[94] and MIF variants[95]. Ileal CD has been related to the following genes: *IL-10*[96], *CRP* gene[97], *NOD2, ZNF365* and *STAT3*[98]. Genes/loci associated with ileocolonic CD are *3p21*, *ATG16L1*[98] and *TCF-4* (*TCF7L2*)[99]. No role for phenotype in IL23R gene has been demonstrated[100] while a detailed genotype-phenotype analysis revealed weak associations of the IL23R rs10024819 variant with ileal involvement and stenoses in carriers of the TT genotype. Finally, the HLADRB1*0701 has been associated with ileal CD, but only in patients that have no CARD15 variants[101]. Colonic CD has been related to the following genes: the HLA region was associated with inflammatory colonic phenotype and TLR4[102], TLR1, -2, -6[103]. *TNF* gene showed a negative association with stricturing behaviour or colonic location[104]. For IBD5 and OCTN1 and 2, results have not been consistent but associations with perianal and ileal disease have been reported.

Genes related to Crohn's disease activity

Genes implicated in disease activity are the following: *HSP70-2* heat shock protein gene[105], *NOD2*[106], *PAI-1* (type 1 plasminogen activator inhibitor[107]), while the combination of NOD2 and PAI-1 predicted complicated disease behavior[108]. Of importance, NOD2 predicted lower weight in children[109], and CNR1 low BMI[110].

Genes related to surgery

NOD2 gene has been related to early pediatric surgery[111], stenosis and need for surgery[112], previous surgeries[113], increased number of surgeries[107] and surgical costs[114]. NOD2 has no relation to the risk of re-operation[115]. Finally, HLA-G has been associated with higher risk for ileocolonic resection[116].

Genes related to dysplasia and cancer

The *FHIT* gene (fragile histidine triad gene) located at 3p14.2 has been identified as a candidate tumor-suppressor gene. The gene spans the t (3; 8) translocation breakpoint of familial renal-cell carcinoma and contains the FRA3B fragile site. It encodes the human diadenosine triphosphate hydrolase, which in vitro cleaves the diadenosine substrate into ADP and AMP. It has been suggested that *FHIT* gene plays a role in the pathogenesis of IBD and the development and progression of a subgroup of IBD-related carcinomas at an early phase[117-119].

Genes related to extraintestinal manifestations and concommitant diseases

Extraintestinal manifestations are common in CD. Genes related to CD extraintestinal manifestations have been reported, as follows. Peripheral arthritis was related with FcRL3[120], HLADRB*103, HLAB*27 HLA-B*44, HLA-B*35, TNFalpha-308A[121]. CARD15 has been related to spondyloarthropathy[122] and uveitis[123] but not with sacroileitis[124]. TNF-1031C was associated with erythema nodosum while certain HLA alleles (HLA-B27, HLA-B35, HLA-B44) were connected with different disease behaviour and extraintestinal manifestations such as arthropathy, eye and skin manifestations. Genes/loci related to other chronic diseases concomitant to CD are 10p12.2 (sarcoidosis and CD)[125], STAT3 (multiple sclerosis and CD)[126], and a parallel genetic fingerprint between leprosy and CD[127].

Pharmacogenetics in Crohn's disease

Pharmacogenetics is of major importance in CD therapeutics and prognosis. Genes have been implicated in influencing the efficacy and side effects of drugs and reflect a complex interplay regarding absorption, elimina-

tion and transport. Future studies need to be large and prospective with uniformly phenotyped patients and correlating genetic associations with functional data. In addition hypotheses such as whether observations about drug response in IBD lead us to IBD etiology or whether the genes that control the drug response are related to genes that control the disease still remain unanswered. Pharmacogenetic studies to date have found no association between CARD15 variants and prediction of response to various IBD therapies. In addition, responses to azathioprine, steroids and infliximab are not related to NOD2[128]. Of note, NOD2 was related to antibiotic failure[129].For mesalazine, variability in drug acetylation was demonstrated many years ago with patients divided in slow and rapid acetylators, because of polymorphisms in the N-acetyltransferase (*NAT*) genes. Two isoenzymes NAT1 and NAT2 have been identified in humans and more than 50% of Caucasians are NAT2 slow acetylators. Mesalazine is acetylated in the liver by NAT1 into N-acetyl-5 aminosalicylates and excreted in the urine[47].

The clinical usefulness of pharmacogenetics in CD is limited to AZA and TPMT at this moment. The human TPMT gene, consisting of 10 exons, is located on chromosome 6p22.3. The hereditary nature of the TPMT deficiency in humans was initially identified in a study of TPMT activity in red blood cells (RBC). This and subsequent studies determined the distribution of TPMT activity in RBC to be trimodal; 90% of persons have high activity, 10% have intermediate activity and 0.3% have low or no detectable enzyme activity. To date, numerous mutant TPMT alleles have been identified, including the three most frequent alleles (TPMT*2, TPMT*3A and TPMT*3C), which account for 80%-95% of intermediate or low TPMT enzyme activity cases. The prevalence of the most frequent SNPs in the TPMT gene has been reported to vary worldwide. However, it is of interest that studies on the prevalence of TPMT SNPs in large IBD cohorts are lacking. Although AZA is an effective drug for maintenance of remission in IBD, it is associated with side effects. Clinically sound pharmacogenetic studies over the last two decades have shown that polymorphisms in the *TPMT* gene locus play a significant role in the occurrence of various side effects of thiopurine drugs including life-threatening bone marrow toxicity (BMT), a serious dose-related toxicity[130-134].

The G2677T variant in the *MDR1* gene predicted gastrointestinal and unspecified intolerance to azathioprine and methotrexate in IBD patients. These findings suggest a role for MDR1/P-gp in the mechanism of action of azathioprine and methotrexate[135,136].

Twin studies have linked polymorhisms of the vitamin D receptor (*VDR*) gene with bone mineral density in healthy women and in addition VDR is an important regulator of calcium metabolism and bone cell function and influences calcium absorption from the intestine. VDR polymorphisms have also been implicated in susceptibility to CD[137].

The HLADR region has been associated with failure to budesonide[138] while DLG5R30Q predicted response to steroids[139]. Other genes such as MIF (macrophage migration inhibition)[140] and MDR have been also related to steroid therapy[136]. In addition, 1082 AA IL-10 genotype was associated with steroid dependency, whereas the allele 113A of the *DLG5* gene conferred resistance to steroids.

Regarding response to infliximab the data for TNF gene are conflicting. Specifically, there are conflicting data regarding the role of FcGR3A, which has been supported by some authors[141,142], but was not confirmed in patients of the ACCENT I study. Response to infliximab is not related to *TNFa-308*[143] or *TNFR1* and *TNFR2*[144] or *NOD*[145] or *CRP* gene[136]. The association between the Fas ligand-843 TT genotype and lack of response to infliximab seemed to be the most relevant observation[136]. The relationship of infliximab response and lymphotoxin alpha gene (*LTA*) is also conflicting[144].

WHAT LIES AHEAD

Gene-to-gene crosstalk and epistasis

With new methodologies like genome wide association studies, microarrays, and fine SNP analysis becoming available during the last decade, our investigative armamentarium has been considerably enriched. As many studies with complex statistics arise, we understand increasingly the real crosstalk present among genes and the need of a genetic panel for disease diagnosis and prognosis. It is now evident that gene-to-gene interaction and epistasis modulate disease activity and susceptibility[146]. Some data have come to light. A genome-wide scan in a Flemish population of IBD affected families supports the existence of *IBD4* on 14q11, and has shown additional evidence for the existence of other susceptibility loci (1p, 4q and 10p). This study has further demonstrated that epistasis and gene to gene interactions (CARD15-TLR4) are also present in IBD and that population heterogeneity is not to be underestimated[147]. Crosstalk has been demonstrated for TLR9 with NOD2, IL23R and DLG5, and epistasis has been shown between IL23R and DLG5. Also potential epistasis between IL23R variants and the three other previously described CD susceptibility genes CARD15, SLC22A4 and SLC22A5 (OCTN 1 and 2) has been shown[116].

Genetic consortium studies and genome wide scans

Over the past few years, a combination of progress in high throughput genotyping technology and growing knowledge about the human genome through the International HapMap project and the Human Genome Project have enabled genome-wide association studies (GWAS) for several complex diseases. To understand the approach to conducting GWAS in this setting it is important to expound on the concept of linkage disequilibrium, which refers to the nonrandom association of alleles at nearby loci. Specifically, linkage disequilibrium refers to adjacent alleles assorting together nonindepen-

Table 2 Predicted future developments in the genetics of Crohn's disease

What lies ahead in the genetics of Crohn's disease
Gene-to-gene crosstalk and epistasis
Genome wide association studies
Microarrays
Fine single nucleotide polymorphism analysis
Genetic consortium studies and genome wide scans
Genome-wide association studies
Genetic consortium studies
Future perspectives
Functional studies to understand the mechanisms
Combining genetic data with functional data
Combination of a panel of clinical, biochemical, serological and genetic factors
Functional consequences of polymorphisms
Molecular and cellular mechanisms leading to Crohn's disease
Predict disease outcomes
Redesigning the methods of treatment

dently from generation to generation because they are tightly linked and thus less likely to become separated by recombination. Genetic consortium studies are of major importance and homogeneity in methodology issues is of paramount value[148-151]. Appropriate study design[152], power analysis[153] and overall data analysis and meta-analysis[154] are mandatory. Accurate estimation of sample sizes required in a genetic association study is essential before commencing genotyping, to ensure that the study is sufficiently powered to detect the subtle genetic effects that contribute to most complex diseases. The extensive genetic variation and complex linkage disequilibrium across even a small genomic region will give rise to several alternative scenarios. Genetic variation across a region studied should be carefully evaluated and consideration should be given to possible linkage disequilibrium and allelic heterogeneity when evaluating power of an association study. As larger datasets are studied and combined, as genotyping platforms provide even greater depth of coverage of the genome and as modest hits are followed up in large independent panels so that the vast majority of true signals should be identified. These robust genetic data will truly provide a solid platform for functional studies to understand the mechanisms by which these genetic variants predispose to CD. Finally studies at post-transcriptional level become more and more urgent[155]. Enriching our understanding of CD genetics is important but when combining genetic data with functional data the outcome could be of major importance. In fact, improved understanding of immune mechanisms, on which manifold genetic and environmental traits might converge, and which ultimately mediate all phenomena in inflammatory bowel disease, holds promise (Table 2).

CONCLUSION

The recent advances in the understanding of CD genetics have been tremendous[156]. Starting with the susceptibility area, whole genome linkage and association scans have already led to the identification of a number of susceptibility genes (*NOD2/CARD15, DLG5, OCTN1* and *2, NOD1, IL23R, PTGER4, ATG16L1* and *IRGM*) of which the *NOD2/CARD15* gene is the most replicated and understood at present. Although it is clear that genetic research in IBD has advanced our understanding of the clinical heterogeneity of the disease, new efforts are required and point towards the complex combination of a panel of clinical, biochemical, serological and genetic factors, in order to achieve the optimal prediction of both clinical behaviour and response to therapy.

Genome-wide association studies have allowed an unprecedented rapid unraveling of the genetic basis of IBD; however there will be much more follow-up work needed in this field. First, ongoing work including meta-analysis of the Crohn's disease genome wide association studies will probably reveal additional Crohn's disease susceptibility genes. It will then be essential to investigate the functional consequences of polymorphisms in these genes so the molecular and cellular mechanisms leading to CD can be better characterized. Finally, genotype-phenotype correlation studies should help clinicians predict disease outcomes with more accuracy, including the risk for complications, need for surgery, and response to therapy, and finally lead to redesigning the methods of treatment of CD patients.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i2.119

World J Gastroenterol 2012 January 14; 18(2): 119-125
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Immune mechanisms of Concanavalin A model of autoimmune hepatitis

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Supported by Program for Excellent Talents of Anhui Province, No. 2006JQ1196
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Received: April 20, 2011 Revised: July 6, 2011
Accepted: July 13, 2011
Published online: January 14, 2012

Abstract

As a chronic inflammatory disease of the liver, the pathogenic mechanisms of autoimmune hepatitis (AIH) have not yet been elucidated, with prognosis and diagnosis remaining unsatisfied. Currently the only viable treatments of AIH are immunosuppressant application and liver transplantation. It is considered that lack of good animal AIH models is the main reason for the shortage of a simple and efficient cure. The Concanavalin A (Con A) model is a typical and well established model for investigating T-cell and macrophage dependent liver injury in mice, which closely mimics the pathogenesis mechanisms and pathological changes of patients, and is regarded as the best experimental model for AIH research so far. In this paper we elucidated the pathogenic mechanisms of AIH and the evolution of relative animal models. We go on to further focus on Con A-induced liver injury from the point of immunological mechanisms and the change of cytokine levels. Finally, we manifested the clinical significance of the AIH animal models and the challenges they would meet during their future development.

Key words: Autoimmune hepatitis; Animal models; Concanavalin A

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Wang HX, Liu M, Weng SY, Li JJ, Xie C, He HL, Guan W, Yuan YS, Gao J. Immune mechanisms of Concanavalin A model of autoimmune hepatitis. *World J Gastroenterol* 2012; 18(2): 119-125 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i2/119.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i2.119

INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic inflammatory disease of the liver, characterized by a loss of self-tolerance leading to the appearance of autoantibodies, pathological changes and dysfunctions (the detailed pathogenic mechanisms of which still remain vague). According to different antibodies profiles, AIH is classified into three categories: AIH type 1 is characterized by the presence of antibodies to nuclear antigens (ANA) and/or anti-smooth muscle antigen (SMA) antibodies; AIH type 2 is characterized by anti-liver kidney microsomal (LKM)-1 and low level of LKM-3 antibodies (with or without ANA or SMA antibodies); AIH type 3 is characterized by autoantibodies against soluble liver

antigen/liver pancreas (with or without ANA or SMA antibodies)[1].

Around the world, the incidence of AIH is 0.1-1.9 cases out of 100 000 persons per year, which is not very high[2]. However, the prevalence of autoimmune hepatitis in Europe is in the range of 11.6-16.9 cases per 100 000 persons[2], and in the United States, the proportion of hepatitis among patients with liver cancer is about 11%[3]. Incidence is also different between men and women. It was reported that women are more vulnerable to AIH[2,4,5].

Unfortunately, we do not have any better choice of medicines other than immunosuppressants, which can be classified into four generations[6,7]. In the 1950s, the first generation immunosuppressants were limited to azathioprine and steroids, which were enriched by polyclonal anti-lymphocyte and anti-thymocyte globulins in the 1960s[6]. For this generation, 70%-80% patients might relapse after withdrawal of treatment[8]. More seriously, they have many side effects[9]. Corticosteroids, Tacrolimus and Cyclosporine are typical of the second generation[6]. In the early 1990s a broad range of third-generation immunosuppressants emerged[6], most of which are monoclonal anti-lymphocyte and anti-thymocyte globulins followed by the fourth generation, such as the IL-2 monoclonal antibody with its highly specific sites of action[7,10]. The second and the third generation immunosuppressants are in most cases successfully used for treatment of AIH[11,12]. But long term applications of these immunosuppressive drugs carries serious risks[13] and sustained remission[9], even at low doses. Non-system steroids may be the best candidates[14]. Patients with liver failure or fulminant presentation who fail to improve under immunosuppressive therapy should be considered as candidates for liver transplantation. Without treatment, nearly 50% of patients with severe autoimmune hepatitis die in approximately 5 years[15]. Taking this into consideration, it is significantly important to develop new specific drugs. Animal models are the basis of drug discovery and development. Up to the time of writing, there are still no universal animal models of AIH which can be used as pathogenic models as well as therapeutic ones.

As the most important AIH research model, the Con A animal model plays a key role in AIH drug development. In this article we attempt to review the evolution of the Con A animal model of AIH, to sum up the mechanisms of Con A-induced liver injury, and to illustrate its statue in AIH drug development. Furthermore, the future challenges of the animal model are also discussed.

EVOLUTION OF AIH MODEL

AIH models have evolved from crude liver homogenates and adjuvants to the genetic engineering level, which can be classified into five phases[16]. The first phase was in 1972 when Buschenfelde *et al*[17] induced chronic active hepatitis in rabbits immunized with human liver proteins combined with complete Freund's adjuvant. This work built a solid foundation for AIH models. The second phase began in 1983, when Mihas *et al*[18] established transient hepatitis in mice by immunization with syngeneic liver proteins together with the polysaccharide of Klebsiella pneumoniae. In the third phase, taking place from 1987 to 1990, many scientists used inbred or neonatal thymectomy mice to establish the T-cell reactive AIH model. They induced transient hepatitis by immunizing C57BL/6 mice with the supernatant of liver syngeneic liver homogenates with complete Freund's adjuvant and used adoptive transfer technology to study the roles of T-cell, which allowed studies of the pathogenesis of AIH[19]. The fourth phase, from 1992 to 2003, had endotoxin and plant lectin-induced hepatitis models receive extensive attention. Three types of inducers were wildly used during this period: Con A[20], D-galactosamine (GalN) with low dosage of lipopolysaccharides (LPS)[21], and high dosage of LPS[22]. In the fifth phase, from 2002 to 2008, the application of genetic engineering technology accelerated the development of AIH model[23]. From one aspect, gene knockout and transgenic animals facilitated the study of the functions of certain genes[24]. From the other, production of designated antibodies using genetic engineering methods made it possible for scientists to get specific types of autoantibodies[25], and also made it possible for the Con A models to mimic a specific subtype of AIH. Significantly, the production of designated autoantibodies is based on known antigens. Scientists have now clarified the antigens to the following autoantibodies: the antigen to LKM-1 is cytochrome P450 2D6[26,27], the antigen to LKM-2 is cytochrome P450 2C9[1], the antigens to Liver Microsomal are cytochrome P450 1A2 and cytochrome P450 2A6[1]. The animal models of type 2 AIH[28] have been reported, but obviously type I animal models have more clinical significance than type Ⅱ[2]. As is widely known, it is difficult to find the antigen of autoantibodies, which is the limitation of the gene engineering AIH model. The features and parameters of the three models are listed in Table 1[29].

From the information in Table 1, it is obvious that the Con A-induced hepatitis model possesses more advantages than the other two. Firstly, the Con A model includes only one inducer, making it easier to be established compared with the GalN/LPS model. Secondly, there is no significant change of the level of transaminase, which is considered a valid index of the severity of liver injury, in the LPS model, while such change is remarkable in the Con A model. Thirdly, in the Con A model, the serum level of many cytokines relevant to inflammation change dramatically, which is favorable for the study of the pathogenic mechanisms of AIH[29]. Furthermore, besides AIH, Con A animal models with different parameters are adaptable to many clinical diseases, such as fulminant hepatitis[30], virus hepatitis[31], hepatotoxin[32,33] and alcoholic liver diseases[34]. In summary,

Table 1 The features of the autoimmune hepatitis model induced by endotoxins and plant lectins

	Con A[29]	GalN/LPS[29]	LPS[29]
Animal	BALB/c-mice (6-8 wk)	BALB/c-mice (6-8 wk)	BALB/c-mice (6-8 wk)
Inducer	Con A	GalN/LPS	LPS
Dosage	20 mg/kg	LPS: 5 μg/kg GalN: 700 mg/kg	10 mg/kg
Application method	Tail vein	Subcutaneous	Subcutaneous
Transaminase level (max)	8 h	8 h	No significant change

Con A: Concanavalin A; GalN: D-galactosamine; LPS: Lipopolysaccharides.

Con A AIH model is easy, convenient, inexpensive and repeatable, as well as a T-cell activated model and could greatly facilitate the study of the mechanisms of AIH-induced liver injury.

IMMUNOLOGICAL MECHANISMS OF CON A-INDUCED LIVER INJURY

Con A is one kind of lectin, which is purified from *Canavalia brasiliensis*[35]. Tiegs *et al*[20] injected Con A, Succinyl Con A with no agglutination activity, and *Vicia faba* lectin with strong agglutination activity to nuclear magnetic resonance imaging mice *via* tail vein, respectively. The results showed that only Con A could induce liver injury, which indicated that the *in vitro* agglutination activity of this lectin does not correlate with its hepatotoxic potential *in vivo*. They also studied the correlation between the hepatotoxic potential of Con A and its sugar-binding site[20]. Con A has specific sugar-binding sites, whose ligands are α-D-mannoside, methyl α-D-mannopyranoside, α-D-glucose, and methyl-α-D-glucose[36]. They co-administrated Con A with α-D-mannoside or methyl α-D-mannopyranoside to mice, which prevented the induction of hepatic injury by the lectin[20]. This suggested that free sugar-binding sites are indispensible for the induction of liver injury by lectin. Sato *et al*[37] also confirmed that Con A/glycogen multilayer films can be decomposed by exposing them to sugar solutions (D-glucose, D-mannose, methyl-alpha-D-glucose and methyl-alpha-D-mannose), as a result of the displacement of sugar residues of glycogen from the binding sites of Con A by the free sugar added in the solution. This suggested that sugar-binding sites are prerequisites of activated Con A. But among Con A, Succinyl Con A and *Vicia faba* lectin, which have the same sugar-binding site, only Con A can lead to high level of transaminase[20]. These two results indicated that the hepatotoxic potential of Con A is not determined by its agglutination activity or sugar-binding site. Other mechanisms may exist.

The mechanisms of the Con A model have interested many scientists. Previews papers describe that the aminotransferase of mice in thymus[38] and CD4[39] neutralized groups decreased significantly compared with the control group, while the CD8 neutralized group show no significant change. What is more, after injection of Con A, the blood level of interleukin 2 (IL-2), IL-4 and interferon gamma (IFN-γ) all increased dramatically[40]. This suggested that the $CD4^+$ T helper (Th) cell was involved in the liver injury[40]. It is reported that $CD4^+$-positive Th cells recognize the Con A-modified major histocompatibility complex (MHC) structures of macrophages and become activated, followed by an inflammation reaction and the release of IL-1 and IL-2 to the blood[41]. In the experiment of CD8 neutralization, there was a minor decrease of the transaminase level, which suggested that the target cell lysis by cytotoxic $CD8^+$ T lymphocyte (CTL) also contributes to liver injury, but not as the major factor. In conclusion, the main mechanism of the Con A model is that Th cell activation increases the relevant cytokine level, which leads to liver injury. Meanwhile, the CTL mediated target cell lysis may be the secondary mechanism.

In the liver, lymphocytes, sinusoid endothelial cells (SECs), Kupffer cells (KCs) and stellate cells are all involved in the immune response[42]. Lymphocytes can be classified into two groups, exogenous and endogenous[43]. Exogenous lymphocytes originate from the thymus[44], bone marrow[44,45], intestinal tract[46], spleen[47] and lymph gland[48], and enter the liver through circulation. Endogenous lymphocytes are enriched in the portal area of the liver, which count for 25% of non-parenchyma cells in the liver[49]. The endogenous lymphocytes are mainly T cells, while B cells only count for 5% of them. This is why lymphocyte infiltration is mainly focused in the portal area[50].

For a long time, there have been debates about whether KCs or SECs plays a major role in immunological liver injury[51-53]. Knolle *et al*[52] established the spontaneous and LPS activated cell model, and found that SECs and KCs both secreted IL-1 and IL-6, which suggested that SECs are also key cells in liver injury. It has been found that fifteen minutes after intravenous injection of Con A, Con A binds to SECs first; 4 h later, Con A begins to bind to the KCs[52]. Using Scanning Electron micrograph, it is clearly seen that 4 h after intravenous injection of Con A, blood cell endothelium attaches to the SECs first[52]. Then lymphocytes or neutrophils are trafficked into the hepatocytes, leading to inflammation[52]. We can conclude that SECs and KCs are both important, but they play their roles in the different phases. After injection, Con A binds to the mannose gland in the SECs surface first, leading to the breakdown of the SECs membrane, bleb formation and cytoplasm disappearance[30]. SECs detachment facilitates the binding of Con A to the KCs. $CD4^+$ Th cells recognize the MHC class Ⅱ and T cell receptor of KCs modified by Con A and are then activated[30]. Such liver injury is mainly mediated by T helper cells, including Th1 and Th2 cells. Figure 1 depicted the mechanisms of T cell activated liver injury.

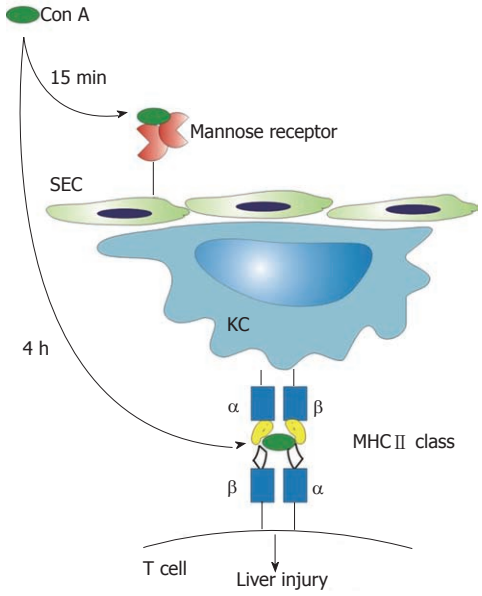

Figure 1 Mechanisms of Concanavalin A induced T cell activated liver injury. Con A: Concanavalin A; KC: Kupffer cell; SEC: Sinusoid endothelial cell; MHC: Major histocompatibility complex.

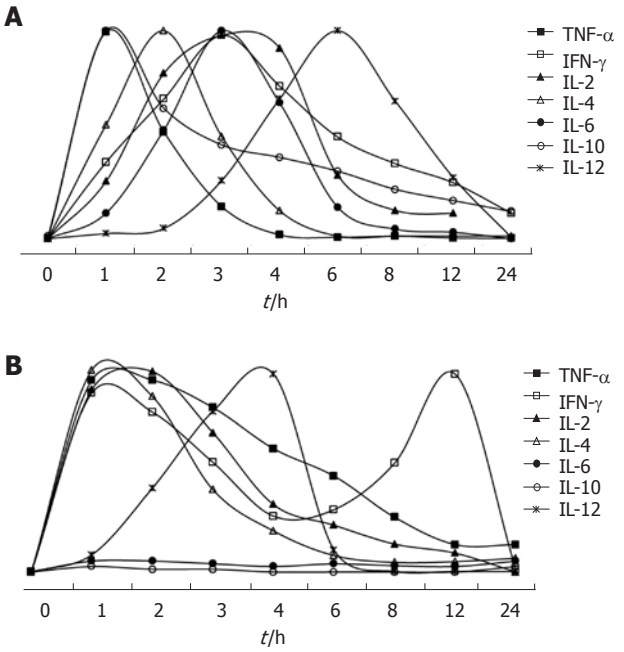

Figure 2 Different cytokines levels within 24 h. A: Plasma level; B: Liver level. TNF: Tumor necrosis factor; IFN: Interferon; IL: Interleukin.

CHANGES IN THE EXPRESSION LEVELS OF RELEVANT CYTOKINES

Some major cytokines involved in the Con A-induced liver injury are IFN-γ[54-55], IL-2[55], IL-4[56], IL-6[56] and tumor necrosis factor α (TNF-α)[56], of which TNF-α and IFN-γ are the major ones.

Figure 2 shows the time when different cytokines reach their peak level in the plasma and liver. In the plasma, TNF-α and IL-10[29,57] first reach their peak level after 1 h, followed by IL-4 after 2 h. IFN-γ, IL-2 and IL-6[29,57] reach their peak after 3 h, followed by IL-12. However, in the liver, TNF-α, IFN-γ, IL-4[29,57] reach their peak level in 1 h, followed by IL-2 and IL-12. There is no significant change for IL-6[57] and IL-10[29,57] in the liver. Especially, the level of IL-10[29] is very low in the liver compared with that in the plasma, which suggested that IL-10 might originate from other tissues, such as the spleen. But one previous paper reported that IL-10 expression in the liver is higher than that in the spleen[57]. As yet, where IL-10 originates remains unanswered.

Comparing the acute and chronic animal models, the expression profiles of IL-10 are quite different. For example, in the acute model induced by Con A, TNF-α, IFN-γ and IL-12 levels increased to 2.11, 1.92 and 8.30 times of their normal level, respectively, after neutralization of IL-10. Reversely, administration of recombinant IL-10 prior to injection of Con A decreased by 47%, 47% and 80% of TNF-α, IFN-γ and IL-12 expression levels respectively. IL-10 is considered to be an anti-inflammatory cytokine in a murine model of Con A[58]. Kato *et al*[59] described that the IL-10 level is increased at 12 h after the Con A injection. After neutralizing antibodies to IL-10, it was intraperitoneally injected into animals of the same model at 6 h before Con A treatment, with serum alanine aminotransferase level being significantly higher than in the control group. Histological studies showed spotty necrosis in the group treated with anti-IL-10 antibodies. These results suggest that IL-10 has an inhibitory effect on liver injury in a murine model of Con A-induced experimental liver injury mediated by cellular immunity[58]. These studies suggested that both endogenous and exogenous IL-10 can protect the liver from acute injury[59].

However, there is evidence indicating that IL-10 could accelerate liver injury in the chronic model[60]. When Con A was administrated intravenously to BALB/c mice once a week, the IL-10 expression level in plasma increased to 7 times higher 20 wk later. Accordingly, in this model, inflammatory infiltration also lasted for 20 wk and activated stellate cells also dramatically increased[60]. All these results suggested that IL-10 aggravated liver injury in the chronic Con A model.

Paradoxically, IL-10 does not play the same role in all chronic models. For example, in the CCl_4 chronic model, IL-10 slows down the process of fibrosis[61]. This may be due to the fact that the mechanisms of liver injury in these two models are different, and the latter does not involve T cell activation. In the acute Con A model, IL-10 may inhibit macrophages and Th1 cells from releasing inflammatory cytokines, which explains why it plays an anti-inflammation role in the acute model[58]. Though IL-10 can inhibit the secretion of anti-inflammation cytokines, secretion of IFN-γ is also inhibited[62]. Some previous studies reported that, to some extent, IFN-γ may relieve liver fibrosis. Therefore, a long duration of IFN-γ deficiency may aggravate fibrosis. As for the CCl_4 model, liver injury is mediated only by free

Table 2 New drugs developed based on the Concanavalin A model

	Classification	Target	Pathway
Hu 23C3[63]	Monoclonal antibody	Human osteopontin	NF-κB
Anti-his H1[64]	Polyclonal antibody	Histone H1	NF-κB
ApoA Ⅱ[65]	High density lipoprotein	Leukocytes and T cells	-
CpG ODN[66]	Oligodeoxynucleotides	DNA binding ability of NF-κB	NF-κB

CpG ODN: CpG-containing oligodeoxynucleotides; ApoA: Apolipoprotein A; Anti-his H1: Antibody against histone H1; NF-κB: Nuclear factor kappa B.

radicals, which is not relevant to the activation of the immune response and the release of inflammation cytokines. In conclusion, the expression profiles in different models, even with the same inducer, are not the same. The various mechanisms, cell types and micro-environments should be taken into consideration in experimental design and execution.

CON A MODEL AND NEW DRUG DEVELOPMENT

In recent years, based on the Con A animal model, many new therapeutic antibodies or proteins have been developed to attenuate liver injury in experimental models (Table 2)[63-66].

Fan *et al*[63] humanized a murine monoclonal antibody 23C3 against human osteopontin by a complementary-determining region grafting method based on computer-assisted molecular modeling, denoted as Hu23C3. They demonstrated that Hu23C3 could have the potential for attenuating Con A-induced liver injury through the nuclear factor kappa B (NF-κB) pathway.

Nakano *et al*[64] intraperitoneally injected a polyclonal antibody against histone H1 immediately after Con A injection; they found that injection of anti-histone H1 antibodies could reduce Con A-induced liver damage, also *via* the NF-κB pathway.

It is reported that Con A-induced hepatitis was attenuated by the administration of apolipoprotein A-Ⅱ, which is the second major apolipoprotein of high-density lipoprotein[65]; this inhibited leukocytes infiltration and the expression of T-cell related cytokines and chemokines.

The survival rate of mice was markedly enhanced by the administration of CpG-containing oligodeoxynucleotides (CpG ODN)[66]. This is because CpG ODN pretreatment inhibits the DNA binding ability of NF-κB, leading to the decrease of systemic/liver levels of TNF-α and IFN-γ. These results suggest that CpG ODN pretreatment protects the mice from Con A-induced liver injury, also *via* NF-κB pathway.

CONCLUSION

In this article we reviewed the evolution of the AIH model and emphasized the importance of the Con A AIH model. Based on the previous papers, we summarized the mechanisms of Con A-induced liver injury, its pathogenic changes and cytokines expression levels. The Con A animal model, which is a typical T cell dependent model, can mimic the mechanisms of clinical AIH diseases. Therefore, we think that it is a good and convenient model for studying the mechanisms of AIH and developing new therapeutic drugs.

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S- Editor Sun H **L- Editor** Rutherford A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i2.126

World J Gastroenterol 2012 January 14; 18(2): 126-135
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Suppression of esophageal cancer cell growth using curcumin, (-)-epigallocatechin-3-gallate and lovastatin

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Supported by A United States National Cancer Institute Grant, No. R01 CA117895; and a grant from the Duncan Family Institute for Cancer Prevention and Risk Assessment, UT MD Anderson Cancer Center
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Received: May 24, 2011 Revised: July 28, 2011
Accepted: August 4, 2011
Published online: January 14, 2012

Abstract

AIM: To determine the effects of curcumin, (-)-epigallocatechin-3-gallate (EGCG), lovastatin, and their combinations on inhibition of esophageal cancer.

METHODS: Esophageal cancer TE-8 and SKGT-4 cell lines were subjected to cell viability methyl thiazolyl tetrazolium and tumor cell invasion assays *in vitro* and tumor formation and growth in nude mouse xenografts with or without curcumin, EGCG and lovastatin treatment. Gene expression was detected using immunohistochemistry and Western blotting in tumor cell lines, tumor xenografts and human esophageal cancer tissues, respectively.

RESULTS: These drugs individually or in combinations significantly reduced the viability and invasion capacity of esophageal cancer cells *in vitro*. Molecularly, these three agents reduced the expression of phosphorylated extracellular-signal-regulated kinases (Erk1/2), c-Jun and cyclooxygenase-2 (COX-2), but activated caspase 3 in esophageal cancer cells. The nude mouse xenograft assay showed that EGCG and the combinations of curcumin, EGCG and lovastatin suppressed esophageal cancer cell growth and reduced the expression of Ki67, phosphorylated Erk1/2 and COX-2. The expression of phosphorylated Erk1/2 and COX-2 in esophageal cancer tissue specimens was also analyzed using immunohistochemistry. The data demonstrated that 77 of 156 (49.4%) tumors expressed phosphorylated Erk1/2 and that 121 of 156 (77.6%) esophageal cancers expressed COX-2 protein. In particular, phosphorylated Erk1/2 was expressed in 23 of 50 (46%) cases of esophageal squamous cell carcinoma (SCC) and in 54 of 106 (50.9%) cases of adenocarcinoma, while COX-2 was expressed in 39 of 50 (78%) esophageal SCC and in 82 of 106 (77.4%) esophageal adenocarcinoma.

CONCLUSION: The combinations of curcumin, EGCG and lovastatin were able to suppress esophageal cancer cell growth *in vitro* and in nude mouse xenografts, these drugs also inhibited phosphorylated Erk1/2, c-Jun and COX-2 expression.

Key words: Chemoprevention; Curcumin; Cyclooxygenase-2; (-)-epigallocatechin-3-gallate; Esophageal cancer; Statin

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Ye F, Zhang GH, Guan BX, Xu XC. Suppression of esophageal cancer cell growth using curcumin, (-)-epigallocatechin-3-gallate and lovastatin. *World J Gastroenterol* 2012; 18(2): 126-135 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i2/126.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i2.126

INTRODUCTION

Esophageal cancer is one of the least studied and deadliest cancers. Tobacco smoke is the most firmly established risk factor for esophageal cancer and has been associated with the development of esophageal squamous cell carcinoma and adenocarcinoma; gastroesophageal reflux with bile acid is another important cause of adenocarcinoma[1-3]. Molecularly, the expression of retinoic acid receptor-β2 (RAR-β2) is frequently lost in premalignant and malignant esophageal tissues, and benzo[a]pyrene, a carcinogen present in tobacco and environmental pollution, and bile acid, a tumor promoter for gastrointestinal cancer, may be responsible for its loss. Restoration of RAR-β2 expression suppressed esophageal cancer cell growth and induced apoptosis *in vitro* and tumor formation *in vivo*; these effects were correlated with decreased expression of activator protein 1 and cyclooxygenase-2 (COX-2) and phosphorylated extracellular-signal-regulated kinases (Erk1/2)[4]. Moreover, the novel retinoid receptor-induced gene-1 (RRIG1), which is a downstream gene of RAR-β2, participates in regulating the effects of RAR-β2 on cell growth and gene expression[5,6]. RRIG1 protein binds to and inhibits a small GTPase RhoA activity. Restoration of RRIG1 expression inhibits RhoA activation and, consequently, reduces tumor cell colony formation, invasion, and proliferation, which are correlated with inhibition of Erk1/2 phosphorylation, COX-2, and cyclin D1 expression[5,6]. These genes together may form a novel molecular pathway that involves RAR-β2-induced RRIG1 expression and suppression of RhoA/Erk1/2/AP-1/COX2[4]. Therefore, targeting this molecular pathway should translate into better control of esophageal cancer.

Cancer chemoprevention was defined as the use of natural, synthetic, or biologic chemicals (such as drugs and food supplements) in the prevention, suppression, or delay of the carcinogenesis process[6]. Several clinical trials of different drugs have been conducted in an attempt to prevent esophageal cancer. The first class of agents tried was the retinoids[7]. The use of retinoids was based on the fact that vitamin A deficiency was found in esophageal cancer patients and the fact that this deficiency induced hyperkeratotic changes in the esophageal mucosa of experimental animals[8,9]. A clinical trial using N-4-(ethoxycarbophenyl) retinamide demonstrated that cancer incidence in the treatment group with severe esophageal dysplasia was reduced by 43.2% compared with that in the placebo group[10]. However, the results from two other trials conducted in Linxian, China, by the National Cancer Institute were inconclusive[11,12]. Our *in vitro* study demonstrated that esophageal cancer cells which do not express RAR-β2 are resistant to all-trans retinoic acid[13]. In animal experiments, dietary N-(4-hydroxyphenyl) retinamide enhanced tumorigenesis in response to N-nitrosomethylbenzylamine in the rat esophagus by increasing tumor initiation events[14]. Moreover, 13-*cis* RA did not reduce NBMA-induced esophageal tumor multiplicity in rats[15].

Non-steroidal anti-inflammatory drugs (NSAIDs) were also tested for the prevention of esophageal cancer[16]. Epidemiological and experimental studies indicated that NSAIDs decreased esophageal cancer incidence[17-21]. Our *in vitro* data showed that aspirin and NS398 induced apoptosis in esophageal cancer cells, which correlated with their ability to inhibit COX-2 enzymatic activity and upregulate the expression of 15-LOX-1 and -2[22-25]. However, during clinical trials of these agents in the chemoprevention of colorectal cancer, it was reported that Vioxx (rofecoxib) and Celebrex (celecoxib) induced cardiovascular events, which raised safety concerns about the high-dose and long-term use of these drugs in cancer prevention[26,27].

However, to date, most preclinical and clinical chemoprevention studies of human cancers have been focused on targeting a single gene, which showed limited activities *in vitro* and *in vivo*[3,16]. In this study, we aimed to target multiple genes in a molecular pathway using combinations of drugs to determine whether this approach is more effective than single-drug treatments in the inhibition of esophageal cancer.

MATERIALS AND METHODS

Cell culture and drug treatment

The human esophageal cancer cell lines SKGT-4 and TE-8 used in our previous studies[13,22] were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 10% fetal bovine serum (FCS), at 37 ℃ in a humidified atmosphere of 95% air and 5% CO_2. For drug treatment, these cells were grown in monolayer overnight and then treated with or without curcumin, (-)-epigallocatechin-3-gallate (EGCG), lovastatin, and combinations of these agents for up to 5 days. The drugs were dissolved in dimethyl sulfoxide (DMSO) and then diluted before use. The concentration of lovastatin was 2 or 4 μmol/L, curcumin was 20 or 40 μmol/L, and EGCG was 20 or 40 μmol/L (all from LKT Laboratories, Inc., St. Paul, MN, United States). The concentrations for the drug combinations were the same as those used individually. For the methyl thiazolyl tetrazolium (MTT) assay, 20 μL of MTT (5 mg/mL, Sigma, St Louis, MO, United States) was added to each well of the 96-well plates and incubated for an additional 4 h. After the growth medium was removed, 100 μL of DMSO was added to the wells to dissolve the MTT crystal, and the optical densities were measured with an automated spectrophotometric plate reader at a single wavelength of 540 nm. The percentage of cell growth was calcu-

lated using the formula: % control = ODt/ODc × 100, where ODt and ODc are the optical densities for treated and control cells, respectively. The data were then analyzed statistically using the Student's *t* test.

Tumor cell invasion assay

Boyden chambers coated with Matrigel were obtained from BD Biosciences (Bedford, MA, United States) for assaying tumor cell invasion ability[6]. Esophageal cancer cells SKGT-4 and TE-8 were first starved in medium without FCS overnight, and the cells (5×10^4) were re-suspended in the FCS-free medium and placed in the top chambers in triplicate. The medium in the top chambers contained lovastatin (4 μmol/L), curcumin (40 μmol/L), EGCG (40 μmol/L), or their combinations. The lower chamber was filled with DMEM and 10% FCS as the chemoattractant and incubated for 48 h. The upper surface was then wiped with a cotton swab to remove the remaining cells. The cells which invaded the Matrigel and attached to the lower surface of the filter were fixed and stained with 1% crystal violet solution. The cells in the reverse side were photographed (5 microscopic fields at 100 × magnification per chamber). The cells in the photographs were then counted, and the data were summarized as mean ± SD and presented as a percentage of the controls (mean ± SD). The data were then analyzed statistically using the Student's *t* test.

Protein extraction and Western blotting

The cells were grown and treated with or without the drugs for 2 d. After that, total cellular protein was extracted as described previously[5,6,13,22-25]. Samples containing 50 μg of protein from each treatment were then separated by 10%-14% on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred electrophoretically to a Hybond-C nitrocellulose membrane (GE-Healthcare, Arlington Heights, IL, United States) at 500 mA for 2 h at 4 ℃. The membrane was subsequently stained with 0.5% Ponceau S containing 1% acetic acid to confirm that the proteins were loaded equally and to verify transfer efficiency. Next, the membranes were subjected to Western blotting by overnight incubation in a blocking solution containing 5% bovine skimmed milk and 0.1% Tween 20 in phosphate-buffered saline (PBS) at 4 ℃. The next day, the membranes were first incubated with primary antibodies and then with horse anti-mouse or goat anti-rabbit secondary antibodies (GE Healthcare) for enhanced chemiluminescence detection of antibody signals. The antibodies used were anti-Ki67 (Vector Laboratories, Burlingame, CA, United States), anti-phosphorylated Erk1/2 (Cell Signaling Technology, Danvers, MA, United States), anti-COX-2 (BD Transduction Laboratories, Lexington, KY, United States), and anti-β-actin (Sigma-Aldrich, St. Louis, MO, United States).

Animal experiments

An animal usage procedure was approved by our Institutional Animal Care and Usage protocol. Esophageal cancer SKGT-4 cells were grown and treated with or without these drugs for 3 d before injection (the doses were the same as above). Nu/nu nude mice (6-8 wk of age) were treated with or without curcumin (50 μg/kg per day), EGCG (50 μg/kg per day), lovastatin (50 μg/kg per day), and their combinations (the same doses used individually) orally for two days and then subcutaneously injected in the right flank through a 22-gauge needle with 2×10^6 tumor cells mixed with 50% Matrigel (BD Biosciences) for a total volume of 200 μL per mouse. The animals were then continuously treated with or without these drugs orally 5 d/wk for an additional 30 dand monitored for tumor formation and growth daily. The tumor mass volumes, measured weekly with a vernier caliper, were calculated as follows: length × width^2/2. At the end of the experiments, the tumor xenografts were taken excised, weighed and the results summarized.

Esophageal cancer tissue samples

Our institutional review board (IRB) approved our protocol for the use of patient tissue samples in this study, which included 156 consecutive patients with available paraffin blocks who had undergone esophagectomy without preoperative chemotherapy or radiotherapy between the years 1986 and 1997 at The University of Texas M.D. Anderson Cancer Center.

Immunohistochemistry

Human esophageal cancer tissue specimens and tumor xenografts from the nude mice were resected and subjected to tissue processing, these samples were embedded in paraffin and 4-μmol/L-thick sections were prepared for immunohistochemical analyses of Ki67, phosphorylated Erk1/2, and COX-2 expression. Briefly, the sections were de-paraffinized twice in xylene for 10 min each and rehydrated in a series of ethanol (100%-50%) and were then subjected to antigen retrieval by cooking in a pressure cooker with 0.01 mol/L citric buffer for 10 min and H_2O_2 treatment to eliminate endogenous tissue peroxidase activity. The tissue sections were then incubated with 100 μL of 20% normal horse or goat serum in PBS and anti-Ki67 (1:50), phosphorylated Erk1/2 (1:50), or COX-2 (1:50) diluted in PBS overnight. The next day, the sections were washed with PBS three times and once with PBS containing 0.1% Tween 20 and further incubated with a second antibody (Horse anti-mouse IgG or Goat-anti rabbit IgG from Vector, Laboratories) for 30 min. After washing with PBS, the sections were then incubated with ABC solution (Vector) in the dark for 30 min and 9-ethylcarbazol-3-amine buffer for 15 min for color development. The sections were counterstained with hematoxylin for 30 s, covered with a cover slip and then reviewed and scored under a microscope as positive or negative staining (10% or more tumor cells with positive staining were counted as positive staining).

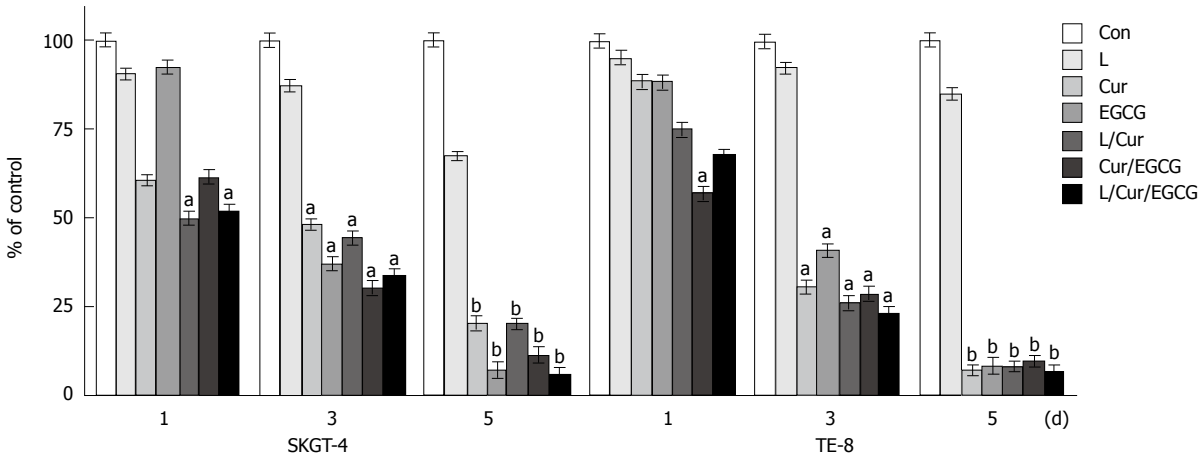


Figure 1 Suppression of esophageal cancer cell growth by curcumin, (-)-epigallocatechin-3-gallate, lovastatin and their combinations. Esophageal cancer SKGT-4 and TE-8 cells were grown in monolayer overnight and then treated with or without curcumin (Cur) (40 μmol/L), (-)-epigallocatechin-3-gallate (EGCG) (40 μmol/L), lovastatin (L) (4 μmol/L) and their combinations for up to 5 d. Methyl thiazolyl tetrazolium assays were then carried out to detect changes in cell viability (see Methods section). The experiments were repeated three times and the results are summarized as a % of the control (Con) (mean ± SD) and analyzed statistically using the Student's *t* test. $^{a}P < 0.05$, $^{b}P < 0.01$.

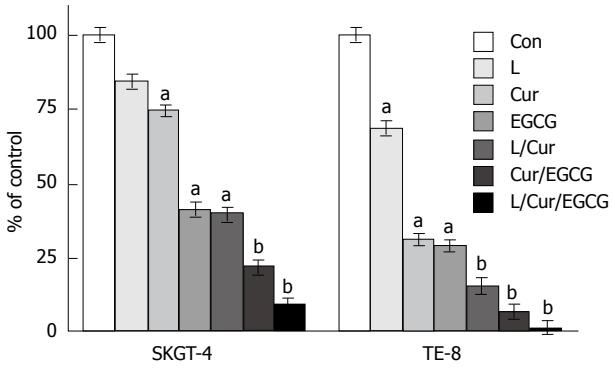


Figure 2 Suppression of tumor cell invasion by curcumin, (-)-epigallocatechin-3-gallate, lovastatin and their combinations. Esophageal cancer SKGT-4 and TE-8 cells were grown and treated with or without curcumin (Cur) (40 μmol/L), (-)-epigallocatechin-3-gallate (EGCG) (40 μmol/L), lovastatin (L) (4 μmol/L) and their combinations in monolayer for 3 d and then subjected to cell invasion assays in Boyden chambers containing Matrigel for 48 h. The invasive cells were stained with 1% crystal violet solution, counted and the results are summarized as a % of the control (Con) (mean ± SD). The data were then analyzed statistically using the Student's *t* test. $^{a}P < 0.05$, $^{b}P < 0.01$.

RESULTS

Reduction of tumor cell viability by curcumin, EGCG, lovastatin and their combinations

We first determined the effects of curcumin, EGCG and lovastatin individually and their combinations on the suppression of esophageal cancer cell growth by treating esophageal cancer TE-8 and SKGT-4 cell lines with two different doses (curcumin at 20 and 40 μmol/L, EGCG at 20 and 40 μmol/L, lovastatin at 2 and 4 μmol/L and these individual drug doses were used for combination treatments) for up to 5 d. The doses selected were based on previous studies[28-37]. Our data showed that lovastatin (4 μmol/L), curcumin (40 μmol/L), EGCG (40 μmol/L) and their combinations significantly reduced tumor cell viability (Figure 1).

Suppression of tumor cell invasion by curcumin, EGCG, lovastatin and their combinations

We next determined the effects of these three drugs on the regulation of esophageal cancer cell invasion capacity and found that lovastatin (4 μmol/L), curcumin (40 μmol/L), EGCG (40 μmol/L), and their combinations significantly reduced tumor cell invasion (Figure 2).

Modulation of gene expression by curcumin, EGCG, lovastatin and their combinations

We then assessed the regulation of gene expression by these three drugs and found that lovastatin (4 μmol/L), curcumin (40 μmol/L), EGCG (40 μmol/L) and their combinations downregulated the expression of p-Erk1/2, c-Jun and COX-2, but upregulated activated caspase 3 expression in esophageal cancer SKGT-4 and TE-8 cells (Figure 3).

Suppression of tumor growth in nude mouse xenografts by curcumin, EGCG, lovastatin and their combinations

We then performed nude mouse xenograft assays of SKGT-8 cells to determine the effects of the drugs individually and their combinations. We found that lovastatin (50 μg/kg per day), curcumin (50 μg/kg per day), EGCG (50 μg/kg per day) and their combinations at the same doses inhibited tumor growth differently (Figure 4). Treatment with a single drug such as curcumin or lovastatin did not have any effects on tumor formation and growth (Figure 4), although these drugs individually and in combination inhibited expression of Ki67, p-Erk1/2 and COX-2 expression in xenograft tissues (Figure 5).

Expression of phosphorylated Erk1/2 and COX-2 in esophageal cancer tissue specimens

To determine the relevance of p-Erk1/2 and COX-2 expression in human esophageal cancer, we analyzed their expression in esophageal cancer tissue specimens

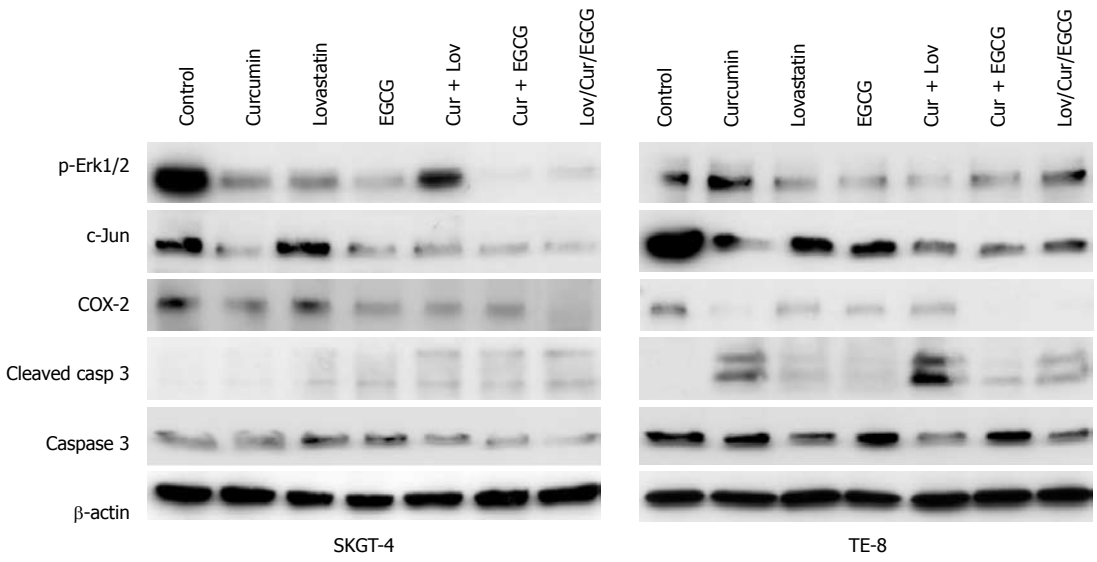


Figure 3 Modulation of gene expression by curcumin, (-)-epigallocatechin-3-gallate, lovastatin and their combinations. Esophageal cancer SKGT-4 and TE-8 cells were grown in monolayer overnight and treated with or without curcumin (Cur) (40 μmol/L), (-)-epigallocatechin-3-gallate (EGCG) (40 μmol/L), lovastatin (Lov) (4 μmol/L) and their combinations for 2 d and total cellular protein was extracted from the cells and subjected to Western blotting analysis of gene expression. Erk1/2: Extracellular-signal-regulated kinases; COX-2: Cyclooxygenase-2.

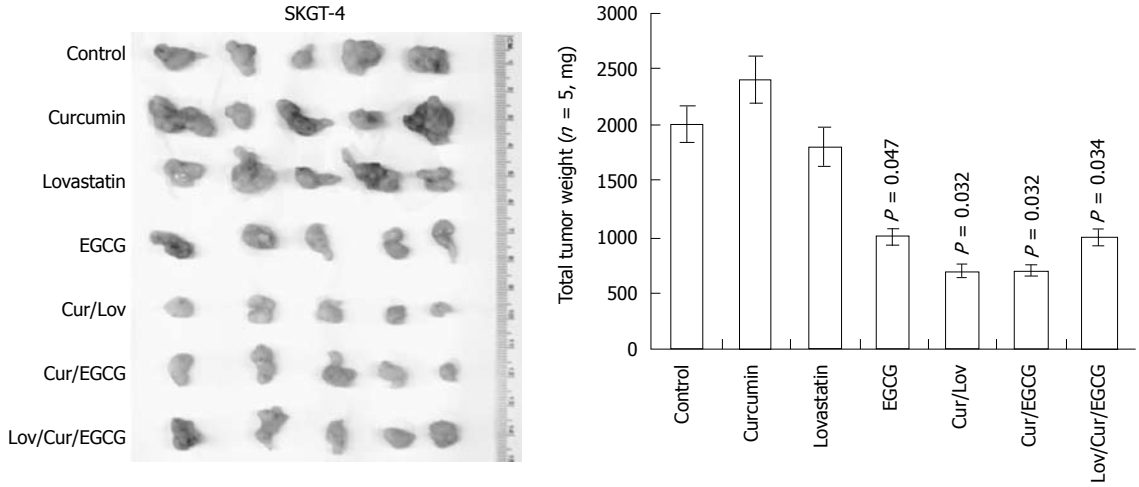
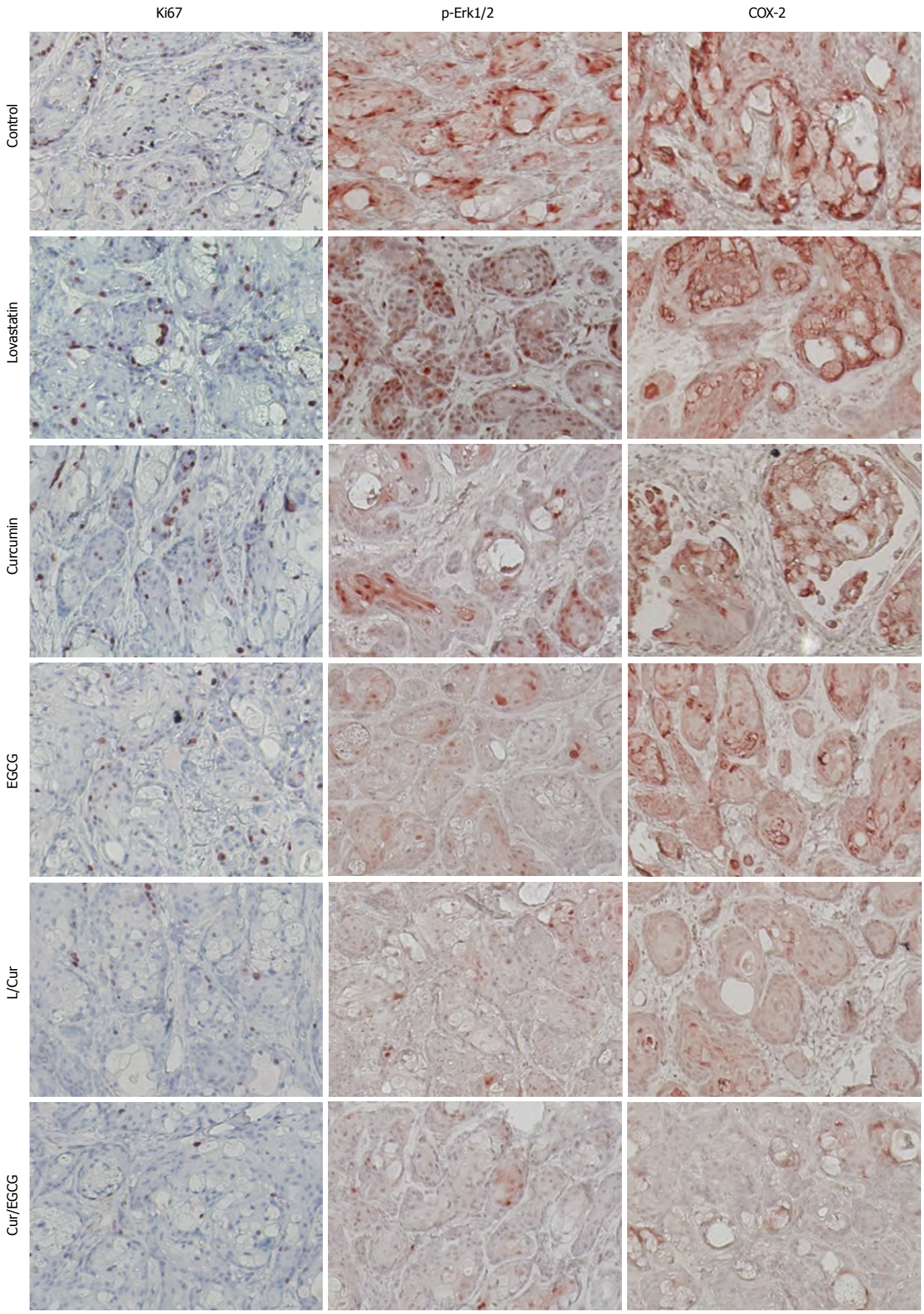


Figure 4 Inhibition of esophageal cancer cell growth by curcumin, (-)-epigallocatechin-3-gallate, lovastatin and their combinations in nude mouse xenografts. Esophageal adenocarcinoma SKGT-4 cells were inoculated subcutaneously into nude mice (5 per group). Two days before tumor cell injection, the mice started treatment with or without curcumin (Cur) (50 μg/kg per day), (-)-epigallocatechin-3-gallate (EGCG) (50 μg/kg per day), lovastatin (Lov) (50 μg/kg per day) and their combinations (the same doses as given individually) for 30 d (5 d/wk by oral gavage). At the end of the experiments, the xenograft tumor mass was isolated and weighed and summarized.

using immunohistochemistry. We found that 77 of 156 (49.4%) tumors expressed phosphorylated Erk1/2 and that 121 of 156 (77.6%) esophageal cancers expressed COX-2 protein. In particular, phosphorylated Erk1/2 was expressed in 23 of 50 (46%) esophageal squamous cell carcinoma (SCC) and in 54 of 106 (50.9%) adenocarcinoma, while COX-2 was expressed in 39 of 50 (78%) esophageal SCC and in 82 of 106 (77.4%) esophageal adenocarcinoma (Figure 6).

DISCUSSION

In the current study, we demonstrated that curcumin, EGCG, lovastatin, and their combinations can significantly reduce the viability and invasion capacity of esophageal cancer cells *in vitro*. Nevertheless, they were much less effective *in vivo* in nude mouse xenografts, especially curcumin and lovastatin individually. At the molecular level, these three agents individually or in combination inhibited the expression of phosphorylated Erk1/2, c-Jun, and COX-2 and induced caspase 3 expression in esophageal cancer cells *in vitro*. In nude mouse xenografts, the expression of p-Erk1/2 and COX-2 was downregulated by these three drugs, especially their combinations. We also analyzed the expression of phosphorylated Erk1/2 and COX-2 in tissue


Ki67
p-Erk1/2
COX-2
Control
Lovastatin
Curcumin
EGCG
L/Cur
Cur/EGCG

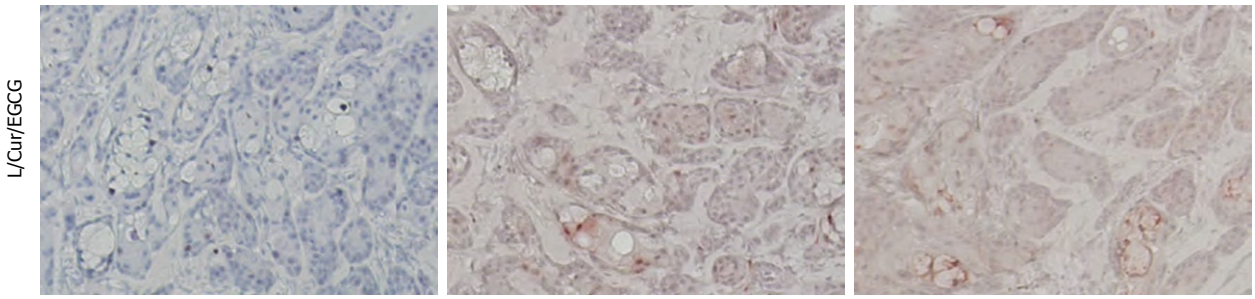


Figure 5 Reduced expression of Ki67, phosphorylated extracellular-signal-regulated kinases and cyclooxygenase-2 in xenografts following treatment of mice with or without curcumin, (-)-epigallocatechin-3-gallate, lovastatin and their combinations. Tumor cell xenografts obtained from the nude mouse experiments were processed and subjected to immunohistochemical analyses of Ki67, phosphorylated extracellular-signal-regulated kinases (Erk1/2) and cyclooxygenase-2 (COX-2) expression. Representative images were obtained in each treatment group. EGCG: (-)-epigallocatechin-3-gallate; L: Lovastatin; Cur: Curcumin.

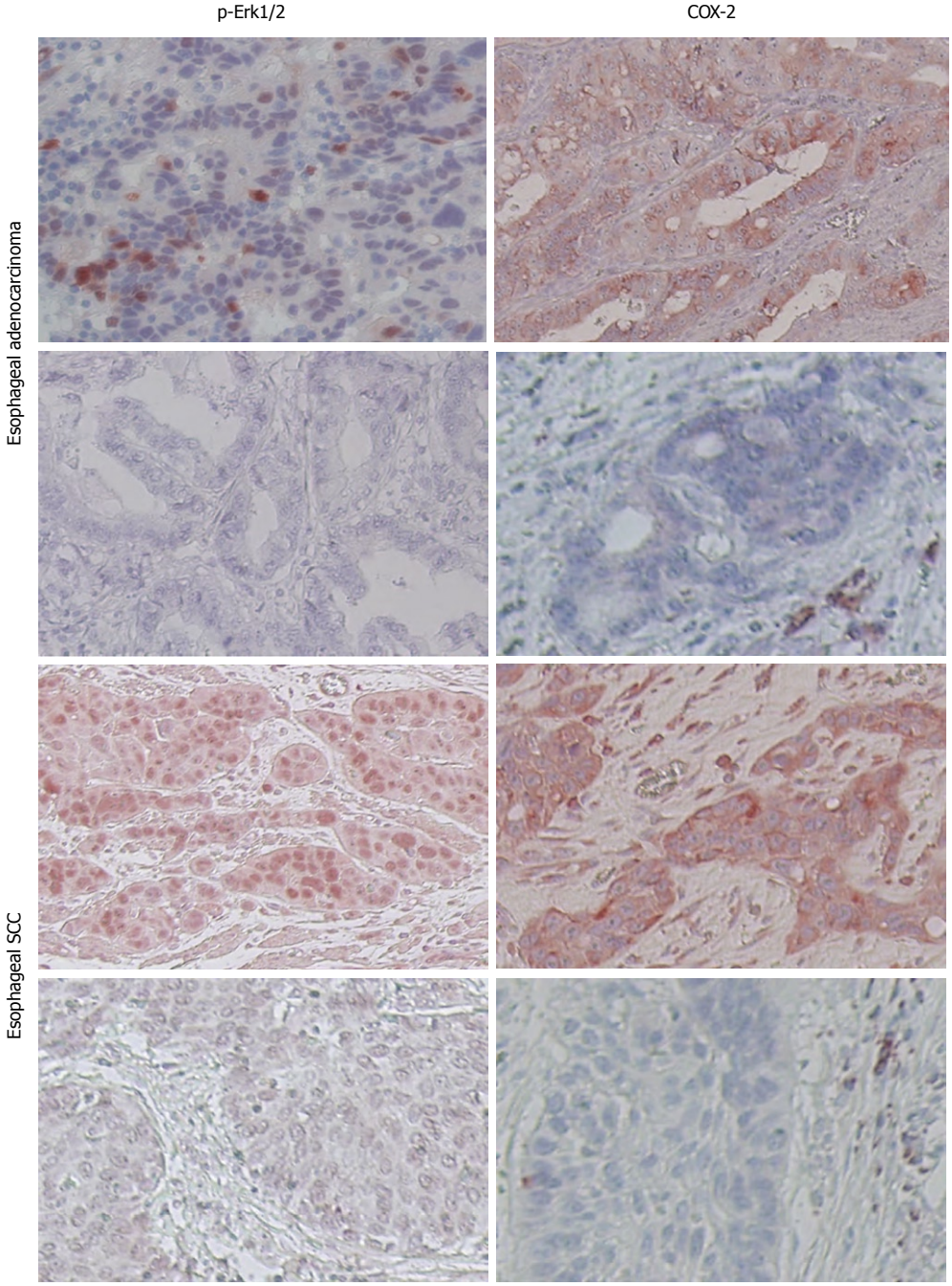


Figure 6 Expression of phosphorylated extracellular-signal-regulated kinases and cyclooxygenase-2 in esophageal cancer specimens. Paraffin sections of esophageal cancer tissues were immunostained with anti-phosphorylated extracellular-signal-regulated kinases (Erk1/2) or cyclooxygenase-2 (COX-2) antibody. Representative images were obtained from these tissue sections. SCC: Squamous cell carcinoma.

specimens from esophageal cancer patients. The data showed that 49.4% of esophageal cancers expressed phosphorylated Erk1/2 and that 77.6% of cancers expressed COX-2 protein. These data suggest that curcumin, EGCG, and lovastatin inhibit esophageal cancer cell growth *in vitro* and in nude mouse xenografts possibly through the suppression of phosphorylated Erk1/2, c-Jun and COX-2 expression.

Previous studies have shown the chemopreventive activity of EGCG in suppressing carcinogenesis in several organs, including the esophagus[29,34]. Molecularly, EGCG can suppress the mitotic signal transduction pathway, e.g., inhibit Erk1/2 phosphorylation and anti-AP-1 activity[38]. A recent study demonstrated that EGCG induced a concentration- and time-dependent reversal of hypermethylation of RAR-β2 in esophageal cancer cell lines, resulting in re-expression of RAR-β2[33]. Furthermore, curcumin has been shown to inhibit different cancers at the initiation, promotion, and progression stages in animal models[31,32,38]. Curcumin also suppressed growth and induced apoptosis in numerous types of cancer cells *in vitro*[38,39]. Although the defined mechanisms of its action require further study, its efficacy appears to be related to the induction of glutathione and glutathione-S-transferase activity, inhibition of lipid peroxidation and arachidonic acid metabolism, and suppression of oxidative DNA adduct formation[32,38,39]. Curcumin can inhibit the activation of NF-κB and the expression of c-Jun, c-Fos, c-Myc, Erk1/2, COX-2, PI3K, Akt, CDKs, and iNOS[31,35,38,39]. Curcumin was also able to suppress cigarette smoke-induced NF-κB activation and COX-2 expression in head and neck SCC and non-small-cell lung cancer cells[31,32]. In esophageal cancer, dietary curcumin can inhibit chemically-induced esophageal carcinogenesis in mice and rats[28,40]. In addition, the statin family of drugs has shown cancer chemopreventive effects[41]. Statins can trigger some tumor cells to undergo apoptosis *in vitro* and suppress tumor growth *in vivo*[30,37,41]. Statins also have an antimetastatic property, which is evident in their suppression of tumor cells invasiveness in Matrigel, as well as in animal experiments[42]. In addition, statins, especially at high concentrations, can inhibit capillary tube formation by endothelial cells *in vitro* and *in vivo*[35,44]. The effects of statins are thought to be mediated through inhibition of Ras and RhoA activity[41]. Based on these previous studies and reports, we determined the effects of their combinations on suppression of esophageal cancer cell growth *in vitro* and in nude mouse xenografts by targeting the RAR-β2/Erk1/2/AP1/COX-2 pathway[3]. Indeed, our current study has demonstrated the effects of their combinations *in vitro*. Molecularly, these three agents were able to regulate the expression of this gene pathway *in vitro* and *in vivo* in nude mice. Nevertheless, individually curcumin and lovastatin had no effect on tumor formation and growth in nude mice, even when the highest dose possible was used. This may be due to the bioavailability of curcumin and the induction of COX-2 expression by high dose lovastatin, reported previously[30,39]. However, the current study did not show the induction of COX-2 expression by high dose lovastatin in esophageal cancer *in vitro* and in nude mice, similar to that seen in prostate cancer[30].

However, there are some limitations in the current study. Firstly, we showed that these three drugs regulated gene expression of the RAR-β2/Erk1/2/AP1/COX-2 pathway, however, previous studies also showed that as chemoprevention agents, these drugs target multiple genes and their pathways in different cancers. Thus, further study is needed to determine the mechanisms of action of these drugs in human cancers. Furthermore, we used established esophageal cancer cell lines to determine the chemopreventive effects of these agents in this study, the results of which may be quite different in comparison to those in premalignant cells *in vivo*. The xenograft assay tested the effects of these agents in suppressing tumor initiation and growth, but not tumor development per se, although the xenograft assay did test the bioavailability of these agents *in vivo*. In addition, we did not test whether the doses of these three agents are clinically achievable, and to reduce costs, we utilized a single dose of each agent and their combinations. Thus, future studies are needed to test these agents in a clinical Phase I trial and in animal experiments where more doses and a time course study will be included.

ACKNOWLEDGMENTS

The authors would like to thank the Department of Scientific Publication of MD Anderson Cancer Center for editing this manuscript.

COMMENTS

Background

Esophageal cancer remains a lethal disease, and is the least studied cancer in the United States. The overall 5-year survival rate for esophageal cancer is only 10%-15%, while the incidence of esophageal adenocarcinoma has significantly increased in the United States and other Western countries. These data indicate an urgent need for the development of novel strategies for prevention, early detection and management of esophageal cancer

Research frontiers

The authors found that the combination of curcumin, (-)-epigallocatechin-3-gallate and lovastatin was able to suppress esophageal cancer cell growth *in vitro* and in nude mouse xenografts.

Innovations and breakthroughs

These three agents inhibited phosphorylated Erk1/2, c-Jun and COX-2 expression.

Applications

Further clinical trials using these agents are warranted.

Peer review

This is the first report of the effects of combining these agents on esophageal cancer cells. The data suggest that these agents might be used individually or in combination for chemoprevention of esophageal cancer. Overall, the study is well designed and the data are convincing.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i2.136

World J Gastroenterol 2012 January 14; 18(2): 136-143
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Characterization of gastric cancer models from different cell lines orthotopically constructed using improved implantation techniques

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Supported by A grant from the Natural Science Foundation of China, No. 30830040
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Received: March 21, 2011 Revised: June 21, 2011
Accepted: June 28, 2011
Published online: January 14, 2012

Abstract

AIM: To develop orthotopic gastric cancer mouse models from different cell lines and characterize the tumor features to assist further in preclinical trials and clinical treatment strategies.

METHODS: Human gastric cancer SGC-7901 and BGC-823 cell suspensions were injected subcutaneously into nude mice to develop solid tumors, and tumor tissue pieces were then implanted under the serous coat of the stomach. An autopsy was performed on all animals of the SGC-7901 and BGC-823 models to observe the primary tumor growth and metastases using pathological and immunohistochemical methods.

RESULTS: Both models showed large tumors *in situ* resulting in pressure and infiltration of the adjacent organs. The gastric cavity became smaller, along with stenosis of the cardia or pylorus. There were biological and statistical differences between the two models. The metastasis rate in involved organs (lymph nodes, kidney, spleen, testis) was significantly higher in the BGC-823 model compared to the SGC-7901 model ($P < 0.05$ or $P < 0.01$). The median survival of the BGC-823 model was shorter than that of SGC-7901 (23 d *vs* 84 d, $P < 0.05$). Histopathologically, the primary tumor and metastatic lesions of the two models showed obvious atypia and mucus in the cytoplasm. Compared with the SGC-7901 model, BGC-823 appeared more poorly differentiated (absence of adenoid structure), had a smaller volume, and richer capillary structure. Immunohistochemical staining revealed cytokeratin 20 and epithelial membrane antigen expression was positive in the SGC-7901 tumors, while negative in BGC-823 ones.

CONCLUSION: Models using the SGC-7901 and BGC-823 cell lines were established which could function in gastric cancer research on carcinogenesis mechanism and drug discovery. The two models showed different tumor behavior and the latter was more malignant than the former.

Key words: Gastric cancer; Orthotopic implantation; Mouse model; Metastasis; Cell line

Peer reviewer: Dr. Courtney Houchen, MD, The University of Oklahoma Health Sciences Center, 975 NE 10th Street, Oklahoma City, OK 73104, United States

Li Y, Li B, Xiang CP, Zhang Y, Li YY, Wu XL. Characterization of gastric cancer models from different cell lines orthotopically constructed using improved implantation techniques. *World J Gastroenterol* 2012; 18(2): 136-143 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i2/136.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i2.136

INTRODUCTION

Gastric cancer is still the second most common malignant tumor in the world, and patients with gastric cancer generally have metastasis when clinically examined. There is a low five-year survival rate and poor quality of life even after tumor resection. We need to develop animal models of gastric cancer for exploring the mechanisms of carcinogenesis and clinical treatment strategy. Since the 1990s, orthotopic implantation techniques have gained popularity in establishing animal models of various tumors[1-4]. Many researchers have constructed orthotopic models of gastric cancer with different methods[5-11]. Of these methods, orthotopic transplantation using tumor pieces is useful because it not only simulates clinical cancer behavior but also promotes metastasis[12]. In the past, researchers mainly employed the "sewing" method to develop stomach cancer models[6,13]. However, there are disadvantages; such as a difficult operation, a time-consuming process, and low survival rates, and the sewing technique is not commonly used. In recent years, the procedure has been improved using tissue adhesive adhering to tumor pieces, which greatly facilitates surgical performance and decreases the mortality rate resulting from surgical operation[5,14]. Based on previous research, we intended to develop gastric cancer models from the SGC-7901 and BGC-823 cell lines and observe their biological characteristics to aid preclinical trials and therapy strategies. The human gastric cell line SGC-7901 was first established from the metastatic lymph node of a 56-year-old female patient suffering gastric adenocarcinoma. The BGC-823 cell line was derived from a specimen from a male patient who was 62 years of age, which was conserved in the Tumor Institute of Tianjin in China. The two gastric cell lines are poorly differentiated. This is the first time that a gastric cancer model of BGC-823 cell line was orthotopically constructed with histological tumor tissue *via* the "adhering" method.

MATERIALS AND METHODS

Cell lines

Human gastric cancer cell lines SGC-7901 and BGC-823 were used for this study. The cells were purchased from the Centre of Cell Cultures of Chinese Academy of Medical Sciences, Shanghai, China, and cultured at 37 ℃ in a humidified atmosphere with 5% CO_2, in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Gibco, Grand Island, NY), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine and 1 mmol/L sodium pyruvate. Cells were maintained by serial passaging after trypsinization with 0.1% trypsin.

Animals

Five- to six-week-old male Balb/c nu-nu mice (weight 18-20 g) were obtained from the Shanghai Experimental Animal Center of the Chinese Academy of Medical Sciences, China. They were kept in cages in a pathogen-free environment (temperature 25 ℃-27 ℃, humidity 45%-50%) and supplied with food and water *ad libitum*. All animal experiments were approved by the ethical committee of the Chongqing Medical University, and conformed to National and International Policies on Human Care and Use of Laboratory Animals.

Subcutaneous tumor specimens

Subcutaneous tumors were grown and then tumor tissue pieces used in surgical orthotopic implantation (SOI). Two cell lines were collected in the log phase and injected subcutaneously at 10^7/0.2 mL into the bilateral croup of Balb/c nu-nu mice. After 2 wk, the resulting tumors from the croup were harvested under strict aseptic conditions following removal of necrotic tissue from the central tumor areas, and cut into small pieces of approximately 1 mm^3.

Orthotopic implantation of tumor fragments

Nude mice were divided into 2 groups of 12 animals each, and were explanted with tumor fragments[5] from the SCG-7901 and BGC-823 cell lines, respectively. All procedures were performed under anesthesia with Sumianxin Ⅱ (0.02 mL per animal; China Academy of Military Medical Science) at the benchtop. After a left-side upper abdominal incision was made, the stomach of the nude mouse was gently exteriorized. One small tissue pocket was prepared in the middle wall of the greater curvature using an ophthalmic scissor, and then one tumor piece was placed into the pocket following fixation with a drop of medical tissue adhesive (gifts from Shunkang Corporation of Biological Adhesive, Beijing, China). To avoid adhesion to adjacent normal tissue, the quantity of the tissue adhesive was strictly measured. The stomach was then returned to the peritoneal cavity, and the abdominal wall was closed with 4-0 absorbable sutures. The mice were given special care and fed in cages as usual after surgery.

Evaluation of tumor growth and metastasis

All mice of the two groups were closely observed. At time of death, an autopsy was carried out to examine the tumor growth. The volume of primary tumor was calculated by the following formula: $V = 0.4 \times ab^2$ (a: maximum diameter; b: minimum diameter)[15]. Primary tumor, lymph nodes (gastroepiploic plexus, hilus pulmonis and mesenterium), and other organs (liver, lung, kidney, testis, spleen, *etc.*) involving infiltration or metastasis were sampled. The sampled tissues were fixed in 10%

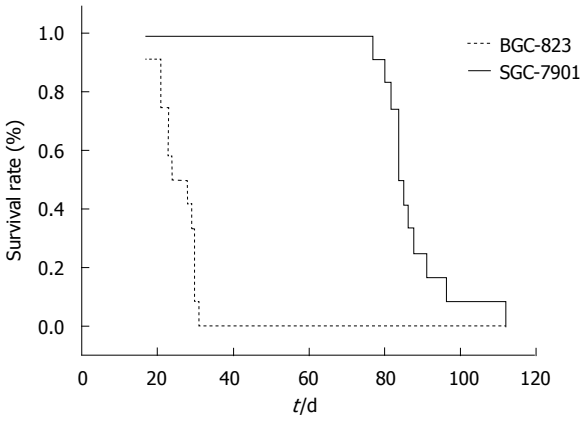

Figure 1 Survival curve of the SGC-7901 and BGC-823 gastric cancer models. The median survival in the BGC-823 model is significantly shorter than that in SGC-7901 (23 d *vs* 84 d, $P < 0.05$).

formalin, embedded in paraffin, sectioned to 3 μm thick, and stained with hematoxylin and eosin for microscopic examination. The ascitic fluid was centrifuged (1000 r, 5 min), and a cytologic smear was prepared for microscopic examination of malignant tumor cells.

Immunohistochemistry

The expression of cytokeratin (CK; High MW), cytokeratin 20 (CK-20) and epithelial membrane antigen (EMA) was studied by using mAbs 34βE12, KS20.8 and GP1.4 (MAB-0052, 0057, 0061; Maixin Inc., Fuzhou, China). An ultrasensitive SP kit (KIT-9710; Maixin Inc., Fuzhou, China) and a DAB kit (DAB-0031; Maixin Inc., Fuzhou, China) were employed according to the manufacturer's instructions. The expression of CK, CK-20 and EMA proteins was defined as positive if the stained area of tumor cells was predominant in their cytoplasm.

Statistical analysis

Data are presented as mean ± SD. The median survival was analyzed using the Wilcoxon Rank-Sum test. The volume of primary tumor was analyzed by the student *t* test. The incidence of metastasis in both groups was analyzed using Fisher exact test. Differences were judged statistically significant at a *P* value < 0.05.

RESULTS

The incidence of tumor growth and general condition

The tumor uptake rate after orthotopic implantation in both of the two groups was 100% (12/12). The median survival for the SGC-7901 and BGC-823 groups were 84 d and 23 d, respectively ($P < 0.05$, Figure 1). Mice of the SGC-7901 group had obvious cachexia (emaciation, retardation, ascites formation, *etc.*), whereas none of the BGC-823 group showed a decline in their general health.

Observation and evaluation of primary tumor growth

The average tumor volume of the BGC-823 group was 1.24 ± 0.73 cm^3, while it was 9.30 ± 3.62 cm^3 in the SGC-7901 group (Figure 2A and B). There was a statistical significance in the difference ($P < 0.05$, Figure 2C). In addition, observations of the primary tumor and related characteristics in the two groups are presented in Table 1 in detail.

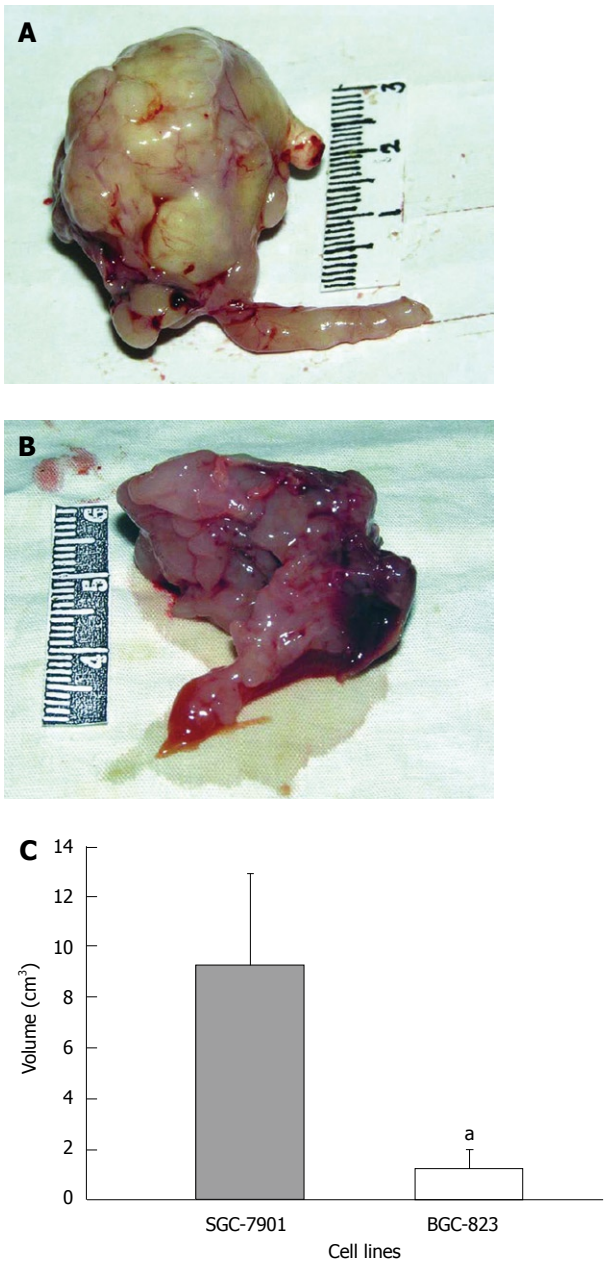

Figure 2 Macroscopic examination of the primary tumor in the two models. A: The tumor of SGC-7901 shows large volume (9.30 ± 3.62 cm^3), irregular lobular shape, and stenosis in the cardia or pylorus; B: The tumor volume of BGC-823 is 1.24 ± 0.73 cm^3, with irregular lobular appearance; C: Comparison of primary tumor volume in two models. The difference is statistically significant. $^aP < 0.05$, student *t* test.

Evaluation of metastases derived from orthotopic implantation tumors

Metastasis, which was always located in lymph nodes, liver, kidney, lung, peritoneum or diaphragm, occurred in both groups of gastric cancer models. Additionally, oth-

Table 1 Comparison of primary tumor and related characters in the two models

Items		SGC-7901 group	BGC-823 group
Primary tumor	Shape	Irregularly lobular	Irregularly lobular
	Color	Grayish yellow	Gray-white
	Texture	Stiffer	Softer
	Vascularity	Rich	Richer
Section of stomach tumor	Gastric cavity	Narrow or vanished	Narrow or vanished
	Greater and lesser curvature	Undistinguishable	Undistinguishable
	Pylorus	Partly or totally obstructed	Totally obstructed
Effects on adjacent organs	Abdominal cavity	Occupying whole abdomen	Occupying upper abdomen
	Adhering extent	Adhering to liver lobes	Adhering to many organs
	Compressed status	Severely compressed	Partly compressed
Ascites	Liquid quantity	Little	Much
	Property	Clear and yellowish	Bloody fluid

Table 2 Comparison of metastatic rates in models of SGC-7901 and BGC-823, *n*/*N* (%)

Groups	Site and incidence of metastasis						
	Lymph nodes[a]	Liver	Kidney[a]	Lung	Spleen[b]	Testicle[a]	Peritoneum or septum
SGC-7901	7/12 (58)	10/12 (83)	5/12 (42)	1/12 (8)	0	0	10/12 (83)
BGC-823	12/12 (100)	12/12 (100)	10/12 (83)	5/12 (42)	7/12 (58)	5/12 (42)	12/12 (100)

[a]$P < 0.05$, [b]$P < 0.01$.

er organs (spleen and testicle) were also found to have metastatic lesions in the BGC-823 group. The incidence of metastasis from various organs in the BGC-823 group was higher than that in the SGC-7901 group, and these differences (except for liver, lung, peritoneum or diaphragm) were considered statistically significant ($P < 0.05$ or $P < 0.01$, Table 2).

Histology

The two groups differed in histological appearance of subcutaneous tumor samples, though the cell lines forming tumors were both poorly differentiated adenocarcinomas. In the SGC-7901 group, tumor cells spread with nest-like structures, characterized by nuclear polymorphism, nuclear hyperchromatism, many red nucleoli, and rich pathological karyokinesis. Mucus was observed in the cytoplasm of part of the tumor cells which resulted in mucus lakes, and adenoid structures and rich blood vessels formed in tumor areas. In the BGC-823 group, histopathologic examination confirmed a different phenotype of the BGC-823 tumor compared to the SGC-7901 tumor. The tumor cells mostly showed medullary growth with characteristics of ample cells, fewer fibroblasts, and rich vascularity. The neoplasm revealed the signs of undifferentiated structure, small size, nuclear hyperchromatism, and rich pathological karyokinesis. Mucus was found in the cytoplasm of part of the neoplastic cells, but there was an absence of glandular differentiation.

Both primary tumors and metastases, whose tumor cells were microscopically identical to the subcutaneous tumor, of the two group models showed similarity in histopathologic characteristics. The stomach cancer of the two models infiltrated the various layers of gastric wall with disruption of the integrity of the mucous layer or muscularis mucosae (Figure 3A and B). Widespread infiltration of tumor cells was found in the subcapsular, the cortical and medullary areas of lymph nodes, while extension into the medullary parts the lymph sinus was diminished (Figure 3C). In some of the lymph nodes, the nodal parenchyma was even totally replaced by neoplastic tumor. Metastases were detected in the liver (Figure 3D) and the kidney (Figure 3E) with nest-like structures or a glandular appearance, occasionally in the adrenal gland or pancreas (not shown). Metastatic lesions were always separated from adjacent normal tissue by fibrous capsules, and lymphoid infiltration in peripheral tumor areas was also seen. Tumors metastasized to the lung and destroyed bronchi or bronchioles (Figure 3F). In addition, local invasion was observed within the abdominal muscle or diaphragmatic muscle. Organs such as the spleen and the testicle (Figure 3G and H) were not spared in the BGC-823 models. Histological examination revealed that the spleen parenchyma was infiltrated and the seminiferous tubules of the testis or ductus epididymidis, and the prostate, were invaded by the metastatic tumor.

Smears of cast-off cells from ascites in both groups confirmed that the malignant cells originated from the primary adenocarcinoma (not shown).

Immunohistochemistry analysis

The expression of CK-20 and EMA protein was positive within the primary tumor and metastases in the SGC-7901 group, whereas CK expression was negative in the same tissues (Figure 4). In the BGC-823 group, CK, CK-20 and EMA immunostaining were negative.

DISCUSSION

The purpose of our study was to develop gastric cancer models from different cell lines with intact tumor

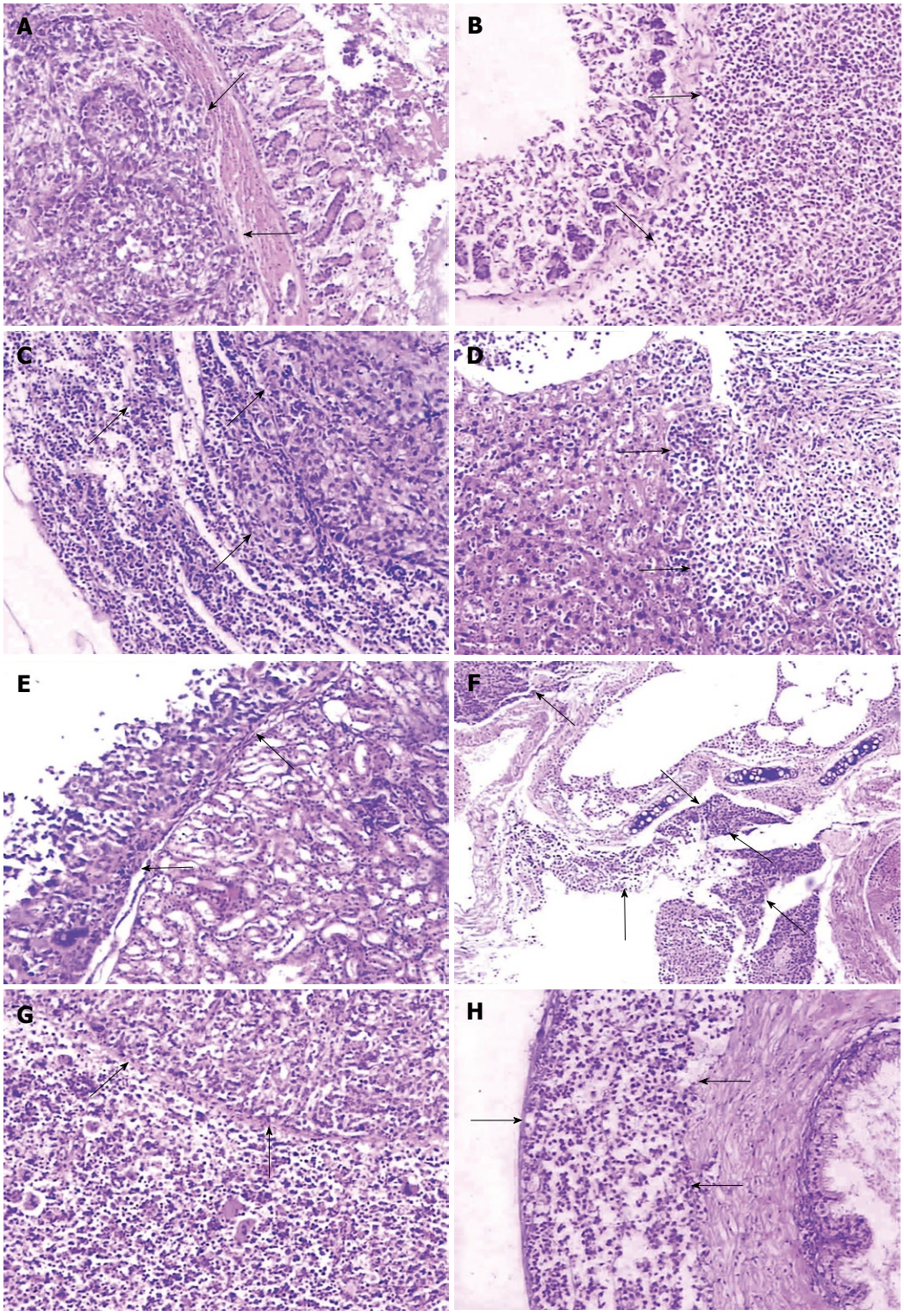

Figure 3 Histology of the primary tumor and metastasis in the two models by hematoxylin and eosin staining. The tumor cells of the SGC-7901 (A) and the BGC-823 (B) model infiltrate the submucosa of the stomach; C: Tumor cells (arrows) are found in the subcapsular and cortical areas of lymph node of the SGC-7901 model; D: Metastases (arrows) detected in the liver of the BGC-823 model; E: Tumor invades into the kidney of the SGC-7901 model, with a fibrous capsule surrounding relatively normal renal tissue (arrows); F: Tumor metastasizes to the lung and surrounds the bronchium in the BGC-823 model; Other organs (G: Spleen; H: Testicle) with metastasis involvement in the BGC-823 model. Tumors are marked with arrows. Hematoxylin and eosin stain; Magnification, × 100.

pieces orthotopically implanted into the stomach wall by tissue glue adhesion. In the present study, we could replicate similar results of the SCG-7901 orthotopic models which have been established by other researchers in China. This is the first time that an animal model has been created from the BGC-823 gastric cancer cell line with SOI of tumor tissue.

Interestingly, we find the two models reveal signifi-

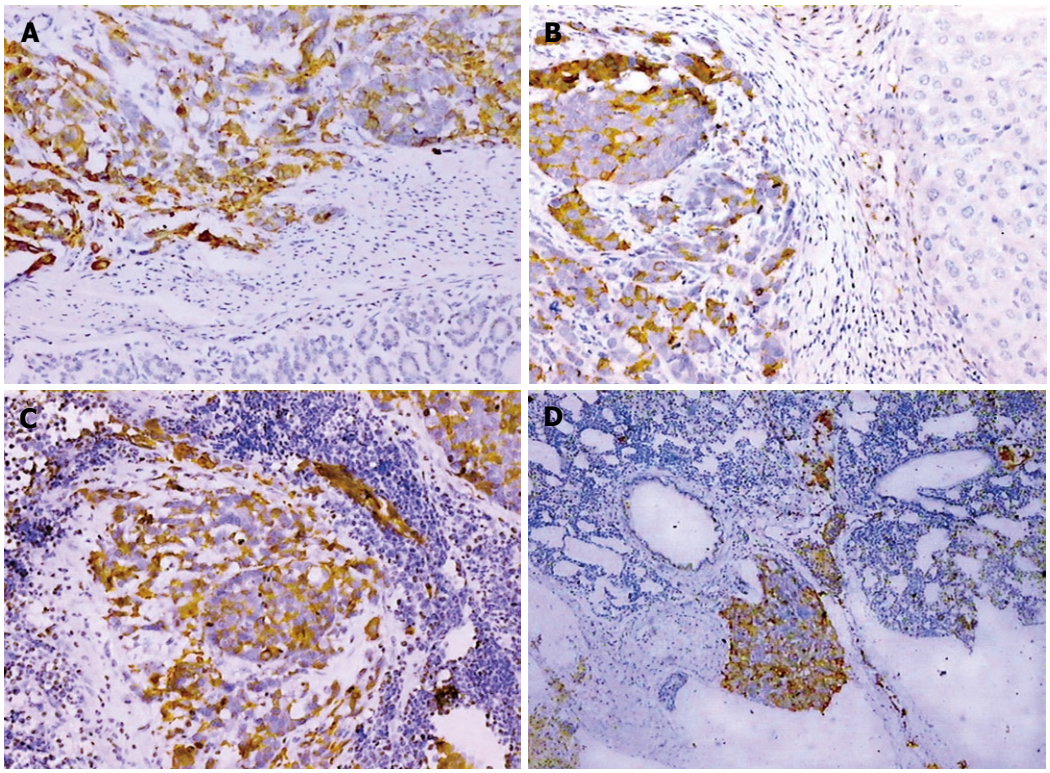

Figure 4 Immunohistochemistry of primary tumor and metastasis in the SGC-7901 model using the self-potential method. Cytokeratin 20 (A: Stomach; B: Liver) and epithelial membrane antigen expression (C: Lymph node; D: Lung) shows as positive, characterized by the brown-yellow stain in the cytoplasm. Visualized by using DAB reagent; Magnification, × 100.

cant differences in biological behavior. It has been reported that heterogeneity resulting from differences of histopathological type, grade, and individual factors reside in different tumor cell lines[5,16]. Thus, animal models derived from different tumor cell lines present different characteristics. Bhargava *et al*[5] orthotopically established gastric cancer models in two sites of the stomach using tumor fragments from three cell lines of differently differentiated extent. The findings showed that the tumor uptake rate and metastases were all different among the three cell lines, between two sites, and at various time points. Fujihara *et al*[13] developed a gastric cancer cell line subtype named OCUM-2M LN, characterized by high incidence of lymph node metastasis, which was obtained from the OCUM-2M cell line by serial passage selection in animals. The ability to metastasize to lymph nodes in subtype OCUM-2M LN was significantly higher than that in parental cell line OCUM-2M. In keeping with these authors above, we also found that heterogeneity among different cell lines contributed to differences of tumor behavior in both gastric cancer models.

Histopathological characteristics of malignant neoplasms were reflected in both cancer models, characterized by neoplastic atypia of varying degrees, and rich blood vessels promoting tumor growth and metastasis. However, the two types of tumor showed differences in pathologic phenotype. The SGC-7901 tumors showed nest-like structures and glandular differentiation, while the BGC-823 tumors presented medullary structures and no adenoid differentiation, which might be explained by the fact that the BGC-823 cell line develops variation and partly changed neoplastic properties during passage[9,13]. Microscopically, mucous secretion in both types of tumor cells, especially in the SGC-7901 tumor, was consistent with the characteristics of mucous adenocarcinoma. Meanwhile, the negative result of CK expression in the two models also excluded the differentiation of squamous carcinoma. The expression of CK-20 and EMA by immunohistochemical staining was positive in the SGC-7901 tumors, but negative in BGC-823. Histopathology and immunohistochemistry analysis all confirmed that the BGC-823 carcinoma had poorer differentiation than SGC-7901. In addition, all epithelial markers in BGC-823 tumors were negative which might suggest an epithelial-to-mesenchymal transition phase. We shall perform immunohistochemical staining with a mesenchymal marker, such as vimentin, to verify this presumption in our next experiments. It is reported that survival prognosis has close correlation with histopathologic grading of tumor[17-19]. The results in our study demonstrated that median survival course in the BGC-823 model was shorter than that in SGC-7901 ($P < 0.05$), which is compatible with histological examination.

Retrospective studies have suggested that there is correlation between tumor volume size and lymph node metastasis or five-year survival[19-22]. In our experimental results, the original tumor in both models presented a large-volume lesion invading adjacent organs and

destroying the stomach cavity. There is no theoretical significance regarding difference of the tumor volume in the SGC-7901 and BGC-823 groups, though the former is obviously larger than the latter ($P < 0.05$) mainly resulting from the different course of primary tumor growth. However, the SGC-7901 tumor texture was harder than that of BGC-823, which was possibly linked with rich stromal component in the former but ample tumor cells and rich blood vessels in the latter. Angiogenesis is a prerequisite for metastatic spread[15,23], which also explains why vascular metastasis is a frequent occurrence in the BGC-823 model.

Metastasis is not only linked with the prognosis of patients but also the dilemma of cancer therapy. In the field of experimental research, metastatic events are assumed to cover the factors of the anatomic site of implantation[12,13,24,25] or status (suspension or fragment) of tumor sample used in implantation[10,23]. Orthotopic transplantation in animal models of various cancers has played a key role in mimicking the clinical tumor behavior[5,23,24]. There has been variable success at achieving good tumor uptake rate and metastatic incidence with implantation procedures using suspension injections and tumor pieces[10,23]. Many studies indicate that the properties on the tumor cell surfaces are preserved by the implantation of tumor pieces, but destroyed after the cell suspensions have been treated by trypsinization resulting in changes of malignant character and further contributing to decline of tumor growth and metastatic rate[13,24]. In the present study, the models of orthotopic implantation into the gastric wall with intact tumor fragments showed not only high uptake rate but also metastasis through various pathways, which was consistent with the previous research. It is well known that the general metastasis in terminal patients with gastric cancer mainly involves direct infiltration, lymphatic metastasis, vascular spread, and implantation dissemination. The aggressive behavior in the two model groups resembles clinical patients suffering from gastric cancer. In the SGC-7901 group, direct infiltration and lymphatic metastasis were frequently observed. However, multiple steps were assumed to be involved in the metastases of the BGC-823 group. In BGC-823 models, we could not identify the accurate pathway of metastasis through local invasion in the liver, kidney, spleen and testicle. The total involvement of multiple steps facilitating metastases requires further studies.

Compared with the SGC-7901 group, metastasis occurrence is earlier ($P < 0.05$), metastasis incidence in different organs higher ($P < 0.05$), and the number of involved organs greater in the BGC-823 group. The deaths in the SGC-7901 group were considered as multi-organ failure caused by cachexia, while the BGC-823 mice mostly died of early metastases and high metastatic rates without visible cachexia. Taken together, the results in our study demonstrate that the BGC-823 gastric cancer model is more aggressive than the SGC-7901 one with the support of the comprehensive factors discussed above.

We have successfully developed two types of gastric cancer model with different tumor behavior by orthotopic implantation, which are characterized by advantages such as low cost and resemblance to clinical gastric cancer. The two models are both suitable for preclinical research on gastric cancer, and their different characteristics may aid with different needs of experimental research. Considering comprehensive factors, surgical orthotopic implantation is still a desirable technique compared with other methods of constructing gastric cancer models[26-29].

COMMENTS

Background

Gastric cancer is still the most common malignant tumor in the world, and detection is difficult before metastasis occurrence, so people need to develop animal models of gastric cancer for exploring the mechanisms of carcinogenesis and clinical treatment strategy. Gastric cancer models of orthotopic implantation with intact tumor tissue have been well established. Moreover, orthotopic implantation performance has been improved from the "sewing" method to the "adhering" one. SCG-7901 orthotopic models have been established by other researchers, whereas this is the first time that an animal model was created from the BGC-823 gastric cancer cell line with surgical orthotopic implantation of tumor tissue.

Research frontiers

Orthotopic implantation technique plays an important role in establishing an animal model of various tumors, including gastric cancer, which can not only simulate clinical cancer development but also facilitate metastasis occurrence. Moreover, the procedure of orthotopic implantation has been improved from the "sewing" method to the "adhering" one. The latter's performance has greatly simplified the surgical operation course. In addition, the BGC-823 gastric cancer model has not been constructed by others, and shows different tumor characteristics compares with the SCG-7901 model.

Innovations and breakthroughs

Orthotopic implantation with "glue paste technique" is a new, popular and convenient method used for the establishment of orthotopic animal models in recent years. The results regarding the BGC-823 gastric cancer model in this study are the first to be reported, and make it possible that researchers can learn the different biological characteristics of gastric cancer models from different cell lines.

Applications

Establishment of the BGC-823 gastric cancer model provides evidence for researchers to learn the biological differences between animal models from different gastric cancer cell lines, which can help them to choose an appropriate model to tailor their research.

Terminology

Surgical orthotopic implantation is defined as tumor cells in the form of suspension or fragments which are orthotopically implanted into the related organs.

Peer review

The study is aimed to develop gastric cancer models from different cell lines and analyze the difference of biological behavior in many aspects, in order to provide evidence for researchers to choose an appropriate experimental carrier and therapy target for clinical trials. The present research is valuable for clinical doctors and researchers.

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S- Editor Sun H **L- Editor** Logan S **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i2.144

World J Gastroenterol 2012 January 14; 18(2): 144-149
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

B1a lymphocytes in the rectal mucosa of ulcerative colitis patients

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Received: March 7, 2011 Revised: June 12, 2011
Accepted: June 19, 2011
Published online: January 14, 2012

Abstract

AIM: To assess B1a cell expression in the rectal mucosa of ulcerative colitis (UC) patients in comparison with healthy controls.

METHODS: Rectal mucosa biopsies were collected from 15 UC patients and 17 healthy controls. $CD5^+$ B cells were analysed by three colour flow cytometry from rectal mucosal samples after mechanical disaggregation by Medimachine®. Immunohistochemical analysis of B and T lymphocytes was also performed. Correlations between, on the one hand, rectal B1a cell concentrations and, on the other, erythrocyte sedimentation rate and C-reactive protein levels and clinical, endoscopic and histological disease activity indices were evaluated.

RESULTS: Rectal B-lymphocyte ($CD19^+/CD45^+$) rate and concentration were higher in UC patients compared with those in healthy controls (47.85% ± 3.12% *vs* 26.10% ± 3.40%, $P = 0.001$ and 501 ± 91 cells/mm^2 *vs* 117 ± 18 cells/mm^2, $P < 0.001$); Rectal B1a cell density ($CD5^+CD19^+$) was higher in UC patients than in healthy controls (85 ± 15 cells/mm^2 *vs* 31 ± 6.7 cells/mm^2, $P = 0.009$). Rectal B1a cell ($CD5/CD19^+$) rate correlated inversely with endoscopic classification (Rs = -0.637, $P < 0.05$).

CONCLUSION: B1a lymphocytes seem to be involved in the pathogenesis of UC, however, the role they play in its early phases and in disease activity, have yet to be defined.

Key words: B1 cell; CD5; Flow cytometry; Rectum; Ulcerative colitis

Peer reviewers: Angelo Zullo, MD, Gastroenterologia ed Endoscopia Digestiva, Ospedale Nuovo Regina Margherita, Via E. Morosini, 30, 00153 Roma, Italia; Stephen M Kavic, MD, FACS, Assistant Professor of Surgery, Department of Surgery, University of Maryland School of Medicine, 22 South Greene Street, Room S4B09, Baltimore, MD 21201, United States

Polese L, Boetto R, De Franchis G, Angriman I, Porzionato A, Norberto L, Sturniolo GC, Macchi V, De Caro R, Merigliano S. B1a lymphocytes in the rectal mucosa of ulcerative colitis patients. *World J Gastroenterol* 2012; 18(2): 144-149 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i2/144.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i2.144

INTRODUCTION

The aetiology of ulcerative colitis (UC) is still unknown, but several studies have demonstrated that there is an abnormal immunologic response to gut antigens[1,2]. One

of the mechanisms which protects the body against intestinal luminal antigens before a specific aggressive inflammatory response is unleashed, is the production of natural antibodies, in particular immunoglobulin A (IgA)[3,4]. A large proportion of IgA is produced in a T-independent way by B cells and in particular, according to several studies, by the B1 sub-population. The majority of B1 cells, also called B1a, express CD5 on their surface. In a previous study[5] we reported that B1a cell ($CD5^+CD19^+$) blood concentrations were reduced in UC patients with respect to those in healthy controls, and that the B1a cell rate was inversely correlated with erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels in UC patients. The aim of the present study was to analyse the rate and concentration of B1a cells in the rectal mucosa of UC patients, to compare these values with those found in healthy controls, and to assess any possible correlation with disease activity.

MATERIALS AND METHODS

Patients

The study population consisted of 15 UC patients and 17 healthy controls. The study protocol was drafted in accordance with the Declaration of Helsinki and all of the patients and controls who participated signed informed consent statements. Patients taking immunosuppressive drugs or corticosteroids were excluded from the study.

The disease activity of the UC patients, undergoing ordinary follow-up colonoscopy, was classified in accordance with the Seo clinical score[6], the modified endoscopic Baron classification[7] and Geboes' histological scoring[8]. Subjects who underwent colonoscopy as a cancer screening procedure and whose result was negative, were enrolled as healthy controls.

Methods

Blood samples and 6 rectal mucosal biopsy specimens, taken 10-15 cm from the anal verge during colonoscopy, were collected from each patient.

Flow-cytometry

A cellular suspension, obtained by fragmentizing 4 rectal biopsies from each patient using a Medimachine (Consul TS, Rivalta di Torino, Italy), was used for flow cytometry. The method utilized was as follows: the collected rectal biopsies were placed in physiologic solution (Na 0.9%) and immediately processed. The biopsies were first washed twice with physiologic solution for 2 min each wash. Each biopsy was minced into < 1 mm^3 pieces which were placed in a sterile microblade-equipped polyethylene chamber (Medicons, BD Biosciences, San Jose, CA, United States) with 1 mL phosphate buffered saline (PBS) 0.01 mol/L. The Medicons contain an immobile stainless steel screen with 100 hexagonal holes, each surrounded by six microblades. When the Medicons are inserted into the Medimachine the tissue comes into contact with the blades by means of a rotating element

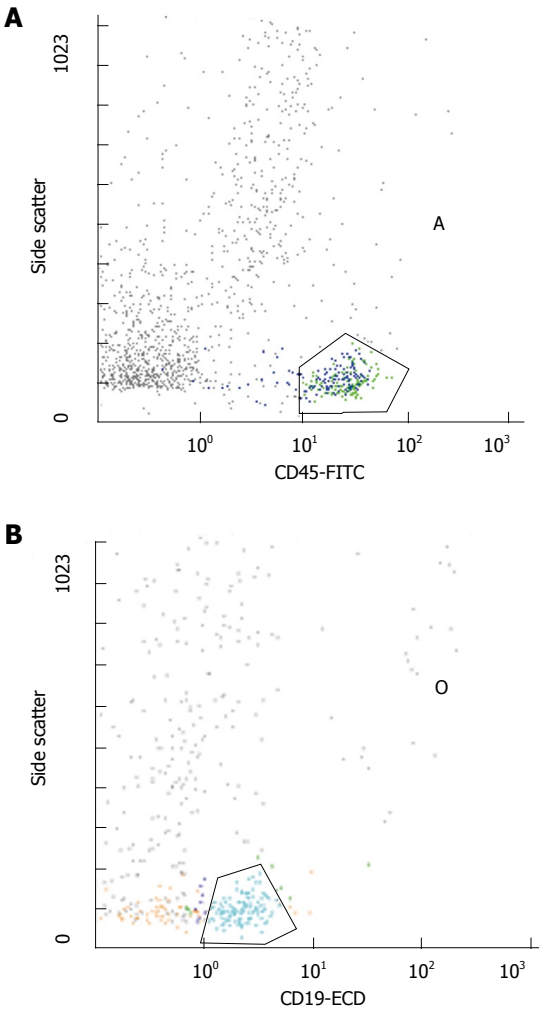

Figure 1 Flow cytometry. A: Immunologic gate for rectal $CD45^+$ cells; B: Immunologic gate for rectal $CD19^+$ cells.

and is disaggregated. A micropump positioned under the screen forces the liquid to pass through the bore-holes, ensuring that the bore-holes remain clean. Medicons with 35 μm separator screens were used.

Fragments were dissociated 4 times for 20 s at a constant speed of 100 r/min. The suspension was filtered using filters with 30 μm diameter holes (Flicons, BD Bioscences, San Jose, CA, United States) and then analysed by flow-cytometry (Figures 1 and 2).

A three-colour flow-cytometric analysis was performed using the following associations: CD45 fluorescein isothiocyanate (FITC), Isotype IgG1 (clone J.33, mouse) (Beckman Coulter Inc, Fullerton, CA, United States), CD5 RD-1 (phycoerythrin), Isotype IgG2a (clone SFCI24T6G12, mouse) (Beckman Coulter Inc.), CD19 ECD (Texas red), Isotype IgG1 (clone J4.119, mouse) (Beckman Coulter Inc.). Flow cytometry was performed as previously described[5].

All mAbs were used at optimal saturating concentrations as recommended by the manufacturers. The cells were washed in a FACS buffer containing PBS/5% FCS/0.05% and sodium azide, then incubated with 10 mg of human

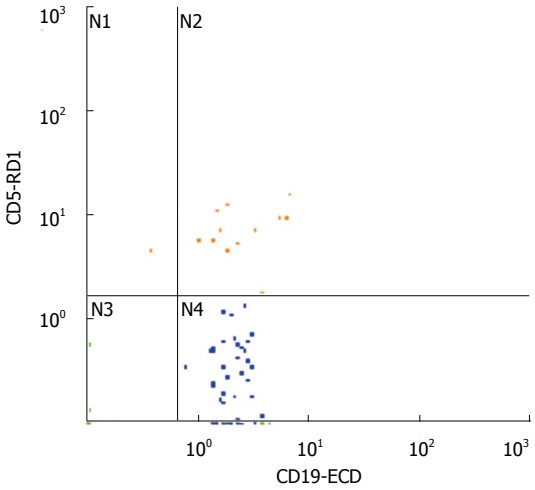

Figure 2 Flow cytometry. $CD5^+/CD19^+$ lymphocytes (B1a cells).

IgG (Sigma Chemical Co., St. Louis, MO, United States) for 30 min at 4 ℃-8 ℃ to block Fc receptors. Cells were washed to remove excess IgG and were triple-stained with either RD-1-conjugated mAb against CD5, or RD-1-control IgG mAb, ECD-conjugated mAb against CD19 or ECD-control IgG mAb and FITC-conjugated mAb against CD45 or FITC-control IgG mAb for 30 min at 4 ℃-8 ℃. Cells were washed twice, re-suspended in a FACS buffer and fixed with 1% paraformaldehyde. At that point, 2 mL of lysing solution (0.17 mol/L NH_4Cl) were added and following 15 min of incubation, samples were analyzed by flow cytometry using a Coulter EPICS XL-MCL (Beckman Coulter Inc). Mononuclear cells were gated depending on their CD45 expression characteristics. Different subsets of cells (CD19) were then gated on the basis of fluorescence 1 and fluorescence 2 staining. An immunologic gate was performed on $CD19^+$ cells to uncover CD5. Isotypic controls were used for all the samples. A two-colour flow cytometric analysis was similarly performed to study the T cells using anti-CD45 FITC mAb (Isotype IgG1, mouse, clone J.33, Beckman Coulter Inc.) and anti-CD3 Phycoerythrin-Cyanin 5 (Pc5) mAb (Isotype IgG1, mouse, clone UCHT 1, Beckman Coulter Inc.).

Immunohistochemistry

Two rectal biopsies from each patient studied underwent immunohistochemical analysis. Samples were fixed in 10% neutral buffered formalin, processed for embedding in paraffin wax, and cut into 5 μm-thick sections. Sections were dewaxed and rehydrated by routine protocols. For anti-CD3 immunohistochemistry, antigen unmasking was performed with 10 mmol/L sodium citrate buffer, pH 6.0, in a microwave oven at 96 ℃ for 30 min. Antigen unmasking was not necessary for anti-CD20 analysis. Sections were incubated in 0.3% hydrogen peroxide for 10 min at room temperature to remove endogenous peroxidase activity, and then in blocking serum for 30 min. Samples were incubated with primary antibodies at room temperature for 45 min. The primary antibodies used were: anti-CD3 (Polyclonal rabbit anti-Human, Dako, Milan, Italy) and anti-CD20 (Monoclonal mouse anti-human CD20cy, Dako) diluted 1:50 and 1:200, respectively. Sections were then washed three times in PBS for 5 min each wash, treated with secondary antibody (Envision System HRP, Dako) for 30 min at room temperature and developed in 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, Milan, Italy). Finally, the sections were counterstained with hematoxylin.

Sections were examined using a Leica DM4500B microscope (Leica Microsystems, Wetzlar, Germany) connected to a Leica DFC320 high-resolution digital camera (Leica Microsystems) and a computer equipped with software for image acquisition and analysis (QWin, Leica Microsystems). The densities of CD3- and CD20-positive cells were evaluated at a magnification of 40 ×, and 10 fields per section were examined. The densities were calculated for each section by dividing the number of positive cells by the area of the rectal mucosa analysed.

Table 1 Comparison of rectal B and T lymphocyte populations and B1 subpopulations in ulcerative colitis patients and controls

	Ulcerative colitis	Controls
$CD19^+/CD45^+$ (%)	47.8 ± 3.1[b]	26.1 ± 3.4
$CD20^+$ (cells/mm^2)	501 ± 91[b]	117 ± 18
$CD3^+/CD45^+$ (%)	53.5 ± 4.2[a]	68.3 ± 3.5
$CD3^+$ (cells/mm^2)	485 ± 100	445 ± 95
$CD5^+/CD19^+$ (%)	15.5 ± 2.0	23.5 ± 4.9
$CD5^+CD19^+$ (cells/mm^2)	85 ± 15[b]	31 ± 6.7

[a]$P < 0.05$, [b]$P < 0.01$ *vs* controls.

Calculation of rectal mucosa B1a cell concentration

The rectal mucosa B1a cell concentration ($CD5^+CD19^+$) was calculated by multiplying the $CD5^+/CD19^+$ ratio, obtained by flow-cytometry, by the B lymphocyte concentration, obtained by immunohistochemistry.

ESR and CRP analysis from blood test

ESR and CRP were measured using the Westergren method and immuno-nephelometry, respectively.

Statistical analysis

Results are expressed as mean ± SE. Statistical analysis was performed using Mann-Whitney *U* test for the comparison between the UC patients and controls and by Spearman's Rank test for correlations. Statistical significance was set at $P < 0.05$.

RESULTS

Adequate material for flow-cytometry was obtained from 13/15 UC patients (8 males and 5 females, median age 54 years, range 19-71 years) and from 13/17 controls (8 males and 5 females, median age 61 years, range 37-88 years). Of the 13 UC patients included in the study, 5 were taking mesalazine and 8 were not.

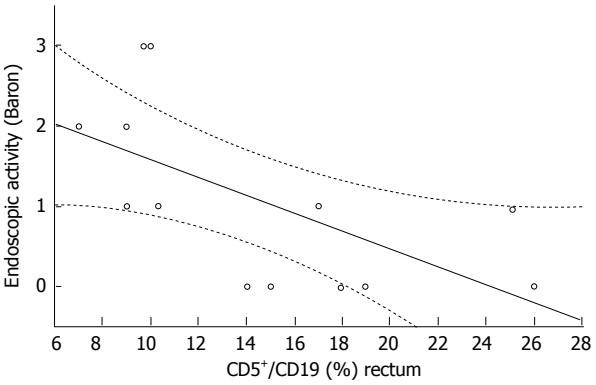

Figure 3 Correlation between B1a cells percentage in ulcerative colitis patients and endoscopic disease activity (*Rs* = -0.68, *P* = 0.01).

Ulcerative colitis was clinically active (Seo score > 150) in 5 patients and endoscopically active (Baron score > 1) in 4. The median histologic activity score was 3 (range 0-5).

Flow cytometry

The percentage of B lymphocytes ($CD19^+/CD45^+$) in the rectal mucosa was higher in UC patients with respect to healthy controls (47.8% ± 3.1% *vs* 26.1% ± 3.4%, *P* = 0.001); while the percentage of rectal T lymphocytes ($CD3^+/CD45^+$) was significantly lower in UC patients with respect to the controls (53.5% ± 4.2% *vs* 68.3% ± 3.5%, *P* = 0.02).

The rectal B1a cell rate ($CD5^+/CD19^+$) did not differ significantly in the two groups (Table 1), and was inversely correlated with endoscopic activity (Rs = -0.68, *P* = 0.01, Figure 3), but not with the clinical SEO disease activity index, ESR and CRP levels, or with age. The mean rectal B1a cell rate was higher, but not significantly different in patients with remission or mild histologic activity (score 0-1), with respect to patients with moderate-severe histologic activity (score 2-5) (22.0% ± 3.0% and 12.7% ± 2.5%, respectively, *P* = 0.1). The rectal B1a cell rate was not significantly different in the patient group taking mesalazine compared with those not taking mesalazine (11.0 ± 2.1 and 17.2 ± 3.0, respectively, *P* = 0.13).

Immunohistochemistry

Histological analysis confirmed that there was an increased concentration of B lymphocytes CD20+ in the rectal mucosa of ulcerative colitis patients with respect to that in controls (cell density 501 ± 91 cells/mm^2 *vs* 117 ± 18 cells/mm^2, *P* < 0.001). T cell density was not significantly different in the UC patients and controls (485 ± 100 *vs* 445 ± 95, *P* = 0.6).

Calculated rectal B1a cell concentration

The calculated B1a cell density was significantly increased in UC patients with respect to that in controls: 85 ± 15 cells/mm^2 *vs* 31 ± 6.7 cells/mm^2, *P* = 0.009.

DISCUSSION

More than 80% of the body's activated B cells are located in the gut, where a continuous interaction takes place between the immune system and the trillion bacteria that reside there[9].

IgA generation by B cells is an important mechanism that regulates this homeostasis, contributing to immune protection but without provoking inflammation. A large proportion of the intestinal IgA against cell wall antigens and proteins of commensal bacteria is specifically induced in response to their presence within the microflora, but is independent of T cells or germinal centre formation. This T cell-independent IgA production is derived from B1 lymphocytes which develop in the peritoneal compartment and are distributed diffusely in the intestinal lamina propria[10]. In mice, peritoneal B cells (B1 cells) do not differentiate during migration through the lymphoid organs and finally home to the gut lamina propria where they switch and differentiate to IgA+ plasma cells[11]. The physiological importance of B1 cells in the maintenance of homeostasis at the mucosal surface has been clearly demonstrated[12].

B cells in inflammatory bowel disease have not been as extensively studied as T cells[13], and data on the role of B1 cells in UC are particularly scanty. Except for a smaller sub-group called B1b, B1 cells are distinguishable from B2 cells by expressing CD5 on their surface[14]. Even in the absence of external antigen stimulation, B1 cells produce natural antibodies (Ab) that provide early, broad protection against pathogens[4]. B1 cells are also known to produce auto-reactive Ab, including Ab to cell membrane components, such as phosphorylcholine[15] and phosphatidylcholine[16] to immunoglobulins (rheumatoid factor) and to single-stranded DNA[17].

B1 cells were thus analysed for their role in autoimmunity and high circulating B1a lymphocyte levels have been reported in some autoimmune diseases, such as systemic lupus erythematosus, primary Sjogren's syndrome[18], rheumatoid arthritis[19], multiple sclerosis[20] and anti-phospholipid syndrome[21]. In the light of recent findings, these cells, and in particular the CD5 molecule on B cells, seem to play a role in preventing autoimmunity[22].

In a previous study we reported that B1a cell ($CD5^+/CD19^+$) concentrations are reduced in the blood of patients with UC even after restorative proctocolectomy[5]. Moreover, B1a cell rate was found to be inversely correlated with ESR and CRP levels in UC patients, indicating that these cells play a protective role against inflammation. Low $CD5^+/CD19^+$ blood percentages in UC and in Crohn's disease (CD) have also been reported by other authors[23,24].

The present study focused on the presence of B lymphocytes, and in particular the B1a sub-group, in the rectal mucosa of UC patients. An increased concentration of B lymphocytes was found in the rectal mucosa of these patients and their percentage within the leukocyte pool was also increased. B1a cell concentrations

in the rectal mucosa of UC patients were significantly higher than those in healthy controls, but not their percentage within the whole B lymphocyte pool ($CD5^+$/$CD19^+$). Senju *et al*[25], who analysed subsets of lamina propria lymphocytes using two-colour flow cytometry, also found no differences in the percentage of $CD5^+$ B lymphocytes in UC, CD patients and controls. They reported that the majority of B cells in the intestinal mucosa did not possess CD5 antigens on their cell surface. In the present study, performed using three colour flow cytometry, we found that the percentage of $CD5^+$ B cells in the rectum was small, but not negligible (between 15% and 23%). The use of anti-CD45 helps to restrict the flow cytometric analysis to leukocytes, excluding from the study the other types of cells homing in the rectal mucosa.

Finding an increased concentration of B1a cells, but not a higher percentage within the whole B lymphocyte population, means that other B lymphocyte subsets were increased in the rectal mucosa of these patients. In addition, finding an inverse correlation between $CD5^+$ B cells and endoscopic disease activity suggests that these cells are recruited to a relatively lower extent when the disease is more severe and possibly, at that point, a more specific immune reaction has already begun. As our findings seem to indicate that there is a loss of tolerance at this disease stage, further studies will clarify this point. The finding of an inverse correlation between rectal mucosa B1a cell rate and endoscopic disease activity is consistent with previous data concerning the inverse correlation of circulating B1a cell rate and blood ESR and CRP levels[5]. The former is a sign of local events, and the latter of a systemic imbalance.

Peterson *et al*[26] reported that the depletion of $CD5^+$ B1 cells has different effects on the induction phase with respect to the effector phase of experimental autoimmune encephalomyelitis. During the induction phase it increases disease incidence, while in the effector phase it reduces disease severity. The role of $CD5^+$ B cells in UC is not yet clear. It remains to be clarified if they play a protective role by producing polyspecific immunoglobulins, an unleashing role by producing autoreactive Ab, or if there is an even more complex modulating role that differs depending on the disease activity. The findings in this and other studies seem to indicate that their role in UC is not marginal, but more in-depth analyses are warranted to better define their function and to determine if and how their modulation has an impact on disease activity.

ACKNOWLEDGMENTS

The authors are grateful to Linda Inverso Moretti for her assistance in editing the English version of this manuscript.

COMMENTS

Background

The aetiology of ulcerative colitis (UC) is still unknown, but several studies have demonstrated that there is an abnormal immunologic response to gut antigens.

Research frontiers

B1 lymphocytes are important in maintaining mucosal surfaces in a state of homeostasis, and it has been found that B1 cell concentrations are reduced in the blood of patients with UC even after restorative proctocolectomy. In the present study, B1a cell expression was assessed in the rectal mucosa of UC patients and compared with that in healthy controls using three color flow-cytometry.

Innovations and breakthroughs

Rectal B1a cell density ($CD5^+CD19^+$) was increased in ulcerative colitis patients compared with healthy controls, but its rate correlated inversely with endoscopic disease score.

Applications

The findings in this and other studies seem to indicate that B1a lymphocytes play a role in the pathogenesis of ulcerative colitis and their modulation could have an impact on the control of disease activity.

Terminology

B1 lymphocytes are distinguishable from B2 lymphocytes because they express CD5 on their surface. Even in the absence of external antigen stimulation, B1 cells produce natural antibodies that provide early, broad protection against pathogens. B1 cells are also known to produce auto-reactive antibodies. CD5 is a 67 kD trans-membrane glycoprotein that interacts in the B lymphocyte with the B cell receptor, negatively regulating growth signaling.

Peer review

This study assessed B1a cell concentration in rectal mucosa of 13 UC patients as compared to controls. A possible correlation between these cells and either endoscopic (modified Baron classification) or clinical (Seo clinical score) activity index was also investigated. Data found that B1a cell concentration is increased in UC, but its distribution (i.e., percentage within leucocytes) did not differ between patients and controls. Moreover, a significant, inverse correlation between B1a cell concentration in rectal mucosa and endoscopic activity index was found.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i2.150

World J Gastroenterol 2012 January 14; 18(2): 150-155
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Human epidermal growth factor receptor-2 gene amplification in gastric cancer using tissue microarray technology

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Received: February 19, 2011 Revised: July 6, 2011
Accepted: July 13, 2011
Published online: January 14, 2012

Abstract

AIM: To assess human epidermal growth factor receptor-2 (HER2)-status in gastric cancer and matched lymph node metastases by immunohistochemistry (IHC) and chromogenic *in situ* hybridization (CISH).

METHODS: 120 cases of primary gastric carcinomas and 45 matched lymph node metastases from patients with full clinicopathological features were mounted onto multiple-punch and single-punch tissue microarrays, respectively, and examined for HER2 overexpression and gene amplification by IHC and CISH.

RESULTS: Twenty-four tumors (20%) expressed HER2 immunohistochemically. An IHC score of $\geqslant$ 2+ was observed in 20 tumors (16.6%). HER2 amplification was detected by CISH in 19 tumors (15.8%) and in their matched lymph node metastases. A high concordance rate was found between HER2 positivity (as detected by IHC) and *HER2* gene amplification (as detected by CISH), since 19 of the 20 IHC positive cases were amplified (95%). All amplified cases had 2+ or 3+ IHC results. Amplification was associated with intestinal phenotype ($P < 0.05$). No association with grading, staging or survival was found.

CONCLUSION: In gastric cancer, HER2 amplification is the main mechanism for HER2 protein overexpression and is preserved in lymph node metastases.

Key words: Human epidermal growth factor receptor-2; Immunohistochemistry; Chromogenic *in situ* hybridization; Gastric cancer

Peer reviewer: Dr. Thomas Wex, PD, Clinic of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University Magdeburg, Leipziger Str. 44, Magdeburg 39120, Germany

Tsapralis D, Panayiotides I, Peros G, Liakakos T, Karamitopoulou E. Human epidermal growth factor receptor-2 gene amplification in gastric cancer using tissue microarray technology. *World J Gastroenterol* 2012; 18(2): 150-155 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i2/150.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i2.150

INTRODUCTION

Alterations of the human epidermal growth factor receptor-2 (*HER2*) gene are implicated in the development and progression of many tumors[1-4]. In breast cancer, HER2 amplification has been found in about 20% of cases and was linked to poor prognosis[5,6]. Breast cancer patients with HER2 amplification have been effectively treated with the monoclonal antibody trastuzumab, a HER2 inhibitor[7-10]. Recently, a number of studies have suggested a response to trastuzumab therapy for other

cancers with HER2 amplification, including germ cell, endometrium and salivary duct carcinoma[11-13].

In gastric cancer, HER2 amplification has been found in 7% to 27% of tumors[14-19]. Reports of trastuzumab therapy in metastatic gastric cancer showed complete tumor regression and disappearance of the metastases in two cases[20,21]. A phase Ⅲ randomized study (Trastuzumab for HER2-positive metastatic gastric cancer) in patients with inoperable, metastasizing and/or recurring gastric cancer with HER2 overexpression or *HER2* gene amplification, documented that 47.3% of the patients who received trastuzumab, along with their chemotherapy, showed a significant regression of the primary tumor and/or the metastases. Moreover, trastuzumab caused a prolongation of the median survival time by 2.4 mo in all patients[22]. Based on these reports, gastric cancer patients with HER2 overexpression and/or amplification could be good candidates for trastuzumab therapy.

HER2 testing can be performed either by immunohistochemical evaluation of protein expression or by evaluating the gene copy number by *in situ* hybridization, most commonly using fluorescence *in situ* hybridization (FISH). However, while immunohistochemistry (IHC) is a relatively inexpensive, easy to perform method for most pathology laboratories, FISH is technically demanding, expensive and requires special equipment[23-25]. An alternative method, chromogenic *in situ* hybridization (CISH), is a combination of *in situ* hybridization with a detection system using a chromogen similar to IHC. Slides are visible under a light microscope and show correlation with morphology. A number of studies compared HER2 testing with IHC, FISH and CISH in breast carcinoma and have shown good correlation between CISH and FISH results[25-30].

We evaluated HER2 overexpression and gene amplification by IHC and CISH, respectively, in 120 cases of gastric carcinoma patients and 45 matched lymph node metastases mounted onto multiple-punch and single-punch tissue microarrays respectively. Our data suggests that, in gastric cancer, HER2 amplification is the main mechanism for HER2 protein overexpression and is preserved in lymph node metastases.

MATERIALS AND METHODS

Patients

The current study involved 120 non-consecutive patients with gastric carcinoma, surgically treated at the 3rd and 4th Departments of Surgery, University of Athens, between 2004 and 2007. Histomorphological data were reviewed from the corresponding hematoxylin and eosin stained slides. Clinical data were obtained from corresponding reports. Clinicopathological information included: gender, age, tumor diameter, histological subtype, tumor location, pT stage, pN stage, pM stage, vascular and lymphatic invasion, survival time, and information on post-operative therapy. Characteristics of patients are summarized in Table 1.

Table 1 Characteristics of patients with gastric cancer

Clinicopathological feature		Frequency *n* (%)
Patient age at diagnosis (yr)	Mean 69.6, min-max 27-96	
Tumor diameter (cm)	Mean 4.6, min-max 1.3-12	
Gender	Male	84 (70)
	Female	36 (30)
Histological type	Intestinal	80 (66.66)
	Diffuse	24 (20)
	Mixed	16 (13.33)
Tumor location	Cardia	37 (30.8)
	Corpus	39 (32.5)
	Antrum	44 (36.66)
pT stage	pT1	15 (12.5)
	pT2	65 (54.16)
	pT3-4	40 (33.33)
pN stage	pN0	36 (30)
	pN1	43 (35.8)
	pN2+3	41 (34.2)
pM stage	pM0	105 (87.5)
	pM1	15 (12.5)
Tumor grade	G1-2	42 (35)
	G3	78 (65)
Venous invasion	Present	37 (30.8)
	Absent	83 (69.2)
Lymphatic invasion	Present	84 (70)
	Absent	36 (30)
Adjuvant therapy	None	32 (26.6)
	Treated	88 (73.4)
	Chemotherapy	55 (62.5)
	Chemo/Radiotherapy	33 (37.5)
5-year survival (%)	(95% CI)	38.9 (25-52)

Specimen characteristics

Paraffin-embedded tissue blocks of primary tumors and matched positive lymph nodes were retrieved from the Department of Pathology, University of Athens. The use of this material was approved by the local Ethics committee. Two tissue microarrays (TMAs) were constructed. The first included punches from primary tumors. In order to exclude bias due to possible tumor heterogeneity, each patient had multiple tumor punches taken from formalin-fixed, paraffin-embedded blocks using a tissue cylinder with a diameter of 1 mm, which were subsequently transferred into one recipient paraffin block (3 cm × 2.5 cm) using a semiautomated tissue arrayer. Each patient had on average 5.1 tissue punches included on this array, including at least 4 tumor punches. The second TMA included single punches from matched metastatic lymph nodes in 45 patients.

Assay methods

IHC: Five μm TMA sections were dewaxed and rehydrated in distilled water. Endogenous peroxidase was blocked using 0.5% H_2O_2. To determine the HER2 expression immunohistochemically, the HercepTestTM (Dako, Glostrup, Denmark) was used according to the manufacturer`s protocol. Following pressure cooker-mediated antigen retrieval sections were incubated with the prediluted primary antibody. Control samples included normal gastric mucosa and breast cancer tissue. Immunostaining was scored by an experienced gastrointestinal pathologist following a 4-step

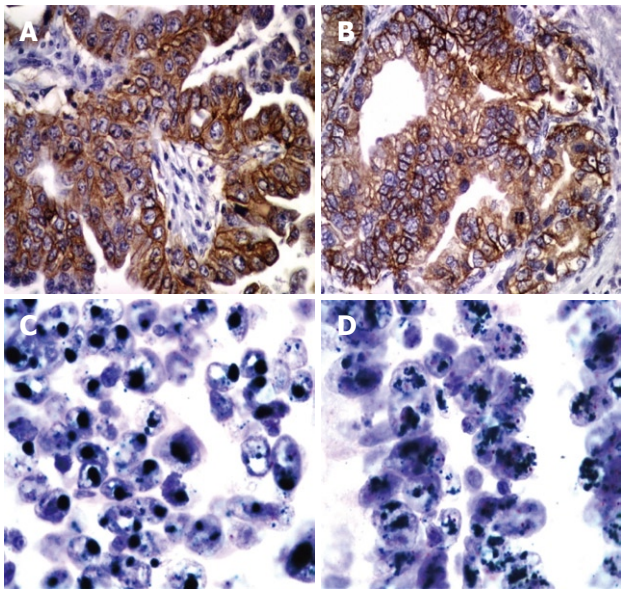

Figure 1 Examples of human epidermal growth factor receptor-2 immunohistochemical expression and amplification in primary and metastatic gastric cancer. Immunohistochemistry shows strong membranous staining of human epidermal growth factor receptor-2 (HER2) in intestinal type gastric cancer (A) and (B) (× 400). Chromogenic in situ hybridization assay shows amplification of HER2 in primary gastric cancer (C) and the corresponding lymph node metastasis (D). Clustered green signals represent the amplified *HER2* gene, while red signals represent centromere 17. Cell nuclei are counterstained with hematoxylin (× 1000).

score (0, 1+, 2+, 3+), according to the consensus panel recommendations on HER2 scoring for gastric cancer[31].

CISH: HER2 CISH was performed using a CISH HER2 probe and Immunodetection Kit (ZytoDot2C SPEC HER2/CEN 17 Probe Kit). TMA sections were deparaffinized and incubated for 5 min in 3% H_2O_2, followed by Heat Pretreatment Solution EDTA in a covered staining jar standing in a boiling water bath at 98 ℃ for 15 min. After washing in distilled water, Pepsin Solution (ES1) was applied and slides were incubated for 5 min at room temperature in a humidity chamber. Sections were then washed in distilled water, dehydrated in increasing ethanol, and air dried. ZytoDot2C SPEC HER2/CEN 17 Probe was applied and sections were covered with a coverslip sealed with a layer of hot glue. Samples were then denaturated at 80 ℃ for 5 min, transferred to a humidity chamber and left to hybridize overnight at 37 ℃. On day 2, immunodetection was performed according to the manufacturer's instructions and sections were counterstained with Hematoxylin and mounted.

Statistical analysis

χ^2 tests and contingency tables were used to analyze the relationship between IHC and CISH, and categorical parameters. Overall survival was estimated by the Kaplan-Meier method and evaluated by log-rank testing. All analysis were carried out using SAS (V9, The SAS Institute, NC, United States).

Table 2 Lauren phenotype, human epidermal growth factor receptor-2 immunohistochemistry and chromogenic *in situ* hybridization in gastric carcinoma

		Diffuse (n = 24)	Mixed (n = 16)	Intestinal (n = 80)
HER2 IHC	0	23	14	59
	1+	0	0	4
	2+	1	0	5
	3+	0	2	12
HER2 CISH	Non amplified	24	14	63
	Amplified	0	2	17

HER2: Human epidermal growth factor receptor-2; IHC: Immunohistochemistry; CISH: Chromogenic *in situ* hybridization.

RESULTS

HER2 immunohistochemistry

HER2 protein expression was observed in 24 of the 120 gastric carcinomas (20%). In more detail, one of the 24 diffuse type carcinomas (4.16%) and 23 of the 96 intestinal and mixed type carcinomas (23.95%) showed HER2 protein expression. Immunostaining was always membrane bound and showed basolateral predominance (Figure 1A and B). Immunostaining in mixed type carcinomas was restricted in the intestinal type component. Quantitative analysis of the immunostaining, according to the consensus panel recommendations on HER2 scoring for gastric cancer, resulted in fourteen 3+ cases (11.66%), six 2+ cases (5%) and four 1+ cases (3.33%)[31] (Table 2). IHC was interpretable in 652 of the 660 spots (98.8%). Reasons for non-interpretable results were missing tissue spots or absence of tumor tissue.

HER2 CISH

Tissue spots were scanned for possible intratumoral heterogeneity by using a 10× objective lens. CISH hybridization signals of the *HER2* gene appeared as dark green-colored dot-shaped signals. The chromosome 17 centromeric regions appeared as bright red-colored dot-shaped signals. Areas of necrosis and overlapping nuclei were avoided. Signal enumeration was performed using the 40× objective lens of a light microscope. HER2 amplification was observed in 19 of the 120 primary gastric carcinomas (15.8%). Amplified cases showed intratumoral heterogeneity with areas of low amplification where HER2 signals appeared as multiple dots or small clusters, and areas of high amplification with presence of large, green *HER2* gene signal clusters (Figure 1C and D). All amplified cases had 2+ or 3+ IHC results (Table 2). HER2 amplification showed significant association with histologic tumor type. Seventeen (21.25%) of the 80 intestinal and two (12.5%) of the 16 mixed type cancers, but 0 (0%) of the diffuse type cancers, were amplified ($P < 0.002$, Table 2). No association between *HER2* gene amplification and tumor grade, size, stage or localization was found.

Table 3 Comparison of human epidermal growth factor receptor-2 immunohistochemistry and chromogenic *in situ* hybridization in primary gastric carcinomas and matched lymph node metastases

	HER2 IHC			HER2 CISH		
	PT	LNM	Concordance	PT	LNM	Concordance
Positive	7	6	85.7%	6	6	100%
Negative	38	39	97.4%	39	39	100%

HER2: Human epidermal growth factor receptor-2; IHC: Immunohistochemistry; CISH: Chromogenic *in situ* hybridization; PT: Primary gastric cancer; LNM: Lymph node metastases.

HER2 alterations in lymph node metastases

Comparative analysis of primary tumors and corresponding lymph node metastases, performed in 45 cases, showed a high concordance in the presence of HER2 overexpression or amplification ($P < 0.0001$, Table 3).

Clinicopathological characteristics

Increasing tumor grade and stage were associated with reduced patient survival ($P < 0.0001$ each). No correlation was observed between patient survival and HER2 overexpression or amplification, even after including postoperative therapy of the patients and location of the tumors in the analysis (Figure 2).

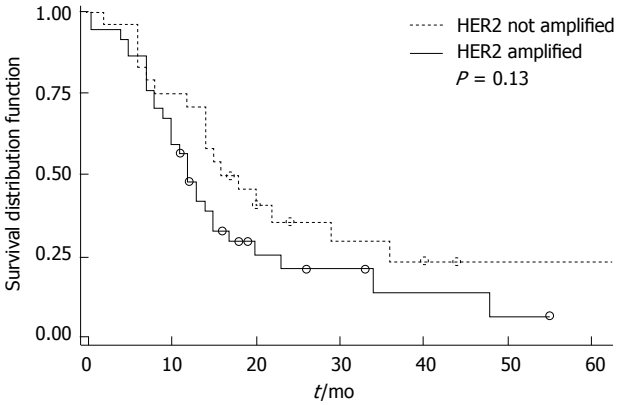

Figure 2 Kaplan-Meier curve for disease-specific survival and human epidermal growth factor receptor-2 amplification in gastric carcinomas. HER2: Human epidermal growth factor receptor-2.

DISCUSSION

The HER2 protein is a frequently analyzed gene product, especially in breast cancer. Recent studies have examined HER2 expression in other tumor types, including gastric cancer[18,32,33]. Immunohistochemical HER2 expression and protein positivity (≥ 2+) was observed in 20% and 14.58%, respectively, of the 120 gastric carcinomas in our study. These results are in keeping with previous reports demonstrating similar frequencies of HER2 overexpression in gastric cancer[18,32,33]. HER2 positivity was observed in 23% of the cases in the study by Yano *et al*[18] and in 22.6% of the cases in the study by Kim *et al*[33]. More recently, a TMA study of 166 gastric carcinomas by Marx *et al*[32] found a HER2 positivity rate of 17% and a strong correlation between IHC and *HER2* gene amplification detected by FISH. In our cases, comparable to others, HER2 overexpression and/or amplification were almost exclusively found in gastric cancers of the intestinal type. This finding supports the presence of different molecular characteristics between the main histologic tumor types that seem to develop through different molecular pathways.

No correlation between HER2 overexpression and/or amplification and tumor localization could be demonstrated in our study. This might be contradictory to previous studies where a high frequency of HER2 expression was reported in cardia carcinomas[34]. However, adenocarcinomas of the gastroesophageal junction, many of which are Barrett carcinomas, are known to have a high rate of HER2 amplification and cannot always be differentiated from cardia carcinomas. This may lead to an artificial increase in the rate of HER2 expression reported in cardia carcinomas.

A high concordance rate was found between HER2 positivity, as detected by IHC, and *HER2* gene amplification, as detected by CISH, since 19 of the 20 IHC positive cases (i.e., ≥ 2+) were amplified (95%). Many of them (73.6%) had high *HER2* gene amplification. However, one HER2 2+ case, detected in a diffuse carcinoma, was not found to be amplified, which may be attributed to a technical error of IHC associated with formalin fixation of the tissue. Alternatively, other mechanisms of HER2 protein overexpression can contribute to inconsistencies between IHC and ISH results.

In the present study we found a high concordance rate of HER2 status between primary tumors and their corresponding lymph node metastases. This finding is in keeping with previous published results, where HER2 amplification status was found to be almost identical in the primary gastric carcinomas and their corresponding lymph node metastases[32] and provides further evidence for the role of HER2 amplification in gastric cancer.

Our findings support gastric cancer HER2 amplification as being the main mechanism that leads to HER2 protein overexpression. Similar findings have previously been reported for esophageal cancer[35]. Moreover, Tapia *et al*[19], in a large-scale TMA study, examined more than 4000 samples from 120 different tumors and could not find any tumors with HER2 overexpression in the absence of gene amplification.

HER2 testing has developed over a number of years, and many retrospective studies using formalin-fixed, paraffin-embedded material and different methodologies have provided inconsistent results[36,37]. Correlation between technical methods can be used to obtain a high concordance rate and to better define the assays with the best ability to identify patient groups that would benefit from a targeted therapy. Although in breast cancer HER2 IHC is an acceptable method of predicting HER2 status, previous studies have shown marked variation in different diagnostic laboratories regarding the interpretation of positive staining[25]. On the other hand, FISH is

technically demanding, expensive and does not produce a permanent archival slide. In our study, CISH testing showed a good correlation to IHC, and therefore seems to represent a reliable methodology for HER2 testing. Moreover, the slides are visible under a light microscope and show good correlation with tumor morphology.

In this study, we used the tissue microarray technique using multiple tissue punches per case to account for possible heterogeneity in terms of protein expression or gene amplification in the primary tumor. Each patient had an average of 4 tumor punches taken. This is particularly important for HER2 testing in gastric cancer since considerable heterogeneity concerning gene amplification has been reported in many studies[38,39]. Such heterogeneity was also noted in our study, since in many amplified cases, areas with low and high amplification were found within the same tumor. Multiple sampling thus helped to minimize possible biases in evaluation, as suggested by Goethals *et al*[40], who recommended that at least four punches of primary tumor are required to account for possible heterogeneity. However, a single tissue punch was sampled in the case of lymph nodes since the issue of heterogeneity may be substantially less important. Our study also benefits from complete clinicopathological and follow-up characterization of patients. In contrast to previous studies[34,41] we could not demonstrate any association between HER2 positivity and clinical outcome.

Our study provides evidence supporting gastric cancer HER2 amplification as being the main mechanism for HER2 protein overexpression and that HER2 amplification is preserved in the lymph node metastases. Therefore, gastric cancer patients with HER2 overexpression and/or amplification seem to be good candidates for anti-HER2 therapy. Moreover, CISH is a reliable and inexpensive method that can be used for HER2 testing of gastric cancer.

COMMENTS

Background

Alterations of the human epidermal growth factor receptor-2 (*HER2*) gene are implicated in the development and progression of many tumors. Breast cancer patients with HER2 amplification have been effectively treated with the monoclonal antibody trastuzumab, a HER2 inhibitor. Recently, a number of studies have suggested a response to trastuzumab therapy for other cancers with HER2 amplification, including gastric cancer, where HER2 amplification has been found in 7% to 27% of the tumors. Reports of trastuzumab therapy in metastatic gastric cancer showed complete tumor regression and disappearance of the metastases in two cases. Based on these reports, gastric cancer patients with HER2 overexpression and/or amplification could be good candidates for trastuzumab therapy.

Research frontiers

The HER2 protein is a frequently analyzed gene product, especially in breast cancer. Recent studies have examined HER2 expression in other tumor types, including gastric cancer. The frequency and significance of HER-2/neu amplification in gastric carcinoma are investigated. In this study, immunohistochemical HER2 expression and protein positivity ($\geq$ 2+) was observed in 20% and 14.58%, respectively, of the 120 gastric carcinomas.

Innovations and breakthroughs

The authors evaluated HER2 overexpression and gene amplification by immunohistochemistry and chromogenic *in situ* hybridization (CISH) respectively, in 120 cases of gastric carcinoma patients and 45 matched lymph node metastases mounted onto multiple-punch and single-punch tissue microarrays respectively. The data suggest that, in gastric cancer, HER2 amplification is the main mechanism for HER2 protein overexpression and is preserved in the lymph node metastases.

Applications

This study provides evidence supporting gastric cancer HER2 amplification as being the main mechanism for HER2 protein overexpression and that HER2 amplification is preserved in the lymph node metastases. Therefore, gastric cancer patients with HER2 overexpression and/or amplification seem to be good candidates for anti-HER2 therapy. Moreover, CISH is a reliable and inexpensive method that can be used for HER2 testing of gastric cancer.

Peer review

The authors studied HER2/neu expression and gene amplification in a cohort of Greek patients with gastric cancer. The manuscript includes 2 aspects. Firstly, they describe the expression/amplification of HER2-neu in the study group. Secondly, they highlight the potential applicability of CISH as a routine method. Data are mostly well documented and conclusions drawn are appropriate.

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S- Editor Sun H **L- Editor** Rutherford A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i2.156

World J Gastroenterol 2012 January 14; 18(2): 156-167
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Protective effect of alcohol consumption for fatty liver but not metabolic syndrome

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Supported by A grant from the Gifu Medical Association; Young Scientists (B) from Japan Society for the Promotion of Science, No. 23790791, in part
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Received: January 10, 2011 Revised: March 23, 2011
Accepted: March 30, 2011
Published online: January 14, 2012

Abstract

AIM: To investigate the effect of alcohol on the metabolic syndrome (MS) and fatty liver in Japanese men and women.

METHODS: A cross-sectional study was conducted in a medical health checkup program at a general hospital. This study involved 18 571 Japanese men and women, 18-88 years of age, with a mean body mass index of 22.6 kg/m^2. A standardized questionnaire was administered. The total amount of alcohol consumed per week was calculated, and categorized into four grades. Fatty liver was examined by ultrasound modified criteria of the revised National Cholesterol Education Program Adult Treatment Panel Ⅲ and the new International Diabetes Federation.

RESULTS: The prevalence of fatty liver decreased in men and women with light to moderate alcohol consumption, whereas the prevalence of MS was not so changed. The prevalence of fatty liver of any grade in men was lower than that in those with no or minimal alcohol consumption. In women with light to moderate alcohol consumption, prevalence of fatty liver was lower than that in women with no or minimal alcohol consumption. By logistic regression analysis, the odds ratio (OR) for MS in women with light alcohol consumption was decreased to < 1.0, but this change was not clear in men. The OR for fatty liver was clearly < 1.0 in men with any level of alcohol consumption and in women with light to moderate consumption.

CONCLUSION: Light to moderate alcohol consumption has a favorable effect for fatty liver, but not for MS in Japanese men and women.

Key words: Alcoholic hepatitis; Epidemiology; Fatty liver; Metabolic syndrome; Alcohol consumption

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Hamaguchi M, Kojima T, Ohbora A, Takeda N, Fukui M, Kato T. Protective effect of alcohol consumption for fatty liver but not metabolic syndrome. *World J Gastroenterol* 2012; 18(2): 156-167 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i2/156.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i2.156

INTRODUCTION

The metabolic syndrome (MS) is defined by abdominal

obesity, hypertension, elevated fasting blood glucose, and dyslipidemia[1]. Importantly, MS is a risk factor for the development of type 2 diabetes mellitus and coronary artery disease, and is associated with an increased risk of cerebrovascular disease and all-cause mortality[2]. The favorable effect of alcohol intake enhances insulin sensitivity, increases high-density lipoprotein-cholesterol (HDL-C), and contributes to a lower risk of type 2 diabetes mellitus[3-6] and cardiovascular disease[7-10]. Some reports have shown that the prevalence of MS is associated with alcohol consumption, irrespective of the amount consumed[11-13]. However, several studies have reported beneficial effects of alcohol consumption on MS[14-16]. Moreover, a study in Korean adults has indicated that light alcohol consumption is associated with a reduced prevalence of MS, whereas substantial alcohol intake leads to a dose-dependent increase in the risk of MS[17]. The effect of alcohol on MS in the general population has been inconsistent in the literature.

Fatty liver is closely associated with MS, and is considered the hepatic manifestation of MS[18]. Findings on the relation between alcohol consumption and fatty liver have also been inconsistent in the literature. Although alcohol consumption certainly may be a cause of fatty liver in some cases[19,20], it potentially plays a protective role against fatty deposition in the liver[21-25].

Therefore recent studies have implied the possibility that the effect of alcohol is different between fatty liver and MS, although fatty liver is closely associated with MS. However, the discrepancy of alcohol effect among the previous studies may be due to differences in the following cofactors: ethnicity, age, body mass index (BMI), drug usage, and lifestyle, such as alcohol consumption, smoking, and exercise. However, no large epidemiological study has investigated the effect of alcohol on fatty liver and MS at the same time.

We performed a cross-sectional study to investigate the effect of alcohol on fatty liver and MS at the same time. In this study, we separated the subjects according to the grade of alcohol consumption and compared the prevalence of fatty liver and MS in each grade. We focused on the discrepancy in the association between alcohol and MS and between alcohol and fatty liver. Additionally, we checked the impact of prevalence of MS without fatty liver, or fatty liver without MS.

MATERIALS AND METHODS

Study design

We performed a cross-sectional study of participants of a medical health checkup program, including abdominal ultrasonography. The study was approved by the ethics committee of Murakami Memorial Hospital, Gifu, Japan. The program was conducted in the Medical Health Checkup Center at Murakami Memorial Hospital. The purpose of the medical health checkup program was to promote public health through early detection of chronic diseases and the evaluation of their underlying risk factors. Known as a "human dock", medical services of this kind are very popular in Japan.

Study population

All of the subjects participating in such health checkup programs at Murakami Memorial Hospital between January 2004 and December 2009 were invited to join this study. Participants who tested positive for hepatitis B antigen or hepatitis C antibody and those who reported a history of known liver disease, including viral, genetic, autoimmune, and drug-induced liver disease, were excluded from the study[26]. We invited 22 119 participants in the health checkup program to enroll in the study. Of these, a total of 19 016 Japanese participants (11 295 men and 7721 women) were enrolled after giving informed consent. We excluded 123 participants (92 men and 31 women) with hepatitis C virus, 312 (214 men and 98 women) with hepatitis B virus, and nine (7 men and 2 women) who were diagnosed with other liver diseases. As a result, this study consisted ultimately of 18 571 participants (10 982 men and 7589 women). The mean age was 46.5 years (SD: 9.9; range: 18-88 years), and the mean BMI was 22.6 kg/m^2 (SD: 3.3; range: 14.0-58.3 kg/m^2).

Data collection

The health checkup programs that were used for the collection of data included the following tests: eye examinations, urinalysis, blood-cell counts, blood chemistry, electrocardiography, chest radiography, barium examination of the upper gastrointestinal tract, and abdominal ultrasonography. The medical history and lifestyle factors of all participants, including physical activity and habits pertaining to smoking and alcohol consumption, were surveyed by a standardized self-administered questionnaire. When the participants had difficulty completing the questionnaire, trained nurses provided assistance. We undertook blood and urine examinations with MODULAR ANALYTICS (Hitachi High-Technologies Corp. Ltd., Tokyo, Japan).

Standardized questionnaire for lifestyle factors

A standardized questionnaire was administered to all participants by the same trained team of interviewers. Habits regarding alcohol consumption were evaluated by asking the participants about the amount and type of alcoholic beverages consumed per week during the past month, then estimating the mean ethanol intake per week. The validity of information related to alcohol consumption was confirmed previously[27]. The total amount of alcohol consumed per week was calculated in grams, and then categorized into the following four grades: non or minimal alcohol consumption, < 40 g/wk; light alcohol consumption, 40-140 g/wk; moderate alcohol consumption, 140-280 g/wk; and excess alcohol consumption, > 280 g/wk[22,24]. Smoking status was also categorized into three groups (never smoker, ex-smoker, and current smoker). On the questionnaire, participants reported the type, duration and frequency of their partici-

pation in sports or recreational activities[28]. When participants performed any kind of sports at least once a week regularly, we categorized them as regular exercisers[29].

Definition of fatty liver

The diagnosis of fatty liver was based on the results of abdominal ultrasonography, which was done by trained technicians with Aloka SSD-650CL (Aloka Co., Ltd., Tokyo, Japan). All ultrasonographic images were stored as photocopies. Gastroenterologists reviewed the photocopies and made the diagnosis of fatty liver without reference to any of the participant's other individual data. Of four known criteria (hepatorenal echo contrast, liver brightness, deep attenuation, and vascular blurring), the participants were required to have hepatorenal contrast and liver brightness to be given a diagnosis of fatty liver[30].

MS

There are several differing criteria for the MS worldwide[1,31-34]. In this study, we used the following two definitions: (1) the revised National Cholesterol Education Program Adult Treatment Panel Ⅲ (rATP Ⅲ) definition[34]; and (2) the new International Diabetes Federation (IDF) definition[32].

According to the rATP Ⅲ definition[1], subjects who had three or more of the following criteria were identified as having MS: (1) triglycerides ≥ 150 mg/100 mL; (2) HDL-C < 40 mg/100 mL for men, and < 50 mg/100 mL for women; (3) elevated blood pressure (systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg); (4) fasting glucose ≥ 100 mg/100 mL instead of ≥ 110 mg/100 mL; or (5) abdominal-obesity-modified waist circumference cutoffs (≥ 90 cm for men and ≥ 80 cm for women) were used instead of the waist circumference cutoffs (≥ 102 cm for men and ≥ 88 cm for women) proposed in the existing definition.

According to the new IDF definition, Japanese people were defined as having MS if the subjects had abdominal obesity (waist circumference cutoffs ≥ 90 cm for men and ≥ 80 cm for women) plus two or more of the following risk factors: (1) elevated triglyceride level ≥ 150 mg/100 mL or on treatment; (2) low HDL–C < 40 mg/100 mL for men and < 50 mg/100 mL for women or on treatment; (3) elevated blood pressure (systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg); and (4) high fasting glucose ≥ 100 mg/100 mL[32].

Sample size

Because preliminary studies indicated that the number in the excess alcohol consumption group was small (n = 22 and 24), we invited as many subjects as possible. Practically, we collected data for waist circumference from 2004. Then, we set the study period from 2004 to 2009.

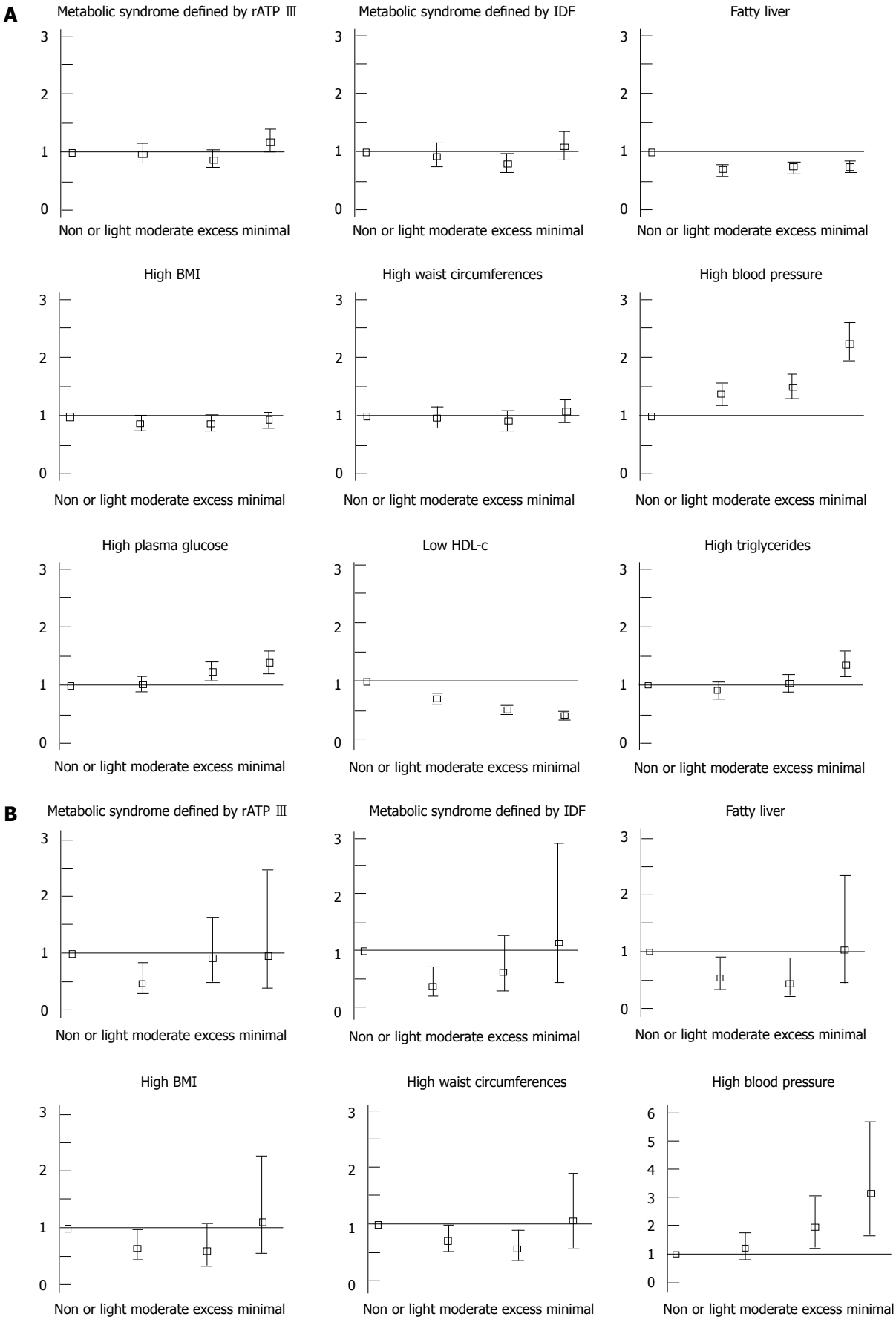
Statistical analysis

The R version 2.4.1 (available from http://www.r-project.org/) was used for statistical analyses. Data was expressed as mean (SD). Two groups of subjects were compared by χ^2 test. The significance of differences between non or minimal alcohol consumption and the others was determined by two-tailed, multiple χ^2 tests with Bonferroni correction ($P < 0.016$ for three comparisons in four groups). The linear association of alcohol consumption with several parameters associated with MS was evaluated by Spearman's rank correlation, and a P value < 0.05 was accepted as significant. We assessed the odds ratio (OR) of the alcohol consumption grade for MS and fatty liver using a multivariate logistic model while controlling for potential covariates. In a multivariate logistic model, we selected age, use of drugs that potentially affect MS, and lifestyle, such as alcohol consumption, regular exercise, and smoking as the potential covariates. The adjusted OR and 95% CIs were calculated.

RESULTS

We investigated the OR of alcohol consumption for MS defined by rATP Ⅲ and fatty liver using a logistic regression model (Figure 1). The OR for MS was decreased to < 1.0 in women with light alcohol consumption, but it was not clear in men. The OR for fatty liver was clearly < 1.0 in men with any level of alcohol consumption and in women with light to moderate consumption. In men and women, the ORs for high blood pressure and high fasting plasma glucose were increased as the level of alcohol consumption increased. Conversely, the OR for low HDL-C was decreased to < 1.0 in men and women. However, the OR for high triglycerides was increased to > 1.0 in men with excess alcohol consumption, and decreased to < 1.0 in women with light consumption. Moreover, the OR for high waist circumference was not significant, and was the same as the OR for high BMI. The actual adjusted ORs are shown in Table 1.

Table 2 indicates the basic characteristics of men and women in the four grades. And liner association between alcohol consumption and several factors associated with MetS were evaluated by Spearman's rank correlation (Table 3). BMI and waist circumferences were lowest in light consumption (Table 2), but Spearman's rank correlation coefficients were not significant (Table 3). Systolic blood pressure, diastolic blood pressure, and fasting plasma glucose were increased as the alcohol consumption increased (Tables 2 and 3). On the other hand, the associations of consumption with diastolic blood pressure, and that with fasting plasma glucose were not statistically significant in women, while systolic blood pressure was also increased as the consumption increased in women. In men and women, low-density lipoprotein (LDL) cholesterol, non HDL cholesterol, and LDL cholesterol/HDL cholesterol ratio were decreased and HDL cholesterol were increased as the consumption increased (Tables 2 and 3). Triglycerides were lower in light consumption and were higher in moderate and excess than those in non or minimal (Table 2). Over all, the trend of the associations between alcohol consumption and MetS


A
Metabolic syndrome defined by rATP Ⅲ
Metabolic syndrome defined by IDF
Fatty liver
High BMI
High waist circumferences
High blood pressure
High plasma glucose
Low HDL-c
High triglycerides
B
Metabolic syndrome defined by rATP Ⅲ
Metabolic syndrome defined by IDF
Fatty liver
High BMI
High waist circumferences
High blood pressure
Non or light moderate excess minimal

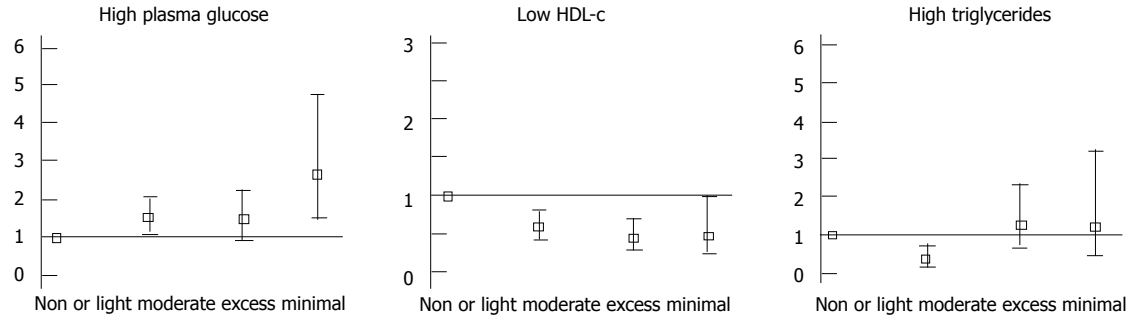


Figure 1 Adjusted odds ratio of the level of alcohol consumption for metabolic syndrome and fatty liver in men (A) and women (B). We assessed the odds ratio (OR) of the level of alcohol consumption for metabolic syndrome (MS) and fatty liver using a multivariate logistic model while controlling for potential covariates. In a multivariate logistic model, we selected age, usage of drugs that potentially affected MS, and lifestyle factors such as wine consumption, regular exercise, and smoking status, as the potential covariates. Bars mean the adjusted OR and 95% CIs. HDL-c: High-density lipoprotein-cholesterol; rATP Ⅲ: Revised National Cholesterol Education Program Adult Treatment Panel Ⅲ; IDF: International Diabetes Federation; BMI: Body mass index.

Table 1 The adjusted odds ratio (95% CI) for the metabolic syndrome and fatty liver

	Men odds ratio (95% CI)	*P* value	Women odds ratio (95% CI)	*P* value
For MetS defined by rATP Ⅲ				
The grade of alcohol consumption				
Light	0.98 (0.83-1.15)	0.77	0.48 (0.27-0.82)	0.008
Moderate	0.88 (0.75-1.04)	0.14	0.9 (0.5-1.65)	0.74
Excess	1.18 (1.01-1.39)	0.043	0.96 (0.37-2.48)	0.93
Age	1.01 (1-1.02)	< 0.001	1.08 (1.05-1.1)	< 0.001
The usage of drugs	4.95 (4.3-5.69)	< 0.001	7.46 (4.96-11.24)	< 0.001
Wine consumers	1.13 (0.67-1.9)	0.66	0.52 (0.24-1.15)	0.11
Regular exercisers	0.6 (0.51-0.71)	< 0.001	0.6 (0.38-0.94)	0.027
Smoking states				
Ex smoker/non smoker	1.21 (1.03-1.41)	0.017	0.89 (0.48-1.65)	0.72
Current smoker/non smoker	1.13 (0.97-1.33)	0.12	1.07 (0.58-1.98)	0.83
For MetS defined by IDF				
The grade of alcohol consumption	0.93 (0.75-1.14)	0.46	0.36 (0.19-0.69)	< 0.001
Light	0.78 (0.63-0.97)	0.029	0.61 (0.3-1.25)	0.18
Moderate	1.08 (0.87-1.33)	0.48	1.14 (0.45-2.9)	0.78
Excess	1 (0.99-1.01)	0.66	1.08 (1.05-1.1)	< 0.001
Age	3.64 (3.05-4.34)	< 0.001	4.85 (3.09-7.63)	< 0.001
The usage of drugs	0.78 (0.37-1.65)	0.52	0.6 (0.26-1.35)	0.22
Wine consumers	0.64 (0.51-0.79)	< 0.001	0.55 (0.34-0.92)	0.021
Regular exercisers				
Smoking states	1.3 (1.06-1.59)	0.01	1.03 (0.54-1.97)	0.93
Ex smoker/non smoker	1.09 (0.89-1.35)	0.39	1.07 (0.54-2.1)	0.85
Current smoker/non smoker				
For fatty liver				
The grade of alcohol consumption				
Light	0.69 (0.6-0.79)	< 0.001	0.54 (0.34-0.88)	0.012
Moderate	0.72 (0.63-0.83)	< 0.001	0.43 (0.21-0.88)	0.021
Excess	0.74 (0.64-0.85)	< 0.001	1.02 (0.44-2.35)	0.97
Age	1 (0.99-1)	0.21	1.06 (1.04-1.08)	< 0.001
The usage of drugs	2.09 (1.83-2.38)	< 0.001	2.17 (1.4-3.38)	< 0.001
Wine consumers	0.85 (0.53-1.35)	0.48	0.59 (0.3-1.15)	0.12
Regular exercisers	0.67 (0.59-0.77)	< 0.001	0.76 (0.52-1.13)	0.17
Smoking states				
Ex smoker/non smoker	1.24 (1.09-1.41)	< 0.001	0.38 (0.18-0.79)	0.01
Current smoker/non smoker	0.92 (0.81-1.05)	0.21	1 (0.57-1.74)	1
For high BMI				
The grade of alcohol consumption				
Light	0.86 (0.75-0.99)	0.034	0.64 (0.43-0.97)	0.036
Moderate	0.85 (0.73-0.98)	0.026	0.59 (0.33-1.06)	0.077
Excess	0.9 (0.78-1.05)	0.18	1.1 (0.53-2.31)	0.8
Age	0.99 (0.98-0.99)	< 0.001	1.04 (1.02-1.06)	< 0.001
The usage of drugs	2.19 (1.91-2.51)	< 0.001	1.77 (1.15-2.74)	0.01
Wine consumers	0.79 (0.48-1.29)	0.34	0.68 (0.39-1.21)	0.19
Regular exercisers	0.93 (0.81-1.06)	0.29	0.69 (0.48-0.99)	0.047
Smoking states				
Ex smoker/non smoker	1.19 (1.04-1.36)	0.013	0.56 (0.32-0.97)	0.037

Current smoker/non smoker	0.99 (0.87-1.14)	0.92	0.95 (0.58-1.57)	0.85
For high waist circumferences				
The grade of alcohol consumption				
Light	0.96 (0.81-1.13)	0.61	0.71 (0.51-0.97)	0.03
Moderate	0.89 (0.75-1.06)	0.21	0.57 (0.36-0.9)	0.016
Excess	1.06 (0.89-1.27)	0.5	1.06 (0.58-1.93)	0.85
Age	1 (0.99-1.01)	0.85	1.07 (1.06-1.09)	< 0.001
The usage of drugs	2.35 (2.01-2.74)	< 0.001	1.64 (1.14-2.36)	0.007
Wine consumers	0.63 (0.32-1.23)	0.18	0.68 (0.43-1.07)	0.095
Regular exercisers	0.71 (0.6-0.84)	< 0.001	0.69 (0.52-0.92)	0.012
Smoking states				
Ex smoker/non smoker	1.26 (1.07-1.49)	0.005	0.93 (0.64-1.35)	0.69
Current smoker/non smoker	1.04 (0.88-1.23)	0.67	0.91 (0.6-1.37)	0.64
For high blood pressure				
The grade of alcohol consumption				
Light	1.33 (1.16-1.53)	< 0.001	1.22 (0.85-1.75)	0.27
Moderate	1.47 (1.27-1.7)	< 0.001	1.97 (1.26-3.07)	< 0.001
Excess	2.24 (1.93-2.59)	< 0.001	3.13 (1.71-5.72)	< 0.001
Age	1.04 (1.03-1.05)	< 0.001	1.07 (1.06-1.09)	< 0.001
The usage of drugs	6.16 (5.33-7.13)	< 0.001	16.09 (10.87-23.83)	< 0.001
Wine consumers	1.09 (0.68-1.73)	0.73	0.72 (0.42-1.23)	0.23
Regular exercisers	0.81 (0.71-0.93)	< 0.001	0.8 (0.57-1.12)	0.19
Smoking states				
Ex smoker/non smoker	1.01 (0.88-1.15)	0.93	0.78 (0.5-1.23)	0.28
Current smoker/non smoker	0.67 (0.59-0.77)	< 0.001	0.73 (0.45-1.18)	0.2
For high plasma glucose				
The grade of alcohol consumption				
Light	1 (0.88-1.13)	0.94	1.52 (1.11-2.08)	0.009
Moderate	1.23 (1.08-1.4)	< 0.001	1.46 (0.95-2.25)	0.085
Excess	1.38 (1.2-1.58)	< 0.001	2.66 (1.49-4.76)	< 0.001
Age	1.03 (1.03-1.04)	< 0.001	1.07 (1.06-1.09)	< 0.001
The usage of drugs	2.05 (1.79-2.33)	< 0.001	1.88 (1.29-2.72)	< 0.001
Wine consumers	1.78 (1.17-2.72)	0.007	0.56 (0.33-0.96)	0.034
Regular exercisers	0.76 (0.68-0.86)	< 0.001	0.92 (0.68-1.24)	0.58
Smoking states				
Ex smoker/non smoker	1.22 (1.08-1.38)	< 0.001	0.68 (0.44-1.05)	0.083
Current smoker/non smoker	0.94 (0.83-1.06)	0.31	0.67 (0.42-1.06)	0.087
For low HDL-c				
The grade of alcohol consumption				
Light	0.7 (0.61-0.8)	< 0.001	0.58 (0.42-0.79)	< 0.001
Moderate	0.49 (0.42-0.57)	< 0.001	0.43 (0.27-0.69)	< 0.001
Excess	0.41 (0.35-0.48)	< 0.001	0.48 (0.24-0.96)	0.039
Age	1.01 (1-1.01)	0.082	1.01 (1-1.02)	0.055
The usage of drugs	3.4 (2.96-3.9)	< 0.001	4.24 (2.97-6.03)	< 0.001
Wine consumers	0.79 (0.47-1.32)	0.37	0.9 (0.6-1.35)	0.61
Regular exercisers	0.71 (0.62-0.82)	< 0.001	0.68 (0.52-0.91)	0.009
Smoking states				
Ex smoker/non smoker	1.06 (0.92-1.22)	0.45	0.64 (0.43-0.95)	0.027
Current smoker/non smoker	1.65 (1.44-1.9)	< 0.001	1.47 (1.03-2.11)	0.036
For high triglycerides				
The grade of alcohol consumption				
Light	0.89 (0.76-1.03)	0.11	0.37 (0.19-0.74)	0.005
Moderate	1.01 (0.87-1.18)	0.88	1.27 (0.68-2.35)	0.45
Excess	1.34 (1.16-1.56)	< 0.001	1.2 (0.45-3.2)	0.72
Age	1 (0.99-1)	0.19	1.06 (1.04-1.09)	< 0.001
The usage of drugs	3.06 (2.67-3.51)	< 0.001	11.13 (7.07-17.53)	< 0.001
Wine consumers	0.83 (0.49-1.4)	0.48	0.92 (0.43-1.99)	0.83
Regular exercisers	0.68 (0.59-0.79)	< 0.001	0.73 (0.44-1.21)	0.22
Smoking states				
Ex smoker/Non smoker	1.26 (1.09-1.46)	< 0.001	0.99 (0.49-2.01)	0.98
Current smoker/Non smoker	1.44 (1.24-1.66)	< 0.001	1.91 (1.02-3.55)	0.042

BMI: Body mass index; HDL-c: High density lipoprotein cholesterol; rATP Ⅲ: Revised National Cholesterol Education Program Adult Treatment Panel Ⅲ; IDF: International Diabetes Federation.

were not so changed between men and women.

This observed inverse association between alcohol consumption and fatty liver might be due to changed habits of alcohol use after previous detection of fatty liver. We analyzed the study population according to previous data. Among 10 981 men, 6547 were new participants and 4434 were repeat participants. Among 7573 women, 5138 were new participants and 2435 were

Table 2 The correlation between components for the metabolic syndrome, liver associated enzymes and alcohol consumptions in participants free from drugs

	Men				Women			
	Non or minimal	Light	Moderate	Excess	Non or minimal	Light	Moderate	Excess
No. of subjects	6154	1734	1616	1478	6893	406	207	84
Age, yr	46.3 (10)	47.5 (9.4)	49.3 (9.4)	49.4 (8.9)	45.1 (9.9)	45.2 (8.8)	45.5 (8.5)	45 (9.3)
Aspartate aminotransferase, IU/L	20.2 (8.7)	19.6 (7.1)	20.8 (10.2)	23.2 (11.6)	17.2 (9)	17.1 (4.9)	17.5 (5.8)	19 (6.2)
Alanine aminotransferase, IU/L	26.1 (16.9)	23.7 (13.3)	23.8 (14.1)	25.5 (14.7)	15.9 (13.1)	15.2 (6.7)	15.6 (6.7)	16 (7)
Gamma-glutamyltransferase, IU/L	24.3 (21.9)	28.9 (24.6)	36 (34.5)	48.8 (51)	13.8 (9.5)	15.8 (12.1)	17.1 (10.6)	22.6 (21.8)
BMI, kg/m^2	23.5 (3.3)	23.2 (2.9)	23.3 (2.7)	23.4 (2.9)	21.4 (3.2)	20.9 (2.7)	20.9 (3.1)	21.3 (3.2)
Waist circumference, cm	81.8 (8.6)	81.5 (7.7)	82.2 (7.5)	82.9 (7.7)	72.3 (8.8)	71.5 (7.9)	71.5 (8.6)	73.9 (9.4)
Systolic blood pressure, mmHg	120.1 (15.6)	121.7 (15.8)	123.9 (15.8)	127.4 (16.1)	111.2 (16.2)	111.2 (15.6)	114.3 (19.4)	116.7 (17)
Diastolic blood pressure, mmHg	75.9 (10.3)	77.2 (10.2)	79 (10.4)	81.3 (10.1)	69.1 (10.3)	69.6 (10.6)	72.2 (11.9)	72.9 (10.9)
Fasting plasma glucose, , mg/dL	100.5 (19.3)	100 (16.4)	102.4 (19.9)	103.5 (19.1)	91.3 (12.7)	91.8 (10.1)	91.5 (9.8)	96.1 (18.7)
HDL-c, mg/dL	46 (11.4)	49.6 (12.5)	52 (13.4)	53.3 (14.2)	59.9 (13.3)	65.7 (14.1)	69 (14.9)	68.6 (14)
LDL-c, mg/dL	128 (30)	122.8 (29.6)	121.5 (29.5)	116.6 (31.6)	118.1 (31.1)	108.4 (29.6)	105.6 (27.7)	102 (26.5)
nonHDL-c, mg/dL	155.4 (33.9)	150.1 (33.4)	150.6 (33)	149.2 (34.7)	139.3 (34.8)	129 (32.2)	127.3 (31.8)	124.4 (28.1)
LDL-c/HDL-c ratio	3 (1)	2.6 (0.9)	2.5 (0.9)	2.4 (0.9)	2.1 (0.8)	1.7 (0.7)	1.6 (0.6)	1.6 (0.6)
Triglycerides, mg/dL	109.5 (83)	108.6 (100.2)	117.7 (107.7)	135.5 (116.7)	64.6 (44.5)	60 (31.9)	69 (53.4)	67.2 (42.2)

Among 18 571 participants (10 982 men and 7589 women), we separate men and women into the following four grades: non or minimal alcohol consumption, < 40 g/wk; light alcohol consumption, 40-140 g/wk; moderate alcohol consumption, 140-280 g/wk; and excess alcohol consumption. Data was expressed as mean (SD). BMI: Body mass index; HDL-c: High density lipoprotein cholesterol; LDL-c: Low density lipoprotein cholesterol.

Table 3 The linear association between the amount of alcohol consumption and several parameters associated with the metabolic syndrome

	Men		Women	
	ρ	*P* value	ρ	*P* value
Age, yr	0.11	< 0.001	-0.09	< 0.001
Aspartate aminotransferase, IU/L	0.09	< 0.001	0	0.77
Alanine aminotransferase, IU/L	-0.02	0.08	-0.02	0.05
Gamma-glutamyltransferase, IU/L	0.34	< 0.001	0.08	< 0.001
BMI, kg/m^2	-0.01	0.16	-0.02	0.1
Waist circumference, cm	0.04	< 0.001	-0.01	0.32
Systolic blood pressure, mmHg	0.14	< 0.001	-0.04	< 0.001
Diastolic blood pressure, mmHg	0.17	< 0.001	-0.02	0.18
Fasting plasma glucose, mg/dL	0.08	< 0.001	-0.01	0.28
HDL-c, mg/dL	0.23	< 0.001	0.13	< 0.001
LDL-c, mg/dL	-0.13	< 0.001	-0.13	< 0.001
nonHDL-c, mg/dL	-0.08	< 0.001	-0.13	< 0.001
LDL-c/HDL-c ratio	-0.25	< 0.001	-0.17	< 0.001
Triglycerides, mg/dL	0.06	< 0.001	-0.06	< 0.001

The liner association between alcohol consumption and several factors associated with the metabolic syndrome were evaluated by Spearman' s rank correlation in 10 982 men and 7589 women, respectively, and a *P* value of < 0.05 was accepted as a significant level. BMI: Body mass index; HDL-c: High density lipoprotein cholesterol; LDL-c: Low density lipoprotein cholesterol.

Table 4 Alcohol consumptions of new and repeat participants

	Men			Women		
	n	Alcohol consumption (g/wk)	*P* value	*n*	Alcohol consumption (g/wk)	*P* value
New participants without fatty liver	4417	113.02 (161.03)	< 0.001[2]	4662	21.37 (67.57)	0.092[2]
New participants with fatty liver	2130	96.14 (157.95)	0.7[1]	476	13.99 (68.47)	0.841[1]
Repeat participants without fatty liver	2986	125.79 (157.24)	< 0.001[3]	2154	21.48 (58.33)	0.853[3]
Repeat participants with fatty liver	1448	102.02 (155.3)		281	18.09 (75.15)	

[1]New participants with fatty liver *vs* repeat participants with fatty liver; [2]New participants with fatty liver *vs* new participants without fatty liver; [3]Repeat participants with fatty liver *vs* repeat participants without fatty liver.

repeat participants. The alcohol consumption of repeat participants with fatty liver was the same as that of new participants with fatty liver (Table 4). Next, we analyzed repeat participants and separated them into four groups according to previous and present fatty liver. We assessed the changed habit of alcohol consumption. At first, the change in alcohol consumption was small in each group (Table 5). Moreover, the level of change was no different among the four groups of men and women.

We calculated the number of subjects with both MS and fatty liver, those who had only MS, and those who had only fatty liver (Table 6). Unexpectedly, more than half of the participants with fatty liver were not diagnosed with MS, even if the definition was changed. 42.5% or 25.5% of men with fatty liver were diagnosed with MS defined by rATP Ⅲ or IDF, respectively. Conversely, 66.0% or 78.8% of men with MS defined by rATP Ⅲ or IDF were diagnosed with fatty liver. The result was similar in women: 43.7% or 38.6% of women with fatty liver were diagnosed with MS defined by rATP Ⅲ or IDF, respectively. Conversely, 49.1% or 55.9% of women with MS defined by rATP Ⅲ or IDF were diagnosed with fatty liver. The prevalence of fatty liver among men with MS decreased along with level of alcohol consumption and prevalence of fatty liver among women with MS decreased in those with light or moderate consumption (Table 6).

DISCUSSION

As far as we know, the present study is the first to investigate the association of alcohol with ultrasonography-

Table 5 Changed alcohol consumption habits among four groups

Previous fatty liver	Present fatty liver	Men		Women	
		n	Change of alcohol consumption (g/wk)	*n*	Change of alcohol consumption (g/wk)
Negative	Negative	2784	–1.69 (109.95)	2112	–5.38 (48.13)
Negative	Positive	269	4.07 (116.14)	75	–20.69 (142.71)
Positive	Negative	202	6.07 (104.09)	42	–20.9 (56.96)
Positive	Positive	1179	–4.45 (100.32)	206	–5.56 (31.87)

Change in alcohol consumption (g/wk) was calculated by present alcohol consumption minus previous alcohol consumption, and expressed as mean (SD). We performed a Tukey test to investigate the statistical significance of the difference between two groups. No significant difference was identified.

Table 6 Positive prevalence of parameters for four levels of alcohol consumption *n* (%)

	Non or minimal	Light	Moderate	Excess	Non or minimal *vs* light	Light *vs* moderate	Moderate *vs* excess
Men							
Fatty liver	2248 (36.5)	457 (26.4)	449 (27.8)	424 (28.7)	< 0.001	< 0.001	< 0.001
MS defined by rATP Ⅲ	1282 (20.8)	331 (19.1)	317 (19.6)	373 (25.2)	0.12	0.33	< 0.001
MS defined by IDF	668 (10.9)	165 (9.5)	143 (8.8)	182 (12.3)	0.12	0.04	0.15
Fatty liver among men with MS defined by rATP Ⅲ	923 (72)	204 (61.6)	188 (59.3)	206 (55.2)	< 0.001	< 0.001	< 0.001
Fatty liver among men with MS defined by IDF	568 (85)	118 (71.5)	100 (69.9)	127 (69.8)	< 0.001	< 0.001	< 0.001
MS defined by rATP Ⅲ among men with fatty liver	923 (41.1)	204 (44.6)	188 (41.9)	206 (48.6)	0.17	0.79	0.005
MS defined by IDF among men with fatty liver	568 (25.3)	118 (25.8)	100 (22.3)	127 (30)	0.85	0.22	0.058
Components of MS							
High waist circumference	968 (15.7)	257 (14.8)	236 (14.6)	257 (17.4)	0.38	0.32	0.15
High blood pressure	1766 (28.7)	591 (34.1)	626 (38.7)	707 (47.8)	< 0.001	< 0.001	< 0.001
High fasting plasma glucose	2332 (37.9)	681 (39.3)	749 (46.3)	729 (49.3)	0.31	< 0.001	< 0.001
Low HDL-C	2133 (34.7)	425 (24.5)	333 (20.6)	284 (19.2)	< 0.001	< 0.001	< 0.001
High triglycerides	1385 (22.5)	360 (20.8)	394 (24.4)	459 (31.1)	0.13	0.14	< 0.001
High BMI (> 25 kg/m^2)	1731 (28.1)	417 (24)	388 (24)	379 (25.6)	< 0.001	< 0.001	0.07
Smoking status							
Current smoker	2012 (32.7)	602 (34.7)	657 (40.7)	740 (50.1)	0.12	< 0.001	< 0.001
Never smoker	2220 (36.1)	446 (25.7)	308 (19.1)	180 (12.2)	< 0.001	< 0.001	< 0.001
Ex smoker	3933 (63.9)	1288 (74.3)	1308 (80.9)	1298 (87.8)	< 0.001	< 0.001	< 0.001
Usage of drugs	938 (15.2)	249 (14.4)	307 (19)	326 (22.1)	0.38	0.00	0.00
Regular exerciser	1096 (17.9)	361 (20.9)	326 (20.3)	272 (18.5)	0.01	0.04	0.64
Wine consumer	45 (0.7)	18 (1)	17 (1.1)	14 (0.9)	0.26	0.30	0.52
Women							
Fatty liver	719 (10.5)	22 (5.4)	9 (4.3)	7 (8.3)	< 0.001	0.01	0.65
MS defined by rATP Ⅲ	632 (9.2)	19 (4.7)	17 (8.2)	6 (7.1)	< 0.001	0.73	0.65
MS defined by IDF	494 (7.2)	12 (3)	10 (4.8)	6 (7.1)	< 0.001	0.25	0.84
Fatty liver among women with MS defined by rATP Ⅲ	322 (50.9)	4 (21.1)	3 (17.6)	2 (33.3)	0.020	0.013	0.65
Fatty liver among women with MS defined by IDF	284 (57.5)	3 (25)	3 (30)	2 (33.3)	0.051	0.16	0.44
MS defined by rATP Ⅲ among women with fatty liver	322 (44.8)	4 (18.2)	3 (33.3)	2 (28.6)	0.024	0.73	0.63
MS defined by IDF among women with fatty liver	284 (39.5)	3 (13.6)	3 (33.3)	2 (28.6)	0.026	0.97	0.84
Components of MS							
High waist circumference	1257 (18.2)	61 (15)	26 (12.6)	16 (19)	0.12	0.05	0.96
High blood pressure	1026 (14.9)	65 (16)	45 (21.7)	22 (26.2)	0.58	0.01	0.01
High fasting plasma glucose	906 (13.1)	70 (17.2)	33 (15.9)	19 (22.6)	0.02	0.29	0.02
Low HDL-C	1772 (25.7)	58 (14.3)	25 (12.1)	10 (11.9)	< 0.001	< 0.001	0.01
High triglycerides	507 (7.4)	12 (3)	18 (8.7)	6 (7.1)	0.00	0.56	0.89
High BMI (> 25 kg/m^2)	792 (11.5)	30 (7.4)	14 (6.8)	9 (10.7)	0.01	0.05	0.96
Smoking status							
Current smoker	372 (5.4)	50 (12.3)	57 (27.5)	25 (29.8)	< 0.001	< 0.001	< 0.001
Never smoker	6086 (88.3)	291 (71.7)	106 (51.2)	37 (44)	< 0.001	< 0.001	< 0.001
Ex smoker	804 (11.7)	115 (28.3)	101 (48.8)	47 (56)	< 0.001	< 0.001	< 0.001
Usage of drugs	575 (8.3)	36 (8.9)	22 (10.6)	6 (7.2)	0.78	0.30	0.87
Regular exerciser	1179 (17.2)	82 (20.3)	34 (16.7)	19 (22.6)	0.13	0.91	0.25
Wine consumer	140 (2)	30 (7.4)	17 (8.2)	11 (13.1)	< 0.001	< 0.001	< 0.001

This table indicates the positive prevalence of parameters for four levels of alcohol consumption: non or minimal, < 40 g/wk; light, 40–140 g/wk; moderate, 140–280 g/wk; and excess, > 280 g/wk. Two groups of subjects were compared by using the unpaired *t* test and χ^2 test. The significance of differences between two side-by-side groups among the four groups was determined by two-tailed, multiple χ^2 tests with Bonferroni correction ($P < 0.016$ for three comparisons in four groups). Smoking status was also categorized into three groups; never smoker, ex smoker and current smoker. Regular exercisers were defined as participants who performed any kind of sports at least once a week. Usage of drugs was defined as participants who receive any drugs that potentially affected metabolic syndrome (MS). HDL-c: High-density lipoprotein-cholesterol; rATP Ⅲ: Revised National Cholesterol Education Program Adult Treatment Panel Ⅲ; IDF: International Diabetes Federation; BMI: Body mass index.

proven fatty liver and MS at the same time in a general population. Our study clearly indicated that the effect of alcohol was different between fatty liver and MS, although they are correlated closely. The effect of alcohol consumption on MS was not consistent, because it differed among components of MS. Alcohol consumption was associated with lower risk for HDL-C, but was associated with higher risk for high blood pressure and high fasting plasma glucose. Waist circumferences were not affected by level of alcohol consumption. Moreover, these results were similar when we used the Japan Society for The Study of Obesity definition for MS[33]. Our study clearly indicated that light to moderate alcohol consumption was associated with lower risk of fatty liver. Moreover, any level of alcohol consumption could have a protective effect on fatty liver in men. Our study was cross-sectional, therefore, the findings might be due to changed alcohol consumption after previous detection of fatty liver. However, our sub-analysis indicated that changes in alcohol consumption were small and were not due to previous detection of fatty liver.

Previous studies have indicated that the presence of fatty liver is a strong predictor of MS[36], and fatty liver correlates with all the components of MS[37]. Among populations with no or light alcohol consumption, liver fat content in participants with MS is significantly increased up to fourfold higher than those without MS[37], and the incidence of fatty liver has been shown to be increased fourfold in men and 11-fold in women with MS[27]. Although fatty liver is considered to be a hepatic manifestation of MS, more than half of Japanese men and women with fatty liver were not diagnosed with MS.

Interpretations

Alcohol consumption is a lifestyle factor, and its effects on health range from beneficial to detrimental. The dose-response relationship between alcohol and all-cause mortality follows a J- or U-shaped curve, which points to lower all-cause mortality among those with light to moderate alcohol consumption compared to excess consumption[38]. This effect is thought to be due mainly to a reduction in cardiovascular disease[7]. This reduction in cardiovascular disease has been attributed to the beneficial impact of alcohol on plasma lipid levels, hemostatic factors[8], and insulin sensitivity[3,6]. Some studies have suggested that as much as half of the cardiovascular benefit attributable to alcohol consumption may be because it increases HDL-C level[7-10]. We found that HDL-C was increased as the quantity of alcohol consumption increased, which was consistent with previous reports[7-10]. On the other hand, alcohol consumption contributes to elevated blood pressure[39,40]. Then, we also found that blood pressure was increased as the quantity of alcohol consumption increased.

In fact, studies about the association between alcohol consumption and obesity have not been consistent. Waist-to-hip ratio increases as the quantity of alcohol consumption increases[41,42], and waist circumference increases with excess alcohol consumption (> 40 g/d)[43]. Alcohol consumption of 30 g/d or more significantly increases BMI and the risk of weight gain[44]. In contrast, another study has reported that light-to-moderate alcohol consumption reduces waist circumference[16]. Moreover, some studies have found no significant association between alcohol consumption and obesity[45,46]. We also found no significant association between alcohol consumption and high waist circumference and BMI (BMI > 25 kg/m^2).

Alcohol consumption may increase triglyceride concentrations[8]. Triglycerides have been reported to be higher in individuals with excess alcohol consumption, but lower in those with light to moderate consumption[8]. Similar results were found in our study, which indicated that the OR for high triglyceride levels was increased to > 1.0 in men with excess alcohol consumption, and was decreased to < 1.0 in women with light consumption. The favorable effect of alcohol consumption contributing to enhance insulin sensitivity has been reported[3-6]. However, another study has provided different evidence that insulin resistance is related to alcohol consumption in a U-shaped manner[47]. In our study, the OR for high fasting plasma glucose was increased as the level of alcohol consumption increased.

Overall, the present study proved that the effect of alcohol consumption for MS was not significant in the Japanese general population. In fact, studies regarding the association between alcohol consumption and MS have not been consistent[11-17], because the relationship heavily depends on the individual components of MS. Moreover, previous studies have claimed that the type of alcohol is important for the association of alcohol consumption and MS[23,48,49]. Modest wine consumption has been reported to be associated with reduced all-cause mortality[48,49] and fatty liver[23]. In our study, however, the association between wine consumption and MS and fatty liver was not significant. Similarly, Djousse *et al*[15] have reported that the association between alcohol consumption and MS is unrelated to the type of alcoholic beverage.

Findings on the relation between alcohol consumption and fatty liver have also been inconsistent[19-25]. Fat deposition in the liver has been shown to be primarily due to an increased influx of fatty acids to the liver; most likely as a result of the increased lipolysis associated with obesity and insulin resistance, and as a result of increased hepatic *de novo* lipogenesis[50]. Reduced fatty acid oxidation and mitochondrial dysfunction and decreased export of fat further contribute to the accumulation of liver fat[51,52]. Alcohol-dehydrogenase-mediated ethanol metabolism generates a reduced form of nicotinamide adenine dinucleotide, which promotes steatosis by stimulating the synthesis of fatty acids and opposing their oxidation[53]. The hepatic lipogenic pathway is activated after the consumption of 24 g/d ethanol[53]. Sterol regulatory element binding protein 1c (SREBP1c)[54] and peroxisome proliferator activated receptor (PPAR)α[55], are altered with alcohol consumption. The involvement

of AMP-activated protein kinase activity in the action of ethanol on the liver has been demonstrated in experimental models of ethanol-induced steatosis[56].

SREBP1c is upregulated, which potentially results in increased conversion of glucose to fatty acids and triglycerides in experimental models of obesity[52]. PPARα is a nuclear receptor that is important in fatty acid uptake and oxidation, and has been shown to be underexpressed in experimental models of non-alcoholic steatosis[57]. In addition, the administration of adiponectin reverses non-alcoholic steatosis in experimental models[57,58].

Thus, several pivotal factors in the pathogenesis of fatty liver may be common in both alcoholic and non-alcoholic subjects. Therefore, the possible mechanism by which alcohol has a protective effect against fatty deposition in the liver remains unclear. Our study also provides clear evidence that light to moderate alcohol consumption has a favorable effect on fatty liver.

Some limitations of our study should be noted. First, although ultrasonography has been validated for detecting fatty liver, it may give an incorrect diagnosis compared to liver biopsy[30]. Second, self-reported information regarding alcohol intake is frequently subject to underreporting, and misreporting could be a source of bias. However, the self-reported information regarding alcohol intake in our study was validated previously[27]. Third, the generalizability of our study to non-Japanese populations is uncertain.

In conclusion, the effect of alcohol consumption was different between MS and fatty liver. The relationship between alcohol consumption and MS depends on the individual components of MS given the inconsistency of the association between alcohol consumption and MS. Light to moderate alcohol consumption has a favorable effect on fatty liver in Japanese men and women. Moreover, any level of alcohol consumption may have a protective effect against fatty liver in men. Unexpectedly, more than half of Japanese men and women with fatty liver were not diagnosed with MS, although fatty liver is considered to be a hepatic manifestation of MS. However, our previous studies have implied that the risk of fatty liver for cardiovascular disease is independent of MS[59]. Thus, fatty liver without MS is an important disease in the general population.

A future longitudinal study is needed to clarify that alcohol consumption has true hepatoprotective effects. Furthermore, the protective mechanism of alcohol against fatty deposition in the liver remains unclear. Thus, basic research is also needed to clarify the mechanisms that underlie modest alcohol consumption and fatty liver.

ACKNOWLEDGMENTS

We thank all of the staff members in the medical health checkup center at Murakami Memorial Hospital. The authors declare no conflict of interest associated with the manuscript.

COMMENTS

Background

The effects of alcohol on the metabolic syndrome (MS) have been inconsistent in previous studies. Fatty liver is closely associated with MS, and is considered to be the hepatic manifestation of MS. Findings on the relation between alcohol consumption and fatty liver have also been inconsistent.

Research frontiers

The favorable effect of alcohol intake enhances insulin sensitivity, and increases high-density lipoprotein-cholesterol, which contributes to a lower risk of type 2 diabetes mellitus, and cardiovascular disease. Moreover, alcohol consumption plays a protective role against fatty deposition in the liver, although alcohol consumption certainly could be a cause of fatty liver in some cases.

Innovations and breakthroughs

The authors investigated the association of alcohol consumption with ultrasonography-proven fatty liver and the MS at the same time in a general population, and clearly indicated that light to moderate alcohol consumption has favorable and unfavorable effects for components of MS, but has a protective effect for fatty liver.

Applications

Light to moderate alcohol consumption has a favorable effect on fatty liver in Japanese men and women. Moreover, any level of alcohol consumption may have a protective effect in men. A future longitudinal study is needed to clarify that alcohol consumption has true hepatoprotective effects. Furthermore, the mechanism of alcohol protection against fatty deposition in the liver remains unclear. Thus, basic research is also needed to clarify the mechanisms that underlie the effect of modest alcohol consumption on fatty liver.

Peer review

The strengths of the study include its large sample size and validated questionnaire.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i2.168

World J Gastroenterol 2012 January 14; 18(2): 168-174
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

^{18}F-fluoro-2-deoxyglucose uptake on PET CT and glucose transporter 1 expression in colorectal adenocarcinoma

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Author contributions: Hong R and Lim SC contributed equally to this work, including research design, data analysis, and writing and editing the manuscript.
Supported by National Research Foundation of Korea Grant funded by the Ministry of Education, Science and Technology through the Research Center for Resistant Cells, No. R13-2003-009
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Received: April 19, 2011 Revised: June 27, 2011
Accepted: July 5, 2011
Published online: January 14, 2012

Abstract

AIM: To evaluate the correlation between the level of ^{18}F-fluoro-2-deoxyglucose (^{18}F-FDG) uptake and glucose transporter 1 (GLUT1) expression in colorectal adenocarcinoma (CRA).

METHODS: Forty four patients with resected CRA and preoperative ^{18}F-FDG positron emission tomography - computed tomography data were investigated in this study. Comparison of maximum standardized uptake value (SUVmax) of the lesion was made with GLUT1 expression by immunohistochemistry and various clinicopathologic factors including tumor volume, invasion depth, gross finding, and lymph node metastasis.

RESULTS: SUVmax was 14.45 ± 7.0 in negative GLUT1 expression cases, 15.51 ± 5.7 in weak GLUT1 expression cases, and 16.52 ± 6.8 in strong GLUT1 expression cases, and there was no correlation between between GLUT1 expression and SUVmax. SUVmax was significantly correlated with tumor volume ($P < 0.001$). However, there was no significant differences in SUVmax and GLUT1 expression among other clinicopathologic factors.

CONCLUSION: GLUT1 expression does not correlates significantly with ^{18}F-FDG uptake in CRA. ^{18}F-FDG uptake was increased with tumor volume, which is statistically significant.

Key words: ^{18}F-fluoro-2-deoxyglucose; Glucose transporter 1; Colorectal cancer

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Hong R, Lim SC. ^{18}F-fluoro-2-deoxyglucose uptake on PET CT and glucose transporter 1 expression in colorectal adenocarcinoma. *World J Gastroenterol* 2012; 18(2): 168-174 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i2/168.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i2.168

INTRODUCTION

Cancer cell growth is an energy-related process supported by increased glucose metabolism[1]. This uptake is mediated by glucose transporter (GLUT) proteins, which are membrane proteins responsible for the transport of glucose across cellular membranes. A family of seven glucose transporters have been cloned[2]. Among these, GLUT1 is the best-known basic, high-affinity glucose transporter, which is restricted to erythrocytes and blood-tissue barriers such as the blood-brain and blood-nerve barriers, in most normal tissues[3,4]. It has long been recognized that cancer cells have increased rates of glucose

metabolism compared with normal cells[5]. Increased GLUT1 expression has been described in many cancers, including breast, lung, kidney, urinary bladder, stomach, colorectum, endometrium, thyroid, head and neck, liver, ovary, salivary gland, and prostate cancer[6] due to a high metabolic rate and fast growth environment, but, generally absent in benign epithelial tissues. The expression of GLUT1 thus would appear to be a potential marker for malignant transformation, and the degree of tumor GLUT1 expression might correlate with biologic behavior in individual patients[7].

Positron emission tomography (PET) using ^{18}F-fluoro-2-deoxyglucose (FDG) is a rapidly developing functional-imaging modality that has shown great promise in the fields of primary, recurrent and metastatic tumor detection, planning and monitoring therapy[8-12]. The cellular mechanism underlying the increased ^{18}F-FDG accumulation in malignant tumors is associated with a higher rate of phosphorylation and diminished rate of dephosphorylation of intracellular phosphorylated glucose, a higher rate of glucose transport across the cell membrane, and higher activity of hexokinase[13]. There have been several studies about possible associations of GLUT1 expression with other clinicopathologic parameters and ^{18}F-FDG PET findings in several cancers, such as carcinoma of lung, pancreas, and breast[1]. However, to the best of our knowledge, it has not been elucidated in colorectal adenocarcinoma (CRA). Therefore, we conducted a prospective study to determine the association between GLUT1 expression and the maximum standardized uptake values (SUVmax) obtained from ^{18}F-FDG PET scans. The relationship between GLUT1 and SUVs with other clinicopathologic factors was also evaluated. Additionally, we evaluated a difference in GLUT1 expression between adenoma and carcinoma in the colorectum.

MATERIALS AND METHODS

Patients

Among patients who had FDG-PET examination and underwent curative surgery for CRA at Chosun University Hospital from January 2007 to December 2010, the present study was conducted on a non-consecutive series of 44 patients where paraffin embedded tissues were relatively well preserved and complete medical records were present. Patients who underwent preoperative chemoradiotherapy and emergency surgery, and patients who had evidence of hereditary non-polyposis colorectal cancer or familial adenomatous polyposis were excluded from the study. The various clinicopathological parameters of the patients were confirmed by reviewing the patient medical records and pathology files. The relationship between clinicopathological parameters for the patients and the immunohistochemical findings with survival was investigated for all 44 patients. Additionally, there were 27 adenomatous cases, including 15 cases of tubular adenoma (TA), 7 villous adenomas (VA), and 5 tubulo-villous adenomas (TVA).

Histopathological analysis

Microscopic examination: each tumor was re-evaluated by retrospective analysis of the medical records and the tissue slide files of the Department of Pathology. Age, gender, tumor size, histological subtypes and the degree of differentiation, the depth of tumor invasion, the status of lymph node metastases and the presence of a distant metastasis were assessed. Stage was defined according to the TNM staging system of the American Joint Committee on Cancer[17]. The examined tissues were fixed in 10% neutral formalin, and the prepared paraffin embedded tissues were sectioned 4-5 μm in thickness. Hematoxylin and eosin staining was performed, and the sections were examined under a light microscope. A representative area suitable for the study purpose was selected, and slides were prepared for immunohistochemical analysis.

Immunohistochemical staining

All of the specimens in this study were tested using a gout polyclonal antibody against GLUT1 (Abcam) according to the manufacturer's protocol. Immunolocalization was performed using the mouse ImmunoCruz Staining System: sc-2050 (Santa Cruz Biotechnology), according to the manufacturer's protocol. The staining process was performed according to a standard protocol. Briefly, the 4 μm sections that were obtained after formalin fixation and paraffin embedding were deparaffinized in xylene and were then rehydrated with distilled water through a graded series of ethanol solutions. The sections were then placed in a glass jar with 10 mmol/L citrate buffer (pH 6.0) and were irradiated in a microwave oven for 15 min. The sections were allowed to cool in the jar at room temperature for 20 min. The slides were then rinsed with Tris buffered saline (TBS). A blocking reagent was added for 10 min after quenching the endogenous peroxidase activity in 0.3% hydrogen peroxide for 10 min. The slides were then washed as described previously, and the slides were subsequently subjected to the primary antibody reaction. Immunohistochemistry was performed on the Nexes ES (Ventana, Tucson, AZ). Slides were incubated with the antibodies for 32 min. The Ventana basic DAB detection kit (catalog No. 760-001) was the secondary detection method. This includes biotinylated immunoglobulin secondary antibody, containing affinity purified goat-antimouse IgG and IgM (b200 lg/mL) and goat-antirabbit IgG (b200 lg/mL) in phosphate buffer with preservative. Incubation was for 8 min. This was followed by conjugated streptavidin horseradish peroxidase. Slides were counterstained with hematoxylin (Ventana catalog No. 760-2021).

Analysis and interpretation of staining

GLUT1 immunostaining was quantified by grading the proportion of cells that were GLUT1 positive. Cells showing strong and distinctive membranous immunoreactivity for GLUT1 were considered positive. Cyto-

Table 1 Summary of clinicopathologic factors of adenocarcinoma

Characteristics	*n* (%)
Age (yr)	
≤ 50	7 (15.9)
51~59	7 (15.9)
60~69	10 (22.7)
≥ 70	20 (45.5)
Sex	
Male	24 (54.5)
Female	20 (45.5)
Pathologic tumor classification (pT)	
pT1	2 (4.5)
pT2	5 (11.4)
pT3	35 (79.6)
pT4	2 (4.5)
Pathologic lymph node classification	
pN0	26 (59.1)
pN1	17 (38.6)
pN2	1 (2.3)
Metastasis classification (M)	
M0	42 (95.5)
M1	2 (4.5)
Gross type	
Ⅰ (polypoid)	8 (18.2)
Ⅱ (ulcerative)	17 (38.6)
Ⅲ (infiltrative)	19 (43.2)

plasmic staining, including a supra nuclear dot pattern or nuclear staining, was regarded as negative[6]. The degree of GLUT1 immunostaining of a specimen was graded according to the proportion of GLUT1-positive cells in it (weakly positive, < 10%; moderately positive, 10%-50%; strongly positive, > 50%)[7].

Statistical analysis

The mean with standard deviation (SD) was calculated for the longest tumor diameter and SUVmax. Mann-Whitney *U* or Kruskal-Wallis test was used to assess differences in the levels of SUVmax and in the staining scores of GLUT1 between the groups. Correlations between SUVmax and GLUT1 expressions and between SUVmax and tumor diameter were analyzed by Spearman's rank test. A value of $P < 0.05$ was considered as statistically significant. The SPSS statistics 17.0 program (SPSS, Korea) was used for statistical evaluation.

RESULTS

The clinical characteristics of the patients are summarized in Table 1. The average age at the time of surgery was 65.73 years and the ratio of male to female participants was 24:20 (54.5%:45.5%). Mean tumor size was 18.92 cm, and mean SUVmax value was 15.47. In normal epithelium, specific GLUT1 expression was not observed. As expected, erythrocyte membranes were strongly GLUT1 positive. In adenoma cases, GLUT1 expression was absent in 23 cases (85.1%) and weakly positive in 4 cases, which were one VA and 3 TVAs. The positive rate of GLUT1 expression was significantly different ($P = 0.008$) among the TA, VA, and TVA. Of 44 cases of CRA, 91% had specific GLUT1 immunostaining in the plasma membrane. The extent of expression varied greatly. Of immunopositive cases, 13 cases (29.5%) showed weak staining (< 10% of tumor cells), 12 cases (27.3%) moderate staining (10%-50% of tumor cells), and 15 cases (34.1%) strong expression (> 50% of tumor cells), which were significantly different from adenomatous cases ($P < 0.001$). In cancer tissue, GLUT1 is usually strongly positive in the center of the necrotic and infiltrative areas (Figure 1). Concerning correlation between GLUT1 expression and SUVmax in PET, the mean SUVmax was 14.45 ± 7.0 in negative GLUT1 expression cases, 15.51 ± 5.7 in weak GLUT1 expression cases, and 16.52 ± 6.8 in strong GLUT1 expression cases, and there was no significant correlation between GLUT1 expression and SUVmax. SUVmax was significantly correlated with tumor volume ($P = 0.002$). However, GLUT1 expression did not correlate with tumor size. There was no significant difference in SUVmax and GLUT1 expression among other clinicopathologic factors including invasion depth, lymph node metastasis and gross type (Table 2).

Table 2 Relation between glucose transporter 1 expression/maximum standardized uptake values and clinicopathologic parameters

Clinicopathologic factors	*n*	GLUT1 expression[1]				SUVmax	
		0	1	2	*P* value	Medium	*P* value
T stage							
T1	2	2	0	0	0.282	6	0.108
T2	5	1	2	2		15.1	
T3	35	12	10	13		24.22	
T4	2	2	0	0		17.5	
N stage							
N0	26	10	7	9		20.3	
N1	17	6	5	6	0.795	25.09	0.346
N2	1	1	0	0		12	
Gross type							
Ⅰ (polypoid)	8	3	4	1		19.94	
Ⅱ (ulcerative)	17	7	3	7	0.473	24.94	0.496
Ⅲ (infiltrative)	19	7	5	7		20.39	
Tumor size (median)		19.59	28.71	20.83	0.14		0.002[2]

[1]GLUT1 expression; [2]Statistically significant, $P < 0.05$. 0: Negative or weak; 1: Moderate; 2: Strong expression. GLUT: Glucose transporter; SUVmax: Maximum standardized uptake values.

DISCUSSION

Among Gluts, Glut-1 and Glut-3 have been proven to show overexpression in both messenger RNA and protein in a variety of cancer cells[14-18]. Therefore, Glut-1 and Glut-3 may play an important role in glucose uptake by these cancers and could be useful biomarkers for malignant transformation[1]. We herein demonstrate that GLUT1 protein expression is a marker for malignant transformation in CRA. For CRA, an initial report showed increased expression of GLUT1 mRNA compared with normal colon[19], and GLUT1 immunostaining was subsequently demonstrated in seven of nine colorectal carci-

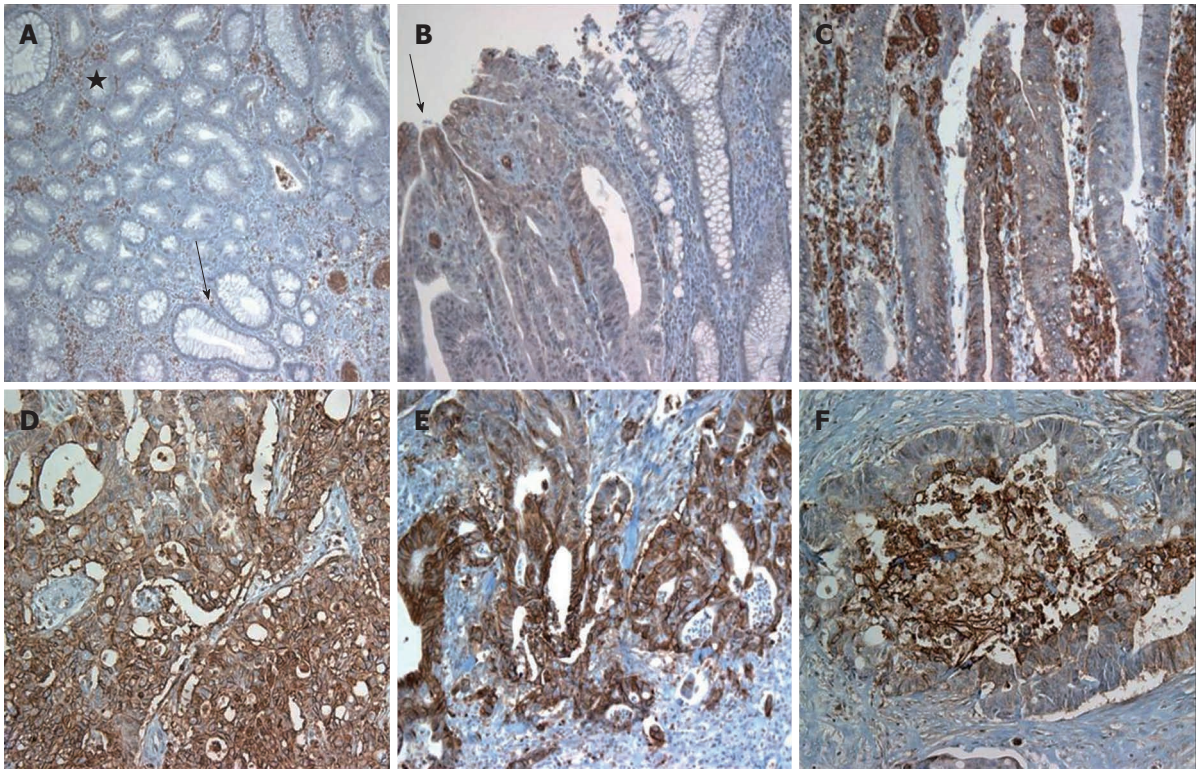

Figure 1 Glucose transporter 1 expression in normal colonic epithelium, adenomas and adenocarcinomas. A: No glucose transporter 1 (GLUT1) expression in tubular adenoma (star) and normal epithelium (arrow), while immunostaining in erythrocytes; B and C: GLUT1 immunostaining in the villous adenoma (B, arrow: Expression at the tip of villous frond); D: Colorectal adenocarcinoma with strong GLUT1 expression; E and F: More strong expression at the infiltrative border (E) and necrotic center (F).

nomas[20]. A recent study of 53 colon carcinomas demonstrated the presence of GLUT1 immunostaining in 83%, and a higher degree of GLUT1 expression correlated with the presence of lymph node metastases[21]. The greater degree of GLUT1 expression in these tumors most likely reflects a greater enhancement of glycolytic metabolism in the more malignant tumors. It has recently been reported that GLUT1 (and/or GLUT3 expression) correlates with poor prognosis and tumor aggressiveness in carcinomas of the lung and bladder, and in squamous cell carcinoma of the head and neck[22-24]. Although the present study did not show these results, these data suggest the possibility that tumors with absent GLUT1 staining might express another GLUT iso-form such as GLUT3, which also might be associated with poor prognosis[22].

In the present study, the normal and most adenomatous colorectal mucosa did not express GLUT1 protein. In benign colorectal neoplasms, GLUT1 expression was absent in TA, and in VA and TVA, there was only rare focal staining at the tips of villous fronds. These results are consistent with a recent report that some VAs have very limited focal GLUT1 expression[21]. GLUT1 expression in VA is consistent with the concept that GLUT1 is a marker of neoplastic progression in the colon, because it is this subtype of colonic adenoma that is believed to have the greatest potential for malignant transformation[7].

In cancer tissue, GLUT1 is usually strongly positive in the luminal border and center of the necrotic and infiltrative areas. Rapid proliferation relative to vascular support exposes tumor cells to persistent hypoxic conditions with potential necrotic or apoptotic effects[6]. Malignant cells, however, can undergo genetic and adaptive changes that allow them to avoid oxygen deprivation-induced death. One of these changes is an increased uptake of glucose and other sugars compared with normal cells[25]. In normal human small intestinal villi, the tips of villi may be a site of relative hypoxia[26]. Because hypoxia is known to stimulate glycolysis and GLUT1 expression[27], the localization of GLUT1 immunostaining to this site in VAs also might reflect an adaptation to enhanced local glycolytic demand[7].

Two possible mechanisms may explain the activation of *GLUT1* gene expression in CRA and other malignancies[7]. First, increased glycolysis and concomitant GLUT1 expression may be a constitutive feature of the malignant phenotype in many cancers. This is consistent with observations that transformation of cultured cells with ras or src oncogenes induces increased glucose uptake and GLUT1 expression[28,29]. Second, local hypoxia in the tumor microenvironment may result in an adaptive increase in glycolytic metabolism and GLUT1 expression[7]. The latter mechanism is also demonstrated in the present study; GLUT1 tended to be expressed stronger at the luminal border and center of tumor nests, increasing with distance from stromal blood vessels.

Higher levels of GLUT1 expression in neoplastic tis-

sue reflect an increased glycolytic metabolism[30]. In previous studies of CRA, a high level of GLUT1 expression was significantly associated with the presence of lymph node metastases[21] and poorer prognosis[7]. These studies suggested that the expression of GLUT1 could be a marker for malignant potential. In our study, the analysis of the association between GLUT1 expression and other clinicopathologic parameters did not show any significant correlation. These results differ from those of previously published data for other tumors[21,31,32], but they are compatible with the results of Avril *et al*[33] for breast cancer. In a study by Haber *et al*[7], the proportion of GLUT1 staining did not correlate with Dukes' stage of the CRA, but Sakashita *et al*[30] demonstrated that in T1 and T2 stage CRA, GLUT1 expression correlated with Duke stage. The discrepancy between the two studies could have been caused by differences in the clinical characteristics of the subjects enrolled. Haber's study included only 6 Dukes' A cases and all other cases were more advanced, while Sakashita's study analysed only T1 and T2 stage cases. So, Sakashita *et al*[30] speculated as follows: in early-stage carcinomas GLUT1 positivity is low, but correlates with the depth of the lesion. In contrast, in the more advanced stages, the tumor cells already show high GLUT expression, and no further increase of GLUT1 expression occurs, even when the cancer invades more deeply.

Cancer cells have higher rates of glucose metabolism than normal cells. Malignant tissues typically demonstrated higher ^{18}F-FDG uptake than benign lesions and normal tissue[34]. PET-CT using ^{18}F-FDG has been known to be a useful tool for several malignant tumors. Several immunohistochemical studies have demonstrated overexpression of GLUT1 in human malignancy and a correlation between GLUT1 expression and neoplastic progression[22]. The overexpression of GLUT1 in human cancers has been reported to be closely related to ^{18}F-FDG uptake on PET-CT[18]. Another report, however, showed no relation between GLUT1 expression and ^{18}F-FDG uptake on PET[33], and there is a controversial report that did not demonstrate a statistically significant correlation between GLUT1 expression and FDG uptake[35].

In present study ^{18}F-FDG uptake related to tumor size, whereas GLUT1 frequency did not. Brown *et al*[36] had mentioned that ^{18}F-FDG uptake and GLUT1 expression appeared to be associated with tumor size, but our data did not support their findings. Tumor size is one of the most important factors affecting the SUVmax[37]. The ^{18}F-FDG uptake might be influenced by the total amount of glucose uptake into the tumor. Therefore, the larger a carcinoma is, the higher is the ^{18}F-FDG uptake by the carcinoma shown on the PET scan. It is well known that SUVmax has a lower than "real" value when the tumor size is < 20 mm because of the limited resolution of current PET scanners[37,38]. In contrast, GLUT1 staining is examined through a microscope, and GLUT1 frequency is determined microscopically. Therefore, GLUT1 frequency shows microscopic activity of glucose uptake into the tumor and is influenced by cell type, cellularity, and pathological structure[39]. At the result, GLUT1 frequency would not be related to tumor size. GLUT1 expression could become strongly positive even in small carcinomas with high cellular density or metabolic activity.

In conclusion, in contrast to other malignant tumor such as lung cancer[39], squamous cell carcinoma of the cervix[1] and head and neck cancer[40], and cholangiocarcinoma[41], GLUT1 expression did not correlate significantly with ^{18}F-FDG uptake and other clinicopathologic parameters in CRA, which suggests that overexpression of GLUT1 cannot fully explain the biologic behavior of CRA. The ^{18}F-FDG uptake was significantly correlated with tumor size only. We identified that GLUT1 is usually strongly positive in the center of the necrotic and infiltrative areas in colorectal cancer. Although overexpression of GLUT1 is very important for ^{18}F-FDG uptake in cancer cells, further investigations should evaluate the contributions of other factors concerning tumor hypoxia and glucose metabolism.

COMMENTS

Background

Cancer cell growth is an energy-related process supported by increased glucose metabolism. This uptake is mediated by glucose transporter (GLUT) proteins, which are membrane proteins responsible for the transport of glucose across cellular membranes. Positron emission tomography (PET) using ^{18}F-fluoro-2-deoxyglucose (FDG) is a rapidly developing functional-imaging modality that has shown great promise in the fields of primary, recurrent and metastatic tumor detection, planning and monitoring therapy. Therefore, the authors conducted a prospective study to determine the association between GLUT1 expression and the maximum standardized uptake values (SUVmax) obtained from ^{18}F-FDG PET scans. The relationship between GLUT1 and SUVs with other clinicopathologic factors was also evaluated. Additionally, the authors evaluated the difference in GLUT1 expression between adenoma and carcinoma in the colorectum.

Research frontiers

This article may present information to the colorectal oncologist for further study about glucose metabolism of colorectal adenocarcinoma (CRA), and to the colorectal oncologist the usefulness of PET CT in evaluating colorectal cancer patients.

Innovations and breakthroughs

In contrast to other malignant tumors such as lung cancer, squamous cell carcinoma of cervix and head and neck, and cholangiocarcinoma, GLUT1 expression did not correlate significantly with ^{18}F-FDG uptake and other clinocopathologic parameters in CRA, which suggests that overexpression of GLUT1 cannot fully explain the biologic behavior of CRA. The ^{18}F-FDG uptake was significantly correlated with tumor size only. The authors identified that GLUT1 is usually strongly positive in the center of the necrotic and infiltrative areas in colorectal cancer. Although overexpression of GLUT1 is very important for ^{18}F-FDG uptake in cancer cells, further investigations should evaluate the contributions of other factors concerning tumor hypoxia and glucose metabolism.

Peer review

Overall the manuscript is reasonably well written and provides additional information on GLUT1 expression and FDG uptake.

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S- Editor Sun H **L- Editor** O'Neill M **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i2.175

World J Gastroenterol 2012 January 14; 18(2): 175-181
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Prognostic relevance of circulating CK19 mRNA in advanced malignant biliary tract diseases

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Supported by Rajavithi Hospital Project Grant and Thailand Research Fund, No. RSA52
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Received: April 9, 2011 Revised: June 17, 2011
Accepted: June 24, 2011
Published online: January 14, 2012

Abstract

AIM: To determine the role of circulating tumor cells (CTCs) in prediction of the overall survival of patients with advanced malignant biliary tract obstruction.

METHODS: We investigated the prognostic value of CTCs by examining two markers, cytokeratin (CK) 19 and human telomerase reverse transcriptase (hTERT) mRNA, in 40 patients diagnosed with advanced malignant biliary tract diseases. Quantitative real-time reverse transcription polymerase chain reaction was used to detect CK19 and hTERT mRNA in the peripheral blood of these patients. Overall survival was analyzed using the Kaplan-Meier method and Cox regression modeling.

RESULTS: Positive CK19 and hTERT mRNA expression was detected in 45% and 60%, respectively, of the 40 patients. Univariable analysis indicated that positive CK19 mRNA expression was significantly associated with worse overall survival (P = 0.009). Multivariable analysis determined that positive CK19 mRNA expression, patient's age and serum bilirubin were each independently associated with overall survival.

CONCLUSION: CK19 mRNA expression levels in peripheral blood appear to provide a valuable marker to predict the overall survival of patients with advanced malignant biliary tract obstruction.

Key words: Circulating tumor cells; Cytokeratin 19; Human telomerase reverse transcriptase; Malignant biliary tract obstruction; Overall survival

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Leelawat K, Narong S, Udomchaiprasertkul W, Wannaprasert J, Treepongkaruna S, Subwongcharoen S, Ratanashu-ek T. Prognostic relevance of circulating CK19 mRNA in advanced malignant biliary tract diseases. *World J Gastroenterol* 2012; 18(2): 175-181 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i2/175.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i2.175

INTRODUCTION

Malignant biliary tract obstruction is a condition that can

result from tumors of the biliary tract, ampulla of vater, duodenum or head of the pancreas. In Thailand, cholangiocarcinoma (CCA) is the most common cause of malignant biliary tract obstruction[1]. Despite recent advances in the diagnosis and treatment of this disease, patient outcome remains poor. The high mortality rate arising from malignant biliary tract obstruction is due to the aggressiveness of tumors that are often discovered at a late stage of disease progression[2]. Palliative therapeutic approaches to endoscopic biliary drainage, such as the use of endoprosthesis stents, are generally recommended for these patients. The two major types of endoprosthesis stents are plastic or polyethylene (PE) stents and self-expanding metal stents (SEMS). Previous studies have demonstrated that partial or total occlusion of PE stents usually occurs 3-4 mo after insertion[3]. Four randomized controlled studies demonstrated that SEMS exhibited a significantly higher patency rate as compared with the PE stents (9 mo *vs* 1.5 mo)[4-7]; however, SEMS is much more expensive than PE stents (1500 USD *vs* 80 USD, in Thailand). A recent study indicated that patients who have a predicted survival duration of more than 4.5 mo should use SEMs for their palliative biliary drainage[8]. In this instance, the higher cost of the SEMs is balanced by a decreased need for repeat intervention that is often necessary in patients who have received PE stents. Therefore, identification of reliable prognostic factors that allow for an accurate prediction of survival duration in patients with advanced malignant biliary tract obstruction is extremely important.

One of the major mechanisms for tumor metastasis is the dissemination of tumor cells from the primary tumor into circulating blood[9]. Previous studies have indicated that detection of circulating tumor cells (CTCs) in the peripheral blood can be used in staging and prognosis stratification for breast and colon cancer patients[10-12]. Until now, however, there has been no study concerning the role of the detection of CTCs as a prognostic factor in patients with malignant biliary tract diseases.

To date, the most common CTCs detection method is quantitative real-time reverse transcription polymerase chain reaction (RT-PCR), a process that can detect mRNA expression levels of the genes coding for these tumor antigens[13]. A high-quality detection marker is required for efficient quantitative real-time RT-PCR-mediated detection of CTCs. Therefore, identification of a good target marker is of the utmost importance for CTC detection. Several gene markers, such as cytokeratin (CK) 19 and human telomerase reverse transcriptase (hTERT), have been evaluated as tumor-specific markers for the detection of CTCs in gastrointestinal cancers[14,15].

hTERT mRNA can be detected in 85% of all cancer cells, including cholangiocarcinoma cells[16]. This is in contrast to most normal cells, which exhibit little or no expression. Our previous study demonstrated that high levels of hTERT mRNA can be detected in the blood circulation of cholangiocarcinoma patients, and it has also been suggested that hTERT mRNA is a promising marker for the detection of cancer cells[17].

CK19 is generally expressed in ductal epithelium (bile ducts, pancreas, and renal collecting tubules) and in the mucosa of the gastrointestinal tract. CK19 immunohistochemistry is used in diagnostic pathology mainly to confirm epithelial immunophenotype in undifferentiated tumors or to establish biliary, pancreatic or renal ductular origin[18]. Most adenocarcinomas arising from the gastrointestinal tract are CK19 positive, including cholangiocarcinoma and pancreatic cancer[18]. Many investigators have used the detection of CK19 mRNA in peripheral blood as a target gene to investigate CTCs[14,19,20]; however, until now there has been no study focusing on the detection of hTERT and CK19 in the peripheral blood of patients with malignant biliary tract diseases.

This study was aimed to evaluate if the levels of CTCs could be used to predict the overall survival of patients with advanced malignant biliary tract obstruction. *CK-19* and *hTERT* were selected as the target genes for CTCs. In addition, this study was performed in accordance with the REporting recommendations for tumor MARKer prognostic studies[21] to ensure the standardization and transparency of the study.

MATERIALS AND METHODS

Patients and samples

We prospectively included the patients with advanced malignant biliary tract diseases who underwent palliative endoscopic retrograde cholangiopancreatography or percutaneous transhepatic biliary drainage at Department of Surgery, Rajavithi Hospital from January 2008 to December 2009. The cutoff date for data analysis was December 31, 2010. The inclusion requirements included patients present with malignant bile duct obstruction that was not amenable to curative resection and patients who were followed up for at least one month after biliary tract drainage. All blood and clinical information was obtained with patient informed consent after approval by the Rajavithi Hospital Ethics Committee.

Pre-treatment fasting blood samples were collected from the peripheral vein into ethylenediaminetetraacetic acid-containing tubes. The first 3 mL blood was discarded to prevent epidermal contamination (2-syringe technique). Sample processing was performed within 1 h of blood withdrawal. Blood was transferred into a 30-mL falcon tube and centrifuged at 1800 r/min at room temperature for 20 min. Plasma was removed, and the peripheral blood mononuclear cell (PBMC) fraction was stored at -80 ℃ until use.

RNA extraction and cDNA synthesis

The total RNA of PBMC fraction samples was extracted using the RNeasy mini kit (Qiagen, GmbH, Germany) following the protocol provided by the manufacturer. RNA integrity was checked by electrophoresis and quantified by absorption at 260 and 280 nm using a spectrophotometer (Beckman Coulter Du® 800, Fullerton, CA). Total RNA was reversely transcribed using random primers and

Table 1 Primer sequences

Primer	Forward	Reverse
hTERT	GCGGAAGACAGTGGTGAACT	AGC TGGAGTAGT CGCTCT GC
CK19	CCCGCGACTACAGCCACTA	GCTCATGCGCAGAGCCT
β-Actin	GTGGGGCGCCCCAGGCACCA	GTCCTTAATGTCACGCACGATTTC

hTERT: Human telomerase reverse transcriptase; CK19: Cytokeratin 19.

Table 2 Clinical characteristics of patients with advanced malignant biliary tract obstruction

	Parameters	*n* (%)
Gender	Male	23 (57.5)
	Female	17 (42.5)
Age (yr)	< 60	18 (45.0)
	> 60	22 (55.0)
Type of cancers	Hilar cholangiocarcinoma	28 (70.0)
	Pancreatic cancer	6 (15.0)
	Common bile duct cancer	2 (5.0)
	Ampullary cancer	2 (5.0)
	Gall bladder cancer	2 (5.0)

the IscriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA, United States) following the protocol provided by the manufacturer. cDNA was stored at -80 ℃ until use.

Detection of CK-19 and hTERT mRNA by quantitative real-time PCR

Expression of *CK19* and *hTERT* genes was analyzed using specific primers (Table 1). In this assay, the housekeeping gene *β-actin* was used as an internal control to normalize variations in integrity and the total amount of cDNA. Quantitative real-time PCR assays were performed in triplicate using SYBR Green master mix (Superarray, Frederick, MD, United States) on the Chromo 4™ System (MJ Opticon Monitor ver. 3.1) (Bio-Rad, United States) for 20 min at 50 ℃. After this, 42 cycling steps for amplification of PCR products were performed (15 s, 94 ℃ for denaturation; 30 s, 60 ℃ for annealing; and 30 s, 72 ℃ for extension). Melting curve analysis was used to assess the specificity of the amplified products. The expression levels of *CK19* and *hTERT* genes from the cDNA were measured by quantitative real-time PCR using the relative quantification method ($2^{-\Delta\Delta Ct}$ method)[22]. The fold-change in gene expression was normalized to a housekeeping gene (*β-actin*) and relative to a calibrator sample. A pool of cDNA derived from the PBMCs of 30 cases of benign (common bile duct stone and gall stone) biliary tract diseases was used as the calibrator source[23]. Evaluation of the $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the calibrator. In this study, we set the positive value as a fold change in gene expression that was greater than 1.5 times relative to the calibrator and the negative value was set as a fold change in gene expression that was lesser than or equal to 1.5 times relative to the calibrator.

Determination of blood chemistries in serum samples

Biochemical studies of serum samples, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), total and direct bilirubin, alkaline phosphatase, carcinoembryonic antigen (CEA) and cancer antigen (CA)19-9, were measured using routine automated methods in the Pathological Laboratory at Rajavithi Hospital.

Cell lines and cell spiking experiments

The human cholangiocarcinoma cell line RMCCA1[24] was incubated in Ham's F12 medium (Invitrogen-Gibco, Carlsbad, CA, United States) containing 10% fetal calf serum (Euroclone-Celbio, Pero, MI) at 37 ℃ in 5% CO_2. To determine the sensitivity of quantitative real-time PCR for detecting cancer cells in PBMCs, cell spiking experiments was performed. The PBMCs obtained from healthy volunteers were counted and diluted in Ham's F12 medium. RMCCA1 cells were serially diluted from 1 × 10^6 cells/mL to 1 cell/mL and added to the PBMCs. Quantitative real-time PCR was then performed to detect CK19 and hTERT mRNA.

Statistical analysis

The primary endpoint of this study was the overall survival of the patients. Survival curves were estimated using the Kaplan-Meyer method, and univariable survival comparisons were calculated according to the log rank test. Multivariable survival analysis was performed using the Cox proportional hazards regression model. The quantitative variables were compared using Mann-Whitney *U* or Student's *t* test, as appropriate. Qualitative variables were reported as counts, and comparisons between independent groups were performed using the Pearson χ^2 test. All tests of significance were two sided and $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Forty-two patients with malignant biliary tract disease were included in this study. Two patients were excluded because of the poor quality of the RNA extracted from their peripheral blood. The average age of these patients was 62 years (range, 41-82 years). With regard to cancer type, 6 (15.0%) were pancreatic head cancer, 2 (5.0%) were ampullary cancer, 2 (5.0%) were gall bladder cancer, 2 (5.0%) were middle and distal common bile duct cancer and 28 (70.0%) were hilar cholangiocarcinoma. The clinical characteristics of the patients are shown in Table 2.

Cell spiking assay

CK19 and hTERT mRNA levels were elevated in the RMCCA1 cell line (Figure 1); therefore, we decided to use this cell line as a positive control for our study. Detection sensitivity of the quantitative real-time PCR assay was determined by serial 10-fold dilutions of RMCCA1 cells in PBMCs. The results demonstrated that CK19 and hTERT mRNA could be detected at levels up to 1000 cells per 10^{10} PBMC dilutions.

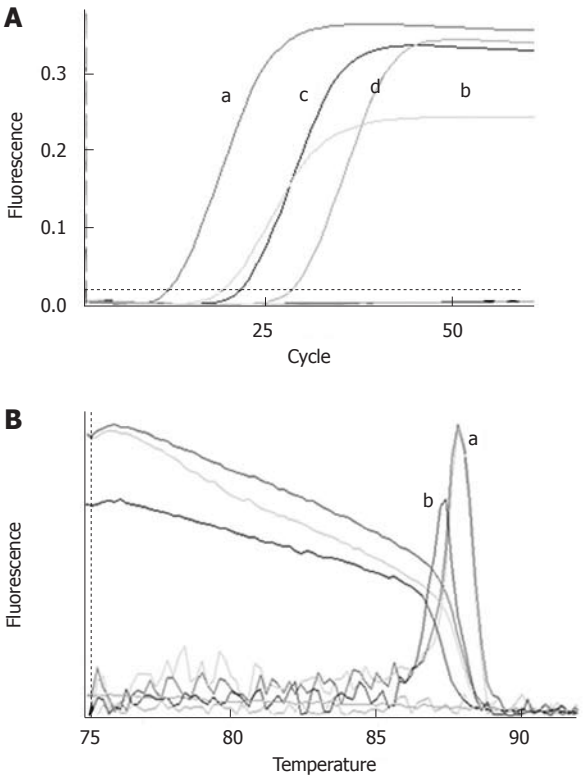

Figure 1 Gene expression levels of cytokeratin 19 and human telomerase reverse transcriptase in RMCCA1 cells (as measured by quantitative real time polymerase chain reaction). A: Amplification plot of cytokeratin (CK)19 mRNA from 10 000 RMCCA1 cells. (a) 1000 RMCCA1 cells; (b) amplification plot of human telomerase reverse transcriptase (hTERT) mRNA from 10 000 RMCCA1 cells; (c) 1000 RMCCA1 cells; and (d) are demonstrated; B: SYBR Green melting curve for quantitative real time reverse transcription polymerase chain reaction (RT-PCR). The melting curves from quantitative real time PCR for CK19 (a) and hTERT (b) consistently gave a single peak with no evidence of non-specific amplification or primer-dimerisation.

Detection of hTERT and CK19 mRNA in PBMCs of malignant biliary tract disease patients

PBMC samples from 40 patients were evaluated for the expression of hTERT and CK19 mRNA. The expression was positive (gene expression more than 1.5 times relative to the calibrator) in 45% (18/40) of samples for CK19 mRNA and 60% (24/40) of samples for hTERT. Figure 2 illustrates the distribution of CK19 mRNA and hTERT mRNA expression in the peripheral blood of these patients.

Relationship between CK19 and hTERT mRNA expression in peripheral blood and clinic pathological features of patients

No statistically significant difference was found among the data obtained from the patients considered as negative or those who were positive for hTERT or CK19 mRNA expression in PBMCs. Factors evaluated included gender, age, serum albumin, globulin bilirubin AST and ALT and alkaline phosphatase levels (Table 3).

Survival analysis

At the time of data analysis, only 1 patient was alive and 39 patients had died. The median overall survival for these patients was 4.0 mo (95% CI: 2.56-4.56). The median survival time was 1.7 mo in patients with positive CK19 mRNA expression, whereas the survival time was 5.3 mo in patients with negative CK19 mRNA expression (Log Rank, $P = 0.009$). We found that the median survival time in patients with a negative hTERT mRNA expression was not significantly different from patients with positive hTERT mRNA expression (5.9 mo *vs* 3.2 mo, Log Rank, $P = 0.183$) (Figure 3).

To identify variables that could be of potential prognostic significance in patients with advanced malignant biliary tract disease, univariable and multivariable analyses were performed using the Cox proportional hazard model to compare the impact of the mRNA expression levels of CK19 and hTERT. Other clinical parameters such as positive or negative hTERT expression, CK19 expression (positive or negative), CEA (cut-off value = 5 ng/mL), CA19-9 (cut-off value = 500 U/mL), total bilirubin (cut-off value = 15 mg/dL) and albumin (cut-off value = 3 g/dL) were also examined. Univariable analysis indicated that only CK19 mRNA expression showed significance as a prognostic factor. Multivariable analysis demonstrated that CK19 mRNA expression ($P = 0.024$), age ($P = 0.026$) and serum bilirubin ($P = 0.002$) were all independent risk factors for survival. The relative risk for CK19 mRNA positive patients was 3.2 times greater than that for CK19 mRNA negative patients (Table 4).

Table 3 Clinical characteristics of patients with negative and positive cytokeratin 19 and human telomerase reverse transcriptase gene expression

	CK19 gene		*p*	*hTERT* gene		*p*
	Negative	Positive	value	Negative	Positive	value
Age (yr)	63.80	61.38	0.42	65.26	60.88	0.16
Sex (male: female)	11:11	12:6	0.35[1]	8:8	15:9	0.52[1]
Total bilirubin (mg/dL)	16.21	16.42	0.95	17.73	15.47	0.53
Albumin (g/dL)	2.88	2.97	0.62	2.83	2.99	0.36
Globulin (g/dL)	3.98	3.76	0.42	3.92	3.84	0.79
AST (U/L)	84.57	112.80	0.23	69.27	117.48	0.12
ALT (U/L)	42.47	65.85	0.12	34.87	66.68	0.28
ALP (IU/L)	449.68	528.85	0.56	436.88	421.91	0.98
BUN (mg/dL)	13.77	26.58	0.13	12.25	23.52	0.18
Creatinine (mg/dL)	0.75	1.30	0.20	0.61	1.19	0.22
CA19-9 (U/mL)	570.20	594.30	0.62[2]	1818.00	259.15	0.11[2]
CEA (ng/mL)	7.47	5.68	0.50[2]	7.21	5.82	0.23[2]

Quantitative variables are presented as the mean value, with the exception of cancer antigen (CA)19-9 and carcinoembryonic antigen (CEA), which are presented as median values. [1]Pearson χ^2 was used to compare two groups; [2]Mann-Whitney *U* test was used to compare groups. CK19: Cytokeratin 19; hTERT: Human telomerase reverse transcriptase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen.

DISCUSSION

The highest incidence of cholangiocarcinoma occurs in Thailand, and the majority of patients included in this study were diagnosed with this disease[1]. In this study,

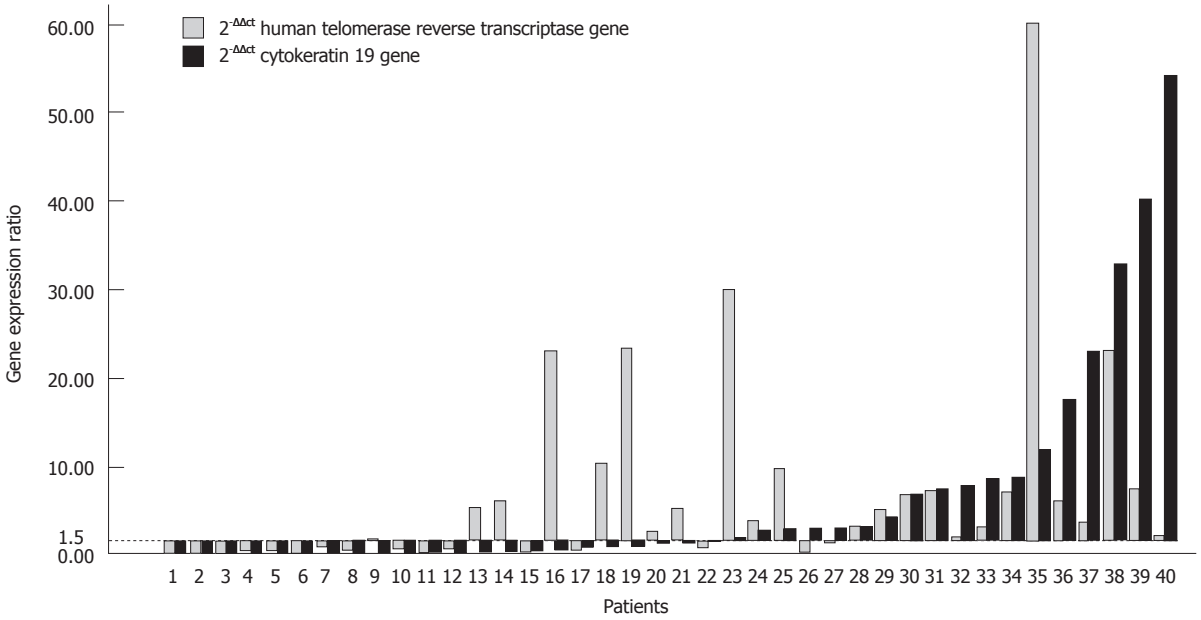

Figure 2 The distribution levels of cytokeratin 19 and human telomerase reverse transcriptase genes in the peripheral blood of 40 patients. The positive value is determined as a fold change in gene expression of more than 1.5 times relative to the calibrator.

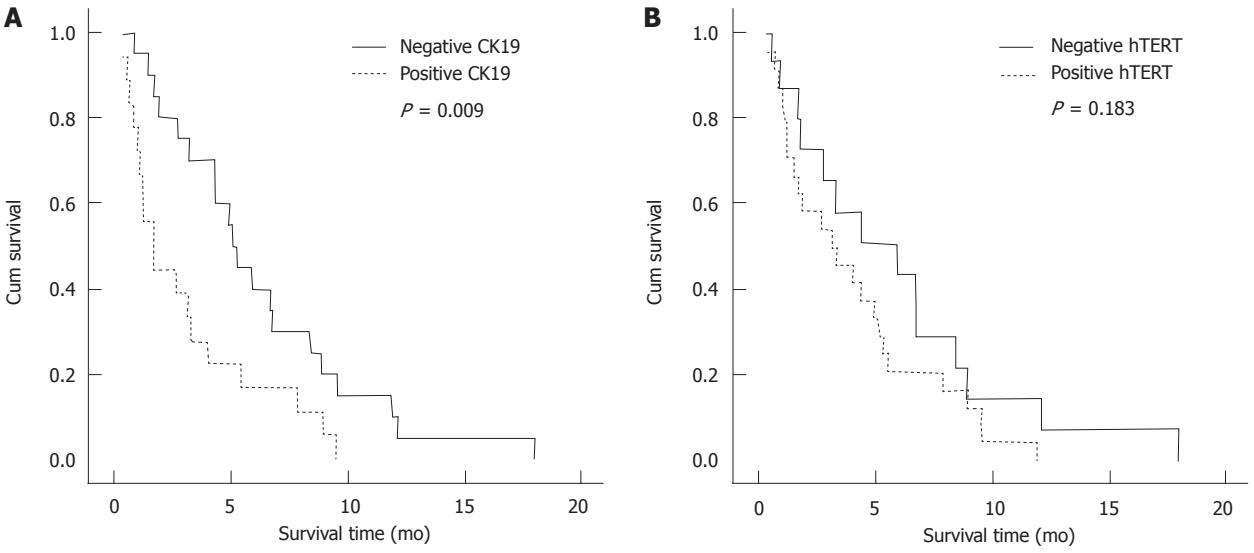

Figure 3 Kaplan-Meier survival curves of patients with positive or negative expression of cytokeratin 19 (A) and human telomerase reverse transcriptase (B) genes measured in the peripheral blood. CK19: Cytokeratin 19; hTERT: Human telomerase reverse transcriptase.

the median survival was 4.0 mo, a finding that is not significantly different from the survival time (3.6-5.0 mo) observed in previous studies of advanced malignant biliary tract disease where the majority of patients were diagnosed with pancreatic cancer[7,8,25]. These results indicate that all cancers that lead to malignant biliary tract obstruction are highly lethal. Most advanced malignant biliary tract disease patients can only be treated with palliative biliary tract drainage.

The choice of stents (PE or SEMs) for endoscopic palliation of jaundice due to malignant biliary tract obstruction is dependent upon the estimation of patient survival[8]. Therefore, there is a need for more accurate tests to predict the survival of patients with advanced malignant biliary tract diseases, as this could significantly improve the treatment outcome for these patients. This is the first cohort paper that studies the level of CTCs as a prognostic factor for overall survival of patients with advanced malignant biliary tract obstruction.

We used quantitative real-time RT-PCR to detect CTCs. As a result of the PCR-based methods, we cannot identify exactly the cell source of the measured markers. Quantitative real-time RT-PCR assesses the expression of target genes from mRNA extracted from the lysates of cells harvested from the peripheral blood of patients. As such, these samples contain not only CTC but

Table 4 Survival analysis using clinical parameters measured by univariable and multivariable analysis

Variables	Univariable analysis		Multivariate analysis	
	P value	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)
CK19 expression	0.011[1]	2.42 (1.22-4.78)	0.024[1]	3.20 (1.17-8.75)
hTERT expression	0.188	1.60 (0.80-3.22)	0.580	1.34 (0.47-3.82)
Age	0.541	0.82 (0.42-1.57)	0.026[1]	0.38 (0.16-0.89)
Total bilirubin	0.106	1.72 (0.89-3.31)	0.002[1]	3.97 (1.69-9.36)
Albumin	0.360	0.73 (0.37-1.35)	0.213	0.59 (0.26-1.35)
CA19-9	0.374	0.73 (0.37-1.43)	0.478	0.74 (0.32-1.70)
CEA	0.381	1.39 (0.68-2.88)	0.124	1.86 (0.84-4.12)

[1]Statistically significant. CI: Confidence interval; CK19: Cytokeratin 19; hTERT: Human telomerase reverse transcriptase; CA: Cancer antigen; CEA: Carcinoembryonic antigen.

also PBMC, circulating endothelial cells and skin cells (e.g., keratinocytes, fibroblasts and melanocytes) that contaminate the sample during blood withdrawal and provide alternate potential sources for the PCR-detected genes[13,26]. Therefore, strict selection of target genes for detection of CTCs is very important. In this study, we used *CK19* and *hTERT* genes as targets for the detection of CTCs. Previous studies have suggested that CTCs are likely to be the principal cell source for *CK19* gene expression, as CK19 expression is mainly restricted to epithelial cells and is limited in normal peripheral blood cells[20,27]. Additionally, we used the 2-syringe technique during blood collection to avoid epithelium contamination from injected site.

In our study, the patients with positive CK19 expression exhibited significantly shorter overall survival compared with the patients with negative CK19 expression (5.3 mo *vs* 1.7 mo; $P = 0.009$). Additionally, multivariable analysis using the Cox regression model also demonstrated that the levels of CK19 expression in peripheral blood, the levels of serum total bilirubin and the age of the patients can all function as independent prognostic factors in patients with advanced malignant biliary tract disease. This is consistent with previously published studies that reported that positive CK19 mRNA expression in peripheral blood was independently associated with a reduction in disease-free survival in patients with breast cancer[20]. In addition, positive CK19 mRNA expression in peripheral blood following chemoradiation was an independent, unfavorable prognostic factor for both overall survival and progression-free survival in patients diagnosed with non-small cell lung cancer[19].

In this study, there were more patients with positive hTERT mRNA expression in peripheral blood than the patients with positive CK19 mRNA expression (60% *vs* 45%); however, detection of hTERT mRNA in the peripheral blood was not identified as an independent prognostic factor in this study. We suggested that the detection of hTERT mRNA expression levels was not a good candidate as a prognostic factor for patients with advanced malignant biliary tract disease. It may be suitable for the distinction between malignant and benign biliary tract diseases in combination with other tumor specific markers.

In this study, neither univariable nor multivariable analysis indicated that serum levels of CA19-9 could be used as a prognostic factor for patients with advanced malignant biliary tract disease. This finding is inconsistent with a previous study that indicated that the levels of serum CA19-9 are of prognostic relevance in patients with biliary tract cancer[28,29]. We suggested that differences among the patients should be considered. Overall survival in a previous study was 16.1 mo[29], whereas the overall survival in our study was 4.0 mo. In addition, the majority of patients in the previous study received chemotherapy, while only two patients in our study received this treatment. This finding reflects a higher disease severity in the patients examined in our study.

Our study demonstrated that the expression of CK19 mRNA in PBMCs prior to palliative procedures was significantly associated with overall survival of the patients with advanced malignant biliary tract disease. We therefore recommend PE stents for patients with positive CK19 mRNA expression in their peripheral blood. The more expensive SEMS should be reserved for patients with negative peripheral blood expression of CK19 mRNA. Further cost-effectiveness studies should be conducted to evaluate the benefit of using CK19 mRNA in helping the physician make decisions regarding the selection of stent-type and the need for endoscopic repeat intervention in patients with advanced malignant biliary tract disease.

COMMENTS

Background

In Thailand, cholangiocarcinoma is the most common cause of malignant biliary tract obstruction. Despite recent advances in the diagnosis and treatment of this disease, patient outcome remains poor. Palliative therapeutic approaches to endoscopic biliary drainage, such as the use of endoprosthesis stents, are generally recommended for these patients. Identification of reliable prognostic factors that allow for an accurate prediction of survival duration in patients suffering from advanced malignant biliary tract obstruction is extremely important.

Research frontiers

This study demonstrated that the levels of circulating tumor cells (CTCs) could be used to predict the overall survival of patients with advanced malignant biliary tract obstruction.

Innovations and breakthroughs

The expression of cytokeratin (CK)19 mRNA in peripheral blood mononuclear cells prior to palliative procedures was significantly associated with overall survival of patients with advanced malignant biliary tract disease.

Applications

This study recommends polyethylene stents for patients with positive CK19 mRNA expression in their peripheral blood. The more expensive self-expanding metal stents should be reserved for patients with negative peripheral blood expression of CK19 mRNA.

Terminology

CTCs is the dissemination of tumor cells from the primary tumor into circulating blood. The detection of CTCs can be used in staging and prognosis stratification for cancer patients.

Peer review

This study provides evidences that high levels of CTCs in advanced malignant biliary tract obstruction patients might be used as a prognostic factor. This is a well performed and clearly presented study.

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S- Editor Sun H **L- Editor** Ma JY **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i2.182

World J Gastroenterol 2012 January 14; 18(2): 182-187
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Quality of life and psychological outcome of donors after living donor liver transplantation

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Supported by National Science and Technology Key Projects, No. 2008ZX10002-026
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Received: May 19, 2011 Revised: July 11, 2011
Accepted: July 18, 2011
Published online: January 14, 2012

Abstract

AIM: To investigate the health related quality of life (HRQoL) and psychological outcome of donors after living donor liver transplantation.

METHODS: Participants were 92 consecutive liver transplant donors who underwent hepatectomy without middle hepatic vein at West China Hospital of Sichuan University between January 2007 and September 2010. HRQoL was measured using the Chinese version of the Medical Outcomes Study Short Form-36 (SF-36), and psychological symptoms were measured using the Symptom Checklist-90-Revised (SCL-90-R). Data collected from donors were compared to previously published data from the general population. Clinical and demographic data were collected from medical records and questionnaires.

RESULTS: The general health score of the SF-36 was significantly lower in females (59.78 ± 12.25) than in males (75.83 ± 22.09). Donors more than 40 years old scored higher in social functioning (85.71 ± 14.59) and mental health (82.61 ± 20.00) than those younger than 40 (75.00 ± 12.13, 68.89 ± 12.98; social functioning and mental health, respectively). Donors who had surgery more than two years prior to the study scored highest in physical functioning (P = 0.001) and bodily pain (P = 0.042) while those less than one year from surgery scored lowest. The health of the liver recipient significantly influenced the general health (P = 0.042), social functioning (P = 0.010), and role-emotional (P = 0.028) of donors. Donors with full-time employment scored highest in role-physical (P = 0.005), vitality (P = 0.001), social functioning (P = 0.016), mental health (P < 0.001), the physical component summary scale (P < 0.001), and the mental component summary scale (MCS) (P < 0.001). Psychological measures indicated that donors were healthier than the general population in obsessive-compulsive behavior, interpersonal sensitivity, phobic anxiety, and paranoid ideation. The MCS of the SF-36 was significantly correlated with most symptom scores of the SCL-90-R.

CONCLUSION: HRQoL and psychological outcome were favorable in living liver transplant donors after donation. Specifically, gender, age, time since operation, recipient health condition, and employment after donation, influenced postoperative quality of life.

Key words: Health related quality of life; Psychology; Living donor liver transplantation; Donor

Peer reviewers: Christopher Christophi, Professor and Head of The University of Melbourne Department of Surgery, Austin Hospital, 145 Studley Road, Melbourne, Victoria 3084, Australia; Giedrius Barauskas, Professor, Department of Surgery, Kaunas University of Medicine, Eiveniu str. 2, Kaunas LT-50009, Lithuania

Jin SG, Xiang B, Yan LN, Chen ZY, Yang JY, Xu MQ, Wang WT. Quality of life and psychological outcome of donors after living donor liver transplantation. *World J Gastroenterol* 2012; 18(2): 182-187 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i2/182.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i2.182

INTRODUCTION

The rapid growth of living donor liver transplantation (LDLT) is attributable to the continual improvement in recipient survival and the shortage of deceased donor liver grafts[1,2]. Evidence supports a significant reduction in mortality of recipients listed for liver transplantation[3,4]. However, the donor of LDLT is exposed to risks inherent to a surgical procedure, and may suffer a considerable psychological burden[5]. Therefore, the safety of the donor operation and the health related quality of life (HRQoL) of the donor after surgery is critical while maintaining graft viability.

In the transplant center at West China Hospital of Sichuan University, liver recipient survival rates at one, three, and five years were 87.4%, 80.5% and 72.7%[6], respectively, which are similar to that reported elsewhere. Since 2001[7] over 250 cases of LDLT have been performed in our center, accounting for 30% of total transplant volume and this ratio is expected to increase in the future. However, the HRQoL and psychological outcome of donors remain unclear. The aim of the current cross-sectional study was to explore the HRQoL and the psychological outcome of donors after LDLT. To our knowledge, this is the first study of HRQoL and psychological outcome for the living liver transplant donor in mainland China. The results of the study may better guide adult-to-adult LDLT practice.

MATERIALS AND METHODS

Patients

From January 2007 to September 2010, 92 consecutive liver donors at West China Hospital of Sichuan University were approached for participation. The investigation extended from September 2010 to March 2011. Inclusion criteria were: age ⩾ 18 years, an understanding of Chinese, and greater than 6 mo recovery from surgery. Exclusion criteria were: severe medical complications and limited ability to self-express. Clinical and demographic data were collected from medical records and self-report questionnaires (completed by interview or mail).

Instruments

HRQoL was assessed using the Chinese version (2002)[8] of the Medical Outcomes Study Short Form-36 (SF-36)[9,10]. The SF-36 is a valid, self-administered questionnaire used internationally to measure 8 domains of health: physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional, and mental health during the last 12 mo. The raw scores of each subscale were transformed into scores that ranged from 0 to 100, with higher scores indicating higher levels of functioning or well-being. The level of HRQoL was assessed by comparing the mean value for the study sample with the mean value for a representative sample of the general population of Sichuan province in China[11]. Scores representing overall physical functioning and mental functioning were calculated from the subscales and presented as the physical component summary scale (PCS) and mental component summary scale (MCS).

The Symptom Checklist-90-Revised (SCL-90-R)[12] is a 90-item self-report symptom inventory used to measure the psychological symptoms patterns of community, medical, and psychiatric respondents. It is a simple questionnaire that has been validated in a number of languages. The Chinese version was adapted by Wang[13]. Each of the items is rated on a five-point scale of distress ranging from "not at all" (1) to "extremely" (5). The nine primary symptom dimensions were labeled as: somatization, obsessive-compulsive behavior, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety, paranoid ideation, and psychoticism. We assessed the level of psychological health of our sample and compared it with the Chinese norm[14].

Ethical considerations

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the West China Hospital of Sichuan University Ethics Committee. All participants were asked to sign an informed consent form.

Statistical analysis

Statistical analysis was performed using SPSS statistical software, version 13.0. Between-group differences in HRQoL and psychological health were examined with independent sample *t* tests, analysis of variance, or nonparametric tests, as appropriate. Multiple comparisons for observed means were tested using the Student-Newman-Keuls procedure when equal variances could be assumed, and by the Games-Howell procedure when equal variances could not be assumed. Pearson correlation analysis were used to analyze the relationships between HRQoL and psychological symptoms. Statistical significance was set at $P < 0.05$.

RESULTS

Donor characteristics

Informed consents for participation were obtained from 92 donors. In the end, 71 (77.2%) validated questionnaires were returned. The results of SF-36 and SCL-90-R completed by interview or mail were not statistically different. All donors received a right hepatectomy without middle hepatic vein, and the vast majority of them reported that they would donate again. All donor relationships with liver recipient and recipient families

Table 1 Donor characteristics

Factors	Frequency	mean ± SD/percent (%)
Age (yr)		38.94 ± 10.44
≤ 40	42	59.2
> 40	29	40.8
Marital status (married/unmarried)	62/9	87.3/12.7
Gender (male/female)	40/31	56.3/43.7
Educational status		
Elementary school	15	21.1
Middle school	46	64.8
University	10	14.1
Occupation		
Worker	12	16.9
Peasant	36	50.7
Civil servant	7	9.9
Others[2]	16	22.5
Complication (yes/no)	5/66	7.0/93.0
Time since operation		
≤ 1 yr	16	22.5
> 1 yr, ≤ 2 yr	34	47.9
> 2 yr, ≤ 3 yr	21	29.6
Employment after donation		
Full-time	53	74.6
Part-time	9	12.7
No employment	9	12.7
Recipients		
Parenthood	9	12.7[1]
Children	9	12.7[1]
Couples	7	9.9[1]
Brothers and sisters	30	42.3[1]
Distant relatives	16	22.5[1]
Recipient health well-being		
Fine	56	78.9
Deterioration or death	15	21.1

[1]The sum of percentages is not equal to 100% due to rounding error; [2]Includes students, unemployed, *etc*.

were improved after donation. The demographics and clinical characteristics of the study population are shown in Table 1. The mean age of participants was 38.94 ± 10.44. Most donors were married (87.3%). More than half of the donors were male (56.3%), peasants (50.7%), and had achieved a secondary education level (64.8%). A total of 7.0% of donors experienced early or late complications including slight biliary leakage, pulmonary infection, and bodily pain. Many (47.9%) donor operations occurred 1-2 years before completing the questionnaires. Most donors worked full- or part-time after donation (87.3%). All donors were related to recipients, and most of them were close relatives (77.6%). The majority of recipients (78.9%) were in good health at the time of investigation.

HRQoL and psychological outcomes

The majority of scores on SF-36 domains did not significantly differ between donors and a representative sample (n = 1603) from the general population of Sichuan province in China (Table 2). Only scores in bodily pain (t = -2.387, P < 0.05) and social functioning (t = -2.246, P < 0.05) were significantly lower in donors compared to the general population, while the average donor physical functioning score was significantly higher than the general population (t = 2.230, P < 0.05).

Table 2 Health related quality of life after donation

SF-36 domains	Donors (71) mean ± SD	General population mean ± SD	t value	P value
Physical functioning	93.66 ± 7.26	90.80 ± 15.07	2.230	0.033
Role-physical	80.88 ± 33.18	79.51 ± 34.70	0.241	0.811
Bodily pain	71.29 ± 27.15	82.41 ± 21.25	-2.387	0.023
General health	67.33 ± 19.11	67.30 ± 21.97	0.010	0.992
Vitality	67.22 ± 18.72	71.44 ± 15.81	-1.234	0.227
Social functioning	79.69 ± 14.11	85.29 ± 18.06	-2.246	0.032
Role-emotional	76.47 ± 39.81	76.45 ± 38.47	0.003	0.998
Mental health	74.13 ± 17.12	73.52 ± 15.68	0.196	0.846

SF-36: Short Form-36.

Table 3 Psychological symptoms after donation

SCL-90-R dimensions	Donors (71) mean ± SD	Chinese norm mean ± SD	t value	P value
Somatization	1.41 ± 0.39	1.37 ± 0.48	0.600	0.553
Obsessive-compulsive behavior	1.50 ± 0.30	1.62 ± 0.58	-2.119	0.042
Interpersonal sensitivity	1.42 ± 0.32	1.65 ± 0.51	-4.183	< 0.001
Depression	1.39 ± 0.35	1.50 ± 0.59	-1.741	0.092
Anxiety	1.35 ± 0.37	1.39 ± 0.43	-0.708	0.485
Hostility	1.54 ± 0.44	1.48 ± 0.56	0.797	0.432
Phobic anxiety	1.11 ± 0.13	1.23 ± 0.41	-5.312	< 0.001
Paranoid ideation	1.25 ± 0.29	1.43 ± 0.57	-3.472	0.002
Psychoticism	1.25 ± 0.34	1.29 ± 0.42	-0.660	0.514

SCL-90-R: Symptom Checklist-90-Revised.

The average SCL-90-R scores of the general population were significantly greater than average donor scores in the areas of obsessive-compulsive behavior (t = -2.119, P < 0.05), interpersonal sensitivity (t = -4.183, P < 0.001), phobic anxiety (t = -5.312, P < 0.001), and paranoid ideation (t = -3.472, P < 0.01) (Table 3). These results indicate that the psychological well-being of liver transplant donors was higher than the general population in these dimensions.

Analysis of HRQoL

The general health domain of the SF-36, was significantly lower for female donors compared to male donors (t = 2.661, P < 0.05). Donors more than 40 years old scored higher in social functioning (t = 2.269, P < 0.05) and mental health (t = 2.184, P < 0.05). Donors who underwent surgery more than two years before the current study scored highest in physical functioning (F = 9.394, P = 0.001) and bodily pain (F = 3.513, P < 0.05), while those undergoing surgery less than one year prior to the study scored lowest. Quality of life differed significantly depending on donor employment status. Donors with full-time employment scored highest in role-physical (F = 5.790, P = 0.005), vitality (F = 9.018, P = 0.001), social functioning (F = 4.786, P < 0.05) and mental health (F = 11.051, P < 0.001). Interestingly, recipient health condi-

Table 4 Donor health related quality of life

Factors SF-36 domains	Groups mean ± SD	Groups mean ± SD	Groups mean ± SD	*t*/*F* value	*P* value
Gender	Male	Female			
General health	75.83 ± 22.09	59.78 ± 12.25		t = 2.661	0.012
Age (yr)	≤ 40	> 40			
Social functioning	75.00 ± 12.13	85.71 ± 14.59		t = 2.269	0.031
Mental health	68.89 ± 12.98	82.61 ± 20.00		t = 2.184	0.038
Time since operation (yr)	≤ 1	> 1, ≤ 2	> 2, ≤ 3		
Physical functioning	82.50 ± 2.89	94.42 ± 6.66[1]	98.33 ± 2.58[1]	F = 9.394	0.001
Bodily pain	52.67 ± 24.35	70.91 ± 27.57[2]	91.33 ± 13.43[1,2]	F = 3.513	0.042
Employment after donation	Full-time	Part-time	No employment		
Role-physical	93.64 ± 6.93	76.67 ± 12.91[3]	75.00 ± 22.36[3]	F = 5.790	0.005
General health	69.45 ± 18.01[4]	76.22 ± 18.54[4]	50.67 ± 16.03	F = 3.538	0.041
Vitality	74.24 ± 15.52	46.67 ± 8.12[3]	48.33 ± 14.38[3]	F = 9.018	0.001
Social functioning	84.09 ± 12.31	75.00 ± 14.43	66.67 ± 12.91[3]	F = 4.786	0.016
Mental health	81.82 ± 11.89	52.00 ± 5.24[3]	53.33 ± 11.50[3]	F = 18.137	< 0.001
PCS	58.51 ± 5.31	52.31 ± 5.01[3,4]	43.59 ± 5.52[3]	F = 11.051	< 0.001
MCS	54.31 ± 6.00	44.56 ± 3.42[3,4]	34.92 ± 2.66[3]	F = 32.748	< 0.001
Recipient health well-being	Well	Poor or death			
General health	71.57 ± 19.10	55.42 ± 9.03		t = 2.121	0.042
Social functioning	82.69 ± 11.77	66.67 ± 17.08		t = 2.763	0.010
Role-emotional	87.18 ± 31.38	41.67 ± 46.29		t = 2.603	0.028

Only statistically significant data are displayed. [1]Compared with group "≤ 1 yr", $P < 0.05$; [2]Compared with group "> 1, ≤ 2 yr", $P < 0.05$; [3]Compared with group "Full-time", $P < 0.05$; [4]Compared with group "No employment", $P < 0.05$. SF-36: Short Form-36; PCS: Physical component summary scale; MCS: Mental component summary scale.

Table 5 Correlation analysis between health related quality of life and psychological symptoms

SCL-90-R dimensions	SF-36			
	PCS		MCS	
	r value	*P* value	*r* value	*P* value
Somatization	0.200	0.290	-0.246	0.190
Obsessive-compulsive behavior	0.173	0.362	-0.421	0.020
Interpersonal sensitivity	-0.067	0.726	-0.545	0.002
Depression	-0.306	0.114	-0.557	0.002
Anxiety	-0.222	0.238	-0.393	0.032
Hostility	-0.335	0.071	-0.456	0.011
Phobic anxiety	0.118	0.535	-0.201	0.266
Paranoid ideation	0.035	0.853	-0.157	0.407
Psychoticism	0.028	0.881	-0.209	0.267

SCL-90-R: Symptom Checklist-90-Revised; SF-36: Short Form-36; PCS: Physical component summary scale; MCS: Mental component summary scale.

tion also influenced donor general health (t = 2.121, P < 0.05), social functioning (t = 2.763, P = 0.010), and role-emotional (t = 2.603, P < 0.05) (Table 4). Marital status, educational status, categories of occupation, complications, or donor-recipient relationship did not significantly affect quality of life.

To reduce the number of outcome variables regarding HRQoL, outcomes among donors were also compared using the PCS and the MCS of the SF-36. PCS (F = 11.051, P < 0.001) and MCS (F = 32.748, P < 0.001) scores were highest in donors with full-time employment and lowest in unemployed donors (Table 4). Other demographic and clinical factors did not affect PCS or MCS scores.

Table 5 presents the correlation coefficients between PCS and MCS scores of the SF-36 and the scores on the SCL-90-R subscales. MCS scores were significantly (all P < 0.05) correlated with obsessive-compulsive behavior (r = -0.421), interpersonal sensitivity (r = -0.545), depression (r = -0.557), anxiety (r = -0.393), and hostility (r = -0.456). There were no significant correlations between PCS scores and SCL-90-R scores.

DISCUSSION

Overall, donors reported a positive experience. The vast majority of donors stated that they would donate again, and almost all believed they had benefited from the donation. All donors were able to return to their (pre-donation) job a few months after donation (while some donors chose to quit their previous job). There were few significant differences in quality of life domains between the donors in the current study and the general population. Interestingly, donors reported a higher level of physical functioning than the general population. This observation has been previously described[15-17].

Female donors scored lower than male donors in the general health domain of the SF-36. This difference may be due to social and psychological factors[18,19]. The rates of psychological distress and physical illness are higher in women probably due to gender roles. Gove[20] points out that the highly structured roles of men tend to be causally linked to good mental health and low rates of morbidity, while the typical nurturing roles of women tend to be associated with a high level of social demand and lack of privacy. Furthermore, occupying a nurturing role impairs one's ability to effectively adopt a patient role[20].

Employment status, a measurement indicative of the

donor's ability to resume societal roles, was significantly related to quality of life. Previous research[21,22] has found that employed liver recipients reported better HRQoL than those unemployed after liver transplantation. However, the relationship between employment and HRQoL of donors remains elusive. While previous research[23,24] reported that most donors are able to return to pre-donation employment status within a few months, the direct relationship between employment status and HRQoL was not detailed until the current study.

Other factors impacting the quality of life included age of donor and time since operation. Older donors reported a significantly higher quality of life in domains such as social functioning and mental health. In addition, quality of life of donors more than one year after surgery was greater than that of donors who had undergone surgery during the previous time. These results suggest that HRQoL recovers with time post operation. In agreement, a study by Chan *et al*[25] found that donor quality of life, particularly the physical component, was most significantly affected during the first three postoperative months while physical and mental quality of life returned to pre-operation levels by a 6 to 12 mo period.

Despite previous reports[15,17] showing no relationship between recipient outcome and donor quality of life, the current study found that recipient well-being was an important factor influencing donor quality of life. The donors in the present study were all genetically and emotionally related to recipients. Throughout the donation process, donors were strongly concerned about the recipients. These emotional ties resulted in a strengthening of the relationships between donors and recipients and their families.

The resection of the right hepatic lobe is a safe operation and resulted in a good psychological outcome for most donors, irrespective of donation-related potential risks. The majority of donors were not anxious, did not feel coerced, and did not consider donation dangerous prior to the operation. Some donors reported excitement in facing a new experience and some said they could handle any consequences of the surgery. Only a few donors reported being anxious, being unsure about the operation, and experiencing increased stress prior to the operation. Some donors verbalized feelings of gratefulness and increased maturity post surgery. Most aspects of donor mental quality of life were significantly related to psychological symptoms. These results indicate the necessity of providing support to donors who experience negative feelings.

In conclusion, LDLT donors were healthy and the overall quality of life and psychological outcome were favorable. Employment after donation is an important factor significantly related to quality of life. Gender, age of donor, time since operation, and recipient health were all found to influence aspects of the quality of life of donors. Right hepatectomy is an acceptable procedure, with encouraging donor outcomes. Donor HRQoL and psychological status should continue to be monitored.

The current study has limitations that should be addressed. The data were collected at a single transplant center and the study design was a cross-sectional analysis which can be less informative than a longitudinal analysis. Nevertheless, the present study yielded important preliminary findings in mainland China. Longer follow-up periods and prospective studies will be necessary to identify long-term quality of life and psychosocial consequences of adult LDLT donors.

ACKNOWLEDGMENTS

The authors would like to thank Professor Ning-Xiu Li for providing the Chinese version of the Short Form-36.

COMMENTS

Background

Living-donor liver transplantation (LDLT) is an effective treatment for end-stage liver disease in selected recipients, especially in Asian countries where the cadaveric graft supply is markedly limited. The graft and recipient survival rates are excellent and equivalent to those after deceased-donor liver transplantation. However, the donor of LDLT is exposed to risks inherent to a surgical procedure, and may suffer a considerable psychological burden. Therefore, the safety of the donor operation and the health related quality of life (HRQoL) of the donor after surgery is critical while maintaining graft viability.

Research frontiers

Currently, Not much researches about HRQoL and psychological well-being of the living liver donors have been reported after LDLT. There is a lack of comprehensive and systemic assessment data on donors' HRQoL and psychological well-being after LDLT. So the present study evaluated the HRQoL and psychological well-being on the LDLT donors and identified some potential factors affecting their HRQoL.

Innovations and breakthroughs

This article is one of the few literatures on the quality of life and psychological well-being of the living liver donors after LDLT. The study is well constructed and well planned, and is a comprehensive and systemic assessment data on donors' HRQoL and psychological well-being after LDLT.

Applications

The present study yielded important preliminary findings on reseaches of the donors' quality of life and psychological well-being. The findings will have a significant impact on future clinical strategies.

Terminology

LDLT is a procedure in which a living person donates a portion of his or her liver to another. HRQoL is a multi-dimensional concept that includes domains related to physical, mental, emotional and social functioning.

Peer review

This is a good study contributing to this important area of liver transplantation. The authors have shown that the right donor hepatectomy is an acceptable procedure.

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S- Editor Sun H **L- Editor** O'Neill M **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i2.188

World J Gastroenterol 2012 January 14; 18(2): 188-191
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Central nervous system vasculitis and polyneuropathy as first manifestations of hepatitis C

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Received: April 11, 2011 Revised: July 9, 2011
Accepted: July 16, 2011
Published online: January 14, 2012

Abstract

Sensory or motor peripheral neuropathy may be observed in a significant proportion of hepatitis C virus (HCV)-infected patients. However, central nervous system (CNS) involvement is uncommon, especially in cryoglobulin-negative subjects. We describe a case of peripheral neuropathy combined with an ischemic CNS event as primary manifestations of chronic HCV infection without cryoglobulinemia. Significant improvement was observed after antiviral therapy. We discuss the spectrum of neurological manifestations of HCV infection and review the literature.

Key words: Hepatitis C; Central nervous system; Polyneuropathy; Interferon-α

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INTRODUCTION

Hepatitis C virus (HCV) infection has become a major cause of liver disease with approximately 170 million people infected worldwide[1]. The severity of the disease varies widely, ranging from asymptomatic carrier state to cirrhosis and hepatocellular carcinoma. HCV chronic infection is often associated with abnormal immunological responses that can result in several extrahepatic conditions such as membranoproliferative glomerulonephritis, Sjögren's syndrome, idiopathic thrombocytopenic purpura, lichen planus, porphyria cutanea tarda, and mixed cryoglobulinemia[2]. Even though these conditions occur relatively infrequently, they significantly increase morbidity and mortality among HCV patients. Although sensory or motor peripheral neuropathy may be observed in a significant proportion of HCV-infected patients, central nervous system (CNS) involvement is uncommon, especially in cryoglobulin-negative subjects[3]. Here, we describe a patient with peripheral neuropathy combined with CNS vasculitis as primary manifestations of chronic HCV infection.

CASE REPORT

A previously healthy 37-year-old Caucasian woman presented to the emergency department in May 2003, with a 9-mo history of malaise, loss of appetite, and substantial weight loss (19.96 kg). Over the previous month, she had developed fatigue and muscle weakness, and become

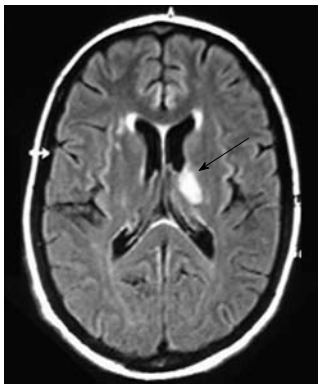

Figure 1 Magnetic resonance imaging of the head showing a 1.5-cm, high-signal lesion in the left thalamus, suggestive of ischemic injury (arrow).

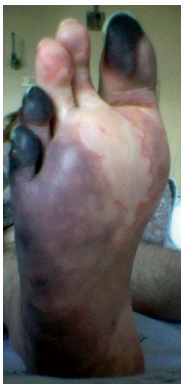

Figure 2 Necrotic lesions on the right forefoot due to severe vasculitis.

unable to perform several activities of daily living, such as hair brushing, climbing stairs and doing household chores. There was no history of blood transfusions or intravenous drug abuse. The patient was conscious and oriented to time and place. On examination, atrophy of the dorsal interosseous muscles, flaccid quadriparesis with hyporeflexia, and symmetrical distal sensory loss were noted. An electroneuromyographic study revealed sensorimotor polyneuropathy.

Over the next 24 h, she became increasingly disoriented. Magnetic resonance imaging of the head showed a T1 low, T2 and fluid-attenuated inversion recovery (FLAIR) high-signal lesion in the left thalamus, approximately 1.5 cm in diameter (Figure 1), which probably represented ischemic injury. In addition, small foci of increased signal intensity at the semioval center and subcortical white matter were identified on T2 and FLAIR sequences. A rheumatologic panel including antinuclear antibody, rheumatoid factor, anti-DNA and cardiolipin antibodies was negative. Thyroid-stimulating hormone, vitamin B12, and aminotransferases levels were within normal limits. Further testing showed negative serology for hepatitis B virus, HIV, syphilis, cytomegalovirus, and human T-lymphotropic virus 1/2. Enzyme immunoassay to detect HCV antibody was positive, as well as serum HCV-RNA by polymerase chain reaction (PCR). A liver biopsy confirmed chronic hepatitis with mild necroinflammatory activity and no fibrosis. We then considered that the diagnosis of CNS vasculitis and peripheral polyneuropathy was probably related to chronic HCV infection. Serum cryoglobulins were persistently negative after seven determinations. The patient was initially treated with intravenous methylprednisolone followed by oral prednisone, with resolution of her symptoms. Subsequently, standard interferon-α (3 mU three times per week) plus ribavirin (1 g/d) were added to steroid maintenance therapy. During HCV treatment, an attempt to reduce prednisone dose resulted in the development of necrotic lesions on the right forefoot (Figure 2), which led to its amputation. In spite of permanent discontinuation of antiviral drugs and the need for increasing corticosteroid dosage, the patient showed sustained virological response, with HCV RNA persistently undetectable in serum by sensitive PCR-based assay. She remains asymptomatic, until last seen, under low dose prednisone.

DISCUSSION

Although the precise frequency of peripheral neuropathy in HCV-infected patients is unknown, it is considered the most common neurological complication in this setting. In a French cohort of 321 subjects with chronic hepatitis C, symptomatic peripheral neuropathy was observed in 9% of the cases[4]. Even though the neurological findings were more frequent among cryoglobulin-positive patients, in this study, a significant proportion of cryoglobulin-negative individuals presented with peripheral nervous system involvement (17% *vs* 8%). Other reports of peripheral neuropathy in HCV-infected patients without detectable cryoglobulins[5-7] indicate that, although the presence of cryoglobulins seems to be an important feature in these cases, there are possibly other factors contributing to the development of peripheral neuropathy. In a study including 51 patients with HCV infection and neuropathy, Nemni *et al*[7] showed that 22% of the subjects had undetectable serum cryoglobulins. Cryoglobulin-negative individuals were more likely to have mono- or multiple neuropathy. Interestingly, the morphological findings in the sural nerve from cryoglobulin-negative and -positive patients are consistent with an ischemic mechanism of nerve damage. The authors stated that the vasculitic process in cryoglobulin-negative HCV subjects was probably secondary to complement pathway activation by HCV itself, or by an interaction between the virus and the host immune system. A direct role of HCV in the pathogenesis of peripheral neuropathy was also proposed, based on the finding of HCV RNA in nerve biopsy specimens[8]; however, this association remains to be confirmed.

Specific CNS involvement is more rarely reported in HCV-infected patients. CNS involvement, however, may present different facets, such as fatigue, depression, cognitive impairment and vasculitis. Although it may be the initial extrahepatic manifestation of HCV infection, well-documented reports on CNS involvement in patients with HCV-associated vasculitis are rare and include mostly cryoglobulin-positive patients[9-11]. Stroke

episodes, transient ischemic attacks, progressive reversible ischemic neurological deficits, lacunar infarctions, or encephalopathic syndrome, commonly attributed to ischemia or rarely to hemorrhage, may occur[12]. Similar to HCV-related peripheral neuropathy, the mechanism behind the CNS vasculitic process in HCV infection is poorly understood, but it has been postulated that recurrent cryoglobulin precipitation with complement fixation and/or HCV-related induction of the innate mechanism of complement activation might be involved in ischemic and inflammatory tissue damage[7]. Although the exact pathway is not known, HCV-induced vasculitis without cryoglobulinemia by the other mechanisms previously discussed for peripheral neuropathy may be responsible for the CNS findings in this case.

The treatment of HCV-associated peripheral neuropathy in cryoglobulin-positive individuals is based on anti-HCV drugs. Combination therapy with interferon (pegylated or not) plus ribavirin may induce a complete clinical response in a significant proportion of patients with HCV-related systemic vasculitis, and consequently, in those with cryoglobulin-related peripheral neuropathy[13,14]. The role of HCV therapy in subjects with cryoglobulin-negative peripheral neuropathy is unclear. Lidove *et al*[5] have reported significant neurological improvement in two cryoglobulin-negative patients treated with interferon monotherapy. However, long-term follow-up was not reported and the possibility of development or worsening of peripheral neuropathy in interferon-based treatments is a major concern in this setting[15]. Data about the safety and efficacy of interferon-based regimens in the treatment of HCV-associated CNS vasculitis are even scarcer. There are a few case reports showing favorable outcomes in cryoglobulinemic subjects treated with corticosteroids or interferon for CNS involvement[12,16,17]. However, such reports cannot support a solid recommendation, especially for those patients with cryoglobulin-negative CNS vasculitis. In addition, it should be emphasized that for cases of severe cryoglobulinemia-associated vasculitis (as those with rapidly progressive renal failure or neurological involvement), it is recommended that antiviral therapy should be delayed for 2-4 mo, while they are submitted to aggressive therapy with plasmapheresis, corticosteroids (intravenous methylprednisolone followed by oral prednisone), and either cyclophosphamide or rituximab[18].

In this report, we describe a patient with peripheral neuropathy combined with an ischemic CNS event as primary manifestations of chronic HCV infection. The absence of other classical risk factors for ischemic stroke, the association with peripheral vasculitis and the improvement observed after steroid therapy suggests a vasculitic origin for the neurological findings. Although this report cannot prove a definite cause-and-effect of HCV infection and the neurological manifestations observed, an important role of HCV is suggested by the significant improvement observed after the HCV sustained virological response. Another interesting finding in the present case was the achievement of sustained viral clearance in spite of the prolonged use of steroids. Although we have not been able to evaluate viral load during therapy sequentially, previous studies have shown that exposure to steroids increases HCV viral load, both in liver transplant patients and in the non-transplant setting[19,20].

In conclusion, our case highlights the need for clinicians to broaden consideration of differential diagnoses, with particular attention to atypical features of common diseases. Testing for HCV should be performed in all cases of neurological signs of uncertain origin, even in the absence of usual risk factors for hepatitis C. Successful antiviral therapy may lead to a significant improvement of neurological manifestations and should be considered in these cases.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i2.192

World J Gastroenterol 2012 January 14; 18(2): 192-196
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Concurrent systemic AA amyloidosis can discriminate primary sclerosing cholangitis from IgG4-associated cholangitis

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Supported by Grants-in-Aid for Clinical Research from National Hospital Organization, Grants-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan

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Received: May 30, 2011 Revised: August 11, 2011
Accepted: August 15, 2011
Published online: January 14, 2012

Abstract

Chronic hepatobiliary inflammatory diseases are not widely acknowledged as underlying disorders of systemic AA amyloidosis, except epidemic schistosomiasis. Among them, primary sclerosing cholangitis (PSC) might initiate amyloid A protein deposition in diverse tissues, giving rise to systemic amyloidosis, due to a progressive and unresolved inflammatory process, and its possible association with inflammatory bowel diseases. Nevertheless, only one such case has been reported in the literature to date. We report a 69-year-old Japanese woman with cirrhosis who was diagnosed with PSC complicated with systemic AA amyloidosis, without any evidence of other inflammatory disorders. As a result of cholestasis in conjunction with biliary strictures and increased serum IgG4, the presence of $IgG4^+$ plasma cells was examined systemically, resulting in unexpected documentation of Congo-red-positive amyloid deposits, but not $IgG4^+$ plasma cells, in the liver, stomach and salivary glands. Elevated serum IgG4 is the hallmark of IgG4-related disease, including IgG4-associated cholangitis, but it has also been demonstrated in certain patients with PSC. Amyloid A deposits in multiple organs associated with an indolent clinical course that progresses over many years might have a diagnostic value in discriminating PSC from IgG4-associated cholangitis.

Key words: Primary sclerosing cholangitis; IgG4-associated cholangitis; AA amyloidosis

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is an intractable fi-

bro-inflammatory disease of the bile ducts that is characterized by biliary strictures without any underlying insults, e.g., immunodeficiency, ischemia, and biliary toxins[1]. PSC usually follows an indolent but progressive course, which results in eventual death or liver transplantation in the majority of patients. A single center study in Germany demonstrated that the estimated median survival from the time of diagnosis to death or time of liver transplantation was 9.6 years; 39.6% of patients underwent liver transplantation, while 14.3% of them developed hepatobiliary malignancies[2]. Definite diagnosis is thus required in cases with suspected lesions, especially to discriminate PSC from IgG4-associated cholangitis (IAC); a recently defined disorder with better prognosis[3-5].

IAC consists of a biliary stricture that responds to or improves with corticosteroid therapy[6], and is recognized as one of a variety of IgG4-related disease that exhibits a wide range of clinical manifestations. The clinical diagnostic criteria for IgG4-related disease consists of three parts: namely, enlarged/thickened lesions in one or more organs; elevated serum IgG4 levels (≥ 135 mg/dL); and histopathological findings[5]. Although IgG4 levels are usually higher in patients with IAC than in those with PSC, raised serum IgG4 levels have been recently reported in 9%-36% of patients with PSC[7,8]. Therefore, the identification of $IgG4^+$ plasma cell infiltrates in the bile duct as well as in other organs[5,9] is important in making a diagnosis.

In this report, we describe a patient with cirrhotic PSC who had elevated levels of serum IgG4. Multiple organ biopsies were performed to obtain a definitive diagnosis and rule out IAC. Unexpectedly, Congo-red-positive amyloid deposits, but not $IgG4^+$ plasma cells, were demonstrated in the liver, stomach and salivary glands. Subsequently, raised levels of serum amyloid A protein (SAA) were confirmed, resulting in a diagnosis of PSC complicated with systemic AA amyloidosis, despite the absence of known genetic susceptibility[10]. This is the second report describing the concurrence of systemic AA amyloidosis in PSC. A sustained acute phase response involving the overproduction of SAA over a period of many years is likely to characterize indolent hepatobiliary inflammation in PSC, but not in IAC.

CASE REPORT

A 69-year-old Japanese woman was referred to our hospital with progressive elevation of cholestatic liver enzymes in October 2009. She had a history of endoscopic sphincteropapillotomy for choledocholithiasis at age 47 years, at which time, PSC was also suspected due to the irregularity of the extra- and intrahepatic bile duct walls, as revealed by endoscopic retrograde cholangiopancreatography (ERCP). Her cholestatic liver tests subsequently remained abnormal despite removal of all gallstones, indicating the presence of PSC. She had never been immunocompromised. Fatigue, pruritis, and abdominal fullness had worsened even after administration of ursodeoxycholic acid (600 mg/d; 13.2 mg/kg body weight) and she was therefore admitted to our hospital in October 2010. On admission, mild jaundice was apparent, and blood tests revealed elevated liver enzymes along with increased acute phase proteins, i.e., aspartate aminotransferase 109 IU/L (normal < 37 IU/L), alanine aminotransferase 80 IU/L (normal < 39 IU/L), alkaline phosphatase 871 IU/L (normal < 359 IU/L), γ-glutamyltranspeptidase 72 IU/L (normal < 75 IU/L), total bilirubin 3.2 mg/dL (normal < 1.2 mg/dL), C-reactive protein 2.66 mg/dL (normal < 0.3 mg/dL), and SAA protein 303.9 mg/L (normal < 8.0 mg/L). With a Child-Pugh score of 10, her functional hepatic reserve was reduced and she had moderate ascites. Anti-nuclear, anti-smooth muscle, anti-mitochondrial, and perinuclear anti-neutrophil cytoplasmic antibodies were negative. Serum IgG was 2890 mg/dL (normal < 1700 mg/dL), including elevated IgG4 level of 251 mg/dL (normal < 105 mg/dL). Although serum IgE was 15 400 IU/mL (normal < 361 IU/mL), antibodies against parasites, including liver flukes, were negative. Viral markers for hepatitis B and C were both negative. Abdominal contrast-enhanced computed tomography showed biliary strictures from common hepatic duct to the second to third branches, accompanied by dilatation of the distal bile ducts. Atrophy of the right hepatic lobe in conjunction with collateral vessel formation around the lower esophagus confirmed cirrhosis. The size and contours of the pancreas were normal. ERCP revealed irregularities of the walls of the lower common, hilar and intrahepatic bile ducts, which were accompanied by multiple strictures and a "pruned-tree" appearance in the intrahepatic bile ducts (Figure 1). Deterioration of liver function likely resulted from progression of PSC, but given the equivocal ERCP results and elevated IgG4 levels, we elected to rule out IgG4-related disease, particularly IAC. The biopsied specimen from the distal common bile duct contained only erosive duct mucosa, preventing the visualization of $IgG4^+$ lymphoplasmacytic infiltration. We therefore performed biopsies of the major duodenal papilla[9], the salivary glands, and the gastric mucosa in order to investigate the possibility of multiple organ infiltration of $IgG4^+$ plasma cells.

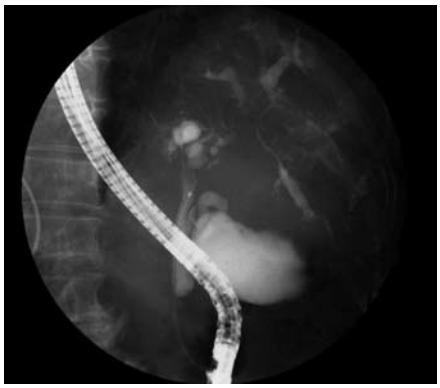

Figure 1 Endoscopic retrograde cholangiopancreatography revealed multiple strictures of the hilar and intrahepatic bile ducts, with a "pruned-tree" appearance, accompanied by dilatation of the distal bile ducts.

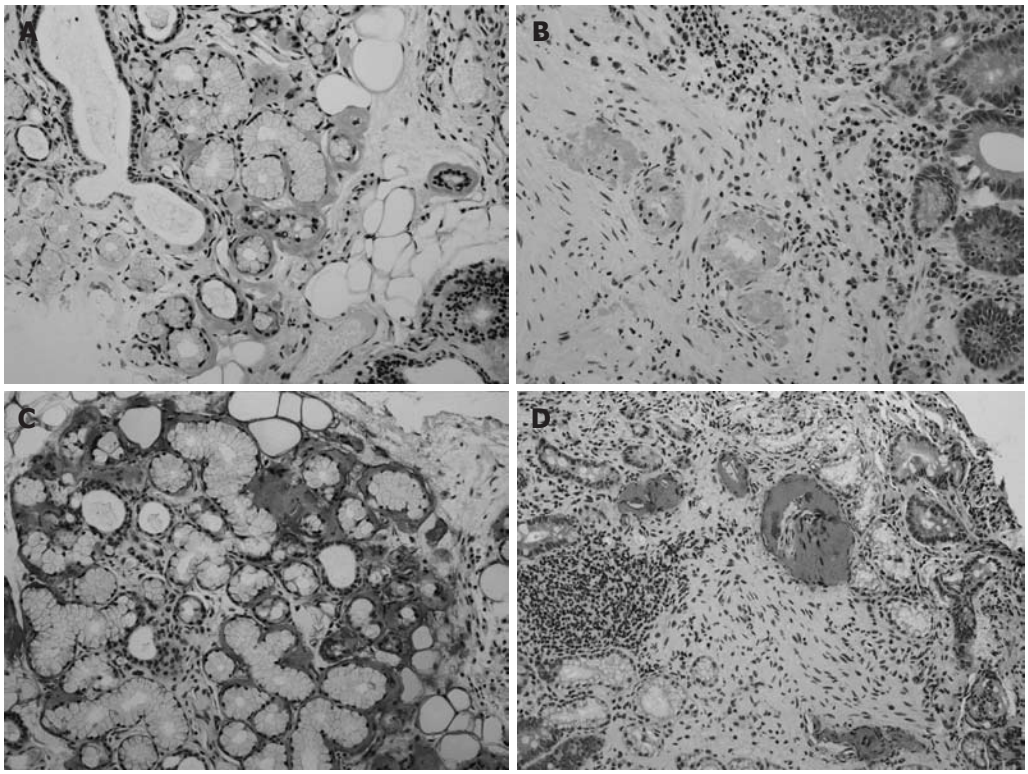

Figure 2 Histology of the salivary glands and the stomach by hematoxylin and eosin stain (200 ×). Amorphous eosinophilic materials in the subglandular and stroma of the salivary glands (A) and submucosal stroma of the stomach (B) were demonstrated. They were found to be Congo-red-positive. Immunohistochemical staining with anti-human amyloid A antibody (200 ×) confirmed a positive AA stain in the salivary glands (C) and stomach (D).

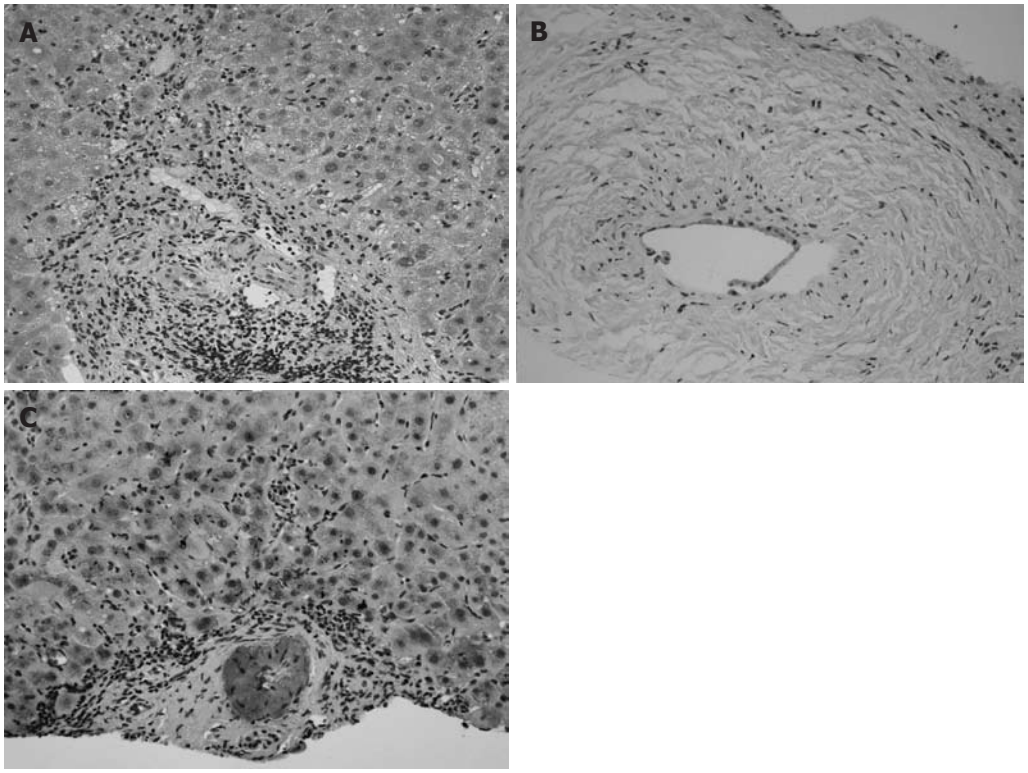

Figure 3 Histology of the liver by hematoxylin and eosin stain. An increase in lymphoneutrophilic infiltrates in the portal tracts, interface hepatitis with ductular proliferation, cholate stasis (400 ×, A), and damaged interlobular bile ducts with collagenous periductal thickening (200 ×, B) were revealed. Amyloid deposition in the vessel walls of the portal tracts was also apparent in immunohistochemical staining (200 ×, C).

Unexpectedly, IgG4$^+$ plasma cells were scarcely found by immunohistochemistry, while Congo-red-positive, amorphous eosinophilic materials were demonstrated in the subglandular and stroma of the salivary glands and in the submucosal stroma of the stomach (Figure 2A and B). Potassium permanganate sensitivity and positive AA immunohistochemical staining confirmed that these materials were AA amyloid deposits (Figure 2C and D). Subsequent liver histology revealed an increase in lymphoneutrophilic infiltrates with some eosinophils in the portal tracts, interface hepatitis and bridging fibrosis, damaged interlobular bile ducts with collagenous periductal thickening, marked ductular proliferation, and cholate stasis (Figure 3A and B). Amyloid deposition in the vessel

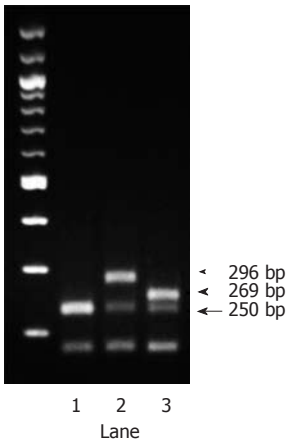

Figure 4 Serum amyloid A1 genotyping by polymerase chain reaction-restriction fragment length polymorphism analysis (2% agarose gel electrophoresis). Arrow (250 bp), arrow head (269 bp) and small arrow head (296 bp) correspond to serum amyloid A1 allele 1.5, 1.3 and 1.1, respectively. Lane 1: 1.5/1.5 homozygosity (our patient); Lane 2: 1.1/1.5 heterozygosity (control patient); Lane 3: 1.3/1.5 heterozygosity (another control patient).

walls of the portal tracts was also apparent (Figure 3C). Oral administration of 30 mg/d prednisolone for 1 wk failed to show any beneficial effect on cholestasis, therefore, a diagnosis of IAC turned out to be implausible. Abnormal uptake was not found in positron emission tomography; again, excluding underlying IgG4$^+$-related lymphoproliferative disease, as well as IgE myeloma. The normal colonoscopic appearance of the colonic mucosa, as well as the histology that only indicated nonspecific inflammation of the intestine, excluded a concurrent diagnosis of inflammatory bowel disease. Currently known patterns of genetic susceptibility to systemic AA amyloidosis were found to be absent[10,11]. SAA1 genotyping by polymerase chain reaction-restriction fragment length polymorphism analysis[12] revealed homozygosity of SAA1.5/1.5 (Figure 4), and sequencing of whole Mediterranean fever (MEFV) exons demonstrated no amino acid substitution mutations (data not shown). Based on all of the above data, we diagnosed the patient with PSC complicated by systemic AA amyloidosis.

DISCUSSION

In this study, we presented a patient with PSC complicated by systemic AA amyloidosis. To the best of our knowledge, this is the second reported case of concurrent PSC and systemic AA amyloidosis, and it included detailed information on pathology as well as on genetic susceptibility to AA amyloidosis.

Sustained overproduction of SAA in association with chronic unresolved inflammation, as demonstrated in our case, is essential for the development of amyloidosis[13]. Nevertheless, susceptibility to AA amyloidosis differs among various diseases. According to Lachmann et al, the most prevailing underlying inflammatory disorder is chronic inflammatory arthritis, followed in descending order by chronic sepsis, periodic fever syndromes, and Crohn's disease[13]. Although PSC is indolent, progressive inflammatory hepatobiliary disease results in cholestatic cirrhosis. Nonetheless, only one other case of PSC has been reported as a cause of AA amyloidosis[14]. Even assuming that about 6% of AA amyloidosis patients do not have underlying inflammatory disorders[13], AA amyloidosis in our case was likely to be secondary to PSC, as coexisting inflammatory bowel disease was excluded. The patient had neither the SAA locus conferring susceptibility to AA amyloidosis in Japanese rheumatoid arthritis, namely SAA1.3, nor MEFV amino acid substitution mutations that are responsible for familial Mediterranean fever, an autoinflammatory disease. Multiple factors affecting amyloid deposition in tissues, such as amyloid P and apolipoprotein E, might have cooperatively contributed to the pathogenesis of AA amyloidosis in this case.

Regarding the diagnosis of PSC, discrimination of IAC is of primary importance owing to better prognosis of the latter with corticosteroid therapy. Serum IgG4 levels in conjunction with cholangiographic features have clinical relevance in this process. In our case, while the elevated serum IgG4 level favored diagnosis of IAC, the "pruned-tree" appearance of the intrahepatic bile ducts coupled with stenosis of the lower common bile duct were equivocal. Moreover, the fact that 9%-36% of patients with PSC also show mildly elevated IgG4[7,8] indicates the need for additional parameters to facilitate differential diagnosis. The presence of IgG4$^+$ plasma cells in the bile ducts and in other organs is suggestive of IgG4-related disease and thus has more diagnostic specificity[5]. In our case, PSC was confirmed by the absence of IgG4$^+$ plasma cells in the examined organs. On the other hand, unexpected demonstration of Congo-red-positive amyloid deposition in the salivary glands, stomach and liver prompted us to consider the distinct implications of these findings on the differential diagnosis, because AA amyloid found in various organs might have diagnostic specificity for PSC. To the best of our knowledge, there have been no case reports describing coexistence of AA amyloid deposits with IgG4$^+$ plasma cells in IgG4-related diseases. A sustained acute phase response in PSC might be a sufficient cause for AA amyloid deposition. Alternatively, mechanistically distinct and/or mutually exclusive inflammatory processes occurring in PSC and IAC, in the latter case reportedly Th-2-dependent[5], might be responsible for the phenomenon. At any rate, estimation of the incidence of AA amyloidosis in PSC[15], as well as in IAC, in a large cohort is necessary to verify our hypothesis.

The proper treatment of PSC complicated by systemic AA amyloidosis remains to be determined. The aim of treatment of AA amyloidosis is generally considered to be the suppression of underlying inflammatory conditions, thereby reducing SAA concentrations[13]. Immunosuppressive agents including anti-tumor necrosis factor therapies are often administered for this purpose, with the exclusion of conditions involving chronic sep-

sis, such as bronchiectasis. Renal dysfunction has been reported as a predominant disease manifestation, and progression to end-stage renal failure has been linked to increased mortality of systemic AA amyloidosis[13]. Regression of AA amyloid deposits (as evaluated by serum amyloid P scintigraphy), that is associated with median SAA concentration during anti-inflammatory therapy, is inversely correlated to the outcomes of renal dysfunction[13]. Although the first case of coexisting PSC and systemic AA amyloidosis reported in the literature experienced relief of symptoms and biochemical improvement as a result of the combination of corticosteroid and azathioprine, the patient experienced progression of amyloidosis-induced nephrotic syndrome followed by its reversal after liver transplantation[14]. Maintaining SAA in a low-normal range through use of anti-inflammatory agents was also difficult in our case; short-term use of prednisolone showed no benefit and colchicine did not decrease serum SAA (data not shown). Administration of tocilizumab, a humanized anti-interleukin 6 receptor antibody that strongly suppresses SAA levels, might be promising for patients with secondary AA amyloidosis who are not responding adequately to treatment for underlying inflammation[16].

In summary, we presented a female PSC patient who had concurrent systemic AA amyloidosis. Is AA amyloidogenesis more specific to PSC than IAC? Could AA amyloid deposition discriminate PSC from IAC? Reexamination of AA amyloid deposition in PSC cases in a large cohort will help answer these and other relevant questions.

ACKNOWLEDGMENTS

We would like to thank following colleagues for their help: Yuka Jiuchi and Yumi Maeda for technical assistance; Yuki Naruke and Yumi Mihara for pathological diagnosis; Satoru Hashimoto, Shinya Nagaoka and Hiroshi Yatsuhashi for the diagnostic decision.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2012 January 14; 18(2): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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World J Gastroenterol 2012 January 14; 18(2): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

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The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

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Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
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Prague, Czech

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American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

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Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

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World J Gastroenterol 2012 January 14; 18(2): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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Name of journal

World Journal of Gastroenterology

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

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World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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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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World Journal of
Gastroenterology ®
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ISSN 1007-9327 CN 14-1219/R Local Post Offices Code No. 82-261

World Journal of Gastroenterology
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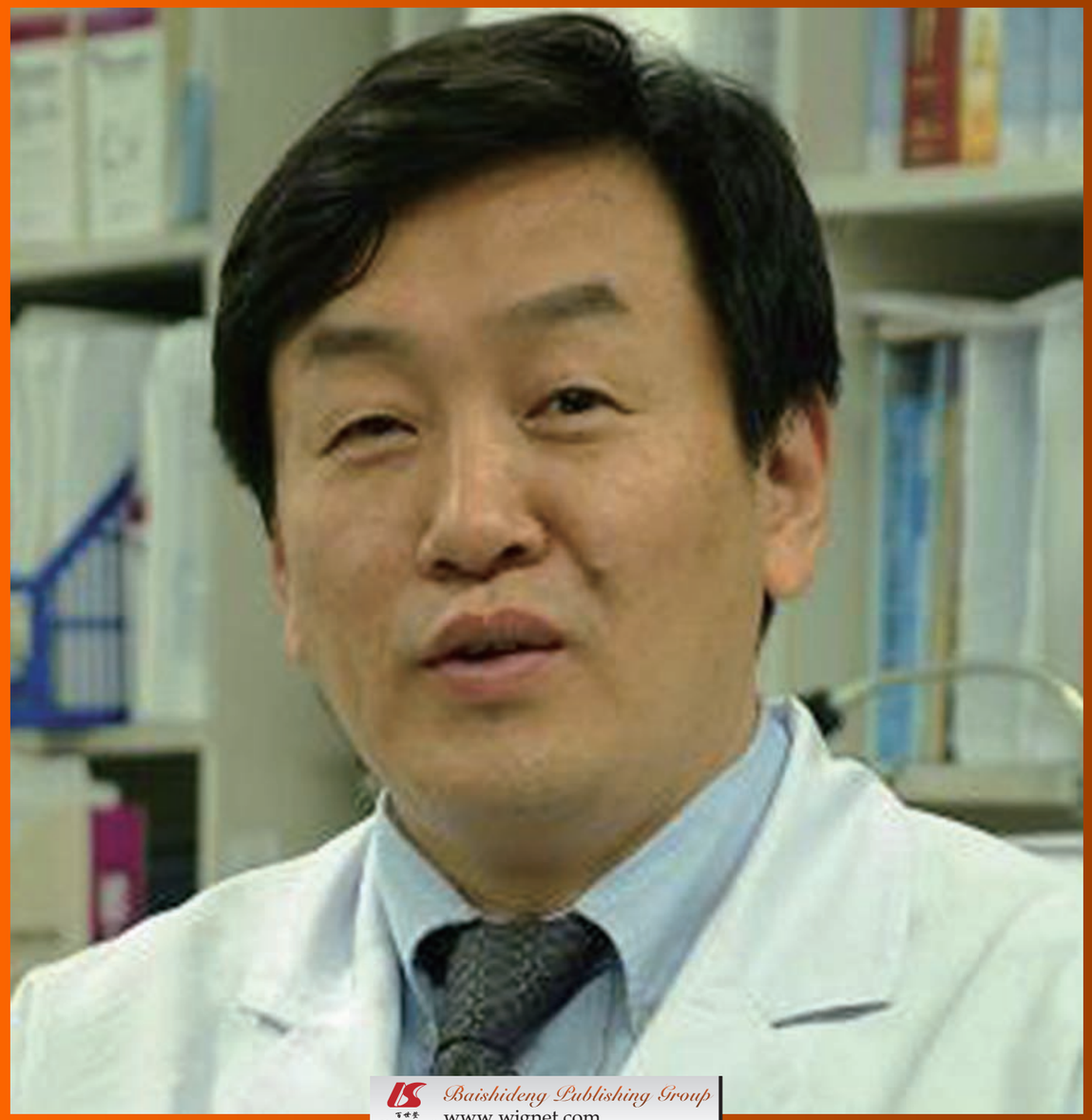

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World Journal of Gastroenterology
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World Journal of Gastroenterology

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

LAUNCH DATE
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RMB 300 Yuan for each issue, RMB 14400 Yuan for one year.

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

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Received: March 30, 2011 Revised: July 14, 2011
Accepted: July 21, 2011
Published online: January 21, 2012

Abstract

There have been increasing concerns regarding the safety of perioperative anti-tumour necrosis factor (anti-TNF) α agents. We performed a literature review to evaluate the post-operative complications associated with perioperative anti-TNF use in patients with inflammatory bowel disease. A comprehensive review was performed with a literature search utilizing Pub Med, Cochrane, OVID and EMBASE databases according to published guidelines. To date, there are only data for infliximab. There are three published studies which have assessed post-operative complications with perioperative infliximab use in patients with Crohn's disease (CD), four studies in ulcerative colitis (UC) patients, and one study on both CD and UC patients. Two out of the three studies in CD patients showed no increased post-operative complications associated with perioperative infliximab. Two out of four studies in UC patients also did not show an increase in post-operative complications, and the combined study with CD and UC patients did not show an increased risk as well. Study results could not be combined secondary to significant differences in study designs, patient population and definition of their endpoints. There appears to be a risk of post-operative complications associated with TNF therapy in some patients. Based on these data, careful patient selection and prospective data collection should be performed.

Key words: Crohn's disease; Ulcerative colitis; Colectomy; Post-operative complications

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Ali T, Yun L, Rubin DT. Risk of post-operative complications associated with anti-TNF therapy in inflammatory bowel disease. *World J Gastroenterol* 2012; 18(3): 197-204 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i3/197.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i3.197

INTRODUCTION

Therapeutic options for the management of inflammatory bowel disease include aminosalicylates, antibiotics, corticosteroids, antimetabolite immunomodulators (e.g., 6-mercaptopurine, azathioprine and methotrexate), and biologic therapies such as anti-tumour necrosis factor (anti-TNF) agents (e.g., infliximab, adalimumab, certolizumab pegol) and natalizumab[1-3]. The anti-TNF therapies are associated with adverse events including serious infections. In large controlled clinical trials of infliximab (IFX), the percentage of patients with serious infections ranged from 4.0% to 4.6%, and the mortality rate was 0.7% to 1.3%[4,5]. IFX-related adverse events, including serious

opportunistic infections such as tuberculosis, listeriosis and histoplasmosis, have also been documented in reports of open-label or retrospective trials[6-9]. However, the TREAT (Crohn's therapy, resource, evaluation, and assessment tool) Registry, a large-scale, ongoing, observational registry designed to examine the safety of Crohn's disease (CD) therapies, found prednisone use (OR: 2.21, 95% CI: 1.46-3.34, $P < 0.001$), narcotic analgesic use (OR: 2.38, 95% CI: 1.56-3.63, $P < 0.001$), and moderate-to-severe disease activity (OR: 2.11, 95% CI: 1.10-4.05, $P = 0.024$) were significantly associated with serious infections. Although the unadjusted analysis showed an increased risk for infection with IFX use, multivariate logistic regression analysis show that IFX was not an independent predictor of serious infections (OR: 0.99, 95% CI: 0.64-1.54)[10].

Despite optimal medical therapy, about two-thirds of patients with CD and one-third of those with ulcerative colitis (UC) eventually require surgery for disease control[11]. Factors increasing the risk of post-operative complications include: preoperative sepsis[12,13] (such as abdominal abscess or systemic infection), impaired nutritional status and intestinal obstruction[14-17]. Common early post-operative complications include abdominal wound infection, anastomotic leakage, pelvic sepsis and small bowel obstruction. Late complications include: anastomotic leak with pelvic sepsis or fistula, anastomotic stricture and pouchitis, and ileo-anal pouch dysfunction[18].

While anti-TNF use has been shown to induce and maintain steroid-free disease remission, improve quality of life and decrease the rate of hospitalizations and surgeries[4,19], concerns have risen regarding the potential harm of these medications. Most recently attention has also been focused on perioperative outcomes for patients who have received anti-TNF therapy and require surgery. The purpose of this review is to analyze the evidence regarding post-operative complications associated with anti-TNF in patients with inflammatory bowel disease.

LITERATURE SEARCH

Separate literature searches were conducted in the Pub Med, Ovid, EMBASE and Cochrane library databases (1950-2010) in accordance with published recommendations[20,21]. Databases were used to search English language literature using search terms such as "post-operative complications", "post-operative sepsis" and "surgical outcomes" in combination with words such as "anti-TNF therapy", "infliximab", "adalimumab", "certolizumab pegol", "natalizumab", "inflammatory bowel disease", "Crohn's disease" and "ulcerative colitis". Exploded terms were reviewed and included or excluded as per their relevance. A comprehensive review of the reference lists of all selected articles was also performed. Two investigators separately performed the literature search. Studies with patients having a diagnosis of ulcerative colitis, Crohn's disease and indeterminate colitis who had received anti-TNF therapy prior to surgical intervention were included. The initial search yielded one hundred and eight articles. Studies published as full articles were included in our systematic review. Only studies involving adult patients (18 years and older) and including control groups were included for the review. Odd ratios were calculated by 2 × 2 tables where the studies failed to include them, with the help of an online calculating system accessed *via* a website (http://faculty.vassar.edu/lowry/odds2x2.html).

No randomized prospective trial was identified. After applying the above mentioned selection criteria, only three retrospective studies assessing IFX associated complications in CD were selected for review[22-24] . Similarly, four retrospective studies involving patients with UC and infliximab were identified[25-28]. One additional study that included patients with both UC and CD was also included in the review and discussed separately[29]. A recently published meta-analysis examining the relationship between IFX and short term complications in patients with UC was also reviewed[30].

AVAILABLE STUDIES IN LITERATURE

Studies with Crohn's disease

No prospective randomized control trial has been published to determine the post-operative complication risks associated with anti-TNF therapy. Three retrospective studies were identified in the literature addressing risk of post-operative complications with IFX[22-24]. Individual study characters (including study design, use of concomitant medications use and dosing of IFX in relation to surgery) are presented in Table 1. Study endpoints and various complications assessed in each study are presented in Table 2, and the results of the risks of complications are presented in Table 3.

Marchal *et al*[22] retrospectively studied a cohort of three hundred and thirteen consecutive patients with CD who received infliximab between 1998 and 2002. A trend towards an increased early infection rate was found in IFX pre-treated patients (6 patients *vs* 1 patient $P = 0.10$), but the authors concluded this was probably due to the increased use of corticosteroids and/or immunosuppressive agents in this group[22]. However, the limited number of patients suggests the possibility of a type Ⅱ error.

In a study by Colombel *et al*[23], a group of investigators identified two hundred and seventy patients who underwent abdominal surgery for CD between 1998 and 2001. This retrospective analysis suggested that perioperative use of IFX use was not associated with an increased risk of post-operative complications. A multiple variable model including both steroid and IFX use was also performed and there was no independent association with either septic or total complications. However, these data were not presented in the published manuscript. Odds ratios were calculated from univariate regression analysis and additional multiple regression analysis was not performed secondary to the limited number of patients.

The investigators of the Appau *et al*[24] study evaluated

Table 1 Crohn's disease study characteristics

Author study center	Study design	Total patients	Study endpoints	Concomitant medication	Case (%)	Control (%)	*P* value	IFX dosing	Last dose IFX prior to surgery < 12 wk (%)
Marchal[22], 2004, Belgium	Case-control	79 total, 40 cases, 39 controls	Early (10 d) and late (3 mo) complications	5-ASA	30	54	N.S.	Episodic 100%	78
				6MP/AZA	45	26	N.S.		
				Steroids	73	41	< 0.00017		
				Antibiotics	25	28	N.S.		
Colombel[23], 2004, Mayo Clinic, Rochester	Retro-spective	270 total, 52 cases	Early (30 d) septic and non-septic complications	5-ASA	-	-	-	Episodic 80%	96
				6MP/AZA	7	-	-		
				Steroids	7	-	-		
				Antibiotics	-	-	-		
Appau[24], 2008, Cleveland Clinic, Cleveland	Case-control	458 total, 329 Non IFX, 60 IFX, 69 pre IFX	30 d post-operative complications	5-ASA	60	58	0.95	-	100
				6MP/AZA	62	15	< 0.001		
				Steroids	65	77	< 0.052		
				Antibiotics	-	-	-		
Kunitake[29], 2008, Massachusetts General Hospital, Boston (combined study)	Case-control	413 total, 101 cases, 312 control	Post-operative complications	5-ASA	-	-	-	-	100
				6MP/AZA	37	6	0.04		
				Steroids	75.30	77	0.79		
				Antibiotics	-	-	-		

IFX: Infliximab; 5-ASA: 5-aminosalicylic acid; AZA: Azathioprine; 6MP: 6-mercaptopurine; N.S.: Not significant.

Table 2 Complications defined in crohn's disease

Study	Study endpoints	Infectious	Non-infectious	Major	Minor
Marchal[22]	Early (10 d) and late (3 mo) complications	Catheter sepsis, wound, upper respiratory, Diarrhea,yeast		Sepsis, leak, peritonitis, abscess, wound failure, severe anemia, bulbar ulcer bleeding	Hematoma, fever, delayed transit, mild infection, intestinal obstruction
Colombel[23]	Early (30 d) septic and non septic complications	Wound sepsis, leak, abscess, fistula, sepsis, pneumonia, bacteremia, urosepsis	CD recurrence, small bowel obstruction, GI bleeding, thromboembolism		
Appau[24]	30 d post-operative complications	Wound infection, sepsis, intra abdominal abscess, 30 d mortality, readmission rate, anastomotic leak and wound complications			
Kunitake[29] (combined study)	Post-operative cumulative complications	Infections, hypomotility, thrombotic, cardiac complications, hepato-renal complications, anastomotic leak, bleeding and death			

CD: Crohn's disease; GI: Gastrointestinal.

Table 3 Post-operative risks of complications with Crohn's disease

Authors	Complications	Cases (%)	Controls (%)	Risks of complications, OR (95% CI)
Marchal[22]	Early Minor (10 d)	15	13	1.2 (0.3-4.3)
	Late Minor (3 mo)	2.50	5.10	1.1 (0.3-4.0)
	Early Major (10 d)	12.50	7.70	1.7 (0.3-7.7)
	Late Major (3 mo)	17.50	12.80	1.4 (0.4-5.0)
Colombel[23]	Septic	17		0.9 (0.4-1.9)
	Non-septic	23		1.0 (0.5-2.0)
Appau[24]	30 d readmission	20	9.4 + 2.9	2.3 (1.0-5.3)
	30 d sepsis	20	9.7 + 5.8	2.6 (1.1-6.1)
	30 d intra-abdominal abscess	10	4.3 + 4.3 (Non IFX + pre IFX group)	5.8 (1.7-19.7)
Kunitake[29] (combined study)	Post-operative cumulative complications	16.80	15.70	1.1 (0.6-2.0)

IFX: Infliximab; OR: Odd ratio; CI: Confidence interval.

30 d post-operative outcomes for CD patients treated with IFX (60 patients) within 3 mo *vs* infliximab-naïve patients (329 patients) prior to ileocolonic resection from 1998 to 2007. In an effort to reduce selection bias, the IFX group was also compared with pre-IFX patients (69 patients) undergoing ileocolonic surgery before 1998. Using multivariate analysis, the IFX group appeared to have an increased risk of 30 d post-operative readmission, sepsis, and intra-abdominal abscess. IFX patients who had a stoma (n = 17) above their anastomosis had a lower incidence of sepsis when compared with those without a stoma. While the sample size for the IFX group was larger than any published data, the sample size of 60 patients is still low; thus, differences in postsurgical outcomes that were found in this study might be further underestimated.

Studies with ulcerative colitis

There have not been any reported differences in the incidence and type of side effects attributed to IFX in patients with CD *vs* UC in general, but one area that has been getting more attention recently is post-operative complications. Four studies evaluating IFX and post-operative complications in UC patients were identified[25-28]. Individual study characters, including study design, use of concomitant medications and dosing of IFX in relation to surgery, are presented in Table 4. Study endpoints and various complications assessed in each study are presented in Table 5, and the results of the risks of complications are presented in Table 6.

The primary aim of the Selvasekar *et al*[25] study was to assess short-term post-operative infectious complications in UC patients who received preoperative IFX and underwent colectomy with ileal pouch anal anastomosis (IPAA) from 2002-2005. The overall post-operative complications in both groups were similar (P = 0.1). This was the first published study addressing post-operative complications and preoperative IFX use in UC. However, this was a single center study with a retrospective design and small sample size. This study was primarily before its approval by the Food and Drug Administration (FDA) in September 2005 and thus it represents off-label use, so ideal dosing was not defined and one might speculate that these were more severely ill patients. Therefore, the use of IFX may simply be a surrogate marker for more severe disease and sicker patients.

The Schluender *et al*[26] study was conducted to assess the 30-d post-operative medical and surgical complications of medically refractory UC patients who received preoperative IFX and underwent colectomy (subtotal or total proctocolectomy with IPAA) from October 2000-October 2005. There was a difference in concomitant 6-mercaptopurine (6-MP)/6-thioguanine (6-TG) use, with 94% in the IFX group also on 6-MP/6-TG *vs* 44% in the non-infliximab group (P = 0.03). However, that there was no significant difference in medical, surgical or infectious complications in patients receiving both IFX and 6-MP compared to patients receiving 6-MP alone was determined. This study was also limited by its single-center single surgeon retrospective design, and small sample size of seventeen patients who received IFX, and therefore lacks the statistical power to detect significant differences related to the two groups. Potential confounders, such as disease severity, were not assessed. However, the article highlighted the significantly increased risks of complications, including infectious complications, in patients exposed to cyclosporine and IFX which has also been reported in another study in steroid refractory UC[31].

The primary aim of a case-matched retrospective study by Mor *et al*[27] was to evaluate post-operative complications after restorative proctocolectomy in UC patients who received IFX preoperatively from January 2000-December 2006. Overall, the prevalence of early post-operative complications (35% *vs* 15%, P = 0.027) was significantly different, and the difference was mostly due to pelvic sepsis (22% *vs* 2%, P = 0.016) in the IFX and non-IFX groups, respectively. On further examination of the patients with pelvic sepsis, an ileo-anal pouch anastomotic leak was identified in eight of ten patients treated with IFX. There was no difference in overall late post-operative complications (52% *vs* 37%, P = 0.23), but pouchitis was more common (39% *vs* 15%, P = 0.037) in IFX *vs* non-IFX groups, respectively. In addition, the rate of three stage procedures was higher in those treated with IFX preoperatively (OR: 2.07, 95% CI: 1.18-3.63) even when adjusting for extent and severity of colitis, as well as steroid and immunomodulators use. The limitations of this study include its retrospective nature and single-center location. Disease severity was assessed in this study, but the retrospective use of hemoglobin and platelet counts are limited markers for severity assessment. Although the study found an increased rate of pouchitis in patients who had received preoperative IFX, risk factors for pouchitis were not controlled, and the diagnosis was not confirmed by endoscopy and histology. In addition, the finding of more frequent three stage procedures in the IFX group must be taken with caution, as the surgeons were not blinded to IFX use and thus may have influenced their decision.

The Ferrante *et al*[28] study evaluated the impact of IFX on post-operative infectious complications in UC or indeterminate colitis patients undergoing restorative proctocolectomy between 1998 and 2008. Short-term post-operative pouch-specific surgical and non-surgical site infectious complications were not significantly different in those who received IFX preoperatively. In multivariate analysis, a moderate-to-high dose of corticosteroids was associated with short-term post-operative pouch-specific complications (P = 0.001), surgical site complications (P = 0.002) and infectious complications overall (P = 0.003). This study, like its predecessors, was a single-center retrospective study with only 22 patients who received IFX within 12 wk of surgery and was too underpowered to detect significant differences between the two groups.

Recently, a meta-analysis was performed by Yang *et al*[30] examined the relationship between preoperative IFX treatment and short term post-operative complications in patients with UC. A total of 5 studies and 706 patients

Table 4 Ulcerative colitis study characteristics

Author and study center	Study design	Total patients	Study endpoints	Concomitant medication	Case (%)	Control (%)	*P* value	IFX dosing	Last dose of IFX prior to surgery < 12 wk
Selvasekar[25], 2007, Mayo Clinic, Rochester	Case-control	301 patients, 47 cases, 254 controls	Post-operative pouch specific and infectious complications	5-ASA	100	60	< 0.0001	Scheduled (majority)	49% (< 8 wk)
				6MP/AZA	91	44	< 0.0001		
				Steroids	47	77	< 0.001		
				Steroids + AZA	70	21	< 0.001		
Schluender[26], 2007, Cedars-Sinai LA	Case-control	151 patients, 17 cases, 134 controls	30 d post-operative surgical and medical complications	5-ASA	-	-	-	2 infusions (median)	Majority (8 wk median)
				6MP/AZA	94	44	0.03		
				Steroids	100	100	1		
Mor[27], 2008, Cleveland Clinic	Case-control	92 patients, 46 cases, 46 controls	Early (30 d) and late post-operative complications	5-ASA	-	-	-	3 infusions (median)	Majority (13.5 wk median)
				6MP/AZA	39	28	0.27		
				Steroids	2 (ranked dose score)	2.24	0.42		
Ferrante[28], 2009, Belgium	Case-control	141 patients, 22 cases, 119 controls	Early (30 d) post-operative complications	5-ASA				Scheduled 2.5 Infusions (median)	Majority (3.9 wk median)
				6MP/AZA	59	55	0.7		
				Steroids (high dose)	9	25	0.163		
Kunitake[29], 2008, Massachusetts General Hospital, Boston (combined study)	Case-control	413 total, 101 cases, 312 control	Post-operative complications	5-ASA	-	-	-	-	100%
				6MP/AZA	37	26	0.04		
				Steroids	75.30	77	0.79		
				Antibiotics	-	-	-		

IFX: Infliximab; 5-ASA: 5-aminosalicylic acid; AZA: Azathioprine; 6MP: 6-mercaptopurine.

Table 5 Complications defined in ulcerative colitis studies

Study	Study Endpoints	Infectious	Medical	Surgical	Pouch
Selvasekar[25]	Post-operative pouch specific and infectious complications	Pouch complications plus; wound infections			Anastomotic leak; pelvic abscess
Schluender[26]	30 d post-operative surgical and medical complications	Not defined	Major: pneumonia, DVT, pancreatitis, ARF, CVA; minor: dehydration, thrombophlebitis, pyoderma gangrenosum, urinary retention	Major: SBO, abscess, bleeding, leak; minor: wound infection, ileus, bleeding	
Mor[27]	Early (30 d) and late post-operative complications	Early: pelvic sepsis, bleeding, thrombosis, ileus; Late: pouchitis, SBO, stricture			
Ferrante[28]	30 d post-operative complications	Surgical infections: pouch complications plus wound infection; non surgical: UTI and respiratory infections			Anastomotic leak; pelvic abscess
Kunitake[29] (combined study)	Post-operative cumulative complications	Infections, hypomotility, thrombotic, cardiac complications, hepato-renal complications, anastomotic leak, bleeding and death			

DVT: Deep vein thrombosis; ARF: Acute renal failure; CVA: Cerebrovascular accident; SBO: Small bowel obstruction; UTI: Urinary tract infections.

were included in their meta-analysis. The analysis failed to find a strong association between preoperative treatment of IFX and short term infectious (OR: 2.24, 95% CI: 0.63-7.95) or non-infectious (OR: 0.85, 95% CI: 0.5-1.45) post-operative complications. However, preoperative IFX use increased short term total post-operative complications (OR: 1.80, 95% CI: 1.12-2.87). These results need to be interpreted with caution. There are significant differences in patient population, characteristics, and study end points in the studies included in the meta- analysis. For

Table 6 Post-operative risks of complications with ulcerative colitis

Authors	Complications	Cases (%)	Controls (%)	Risks of complications, OR (95% CI)
Selvasekar[25]	Pouch specific	19	7	2.6 (0.9-7.5)
	Infectious	28	10	2.7 (1.1-6.7)
Schluender[26]	Medical	6	10	0.6 (0.1-4.8)
	Surgical	30	18	1.9 (0.6-5.9)
	Infectious	18	8	2.4 (0.6-9.6)
Mor[27]	Early	35	15	3.5 (1.5-8.3)
	Late	52	37	2.2 (0.9-5.3)
	Sepsis	22	1	13.8 (1.8-105)
Ferrante[28]	Pouch specific	0	17	0.9 (0.8-0.9)
	Surgical infections	11	23	0.2 (0.03-1.6)
	Total Infectious	11	28.60	0.3 (0.07-1.4)
Kunitake[29] (combined study)	Post-operative cumulative complications	16.80	15.70	1.1 (0.6-2.0)

OR: Odd ratio; CI: Confidence interval.

example, only one study[29] included patients who received their last dose of IFX within 12 wk prior to surgery and the remaining studies included patients where such characteristics and important distinctions were not accounted for. Even the definitions of infectious and non-infectious complications were different in the various studies[25-29]. In the presence of such confounders, it is hard to draw any meaningful results from this meta-analysis.

Combined Crohn's disease and ulcerative colitis study

Kunitake *et al*[29] retrospectively studied 413 patients (45% with CD, 38% with UC and 17% with IC) who underwent abdominal surgery between 1993 and 2007, evaluating the association of preoperative IFX use with post-operative complications. There were trends for higher rates of post-operative death, anastomotic leak, thrombotic complications, hypomotility, hepatorenal complications, and post-operative bleeding in the IFX group, but this did not reach statistical significance. Given its retrospective design, this study shared the same weaknesses as its predecessors. A sample size of approximately 250 patients in each arm to identify a 5% difference in post-operative outcomes was required. This study, while the largest to date in the literature, could only identify 101 patients who underwent IFX treatment within the 12 wk preceding their abdominal surgery. While no statistical significance was observed between the groups, some of the trends seen in the IFX group, such as the higher death rate, were indeed worrisome, especially as the deaths in both groups were because of failure of multiple organ systems due to intra-abdominal sepsis.

RISK OF POST-OPERATIVE COMPLICATIONS

Surgery plays an integral role in the treatment of inflammatory bowel disease, both to control symptoms and to treat complications. The rate of surgery within 3 years of diagnosis of CD varies from 25 percent to 45 percent. Twenty-five percent to 38 percent of patients require a second surgery by 5 years after the first, and about one third of patients who need a second surgery eventually require a third[32]. Indications for surgery include complications such as intra-abdominal abscess, medically intractable fistula, fibrotic stricture with obstructive symptoms, toxic megacolon, hemorrhage, and cancer[33]. Approximately 25 percent of UC patients ultimately require colectomy for the management of their disease[34]. Common indications for surgical therapy of UC are medically refractory disease, intractable disease with an impaired quality of life, and unacceptable side effects from medical therapy. Other indications for surgery include uncontrolled hemorrhage, toxic megacolon, perforation, dysplasia or carcinoma, systemic complications, and growth retardation.

Surgery in inflammatory bowel disease (IBD) patients is associated with morbidity and mortality. The common early post-operative complications include abdominal wound infection, anastomotic leakage, pelvic sepsis and small bowel obstruction. Late complications include anastomotic leak with pelvic sepsis or fistula, anastomotic stricture and, with IPAA, pouchitis and pouch dysfunction[18]. Post-operative infections increase length of hospital stay, cost, mortality and morbidity rates[35]. In one study, the post-operative infections in colectomy patients resulted in an approximately 2 wk longer hospital stay, with an increase of more than 300 percent in the total direct cost[36].

Patients receiving immunosuppressant medications, including biologic therapies, are at increased risk of bacterial, fungal and protozoal infections due to multiple effects on the immune system[7,37,38]. TNF α functions to stimulate angiogenesis and fibroblast proliferation, as well as to increase collagenase and prostaglandin synthesis[39]. In addition, the TNF α receptor has been shown to be necessary for an effective immune response to mycobacteria in animal models[7,39,40]. In addition to the above, reports of histoplasmosis, coccidiomycosis, listeriosis, pneumocystosis, and other fungal, bacterial and mycobacterial infections have been observed in patients receiving IFX.

The perioperative management of a patient with IBD requires a multifaceted approach involving both tradi-

tional and disease-specific considerations. As with any surgical candidate, preoperative risk assessment is crucial. Whether to suspend immunomodulating therapy during the perioperative period has been a challenging question. The difficulty lay in striking a balancing between that of maintaining disease control and that of optimizing wound healing, along with minimizing the post-operative risk of infection and other morbidity. As the use of biologic therapy in IBD continues to grow, we are faced with the additional task of providing recommendations for their use as well, and there is little information on which to base any definitive guidelines[41].

There have been several studies now published evaluating IFX use and perioperative complications. However, as described above, they suffer from similar weaknesses such as their retrospective design, small sample size (most studies included only 40-60 patients receiving IFX), dissimilar study populations (different IBD phenotypes, surgical procedures, and time periods on infliximab), single referral center populations, and use of the medication prior to FDA approval and thus generating selection bias with more ill patients receiving IFX and non-standardized usage of IFX. Potential confounders are generally not well addressed, especially severity and duration of disease, malnutrition, comorbidities, race and type of surgical procedure. Studies evaluating effect of IFX in CD are not adjusted for disease severity. Disease severity determined retrospectively is often inaccurate and surrogate covariates may be inadequate substitutions for validated methods of assessing disease activity. However it is interesting to note that in the studies of UC, those that were adjusted for disease activity showed a relationship between IFX and complications, while the converse was true for those studies that did not. Inappropriate use of anti-TNF therapy among some patients may also confound existing studies owing to their retrospective nature. Studies of the pharmacokinetics of IFX in IBD suggest that the elimination half-life is between 7 and 18.5 d[42,43]. By 12 wk (84 d, or 4.5 half-lives), most IBD patients should have undetectable levels of IFX. A window of 12 wk used by several of these studies may not reflect outcomes associated with the presence of drug at the time of surgery. Ideally, the duration between last infusion and surgery should be included as a continuous variable, but it is difficult to do this in retrospective analyses. Referral practice patterns also may account for the heterogeneous findings of these studies, as these may differ substantially in various regions of the world. Using anti-TNF therapy late in the course of disease in patients less likely to benefit from it (e.g., fixed stricture in CD) may introduce risk without clear benefit. Also, continuing to use anti-TNF therapy after it is clear that it has failed may send a higher risk patient to the operating room. These factors are difficult to measure and could be better addressed in a prospective, randomized study.

CONCLUSION

To date, all of the studies of anti-TNF therapy are of infliximab and are mixed in their reports of the risk of post-operative complications associated with it. The studies are limited by small numbers of patients, disparate comparison groups, different definitions of measured outcomes and varying timeframes of drug exposure and follow-up. Larger controlled studies, with well-defined standardized criteria, are needed to clarify this issue. In the meantime, physicians must be aware of these potential risks so that prudent decisions about patient selection and timing of interventions can be made.

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S- Editor Tian L **L- Editor** Rutherford A **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i3.205

World J Gastroenterol 2012 January 21; 18(3): 205-211
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Enhanced recovery for non-colorectal surgery

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Received: March 30, 2011 Revised: June 15, 2011
Accepted: June 22, 2011
Published online: January 21, 2012

Key words: Enhanced recovery; Length of stay; Complications

Peer reviewers: Fausto Catena, MD, PhD, Department of General, Emergency and Transplant Surgery, St Orsola- Malpighi University Hospital, via Massarenti 9 Bologna 40139, Italy; Paulino Martinez, Department of Colon and Rectal Surgery, Hospital San Jose de Celaya, Celaya 38010, Mexico

Gravante G, Elmussareh M. Enhanced recovery for non-colorectal surgery. *World J Gastroenterol* 2012; 18(3): 205-211 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i3/205.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i3.205

Abstract

In recent years the advent of programs for enhanced recovery after major surgery (ERAS) has led to modifications of long-standing and well-established perioperative treatments. These programs are used to target factors that have been shown to delay postoperative recovery (pain, gut dysfunction, immobility) and combine a series of interventions to reduce perioperative stress and organ dysfunction. With due differences, the programs of enhanced recovery are generally based on the preoperative amelioration of the patient's clinical conditions with whom they present for the operation, on the intraoperative and postoperative avoidance of medications that could slow the resumption of physiological activities, and on the promotion of positive habits in the early postoperative period. Most of the studies were conducted on elective patients undergoing colorectal procedures (either laparotomic or laparoscopic surgery). Results showed that ERAS protocols significantly improved the lung function and reduced the time to resumption of oral diet, mobilization and passage of stool, hospital stay and return to normal activities. ERAS' acceptance is spreading quickly among major centers, as well as district hospitals. With this in mind, is there also a role for ERAS in non-colorectal operations?

INTRODUCTION

In recent years the advent of programs for enhanced recovery after major surgery (ERAS) has led to modifications of long-standing and well-established perioperative treatments. These programs are used to target factors that have been shown to delay postoperative recovery (pain, gut dysfunction, immobility) and combine a series of interventions to reduce the perioperative stress and organ dysfunction[1]. With due differences, the programs of enhanced recovery are generally based on the preoperative amelioration of the patient's clinical conditions with whom they presents for the operation, on the intraoperative and postoperative avoidance of medications that could slow the resumption of physiological activities, and on the promotion of positive habits in the early postoperative period (Table 1)[2]. Common factors are postoperative pain control through continuous mid-thoracic epidural anaesthesia and avoidance of regular opioids drugs, stimulation of gut motility, no mechanical bowel preparation, early physical reactivation, and limited use of catheters, tubes and drains[1].

Most of the studies were conducted on elective patients undergoing colorectal procedures (either laparotomic or laparoscopic surgery) and a growing number of articles and reviews have analysed the data produced (Figure 1). Results showed that ERAS protocols significantly

improved the lung function and reduced the time to resumption of oral diet, mobilization and passage of stool, hospital stay and return to normal activities[3]. Any delay in hospital discharge or early readmission was due to the development of major complications[4,5]. Higher American Society of Anesthesiologists (ASA) score, advanced age, and rectal surgery were associated with delayed mobilization, morbidity and prolonged stay[6]. ERAS' acceptance is spreading quickly among major centers as well as district hospitals. With this in mind, is there also a role for ERAS in non-colorectal operations?

NON-COLORECTAL SURGERY

Compared to colorectal surgery, fewer studies have investigated ERAS in other operations (Table 1)[3-24].

Radical cystectomy

Radical cystectomy is one of the urological procedures which has the highest rate of complications and longest hospital stay[25]. Overall complication rate is 21%-34%, early reoperation rate 6%-7%, mortality rate 0.4%-2.7%, and the hospital stay is 17.4 ± 4.7 d. The most frequent complications are pelvic lymphoceles (8.1%), wound dehiscence (6%-9%), deep venous thrombosis (4.7%), ileus (3.9%), and pulmonary embolism (2%-4%)[26-28]. Over the years, improvements in the surgical technique, anaesthesia and perioperative care have already resulted in reduced morbidity and shorter hospital stays[28]. Age is not a contraindication, and the operation can be administered even to elderly patients with similar complications rates to younger patients[26,29,30]. More important for the prediction of postoperative complications are the preoperative cardiac history, ASA score and the number of intraoperative blood transfusions. For preoperative mortality, the ASA score, blood transfusions and preoperative nutritional deficiency are important[28,31,32].

Arumainayagam *et al*[25] developed the only ERAS protocol available for radical cystectomies. The protocol consisted of stopping the use of mechanical bowel preparation before the cystectomy, implementation of early enteral feeding (with nutritional supplements) and mobilization as tolerated. Patients with ileal conduits after radical cystectomy were encouraged self management of the stoma and catheter care on postoperative day 2. The application of the ERAS protocol produced a significant reduction in the length of stay (13 d *vs* 17 d) but had no effect on the time to first defecation (6 d), morbidity, mortality and readmission rates[25]. Obviously the ERAS protocol does not affect these rates, as it has no influence on risk factors for morbidity, mortality and readmission rates. It would be interesting to evaluate if preoperative nutritional improvement, and not only for early enteral feeding, might decrease the postoperative complications rates in order to better prepare the body for surgical stress.

Liver resections

Liver resections have morbidity rates of 25%-48% and mortality of 1%-7%[33-35]. The length of stay with a traditional perioperative pathway ranges from between 8 and 14 d. The length of stays and intensive therapy unit stays are shorter if resections are conducted with laparoscopic surgery[36,37]. Factors associated with postoperative morbidity are neoadjuvant chemotherapy, vascular clamping, intraoperative blood loss with transfusion[35,38], comorbid conditions, pre-existent liver disease and small remnant liver volume[39]. Factors associated with postoperative mortality are the presence of blood transfusions and extended resections[40]. Age is not associated with an increase of morbidity or mortality[41].

The application of an ERAS protocol to liver surgery was evaluated by van Dam *et al*[1]. Their protocol was similar to those of colorectal surgery, including: nutritional supplements up to two hours before surgery, thoracic epidural analgesia, short acting anesthetics, avoidance of excessive IV fluids, warm fluids, and one night in the recovery ward before being admitted to the normal surgical ward. Among the criteria for discharge was the normalization or decreasing of serum bilirubin. Results achieved confirmed hospital stays shorter than 2 d and with no significant differences in the rates of morbidity, mortality and readmissions. In fact, as for radical cystectomies, the ERAS protocol did not alter any of the risk factors for these outcomes. A different study performed on liver resections undergoing ERAS evaluated the addition of laxatives to the protocol[42]. Although routing postoperative laxatives resulted in an earlier first passage of stool, the overall rate of recovery remained unaltered[42].

Upper gastrointestinal surgery

Gastric and oesophageal resections are operations associated with long hospital stays and postoperative morbidities. The average length of hospital stay after oesophagectomy ranges from 11 to 26 d following open surgery, and 7 to 13 d following laparoscopic surgery[43]. Postoperative pulmonary complications have been reported in 15%-30% of cases and are the most common cause of major morbidity and mortality[44]. Risk factors include impairment in lung function, cardiac reserve, preoperative physical activity and body composition[44]. Furthermore, a history of pulmonary disease, age, and preoperative physical activity also significantly predicts postoperative death[45]. For gastric resections, old age does not seem to affect morbidity rates (25%-29%)[46,47] but still influences mortality, which is higher amongst elderly patients (3% *vs* 10%)[29]. Advanced age, low albumin, ASA score, palliative resections and resection of two or more additional organs were independent risk factors for mortality[47].

ERAS protocols have been applied on both transthoracic oesophagectomies (Ivor-Lewis procedure) and laparoscopic gastric resections[43,48]. The ERAS protocol for oesophagectomies involved extubation in the operating theatre or immediately on arrival in the intensive care unit, early mobilization, negative fluid balance, intense respiratory physiotherapy and epidural analgesia. Patients remained in intensive care for three days, most drains

Table 1 Types of Enhanced Recovery protocols adopted

	Preoperative	Intraoperative	Postop (first 24 h)	Day 1	Day 2	Day 3	Day 4	Additional comments
Kahokehr *et al*[7,8]	Routine nutritional assessment; nutrition supplementation; NBM 2 h preinduction; carbohydrate loading; no bowel preparation; functional assessment and goal setting	Thoracic epidural; short acting anesthetics; intraoperative fluids: 1000 mL of crystalloid and 500 mL of colloid; prophylactic antiemetics at induction (dexamethasone); no drains or NG tubes	All Ⅳ fluid stopped before patient discharged to ward; prophylactic antiemetics; early oral feeding; nutritional supplementation; no opioids	Removal of urinary catheter	Removal of epidural			Early mobilization and physiotherapy
King *et al*[9-11], Blazeby *et al*[12], Faiz *et al*[13]	Optimized premorbid health status; functional assessment and goal setting; Meeting with stoma nurse. Nutrition supplementation; bowel preparation (for left colonic, sigmoid and rectal tumours)	Thoracic epidural; intraoperative fluids: 2000 mL of crystalloid; minimal-access surgery; local anaesthetic infiltration to the largest wound; no drains or NG tubes	Free fluid; 1 high-protein/high-calorie drink; patient sat out in chair	All Ⅳ fluid stopped; regular paracetamol; 3 high-protein/high-calorie drink; normal diet offered; patient sat out in chair; start walking; removal of urinary catheter for colonic resections; laxatives	Removal of epidural; regular NSAIDS; Morphine for breakthrough	Removal of urinary catheter for rectal resections		Aim for discharge on day 3 for colonic or day 5 for rectal resection; Provision of hospital contact numbers, review on ward if problems within 2 wk; review in outpatient clinic on day 12
Jottard *et al*[14]	Functional assessment and goal setting; nutrition supplementation; no bowel preparation	Thoracic epidural; anti-thrombotic and infection prophylaxis; standard anesthetic protocol; prevention of intraoperative hypothermia; no drains or NG tubes	Free fluid	All Ⅳ fluid stopped; normal diet offered				Use of anti-emetics; early mobilization; postoperative nutritional care
Maessen *et al*[4,5], Nygren *et al*[3], Hendry *et al*[6]	Functional assessment and goal setting; nutrition supplementation; no bowel preparation	Thoracic epidural; prevention of intraoperative hypothermia; Transverse/curved incision	Oral analgesia; Patient sat out in chair; nutritional supplements; free fluid > 800 mL	All Ⅳ fluid stopped; nutritional supplements > 400 mL; normal diet offered; patient sat out in chair > 6 h	Removal of epidural; removal of urinary catheter			
Soop *et al*[15]	Nutrition supplementation	Thoracic epidural	Prophylactic antiemetics	Regular paracetamol and NSAIDS; patient sat out in chair for 2 h	Patient sat out in chair for 4 h	Patient sat out in chair for 3 h	Epidural removed (at least)	
Raymond *et al*[16]	Functional assessment and goal setting; nutrition supplementation	Thoracice epidural; Intra-operative targeted fluid management; No NG tube						Early mobilization/resumption of diet
Turunen *et al*[17]	Functional assessment and goal setting; preoperative feeding; bowel preparation	Thoracic epidural; high-oxygen P; prevention of hypothermia; no drains or NG tubes		Removal of urinary catheter				Early mobilization/resumption of diet; no routine opioids, regular; paracetamol and NSAIDS; fluid restriction
Senagore *et al*[18]		No NG tube	PCA; free fluids	Removal of urinary catheter; normal diet offered; regular NSAIDs, gabapentin, hydroxycodone if needed; no drains				
Wennstrom *et al*[19]	Functional assessment and goal setting; no bowel preparation; preoperative oral hydration	Thoracic epidural; short acting anaesthetics; no opioids	Free fluid; patient sat out in chair		Epidural removed; urinary catheter removal			

Mohn *et al*[20]	Functional assessment and goal setting; nutrition supplementation; bowel preparation.	Thoracic epidural; total intravenous anesthesia; intra-operative targeted fluid management; restricted postoperative intravenous fluids; routing antiemetics postoperatively; short midline incisions; No drains or NG tubes	Patient sat out in chair	Removal of urinary catheter; patient sat out in chair; normal diet offered; regular paracetamol and nsaids, opioids for breakthrough	Epidural removed		Regular laxatives twice daily; antithrombotic prophylaxis
Teeuwen *et al*[21]	Nutritional supplements; bowel preparation in left-sided resections; thrombotic prophylaxis	Thoracic epidural; transverse incisions except in Crohn's disease and rectal surgery; intra-operative targeted fluid management (hypotension treated with vasopressors); no drains except in rectal surgery; no NG tubes; prophylactic antiemetics	Free fluids; nutritional supplements; patient sat out in chair	Normal diet offered; intravenous fluid administration; start walking	Epidural removed; urinary catheter removal; regular paracetamol; NSAIDs opioids for breakthrough		
Ahmed *et al*[22,23]	Functional assessment and goal setting; nutritional supplements; no bowel preparation	High inspired oxygen; concentration; transverse incisions; no drains or NG tubes	Free fluids; soft diet offered; patient sat out in chair	Start walking			Regular paracetamol NSAIDs, opioids for breakthrough
Kirdak *et al*[24]	Thrombotic prophylaxis; bowel preparation; nutritional supplements	Thoracic epidural; pelvic drains with rectal dissections; urinary, central venous, and nasogastric catheters were routinely used	Start walking	NG tubes and urinary catheters removed (except pelvic dissection); soft diet offered; start walking; patient sat out in chair	Removal of urinary catheter (low pelvic operations) and drains	Epidural removed; regular paracetamol; central venous catheters removed; normal diet	

NBM: Neuronal basal medium; NG: Nasogastric; NSAIDS: Nonsteroidal antiinflammatory drugs; PCA: Pyrrolidone carboxylic acid.

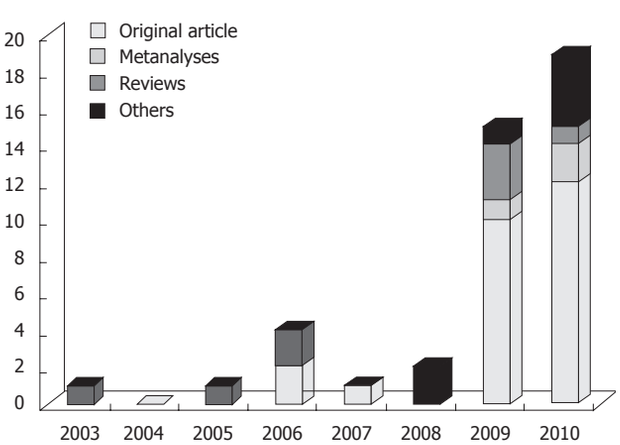

Figure 1 Number and types of articles published on Enhanced Recovery.

and tubes were removed on day 4, oesophageal radiology studies were performed on day 5 and the nasogastric tube consequently removed. Particular attention was dedicated to early signs of potential complications (pulmonary infections or anastomotic leaks). With this protocol the authors achieved a significant reduction of pulmonary complications (31% *vs* 38%), mortality (1% *vs* 5%) and hospital stay (9 d *vs* 13 d)[43]. Even in this case, as well as for radical cystectomy, no evaluation aimed for an improvement of the preoperative nutritional status, which might decrease the postoperative complications rates in order to better prepare the body for surgical stress.

Patients receiving an ERAS protocol for laparoscopic gastric surgery had their anastomosis tested on the first postoperative day with a water soluble swallow study. If the anastomosis was intact, free fluids were started and an early diet on day 2 consisting of small, frequent low-sugar meals[48]. The urinary catheter was removed on day 1. No epidural analgesia was used for pain relief, nor any abdominal drains or nasogastric tubes. Discharge was planned for day 3. Results showed a short length of stay (4 d, range 2-30) and reduced readmission rate (6.2%). Only two patients developed postoperative complications, namely a wound abscess and an urinary infection[48].

Gynecological surgery

Hysterectomy is a common gynecological procedure that is performed through various routes. Overall morbidity is present in 16% of cases, but their frequency depends on the route adopted to remove the uterus; they are usually rare after open abdominal hysterectomies (hemorrhages: 2.4%, genitourinary disorders: 1.9%, infection: 1.6% and urinary tract infections: 1.6%), even lower with the vaginal route but higher for laparoscopic abdominal hysterectomy[49]. The major causes of morbidity in patients who undergo abdominal hysterectomies are medical rather than surgical and the most important factor associated with them is the presence of comorbidities[50,51]. Readmissions are confined to 5.4%-7.2% of cases.

One article focused on ERAS protocol in gynecological operations. The protocol consisted of intensified information on pre-hospitalization consultations and admission. Intravenous lines, urinary catheters and tampons were removed before the patient left the recovery unit. Mobilization and normal food intake, including per oral analgesics, started a few hours after the operation. Routine postoperative enemas were discontinued[52]. A significant reduction of the length of stay was also confirmed in this study, but no mention was made about the rate of postoperative complications or readmission[52].

Post-bariatric body contouring surgery

Patients undergoing post-bariatric body contouring surgery have a higher risk for postoperative complications (28%)[53,54] and among these the most common are those involving the wound healing process (infections, seromas, hematomas and delayed healing)[55-58]. The causes are multifactorial and include the percentage excess weight loss[53], total tissue resection weight[59], preoperative body mass index[60] and the recently discovered "high-calorie malnutrition"[61]. This syndrome involves the preoperative deficiency of vitamins and minerals that are important for the healing process. Differently from the other risk factors, high-calorie malnutrition syndrome is common to the overweight, obese and post-obese patients, and its perioperative corrective could improve the wound healing in all these subcategories. This is shown in the study by Agha-Mohammadi where the rate of complications in post-bariatric and obese patients was similar to normal-weight patients after perioperative nutritional supplementation of many primary ingredients necessary for wound healing and immune system competency[61]. In this case the length of stay was not evaluated because most plastic surgery procedures can be conducted during short admissions or even day surgery.

CONCLUSION

The application of ERAS protocols to non-colorectal surgery is more complex due to the paucity of literature available and to the outcomes that might be different according to the peculiarities posed by each discipline and its specific problems. Generally, a principle of ERAS is that the reduction of hospital stay should be balanced against the possibility of increased readmission rates. To achieve this objective, the rate of postoperative complications should be reduced so that patients can be safely sent home earlier with no risk to their health and no need for readmission. These two principles, reduction of the length of stay and of the postoperative morbidity, should both be targeted in a comprehensive ERAS program. However, most of the studies analyzing ERAS protocols in non colorectal-surgery focused mainly on only one factor, the length of hospital stay, which is the most evident in terms of hospital costs and productivity. The analysis conducted on non-colorectal studies showed that most protocols tried to optimize the perioperative administration of drugs, fluids and tubes following the path traced by colorectal studies. These factors obviously impacted on the overall length of stay but did not act on risk factors for postoperative morbidity, with an exception made for the post-bariatric study. Not surprisingly, the incidence of postoperative complications remained the same in most articles except for the post-bariatric study. To further improve the already positive results achieved by most ERAS programs it is advisable to focus more on the clinical conditions with which the patient arrives to the operation, redefining the situation by which the body faces the surgical stress and improving its ability to deal with it. This could not only reduce the length of stay, but also the complication and readmission rates.

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S- Editor Tian L **L- Editor** Rutherford A **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i3.212

World J Gastroenterol 2012 January 21; 18(3): 212-224
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Hepatitis C virus induced insulin resistance impairs response to anti viral therapy

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Received: April 14, 2011 Revised: June 20, 2011
Accepted: June 21, 2011
Published online: January 21, 2012

Key words: Hepatitis C virus-chronic hepatitis C; Insulin resistance; Sustained virological response; Homeostasis model assessment

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El-Zayadi A, Anis M. Hepatitis C virus induced insulin resistance impairs response to anti viral therapy. *World J Gastroenterol* 2012; 18(3): 212-224 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i3/212.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i3.212

Abstract

Hepatitis C virus (HCV) infection is an important risk factor for insulin resistance (IR). The latter is the pathogenic foundation underlying metabolic syndrome, steatosis and cirrhosis, and possibly hepatocellular carcinoma (HCC). The interplay between genetic and environmental risk factors ultimately leads to the development of IR. Obesity is considered a major risk factor, with dysregulation of levels of secreted adipokines from distended adipose tissue playing a major role in IR. HCV-induced IR may be due to the HCV core protein inducing proteasomal degradation of insulin receptor substrates 1 and 2, blocking intracellular insulin signaling. The latter is mediated by increased levels of both tumour necrosis factor-α (TNF-α) and suppressor of cytokine signaling 3 (SOC-3). IR, through different mechanisms, plays a role in the development of steatosis and its progression to steatohepatitis, cirrhosis and even HCC. In addition, IR has a role in impairing TNF signaling cascade, which in turn blocks STAT-1 translocation and interferon stimulated genes production avoiding the antiviral effect of interferon.

INTRODUCTION

Infection with hepatitis C virus (HCV) is a common problem worldwide, affecting millions of people across all populations. Most acutely infected patients develop chronic hepatitis and become a potential source of virus transmission, and as many as 1 in 5 will develop cirrhosis and its complications[1]. Besides, HCV is an increasingly recognized important cause of extrahepatic manifestations, including insulin resistance (IR)[2].

IR is a complex pathophysiological condition where higher-than-normal concentrations of insulin are needed to maintain a normal glycemia and adequate glucose utilization in insulin target tissues[3]. IR is of global importance since is closely linked to the epidemic condition of obesity and it precedes and predicts the development of type 2 diabetes mellitus (T_2DM) and increases the risk of life-threatening complications such as cardiovascular diseases, renal failure, and infections. However, these complications are not major causes of death in cirrhotic patients with IR[4]. In contrast, the development of in-

trahepatic complications, including HCC, is known to be associated with IR[5].

IR is extremely common in patients with chronic HCV infection and has been associated with increased disease severity, extrahepatic manifestations and decreased response to antiviral therapy[6]. Understanding the basis of such associations is of paramount importance to inform treatment strategies for patients with HCV. This review summarizes recent information on the different issues of HCV infection and IR.

CAUSES OF INSULIN RESISTANCE

It represents interplay between genetics (inherited) and environmental (acquired) factors. Genetic factor include abnormal insulin, abnormal insulin receptor and abnormal signaling proteins. The main acquired factors of IR include: abdominal obesity, aging, hyperglycemia, medications and recently HCV infection.

Abdominal obesity

Obesity is associated with IR, hepatic steatosis and over expression of tumor necrosis factor-α (TNF-α). All of these factors increase the risk of fibrosis and decreased antiviral efficacy. Also, obesity decreases interferon bioavailability and impairs immune stimulating properties of interferon. Hepatic steatosis, nonalcoholic steatohepatitis and fibrosis are associated with release of reactive oxygen species (ROS), which contribute to decreased HCV response to interferon (IFN)[7,8].

Moreover, obesity is associated with decreased number and downregulation of insulin receptors and impairment of postreceptor signaling. Overflow of free fatty acids (FFAs) from adipose tissue interferes with intrahepatic insulin signaling pathway *via* increased levels of proinflammatory cytokines such as TNF-α[9,10], and proteasomal degradation of the insulin receptor substrates (IRS) 1 and 2 (Figure 1)[11].

Aging

Aging is associated with increased levels of FFAs and triglycerides, in addition to glucose transporter-4 (GLUT4) decrease and mutation.

Hyperglycemia

Overflow of FFAs from adipose tissue to systemic circulation impairs insulin-mediated glucose uptake by the muscles resulting in hyperglycemia and peripheral IR.

Medications

Some medications can induce IR as glucocorticoids, cyclosporine, growth hormones, thyroid hormones, sex hormones, analogues, thiazides, β-blockers, protease inhibitors for HCV and nucleoside analogues for hepatitis B virus.

Hepatitis C virus infection

Recently has evolved as a significant risk factor for the development of IR.

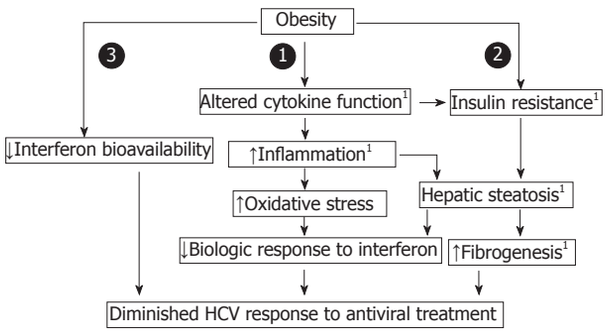

Figure 1 Obesity is associated with insulin resistance and decreased antiviral efficacy. [1]Indicates potential for coexistent viral potentiation. HCV: Hepatitis C virus.

ROLE OF ADIPOKINES IN INSULIN RESISTANCE

Adipokines

Adipokines are polypeptides secreted in the adipose tissue in a regulated manner and they include adiponectin, leptin, resistin, retinol-binding protein 4, visfatin, omentin, vaspin, chemerin, apelin, TNF-α, interleukin 6 (IL-6), and monocyte chemoattractant protein-1[12].Chronic HCV infection is associated with changes in the serum level of some adipokines[13].

Adiponectin

Decreased serum levels of adiponectin have been reported in chronic hepatitis C (CHC), especially in patients with steatosis. Deficiency of adiponectin is associated with obesity, IR, glucose intolerance, triglyceride (TG) accumulation and steatosis, and metabolic syndrome[13].

Leptin

Leptin insufficiency is associated with increased body weight, increased FA synthesis, decreased FA oxidation, decreased TG excretion, increased steatosis, and impaired insulin sensitivity and secretion[13]. In a recent controlled study of HCV-infected nondiabetic males compared to matched uninfected controls, circulating levels of the adipokines leptin and adiponectin were independently associated with IR, but not with the presence of HCV, and it was concluded that HCV-associated IR does not seem to be mediated by adipokines or proinflammatory cytokines[14].

Tumor necrosis factor-α

TNF-α is also considered one of the adipokines secreted in adipose tissue. HCV may also induce IR by triggering the production of proinflammatory cytokines, because in hepatitis C, circulating TNF-α levels are increased[15,16]. TNF-α can induce IR by several mechanisms, both direct and indirect. TNF-α interferes with the insulin signaling pathway, *via* induction of the suppressor of cytokines 3 (SOCS-3) that inactivates phosphatidylinositol-3-kinase (PI3K). The latter inhibits GLUT-4 translocation to cell membrane and intracellular glucose entry[13]. Increased

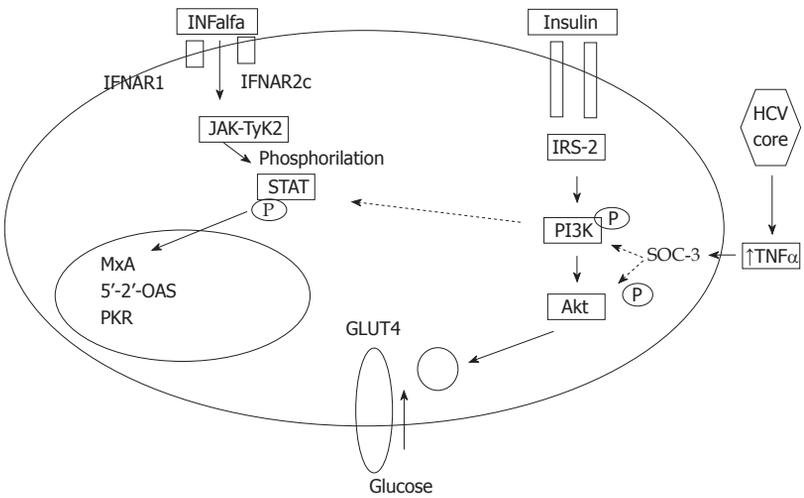

Figure 2 Interaction between hepatitis C virus core, insulin- and interferon-α signaling pathways continuous lines represent activation. Dotted lines represent inhibition. Hepatitis C virus core protein induces expression of tumour necrosis α (TNF-α), which in turn activates suppressor of cytokines-3 (SOCS-3). Activation of SOCs-3 leads to proteasomal degradation of insulin receptor substrate and inactivates phosphatidyl-inositol-3-kinase (PI3K), leading to inhibition of translocation of glucose transferase (GLUT-4) to cell membrane, blocking intracellular glucose entry, with subsequent hyperglycemia, hyperinsulinemia and peripheral insulin resistance. Activation of SOCS-3 also leads leading to inhibition of Tyr-phosphorylation of signal transducers and activators of transcription 1 leading to impaired TNF-α signaling. HCV: Hepatitis C virus; IFNAR2c: Interferon receptor chain 2; IRS2: Insulin receptor substrate 2; JAK: Janus kinase; TYK2: Tyrosine kinase 2; STAT: Signal transducer and activator of transcription.

TNF-α-mediated expression of SOCS-3 has been identified in obese, genotype 1 patients with chronic HCV infection[17].

In addition, TNF-α triggers lipolysis of FFAs from adipose tissue, leading to increased serum levels of free fatty acids and it has also a direct inhibitory effect on insulin action in the liver. These effects lead to reduced glucose uptake in muscle, and to increased hepatic glucose production[18,19]. TNF-α is also suggested to regulate expression of several adipocyte genes known to modulate insulin sensitivity/resistance[20], therefore, it is suggested that TNF-α acts as a possible link between HCV and diabetes[21].

Apeline

Apeline, an adipocytokine derived from adipose tissue, has been recently suggested to be associated with fibrosis progression and development of cirrhosis in HCV infection. In addition, apeline levels are more elevated in IR subjects than in non-IR. Both TNF-α and apeline may rather express complementary effect in fibrosis progression as well as impaired response to IFN therapy.

HEPATITIS C VIRUS AND INSULIN RESISTANCE

Hepatitis C virus induces insulin resistance

The causal relationship of HCV infection and IR development has been demonstrated by the increased prevalence of IR in chronic HCV infection. Whereas the overall prevalence of IR is 10%-25% of the population[22], the prevalence IR in HCV infection reaches figures ranging between 30% to 70%[23,24]. Moreover, IR with HCV infection is increased at early stages of liver disease without liver fibrosis, and is on average significantly higher than that found in patients with chronic hepatitis B, matched for age and body mass index[25].

The causal relationship of HCV infection and IR development has also been demonstrated by improvement of IR after successful therapy in CHC patients, whereas no improvement is observed in nonresponders[26-28].

Mechanism

HCV virus, through both direct and indirect pathways, affects the insulin signaling pathways, promoting IR at a cellular level. Insulin effects are elicited after binding of insulin to its receptor that is linked to a complex signaling pathway that involves sequential activation of IRS, PI3K, Akt, a protein kinase which is a downstream of PI3K activation, and protein kinase C. This cascade of events eventually results in stimulation of glucose uptake after translocation of the GLUT4 to the plasma membrane. IR results from defects at any level of the insulin receptor-related signaling pathway[29] (Figure 2).

Following inflammatory response in the liver to HCV infection, a profound impairment of insulin signaling occurs at the level of IRS tyrosine phosphorylation and PI3K activation[30]. HCV core protein induces expression of TNF-α, which activates SOCS-3, leading to subsequent proteasomal degradation of IRS1 and IRS2, resulting in the development of IR. Meanwhile, SOCS-3 inactivates PI3K, which in turn inhibits translocation of GLUT-4 to cell membrane, thus blocking intracellular glucose uptake[10,11,31] (Figure 3).

It has been also suggested that increased levels of pro-inflammatory cytokines such as interleukin 1, TNF-α, IL-6 and leptin, and reduced levels of adiponectin may directly contribute to the occurrence of HCV-related IR[23].

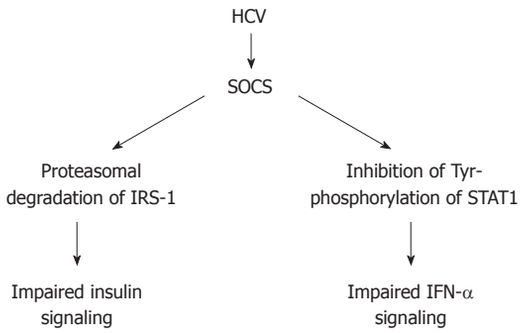

Figure 3 Proposed dual role of the upregulation of members of the suppressor of cytokines family by hepatitis C virus: simultaneous impairment of the insulin and IFN-a signaling systems. HCV: Hepatitis C virus; SOC: Suppressor of cytokines; IRS-1: Insulin receptor substrate 1; STAT1: Signal transducers and activators of transcription1; IFN-α: Interferon-α.

Viral load and insulin resistance

The relationship between the severity of IR and HCV replicative levels has been very difficult to prove. However, recent work seems to suggest so[32]. In addition, it has been shown that IR is modified by treatment and that the incidence of T2DM in patients achieving sustained virological response (SVR), defined as undetectable HCV RNA 24 wk after completing treatment, is significantly lower than that seen in nonresponders[26-28]. However, it is still not clear whether HCV replication directly increases IR, or whether hyperinsulinemia stimulates viral replication, as suggested by in vitro data[33]. It is to be noted that the global level of IR is likely to depend on the contribution from the adipose tissue and the muscle, two extrahepatic compartments that are not infected by HCV.

Viral genotype and insulin resistance

Although the interference with the insulin effects shows some HCV genotype-specificity, IR has been reported to occur in all HCV genotypes, but to a different extent[34]. HCV genotype 3a, in addition, may alter the intrahepatic insulin signaling through a downregulation of peroxisome proliferator-activated receptor[35]. In HCV genotype 1b infections, substitutions of amino acids 70 and/or 91 in HCV-1b core were found to be significant determinants of severe IR, in patients without cirrhosis and diabetes mellitus, which suggests a real connection between HCV-1b infection and IR at early stages of liver disease[36].

Hepatitis C virus, iron overload, oxidative stress and insulin resistance

Hepatic iron overload has been repeatedly reported in patients with chronic HCV infection[37-39]. Although the mechanisms of hepatic iron overload remains unclear, recent studies showed a decreased hepatic expression of hepcidin, a negative regulator of duodenal iron absorption, in patients with HCV infection[40-42], in addition to increased hepatic expression of transferring receptor 2, a mediator of iron uptake, which is responsible for the hepatic iron overload[43].

The mechanisms through which iron causes IR are not clear. Hepatic iron overload produces oxidative stress and is a factor responsible for the development of HCV-associated IR[44-47]. Oxidative stress, itself, is an independent factor in the development of IR in patients with signaling[47], and has been associated with altered IFN-α signaling *via* decreased phosphorylation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway[48]. In addition, a cross-talk between iron metabolism and insulin-glucose metabolism has recently been documented[49]. Iron has been found to reduce hepatic extraction/metabolism of insulin and to interfere with insulin action on the liver, leading to peripheral hyperinsulinemia[50,51]. In contrast, hyperinsulinemia may cause rapid stimulation of iron uptake into the liver, because insulin is known to redistribute transferrin receptors from an intracellular membrane compartment to the cell surface[52].

EFFECTS OF HEPATITIS C VIRUS-INDUCED INSULIN RESISTANCE

Increased incidence of type 2 diabetes mellitus

An important clinical implication of IR in chronic HCV infection is the strong relationship of IR and T2DM development[53]. One recent meta-analysis has confirmed that chronic HCV is associated with an increased risk of developing T2DM compared with both non-infected controls and patients with chronic HBV infection, which is independent of the presence of cirrhosis[54]. Nearly 30%-70% of patients with CHC display some form of IR[23]. accordingly worldwide, 47 million patients may have HCV-associated DM[21].

On the other hand, IFN therapy is often implicated in the literature as having a role in the development of diabetes in HCV patients. However, this association is rare, and the few cases of DM developing during IFN therapy had T1DM, in line with other autoimmune manifestations induced by IFN[55,56].

Insulin resistance-induced hepatic steatosis, fibrosis and hepatocellular carcinoma

HCV, IR and steatosis: The overall prevalence of steatosis in patients with HCV infection is approximately 55% ranging from 35% to 81% in various studies, which is approximately 2-3 folds higher than the prevalence of steatosis in other liver disease[57]. One-to two-thirds of liver biopsies from CHC patients have histological evidence of steatosis, which has been associated with being overweight, hepatic fibrosis and increased TG levels[58,59].

The relationship between IR and HCV infection is complex and bidirectional; HCV induces steatosis[57], and the latter could also cause IR[60]. In addition to inflammation, HCV proteins also play a role in the development of IR and oxidative stress, the two key pathways in the pathogenesis of non alcoholic fatty liver disease (NAFLD)[61]. On the other hand, insulin is an anabolic

hormone and promotes hepatic lipogenesis[62], and inhibits lipolysis[63]. Therefore, the initial step in HCV-related metabolic disorders remains unclear.

The current evidence suggests that HCV-associated hepatic steatosis is mainly virus-induced in genotype-3a infected patients[64]. which seem to be mediated by an impaired very low-density lipoprotein (VLDL) secretion, most likely *via* an impaired activity of the liver microsomal triglyceride transfer protein (MTP)[65]. On the contrary, the host-factors (mainly IR) play a major role in steatosis in non-3 genotypes[64]. In addition to inflammation, HCV proteins also play a role in the development of IR and oxidative stress, the two key pathways in the pathogenesis of NAFLD[65]. It is well known that IR causes impaired metabolic clearance of glucose and hyperglycemia. In addition, peripheral IR also increases adipose tissue lipolysis, leading to increased plasma and hepatic uptake of FFAs. Increased hepatic uptake of FFAs impairs β-oxidation in mitochondria, together with decreased excretion of VLDL, resulting in TG retention with subsequent development of hepatic steatosis[66,67].

A direct relationship between HCV replication and steatosis has been extensively documented by both clinical and experimental data[68,69]. HCV core protein also regulates secretion of VLDL, TG, and apoliprotein B through regulation of MTP peroxisome proliferator-activated receptor γ (PPAR-γ), and sterol regulatory element binding protein-1c (SREBP-1c)[70-72]. The latter is a protein central to insulin signaling, also involved in up-regulation of de novo lipogenesis and inhibition of fatty acid β-oxidation; two events that can favour intracellular accumulation of triglycerides.

Recently, there has been considerable interest in the role of micro-RNAs (miRNA) in the genesis of both fatty liver and HCV replication, in particular, mir122, the most abundant liver miRNA, has been shown to affect the development of steatosis by increasing lipogenesis and by enhancing HCV virus replication[73].

HCV, IR and fibrosis: Both IR and hepatic steatosis have been closely associated with progression of hepatic fibrosis in patients with HCV infection[57]. A recent meta-analysis using 3068 patients recruited at 10 centers in 5 countries suggests that steatosis and diabetes are both independent factors of fibrogenesis in patients with genotype 1 infection[74]. Significant fibrosis has also been associated with IR independent of steatosis in CHC genotype 1 and 4 infections.

Recently, several reports have suggested that IR may contribute to the progression of fibrosis[75-77]. IR may directly stimulate the proliferation of hepatic stellate cells (HSCs) promoting collagen I synthesis[78] or IR-induced hepatic lipid accumulation and generation of ROS can activate HSCs, initiating progression of fibrosis to cirrhosis[79].

HCV, IR and HCC: Subjects with HCV and diabetes have a higher risk of developing HCC[80]. In a very large, general population-based, cohort study comprising a total of 23 820 residents in Taiwan and followed up for 14 years[81], diabetes was associated with HCC especially among individuals with HCV infection, with lower risk values among carriers of the hepatitis B virus or uninfected individuals. Recent evidence strongly suggests that steatosis and diabetes may also significantly enhance the risk of HCC[82,83].

HCV core protein was reported to induce HCC in transgenic mice, providing a direct experimental evidence for the contribution of HCV core protein in the development of HCC in human HCV infection[84]. More recent reports support the oncogenic potential of HCV and linked clinically the substitutions of amino acid 70 and/or 91 in HCV-1b core region to HCC[85,86]. This may suggest the presence of IR-dependent pathway as a mechanism of HCV-1b core region-associated hepatocarcinogenesis, and the importance of eradicating such mutant HCV in reducing the development of HCC[36].

IR is recognized as an independent risk factor for the development of HCC worldwide[87-89], and the development of diabetes-related HCC suggests that IR has direct effects on hepatocarcinogenesis, although precise mechanisms for this effect remain unclear. IR causes lipid accumulation, which results in changes in serum adipocytokine levels, including reduction of adiponectin, which has suppressive effects for hepatocarcinogenesis[90] Hepatic lipid accumulation also increases oxidative stress, which may be responsible for the development of HCC[88,89]. Hyperinsulinaemia and increased levels of insulin growth factors have been shown to promote cell proliferation[90,91], and IL-6. Another possibility is that insulin has a mitogenic effect, through activation of a mitogen-activated protein kinase pathway, suggesting that insulin may be directly linked to hepatocarcinogenesis[92]. However, the specific cytokines and mechanisms that promote neoplasia during IR have not yet been fully defined.

Insulin resistance-induced interferon resistance

IR is associated with a poor response to anti-viral treatment in patients with HCV infections, both for initial virological response[93,94] and SVR[76,95]. This negative association has been reported to occur both in patients infected with the genotype 1[76,96] and in those with genotypes 2 and 3[97].

Although the reason for such association is largely unknown, several possibilities have been suggested. In one study, obese HCV patients have approximately an 80% lower chance of achieving SVR compared with non-obese patients[98]. Obese HCV patients with steatosis are thought to have increased lipid droplets in hepatocytes, which can act as a functional barrier for the interaction between antiviral drugs and hepatocytes[8,99]. Alternatively, lipids are important for HCV replication[100],and accumulation of hepatic lipid droplets may increase HCV replication and results in poor responses to anti-viral treatment[100]. Obese people are known to have a poor lymphatic circulation[101], this could result in suboptimal

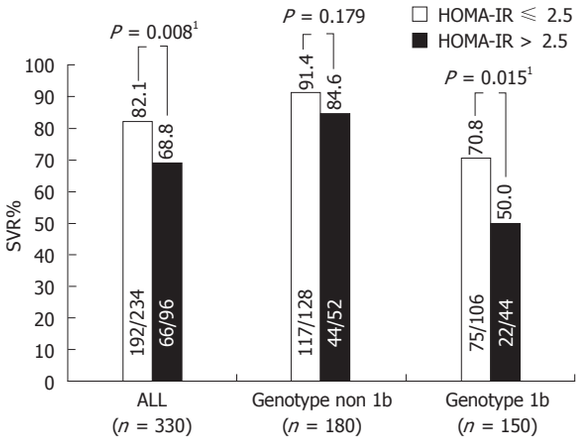

Figure 4 Insulin resistance predicts response to peginterferon-α/ribavirin combination therapy in chronic hepatitis C patients[106]. [1]Statistical significance. SVR: Sustained virological response; HOMA-IR: Homeostasis model assessment-insulin resistance.

serum levels of pegylated interferons (PEG-IFNs) and a reduced response to antivirals, as certain PEG-IFNs may be preferentially absorbed through blood capillaries or the lymphatic circulation. Obesity may also affect the antiviral response by modulating the IFN signaling pathway, as a recent study showed that obese HCV genotype 1 patients had increased mRNA expression of SOCS-3 compared with normal controls[102] (Figure 2).

However, in a recent large-scale study, IR but not steatosis or fibrosis was the most important predictor for response to PEG-IFN and ribavirin therapy[103]. Moreover, the molecular link between insulin signaling and reduced response to IFN-α has been demonstrated in several studies[104-106]. HCV core protein stimulates the SOCS-3, which is a negative regulator of IFN-α signaling. SOCS-3 upregulation inhibits expression of interferon stimulated genes as 2',5'-oligoadenylate synthetase and protein kinase receptor (PKR), through inactivation of the JAK-STAT pathway[105].

Downregulation of PPAR-γ and an upregulation of SOCS-7 were also observed upon expression of the HCV genotype 3 core protein[107], and it was suggested that the activation of SOCS family members may be a mechanism common to all major HCV genotypes[108]. However, activation of SOCS family members is not the only mechanism suggested to account for HCV-induced IR, as it has been shown that HCV core protein also suppresses insulin signaling through a proteasomal activator 28 γ-dependent pathway[109]. This is worthy of note, because this activator plays a role also in the development of steatosis and HCC[110].

Insulin resistance-associated extrahepatic manifestations

In patients with extrahepatic manifestations of HCV, fasting insulin levels and homeostasis model assessment (HOMA) for IR are significantly higher than for patients without extrahepatic manifestations[111]. Among various extrahepatic manifestations, IR is associated with oral lichen planus[112], oral squamous cell carcinoma and multiple primary cancers including gastric cancer[113]. Although reasons for this association remain unclear, a high prevalence of precancerous lesions and cancers are seen in patients with T_2 DM[114,115], suggesting that IR or hyperinsulinemia may enhance carcinogenic activities.

THERAPEUTIC IMPLICATIONS

The clinical implications of the presence of IR in patients with CHC have become evident in many studies that consistently showed that these cofactors are related to both disease progression and a poorer response to antiviral therapy.

Effect of insulin resistance on sustained virologicresponse

The negative impact of IR on response to antiviral therapy has been demonstrated in several studies[93-97]. Romero-Gómez *et al*[76], showed marked differences in the rates of SVR in HCV infected patients with and without IR, assessed by HOMA-IR. In this study, 23 of 70 (32.8%) patients with genotype 1 CHC and IR (HOMA-IR > 2) achieved a SVR *vs* 26 of 43 (60.5%) genotype 1 CHC patients without IR. These findings were independently confirmed[65], and extended to genotypes 2 and 3[103].

In a recent large-scale study in CHC patients, pretreatment HOMA-IR was associated with SVR to combination therapy with (PEG-IFN)/ribavirin, in particular among "difficult-to-treat" patients (genotype 1b and high baseline viral loads). These findings suggest that pretreatment measurement of HOMA-IR, in combination with tests of HCV genotypes and viral load, may be used as the determinants for selecting regimens in CHC patients (Figure 4)[103].

Effect of improving insulin resistance on sustained virologicresponse

Since IR is considered a factor that can be modified and improved by various interventions, it would be valuable to evaluate by prospective studies whether the improvement of IR before initiation of the combination therapy for CHC can significantly increase the SVR rate. It has been shown that weight reduction may have an impact on both liver histology and biochemistry in patients with CHC[116,117]. Moreover, a strict low calorie diet for three months, aiming to achieve a 10% reduction in body mass index before starting treatment has determined higher rates of response to Peg-IFN plus ribavirin therapy[118].

Amelioration of IR may improve the response to antiviral treatment. However, the impact of insulin-sensitizing agents, biguanides and thiazolidinediones, on SVR rates has not yet been established.

Recently, metformin, a biguanide agent that decreases the production of glucose in hepatocytes and increases the utilization of glucose within skeletal muscle, has been reported to ameliorate HCV-associated IR, and increase the SVR rate in HCV genotype 1- infected patients with normalization of HOMA-IR at week 24 of therapy[119].

Thiazolidinediones improve insulin sensitivity through

activation of the peroxisome proliferator-activated receptor-γ (PPAR-γ) in adipocytes and skeletal muscle[120]. Pioglitazone, a thiazolidinedione agent, has been found to decrease SOCS-3 expression in diabetic patients[121], and has been also reported to ameliorate IR and increase SVR rates in Egyptian patients with HCV genotype 4 infections[122]. In a recent randomized, double-blind, placebo-controlled study, adding pioglitazone 30 mg once daily simultaneously to the standard of care clearly increased the on-treatment virological response, but failed to increase the sustained virological response after the end of treatment[123]. A recent prospective, multicenter study aimed to investigate the efficacy and safety of pioglitazone, 15 mg once daily, added to the Peg-IFN-α2a, 180 μg once weekly/ribavirin and 1200 mg once daily combination therapy in chronic hepatitis C patients who were previously nonresponders to a Peg-IFN-α/ribavirin combination without the insulin sensitizer. All patients had a baseline HOMA > 2 as additional inclusion criterion and diabetic patients were however excluded. Unfortunately, none of the first five patients enrolled into the trial had a satisfactory virological response after 12 wk of retreatment, despite the fact that in at least three of them the IR score improved, and thus the study was prematurely terminated[124].

Because both metformin and thiazolidinedione agents have severe adverse effects, neither is recommended for patients with liver cirrhosis. Biguanides predispose cirrhotic patients to lactic acidosis[125], while thiazolidinediones may cause significant hepatotoxicity[126]. Therefore further validation for safety is required.

It is still unclear whether one should start the antiviral retreatment together with the insulin sensitizer or only once the HOMA-IR score has decreased to a level predicting a sufficient SVR rate[76]. Also, insulin sensitizing therapy might need to be tailored according to HCV genotype, and PPARs agonists should probably be considered only in insulin-resistant patients with HCV genotype 3a[127]. This approach, however, should also take into consideration the known effects of PPAR agonists on serum lipid profile and their potential consequences on the HCV life cycle. HCV circulates bound to lipoproteins in complexes known as lipoviroparticles[128]. As a result, HCV entry into hepatocytes appears to be mediated and facilitated, among others, by the low density lipoprotein (LDL) receptor[129]. In keeping with this, at least two recent studies have suggested that baseline LDL-associated cholesterol levels may affect response to antiviral therapy[130,131]. In fact, higher levels of cholesterol-and ApoB-rich lipoproteins could facilitate viral clearance by impeding HCV interaction with cell surface receptors. Thus, drugs like thiazolidinediones that modify the circulating lipoprotein profile may have unexpected and potentially unwanted effects on the HCV life cycle. Although highly speculative, these hypotheses deserve being appropriately evaluated in clinical trials.

Finally, dipeptidyl peptidase (DPPIV) inhibitor is a new therapeutic agent[132] that has shown its clinical efficacy in T_2DM[133]. It may be suited for ameliorating HCV-associated IR, as activation of DPPIV was considered a factor responsible for HCV-associated IR[134].

Effect of sustained virologicresponse on insulin resistance

Evidence that effective antiviral treatment will result in improved glucose homeostasis in patients with T_2DM is at best preliminary. It has been shown that IR is modified by treatment and that the incidence of T_2DM in patients achieving SVR is significantly lower than that seen in non-responders[25,26]. Kawaguchi *et al*[28], reported a significant decrease in HOMA-IR values in patients who attained a SVR, while no changes occurred in HOMA-IR non-responders and relapsers.

Effect of sustained virologicresponse on steatosis

In HCV genotype 3 infections, the severity of steatosis is directly related to the HCV RNA viral load, and steatosis often resolves with the loss of viremia after antiviral treatment[8,135,136], and reappears after the end of therapy in relapsers[7].

On the contrary, in genotype 1 infection, the severity of steatosis is independent of the HCV viral load and antiviral therapy alone does not improve steatosis in these patients[7,137]. Similar data have been obtained for genotype 4 infections, while little data are available for genotype 2[138].

Therapeutic effects of phlebotomy

In order to reduce hepatic iron deposition, both dietary iron restriction and phlebotomy are effective. It has been shown that dietary iron restriction (less than 7 mg/d) decreases serum alanine aminotransferase levels in patients with HCV infection[138], and phlebotomy reduces oxidative stress as well as IR in patients with HCV infection[47,138-140].

A number of studies have shown that phlebotomy to induce iron depletion may lead to regression of fibrosis. In the three trials in which paired liver biopsies before and after treatment with IFN or phlebotomy plus IFN were evaluated, all reported histological improvements in the phlebotomy group[141-144]. Di Bisceglie *et al*[145] compared treatment with phlebotomy alone to phlebotomy plus IFN, and found that, after 1 year, the phlebotomy-only group demonstrated mild histological improvement that did not reach statistical significance.A recent meta-analysis of six prospective randomized controlled trials found that phlebotomy also improves therapeutic response to interferon in patients with CHC[146].

Additionally, it has been reported that hepatic iron concentration is correlated with the risk of developing HCC[147]. Moreover, a long-term combination treatment with phlebotomy and dietary iron restriction has been found to reduce the risk of development of HCC in patients with HCV infection[143]. If these observations are correct, it is possible that therapeutic phlebotomy in patients with CHC may provide a benefit to patients even in the absence of an SVR to current therapy[147].

Effect of anti-diabetic agents

Anti-diabetic agents such as exogenous insulin and sulfonylurea agents, are effective for decreasing plasma glucose leading to prevention of diabetes mellitus-associated complications including cardiovascular diseases[148-150]. However, the use of exogenous insulin or sulfonylurea agents may worsen hyperinsulinemia. Recently, an association has been reported between exogenous sulphonylurea or insulin treatment and the development of HCC in patients with HCV infection[151,152]. The use of exogenous insulin has also been reported to be associated with the development of colon cancer[153], and other malignancies[154,155]. Although a causal relationship between exogenous insulin and the development of HCC remains controversial[122], the reduction of serum insulin levels is a first line therapeutic strategy for IR[156].

CONCLUSION

IR is one of the pathological features in patients with HCV infection. IR plays a crucial role in the development of various complications and events associated with HCV infection. Mounting evidence indicates that HCV-associated IR may cause hepatic steatosis, hepatic fibrosis, resistance to anti-viral treatment, hepatocarcinogenesis and proliferation of hepatocellular carcinoma; and extrahepatic manifestations.

Thus, HCV-associated IR is a therapeutic target at any stage of HCV infection. However, therapeutic guidelines for preventing the distinctive complications of HCV-associated insulin resistance have not yet been established. Insulin-sensitizing agents are reported to improve sustained virologic response rates, but further validation for safety is required. Little is known regarding the effect of anti-diabetic agents on HCV infection, and a possible association between use of exogenous insulin or a sulfonylurea agent and the development of HCC has recently been reported.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i3.225

World J Gastroenterol 2012 January 21; 18(3): 225-236
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Stress-induced visceral analgesia assessed non-invasively in rats is enhanced by prebiotic diet

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Supported by Central Labs for Frontier Technology Kirin Holdings Co., Ltd Japan and the National Institute of Health grants, P50 DK-64539 (to Taché Y and Larauche M), Center Grant DK-41301 (Animal Core, to Yvette Taché), R01 DK-33061 (to Yvette Taché); and Veterans Administration Research Career Scientist Award

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Received: May 15, 2011 Revised: July 6, 2011
Accepted: October 27, 2011
Published online: January 21, 2012

Abstract

AIM: To investigate the influence of repeated water avoidance stress (rWAS) on the visceromotor response (VMR) to colorectal distension (CRD) and the modulation of the response by a prebiotic diet in rats using a novel surgery-free method of solid-state manometry.

METHODS: Male Wistar rats fed a standard diet with or without 4% enzyme-treated rice fiber (ERF) for 5 wk were subjected to rWAS (1 h daily x 10 d) or no stress. The VMR to graded phasic CRD was assessed by intraluminal colonic pressure recording on days 0 (baseline), 1 and 10 (45 min) and 11 (24 h) after rWAS and expressed as percentage change from baseline. Cecal content of short chain fatty acids and distal colonic histology were assessed on day 11.

RESULTS: WAS on day 1 reduced the VMR to CRD at 40 and 60 mmHg similarly by 28.9% ± 6.6% in both diet groups. On day 10, rWAS-induced reduction of VMR occurred only at 40 mmHg in the standard diet group (36.2% ± 17.8%) while in the ERF group VMR was lowered at 20, 40 and 60 mmHg by 64.9% ± 20.9%, 49.3% ± 11.6% and 38.9% ± 7.3% respectively. The visceral analgesia was still observed on day 11 in ERF- but not in standard diet-fed rats. By contrast the non-stressed groups (standard or ERF diet) exhibited no changes in VMR to CRD. In standard diet-fed rats, rWAS induced mild colonic histological changes that were absent in ERF-fed rats exposed to stress compared to non-stressed rats. The reduction of cecal content of isobutyrate and total butyrate, but not butyrate alone, was correlated with lower visceral pain response. Additionally, ERF diet increased rWAS-induced defecation by 26% and 75% during the first 0-15 min and last 15-60 min, respectively, compared to standard diet, and reduced rats' body weight gain by 1.3 fold independently of their stress status.

CONCLUSION: These data provide the first evidence of psychological stress-related visceral analgesia in rats that was enhanced by chronic intake of ERF prebiotic.

Key words: Stress-related visceral analgesia; Water avoidance stress; Colorectal distension; Enzyme-treated rice fiber prebiotic; Short chain fatty acids; Defecation; Rat; Solid-state manometry

Peer reviewer: Damian Casadesus Rodriguez, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

Larauche M, Mulak A, Yuan PQ, Kanauchi O, Taché Y. Stress-induced visceral analgesia assessed non-invasively in rats is enhanced by prebiotic diet. *World J Gastroenterol* 2012; 18(3): 225-236 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i3/225.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i3.225

INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional bowel disorder of unclear etiology, characterized by recurrent abdominal pain and altered bowel habits without structural abnormalities affecting 10%-15% of the US[1,2] and 5%-10% of the Asian adult population[3,4]. Stress is an important factor in the onset, maintenance and exacerbation of IBS symptoms[5-7]. Recently, interactions between intestinal microbiota, mucosal barrier function, and immune system have been identified to play a role in IBS pathogenesis[8,9]. An imbalance in the gastrointestinal microbial population induced by infection, dietary changes or antibiotics can produce low grade inflammation as observed in a subset of IBS patients[10]. Alterations in gut transit, which can be related to dietary factors, stress or antibiotics, may also contribute to the abnormalities observed in enteric microbiota metabolic activity including fermentation processes[11]. Prebiotics as indigestible food constituents provide specific substrates ready to be metabolized by the beneficial gut microbiota thereby stimulating their growth or activity[8,12]. Prebiotics are also a source of short chain fatty acids (SCFAs) that provide energy to the epithelium and exhibit anti-inflammatory properties[13].

Visceral hypersensitivity, a key feature of IBS[14], can be associated with a low grade colonic mucosal inflammation in a subset of patients[15]. Therefore, the concept that anti-inflammatory action of probiotics and prebiotics could be beneficial in visceral pain has emerged[16]. In fact, there are reports that *Escherichia coli Nissle 1917* inhibits the visceral hypersensitivity associated with trinitrobenzene sulphonic acid colitis[17]. Similarly, *Lactobacillus paracasei* prevents the visceral hypersensitivity associated with inflammation in mice with gut microbiota altered by antibiotics[18]. Recently, a new prebiotic, enzyme-treated rice fiber (ERF) made from rice bran containing dietary fiber and fat soluble fraction has been shown to reduce inflammation and clinical symptoms in the murine dextran sodium sulfate colitis model by modulating the colonic microbiota environment and regulating immune cell differentiation[12] and to prevent 3-d intermittent restraint stress-induced IBS-like symptoms in rats[19].

Existing evidence from several groups including ours indicates that stress induces alterations of colonic functions (increased permeability, mucus secretion, motility, myenteric nerves activation and serotonin release) and the development of visceral hypersensitivity in rats[20-22]. In particular, we previously developed a psychological stress model of intermittent water avoidance stress (WAS)[23] which upon repeated exposure (rWAS) in rats induces sustained visceral hyperalgesia when monitored by electromyography (EMG)[24,25]. In rats, rWAS also induces colonic epithelial alterations associated with cytokines increase, mast cell activation and antigen sensitization[24,26-28], which may all contribute to greater visceral sensitivity[29]. Administration of probiotics chronically throughout the 10 d of rWAS prevents the stress-related colonic epithelial alterations and antigen load in the mucosa[30]. By contrast, we have recently shown in mice that exposure to rWAS can induce stress-related visceral analgesia in response to colorectal distension (CRD) when monitored non-invasively using a novel method of solid-state manometry, which avoids prior surgery to implant recording electrodes and subsequent single housing which are required in commonly used EMG monitoring of visceral pain[31].

Therefore in the present study, we first examined whether exposure to rWAS induced visceral analgesia in rats using the non-invasive monitoring of the visceromotor response (VMR) to graded phasic CRD. Second, we tested whether the chronic feeding of 4% ERF prebiotic would affect rWAS-related alterations of visceral sensitivity and colonic motor functions and potential underlying mechanisms by monitoring histological changes in the colonic mucosa and cecal content of SCFAs.

MATERIALS AND METHODS

Animals

Male Wistar rats (Harlan Laboratories, Indianapolis, IN) were fed *ad libitum* a standard diet (composition detailed in Table 1) with or without 4% ERF (formulated in pellets for 4 wk, then in powder form) (Kirin Holdings Company, Laboratory, Japan) starting from weaning (~ 21 d old, weight ~ 50-74 g) and throughout the 48 experimental days. Rats were housed in pairs, under standard conditions of temperature and humidity, on bedding with enrichment. At the end of the experiment, animals were euthanized by an intraperitoneal overdose of anesthesia (urethane 25%) and thoracotomy. Experimental protocols were performed in accordance with animal protocol No. 12049-09 approved by the Greater Los Angeles Institutional Animal Care and Use Committee.

Preparation and chemical composition of enzyme-treated rice fiber

The ERF diet was prepared as detailed previously[12] and composed roughly of 70% dietary fiber by weight[19,32] formulated to have 4% of ERF in standard food (Table 1). In the standard diet, 4% ERF was replaced by cellulose (30 g/kg) (Table 1).

Repeated water avoidance stress

The procedure of rWAS was performed as described before[23]. Each adult Wistar rat (232-311 g) was placed on a pedestal (10 cm × 8 cm × 8 cm) affixed to the center of a Plexiglas® standard rat cage floor (45 cm length × 25 cm width × 25 cm height) for 1 h daily for 10 consecutive days between 8 and 10 am. The Plexiglas® rat cage

Table 1 Chemical composition of standard and enzyme-treated rice fiber diets

Content (g/kg)	Standard	ERF[1]
Casein[2]	146.0	140.4
Vitamin mix	10.0	10.0
Mineral mix	35.0	35.0
Choline chloride	2.0	2.0
Cellulose[2]	30.0	-
ERF	-	40.0
Corn oil	50.0	50.0
Corn starch	727.0	722.6

[1]Enzyme-treated rice fiber (protein, 14.9%; dietary fiber, 74.5%); [2]According to AIN 93G formula, protein and dietary fiber contents in two diets were adjusted to the same value using casein and cellulose, respectively. ERF: Enzyme-treated rice fiber.

was filled with room temperature water (25 ℃) up to 1 cm from the top of the pedestal. Non-stressed rats were kept in their home cage and handled daily (5 min).

Measurement of colonic motor function and visceral pain

Fecal pellet output: In rats subjected to rWAS protocol, fecal output was monitored as the total number of pellets expelled for the 1-h period of rWAS exposure on each of the 10 d with a time course response monitored every 15 min.

Assessment of visceral pain response to CRD: Visceral sensitivity to CRD was assessed using the non-invasive manometric method that we recently developed and validated for use in mice and rats[31,33]. Briefly, a PE50 catheter was taped 3.5 cm below the pressure sensor of a miniaturized pressure transducer catheter (SPR-524 Mikro-Tip catheter; Millar Instruments, Houston, TX). A custom-made balloon (2 cm wide × 5 cm long)[33,34], prepared from an infinitely compliant high-density polyethylene 16 micron gauge plastic bag (STOUT, C4348N16, Wichita, KS), was tied over the catheter at 1 cm below the pressure sensor with silk 4.0 (Henry Schein Inc., Melville, NY).

On experimental day, rats were briefly anesthetized with isoflurane (3% in O_2) and the lubricated "balloon-pressure sensor" catheter was introduced into the rectum and distal colon such that the distal end of the balloon was positioned at 1 cm from the anus and the catheter secured to the tail with tape. Each animal was placed in a Bollman cage covered with a light tissue blanket and left to rest for 30 min before the CRD procedure. Each balloon was connected to the barostat and the miniaturized pressure transducer to a preamplifier (model 600; Millar Instruments, Houston, TX). The intracolonic pressure (ICP) signal was acquired using CED Micro1401/SPIKE2 program. The CRD protocol consisted of two CRDs at 60 mmHg to unfold the balloon immediately followed by two series of graded phasic distensions to constant pressures of 10, 20, 40 and 60 mmHg. Each CRD lasted 20 s and was applied at a 4 min inter-stimulus interval (Figure 1). A similar CRD paradigm has been used previously to assess visceral pain-related responses in rats[35].

Data analysis

The phasic component of the intracolonic pressure (pICP) was extracted from the ICP signal recorded[31,33]. The VMR was defined as the increase in area under the curve (AUC) of pICP during CRD over the mean value of pre- and post-distension 20 s periods. To examine the pressure-response relationship and adjust for inter-individual variations of the signal[35], ICP amplitudes were normalized for each rat to the highest pressure (60 mmHg) in the 1st set of CRD. This value served as 100% response (control) in the baseline period of data collection before exposure to WAS (day 0) and represented the baseline VMR. The VMR to the subsequent CRDs was expressed either as % from their baseline values or mean change from the baseline response (Δ VMR in %) at different pressures of distension as validated in our previous studies[25,31,33]. In some cases, to determine correlations between visceral pain responses and inflammatory scores or cecal organic acid content, the non-normalized cumulative AUC response during the CRD procedure on day 11 (for all pressures of distensions) was used[36]. For subgrouping analysis, each rat exhibiting VMR to CRD at 60 mmHg with a value higher than that obtained at baseline (> 10%) was categorized as hyperalgesic, while each rat presenting value lower than that obtained at baseline (< 10%) was categorized as analgesic.

Experimental protocol

Rats were fed 4% ERF diet (2 groups) or standard control diet (2 groups) starting at the weaning period for 37 d until they reached the age of 6-6.5 wk (weight ~ 232-311 g) and handled every 3-4 d (petting, 5 min). All experimental protocols were performed in the morning and conducted in conscious non-fasted rats. All groups were habituated to the experimental conditions for VMR monitoring (training to Bollman cages, 90 min per day for 3 consecutive days before day 0), then tested for baseline CRD response (day 0). Thereafter rats were exposed daily to 1 h WAS for 10 consecutive days or no stress (days 1-10), while still maintained under standard or 4% ERF diet. Non-stressed animals were kept in their home cages and handled daily. Body weight was monitored in the morning, every 2-4 d for 37 d and daily during the stress period before each stress session as well as in non-stressed groups. Body weight gain was expressed as % of initial body weight. Fecal pellet output (FPO) was monitored during each rWAS session. On days 1 (acute WAS) and 10 (rWAS), 45-50 min after the end of the stress session, and on day 11, 24 h after the last rWAS session, rats were subjected to CRD protocol and VMR was monitored in all groups (Figure 1). Immediately after the last CRD, animals were euthanized and cecum and distal colon, at a level caudal to the balloon placement, were collected.

Colonic histological assessment

The distal colonic segment was rinsed with cold phosphate buffer saline, fixed in 4% paraformaldehyde and kept at 4 ℃ until processed. After fixation, paraffin-embedded specimens were sectioned at 5 μm (whole thickness) and stained with HE. Inflammatory scores were

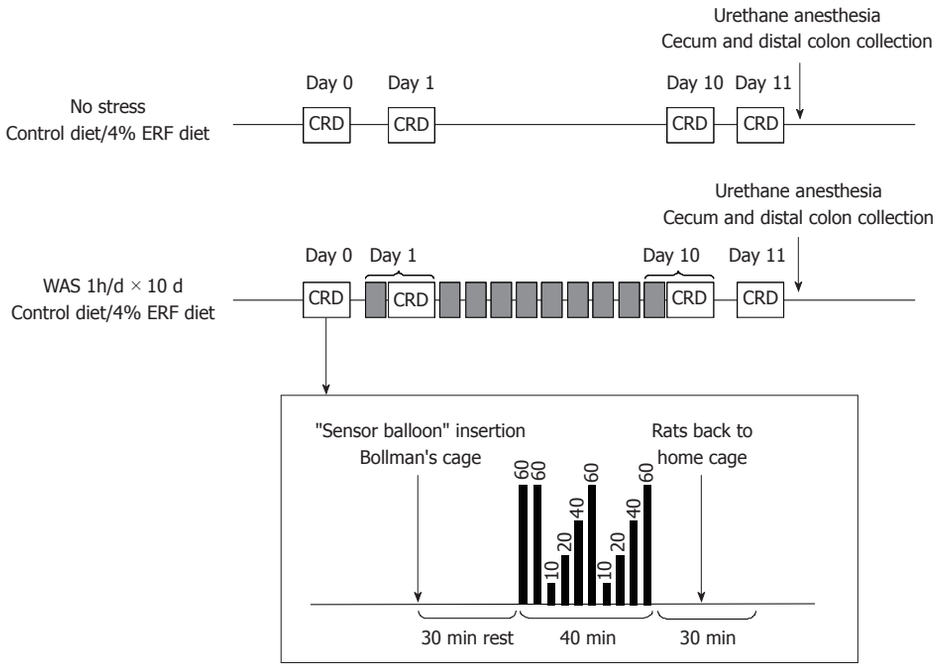

Figure 1 Treatment and colorectal distension (black column with number in mmHg) protocol for the assessment of visceral pain in Wistar rats exposed to daily intermittent water avoidance stress. Grey boxes represent the daily sessions of water avoidance stress (1 h). ERF: Enzyme-treated rice fiber; CRD: Colorectal distension.

Table 2 Baseline visceromotor response to colorectal distension at day 0

CRD (mmHg)	Standard diet[1]		ERF diet[1]	
	No stress (n = 7) VMR (%)[2]	WAS (n = 7) VMR (%)[2]	No stress (n = 9) VMR (%)[2]	WAS (n = 8) VMR (%)[2]
10	-0.3 ± 1.2	0.9 ± 1.9	2.8 ± 2.8	0.89 ± 1.0
20	5.1 ± 1.8[a]	26.8 ± 12.2	30.6 ± 12.2	30.7 ± 7.0
40	71.4 ± 6.2	77.3 ± 11.8	71.1 ± 11.2	88.7 ± 7.8
60	100 ± 0	100 ± 0	100 ± 0	100 ± 0

[1]Rats were fed standard or 4% enzyme-treated rice fiber (ERF) diet (2 groups each before to be assigned to no stress or water avoidance stress, WAS) starting at the weaning period for 37 d before performing the baseline visceromotor response (VMR) to colorectal distension (CRD); [2]Each value represents the mean ± SE of n as indicated in parenthesis; [a]$P < 0.05$ *vs* all other groups at 20 mmHg - 2-way ANOVA, Bonferroni post test.

established following a scale of 0 to 3: 0: normal, 1: mild, 2: moderate, 3: severe based on the occurrence of inflammatory cell infiltration and mucosa microscopic aspect (submucosal edema, vascular dilation, mucosal erosion, atrophy).

Cecal content of organic acids

The cecum was cut open and its content flash frozen less than 15 min post collection and kept at -80 ℃ until assayed for SCFAs levels as previously described[12]. Briefly, 0.3 g of the cecal content was weighed and 1 mL of Milli-Q water added. The mixture was incubated at 4 ℃ for 60 min. After centrifugation at 12 000 r/min at 4 ℃ for 10 min, the obtained supernatant was filtered using a 0.22 μm filter. Organic acids were separated with the Shimpack SPR-H 250 L (Shimadzu Co. Ltd., Kyoto, Japan). The mobile phase was 4 mmol/L of p-toluene sulfonic acid, and the detector was electric conductivity (Shimadzu CDD-6A, Kyoto Japan).

Statistical analysis

Each experimental group included 5-10 rats. Data were analyzed using one-way ANOVA or 2-way ANOVA followed by Bonferroni *post hoc* test or by paired or unpaired Student *t* test as specified to assess the difference between treatment groups. Contingency statistical analyses for correlation between visceral sensitivity and cecal organic acid content were performed using two-tailed Fisher's exact test. Based on the Grubb's test results, three out of the total number of 34 rats on two different days of CRD were found to be outliers and excluded from VMR data analysis. Due to technical issues (i.e., expulsion of the balloon before CRD or probe failure during CRD), 5 rats were excluded from some parts of the analysis immediately after stress (day 1: 2 rats) or 24 h later (day 11: 3 rats). A P value < 0.05 was considered significant.

RESULTS

Visceral pain

Rats were fed a standard diet or 4% ERF diet for 48 d and CRD was performed on days 37 (baseline, day 0), 38 (day 1), 47 (day 10) and 48 (day 11) with or without (no stress) 1-h daily WAS from days 38 to 47. At day 0, the VMR to baseline CRD in each of the four groups of rats was similar at all pressures of distensions, except at 20 mmHg in rats fed standard diet to be assigned to no stress which presented a lower VMR [$F(3.108)=184.7$, $P < 0.001$] compared to the groups fed ERF diet ($P < 0.05$) (Table 2).

Table 3 Visceromotor response after water avoidance stress or no stress in rats fed standard or enzyme-treated rice fiber diet

Time[1] Post WAS	CRD pressure (mmHg)	Standard diet[1]				ERF diet1			
		No stress		WAS		No stress		WAS	
		VMR (%)[2]	*n*	VMR (%)[2]	*n*	VMR (%)[2]	*n*	VMR (%)[2]	*n*
45 min day 1	40	15.8 ± 17.6	7	- 32.4 ± 15.2[a]	7	26.0 ± 23	8	- 29.5 ± 12.3[b]	8
	60	1.0 ± 9.7		- 30.0 ± 10.8[a]		12.9 ± 13.8		- 28.7 ± 6.0[b,c]	
45 min day 10	40	-26.4 ± 11.7	7	- 36.2 ± 17.8[b]	7	3.7 ± 21.3	8	- 49.3 ± 11.6[b,c]	9
	60	-23.7 ± 18.6		- 20.6 ± 12.2		21.6 ± 23.5		- 38.9 ± 7.3[b]	
24 h day 11	40	7.5 ± 38.7	5	- 28.2 ± 15.0	7	- 4.1 ± 21.5	6	- 49.8 ± 12.6[b]	9
	60	30.4 ± 15.4		- 24.3 ± 9.6[c]		5.8 ± 15.1		- 34.3 ± 10.8[b]	

[1]Rats were fed 4% enzyme-treated rice fiber (ERF) diet or standard control diet starting at the weaning period for 37 d, then tested for baseline visceromotor response (VMR) to colorectal distention (CRD) response (day 0) and thereafter exposed daily to 1 h water avoidance stress (WAS) for 10 consecutive d or no stress (days 1-10), while still maintained under standard or 4% ERF diet. Non-stressed animals were kept in their home cages. On days 1 (acute WAS) and 10 [repeated water avoidance stress (rWAS)], 45-50 min after the end of the stress session, and on day 11, 24 h after the last rWAS session, rats were subjected to CRD protocol and VMR was monitored in all groups; [2]Each value represents the mean ± SE of % VMR changes from baseline (day 0) in number of animal indicated by n in columns). Minus values represent analgesia. [a]$P < 0.05$, [b]$P < 0.01$ *vs* baseline; [c]$P < 0.05$ *vs* respective diet non stressed group, 2-way ANOVA, Bonferroni post hoc test.

Repeated water avoidance stress induces an immediate analgesia compared to baseline in standard diet fed rats

Non-stressed rats on standard diet exhibited no statistically significant changes in VMR compared to baseline at all pressures of CRD when tested on days 1 ($n = 7$), 10 ($n = 7$) and 11 ($n = 5$) after baseline recording [$F(3.99) = 1.248$, $P = 0.28$] (Table 3 and Figure 2). By contrast, acute WAS (day 1) decreased the VMR to CRD (analgesia) at 40 mmHg and 60 mmHg by 32.4% ± 15.2% and 30.0% ± 10.8% respectively compared to baseline ($n = 7$, $P < 0.05$, Bonferroni post hoc test) (Table 3 and Figure 2A). After 10 consecutive days of rWAS, rats tested within 45-50 min after the last session of stress exhibited a decreased VMR to CRD (analgesia) at 40 mmHg by 36.2% ± 17.8% compared to baseline ($n = 7$, $P < 0.01$) but not at 60 mmHg ($n = 7$, $P > 0.05$) (Table 3 and Figure 2B). The VMR to CRD was not significantly different from baseline values on day 11, 24 h after the last stress session ($n = 7$, $P > 0.05$) (Table 3 and Figure 2C). Two-way ANOVA showed a significant influence of rWAS treatment [$F(3.88) = 2.761$, $P = 0.0468$] and time [$F(3.88) = 77.56$, $P < 0.0001$]. However, there was no interaction between treatment and time. In rats fed standard diet, 85.7%, 66.7% and 85.7% of animals developed visceral analgesia in response to rWAS when tested at day 1, 10 or 11, respectively, while 100% exhibited visceral analgesia in rats fed ERF diet consistently over the 3 d of testing.

ERF diet potentiates the immediate and induces delayed visceral analgesia to rWAS compared to baseline in rats

In non-stressed rats fed 4% ERF diet tested on days 1 ($n = 8$), 10 ($n = 8$) and 11 ($n = 6$) after baseline recording, the VMR was not significantly different to the baseline at all pressures of CRD [$F(3.104) = 0.6390$, $P = 0.59$]; $P > 0.05$) (Table 3 and Figure 2). By contrast, one session of WAS decreased the VMR to CRD compared to baseline at 40 and 60 mmHg by 29.5% ± 12.3% and 28.7% ± 6.0% respectively ($n = 8$, $P < 0.01$ each) (Table 3 and Figure 2A). Likewise when rats were exposed daily to rWAS and tested on day 10 (Figure 2B) the VMR to CRD was decreased significantly at 20, 40 and 60 mmHg by 25% ± 7.8%, 49.3% ± 11.6% and 38.9% ± 7.3%, respectively compared to baseline ($n = 9$, $P < 0.05$, $P < 0.001$ and $P < 0.001$, respectively) and the decreased VMR was maintained on day 11 (Table 3 and Figure 2C).

ERF diet compared with control diet decreases the percentage of rats developing hyperalgesia in response to rWAS at 60 mmHg CRD over the 10 d of stress

We further performed subgrouping analysis of VMR in animals developing hyperalgesia vs analgesia compared to their basal VMR at 60 mmHg and combined this number over the 10 d of stress (three testing days) for each group. In the non-stressed rats fed either standard or ERF diet, there was no statistical difference ($P > 0.05$, Fisher's exact test) in the percentage of rats that exhibited hyperalgesia throughout the whole 10 d of stress (40%, 28.6% and 80% of rats fed the standard diet, $n = 7$/group *vs* 57%, 57% and 60% fed ERF diet, $n = 7$/group, at days 1, 10 and 11, respectively). By contrast in rWAS groups, while 14.3%, 33.3% and 14.3% fed standard diet developed hyperalgesia at day 1, 10 and 11 ($n = 6$-8/group) respectively, none did in the ERF diet fed group ($n = 8$-9/group) ($P = 0.0478$, Fisher's exact test).

Cecal organic acids analysis

Cecum content was collected on day 48 immediately after the last CRD. In non-stressed rats, 4% ERF diet significantly increased cecal butyrate content compared with standard diet (9.2 ± 0.8 mmol/L per g *vs* 6.1 ± 0.8 mmol/L per g, $P < 0.05$; Figure 3A). In rWAS exposed rats fed ERF diet, cecal butyrate content values were still maintained at similar levels (9.5 ± 1.1 mmol/L per g), however the values did not reach significance when compared with rWAS + standard diet group (7.3 ± 1.4 mmol/L per g) due to a trend to increased butyrate in the rWAS + standard diet group. No statistical differences were detected for the other cecal organic acids even though there was a tendency for isobutyrate to be lower in rats fed ERF diet and exposed or not to rWAS compared to standard diet

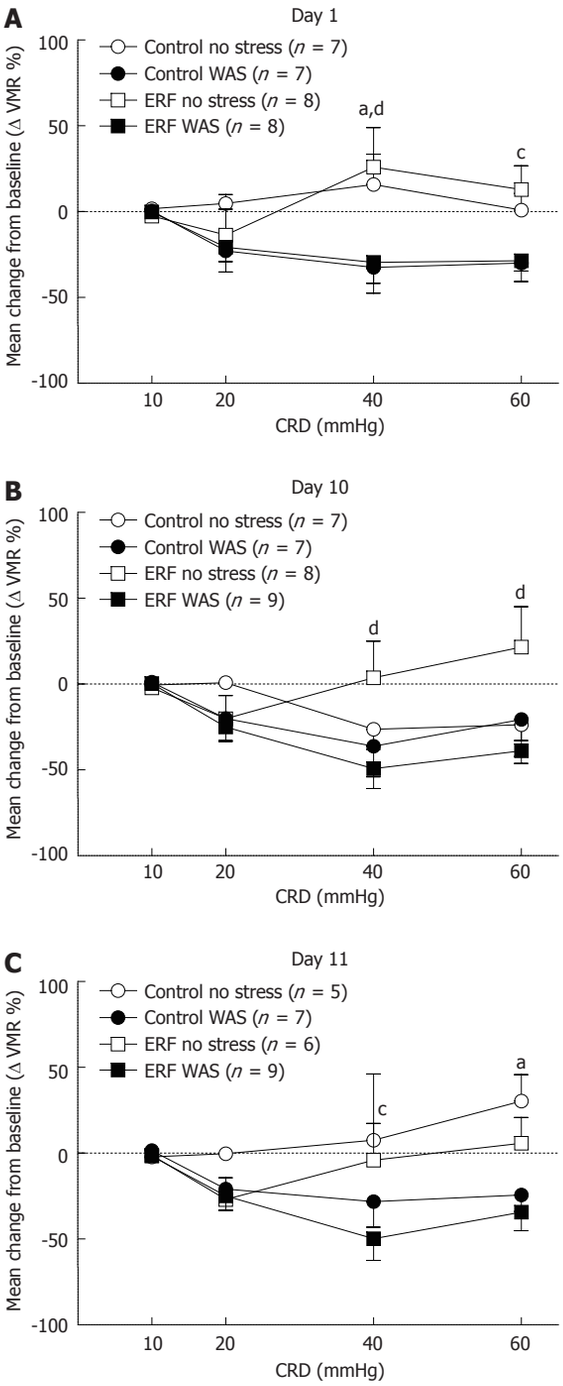

Figure 2 Influence of water avoidance stress or no stress on visceral sensitivity to colorectal distension in rats fed standard or prebiotic (4% enzyme-treated rice fiber) diet ad libitum. A: Immediate visceral motor response (VMR) at day 1; B: Immediate VMR at day 10; C: VMR 24 h at day 11. Data are expressed as mean ± SE of number of animals indicated in parenthesis for each group. [a]$P < 0.05$ standard diet no stress *vs* standard diet water avoidance stress (WAS); [c]$P < 0.05$, [d]$P < 0.01$ enzyme-treated rice fiber (ERF) no stress *vs* ERF WAS; 2-way ANOVA followed by Bonferroni post hoc test. CRD: Colorectal distention.

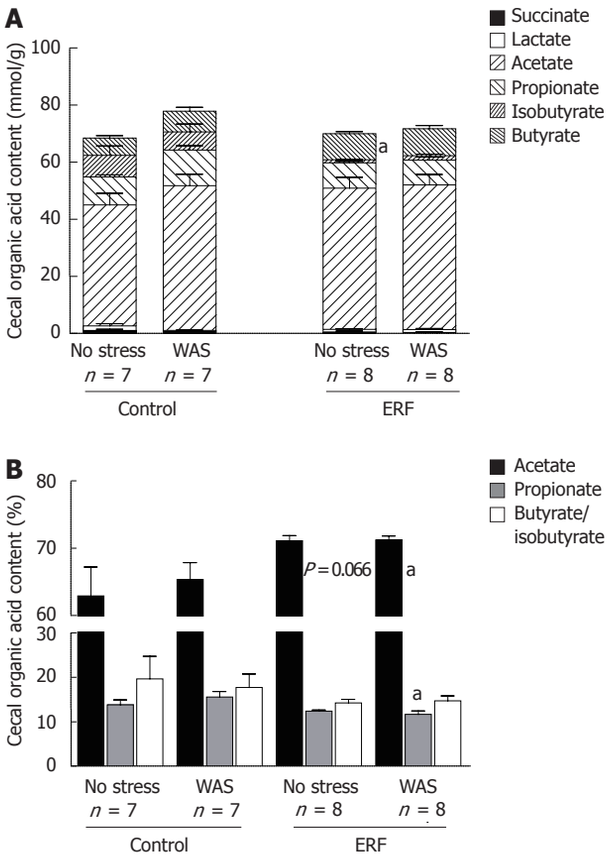

Figure 3 Cecal organic acid content in mmol/L per g (A) or % (B) in rats fed either standard or 4% enzyme-treated rice fiber prebiotic diet and exposed or not to repeated water avoidance stress in rats. Data are expressed as mean ± SE, of number of rats indicated as *n* below columns. [a]$P < 0.05$ *vs* control diet same stress status group; unpaired student *t* test. WAS: Water avoidance stress; CRD: Colorectal distention.

fed rats (1.51 ± 0.64 mmol/L per g *vs* 6.30 ± 2.79 mmol/L per g, $P = 0.10$; 1.04 ± 0.11 mmol/L per g *vs* 7.52 ± 3.37 mmol/L per g, respectively, $P = 0.06$).

The ratio of SCFAs, acetate:propionate:butyrate/isobutyrate, in the cecal content of rats fed ERF diet compared to standard diet, stressed or not, showed that ERF diet increased the percentage of acetate in stressed rats (71.2% ± 0.6% *vs* 65.4% ± 2.5%, $P < 0.05$) as well as in non-stressed rats, although not significantly (71.2% ± 0.7% *vs* 63.0% ± 4.3%, $P = 0.07$) (Figure 3B). ERF diet also decreased the percentage of propionate in stressed animals (11.7% ± 0.8% *vs* 15.6% ± 1.2%, $P < 0.05$; Figure 3B).

Interestingly, lower concentrations in cecal content of isobutyrate and total butyrate (isobutyrate + butyrate), but not butyrate alone, were correlated with lower visceral pain response (overall AUC during CRD on day 11) ($R_p = 0.41$, $P = 0.0368$ and $R_p = 0.42$, $P = 0.0335$, respectively) in stressed and non-stressed animals fed either ERF or standard diet.

Colonic histological assessment

In animals fed standard diet, rWAS evoked slight colonic inflammation with mild invasion of inflammatory cells and submucosal edema that was rarely observed in non-stressed animals. The inflammatory score was statistically different between these groups (1.3 ± 0.3 *vs* 0.3 ± 0.2 arbitrary units, $P < 0.05$, unpaired *t* test) (Figure 4A, B and E) and statistical analysis confirmed that positive inflammatory scores occurred less frequently than expected by chance in stressed animals ($P < 0.05$, two-tailed Fisher's exact test). The inflammatory score of 4% in ERF-fed

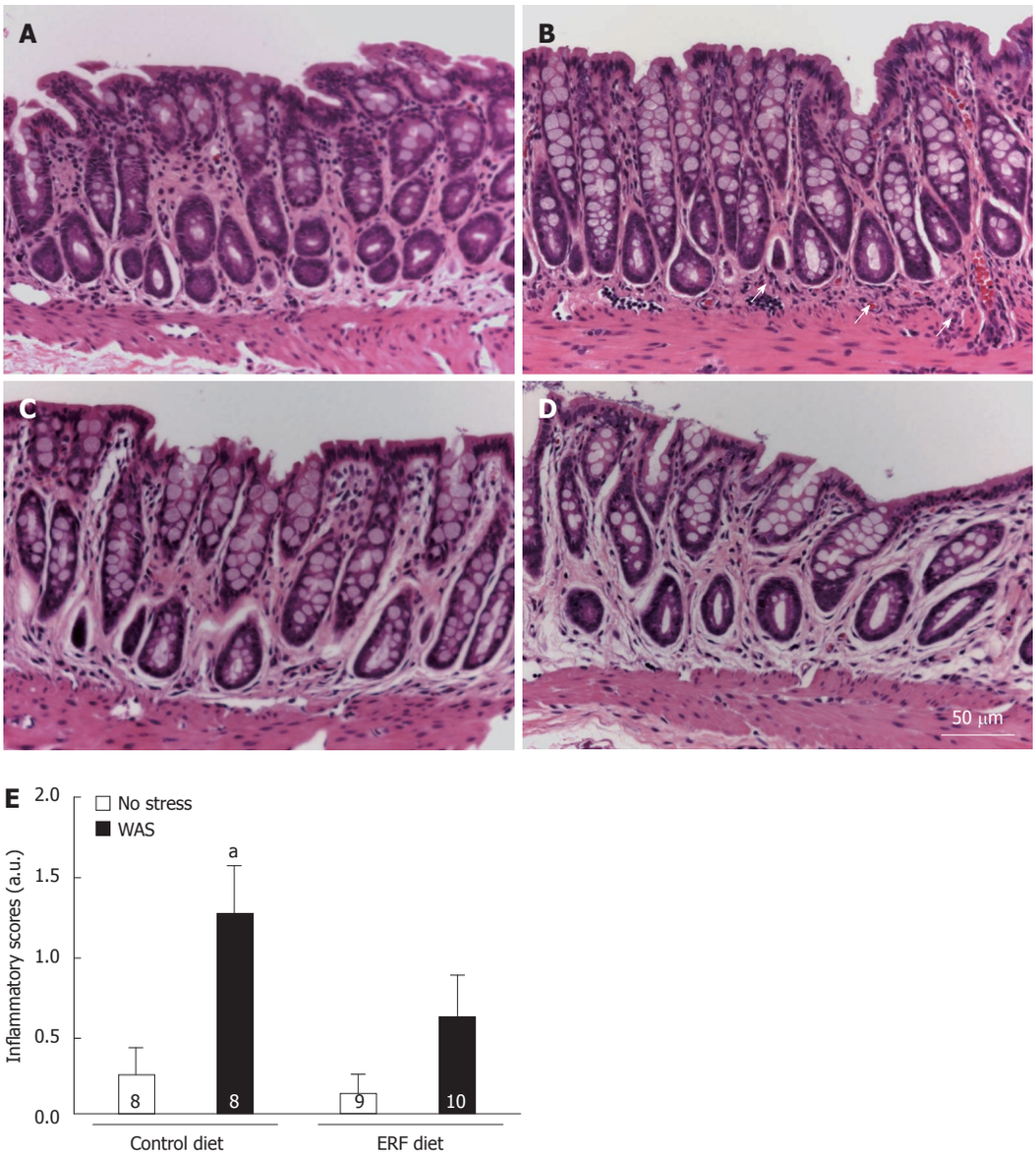

Figure 4 Inflammatory scores in distal colon of rats fed either standard or 4% enzyme-treated rice fiber diet and exposed or not (no stress) to repeated water avoidance stress. A-D: Representative histological sections of the rat distal colon stained with hematoxylin and eosin. The arrows point to an area in the lamina propria with inflammatory cell infiltration in stressed rats fed with standard diet (B) that was not observed in non-stressed rats fed with standard (A) or 4% enzyme-treated rice fiber (ERF) (C) diet and stressed rats fed with 4% ERF diet (D). E: Inflammatory scores. Data are expressed as mean ± SE, of number of rats indicated as n in each column. [a]$P < 0.05$ *vs* opposite diet same stress status group; unpaired student *t* test. WAS: Water avoidance stress; a.u.: Arbitrary unit.

animals exposed to stress was similar to that of non-stressed animals (fed ERF or standard diet, $P > 0.05$ paired and unpaired *t* test, respectively) (Figure 4C-E). Of the animals exposed to rWAS, 4 out of 10 in the ERF-fed group (40%) and 7 out of 8 in the standard diet-fed group (87.5%) exhibited positive inflammatory scores ($P = 0.07$, two-tailed Fisher's exact test).

There was no correlation between inflammatory scores and visceral pain responses ($R_p = 0.17$, $P > 0.05$).

Influence of ERF diet on rWAS-induced defecation

No diarrhea was observed in either group and all the feces expelled during the stress period were formed. Time course study showed that in the standard diet, the average defecation response to WAS took place during the first 0-15 min (1.05 ± 0.12 FPO/15 min per 100 g body weight) followed by little to no defecation during the remaining 15-60 min of exposure (0.22 ± 0.02 FPO/15 min per 100 g body weight). This response was significantly increased in ERF compared with the standard diet group (1.42 ± 0.07 and 0.31 ± 0.03 FPO/15 min per 100 g of body weight, respectively) [$F(3.320) = 85.73$, $P < 0.0001$] (Figure 5A and B). Even though for both diet groups, over the 10 d of WAS, most of the defecation occurred in the first 15 min of exposure to stress, the average defecation in both the 0-15 min and 15-60 min periods were significantly higher in the ERF diet than the standard diet group (1.42 ± 0.07 *vs* 1.05 ± 0.12 and 0.31 ± 0.03 *vs* 0.22 ± 0.02 FPO/15 min per 100 g body weight, respectively; $P < 0.05$; Figure 5A and B).

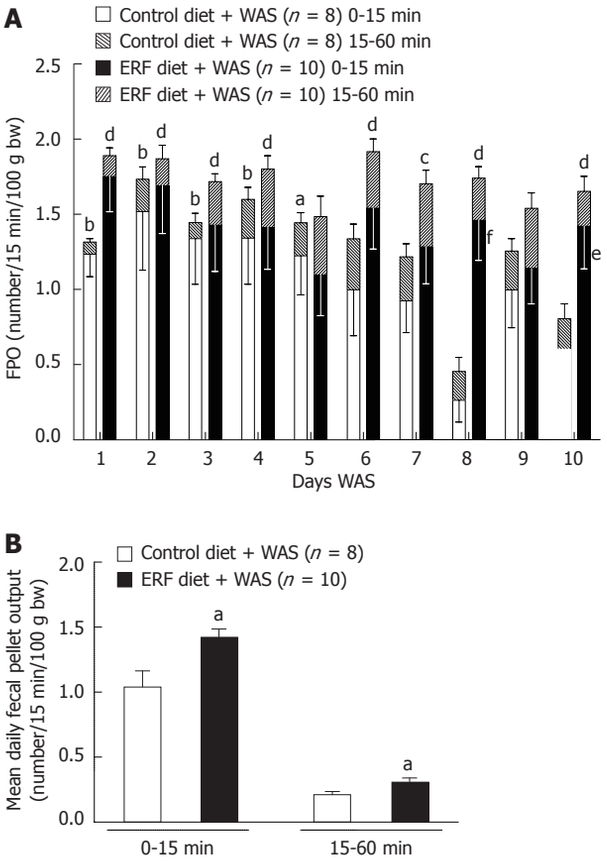

Figure 5 Defecation induced by repeated water avoidance stress in rats fed either standard or 4% enzyme-treated rice fiber prebiotic diet. A: Time-course of hourly defecation expressed as number/15 min per 100 g of body weight for 0-15 min and 15-60 min time periods over the 10 d of 1-h water avoidance stress (WAS). [a]*P* < 0.05, [b]*P* < 0.01 *vs* standard diet interval 0-15 min; [c]*P* < 0.05, [d]*P* < 0.01 *vs* enzyme-treated rice fiber (ERF) diet interval 0-15 min; [e]*P* < 0.05, [f]*P* < 0.01 *vs* standard diet interval 0-15 min; 2-way ANOVA followed by Bonferroni post hoc test; B: Mean daily defecation expressed as number/15 min/100 g of body weight for 0-15 min and 15-60 min intervals. [a]*P* < 0.05 *vs* control diet same time interval; unpaired student *t* test. Data are expressed as mean ± SE, *n* as indicated in parenthesis for each group. FPO: Fecal pellet output.

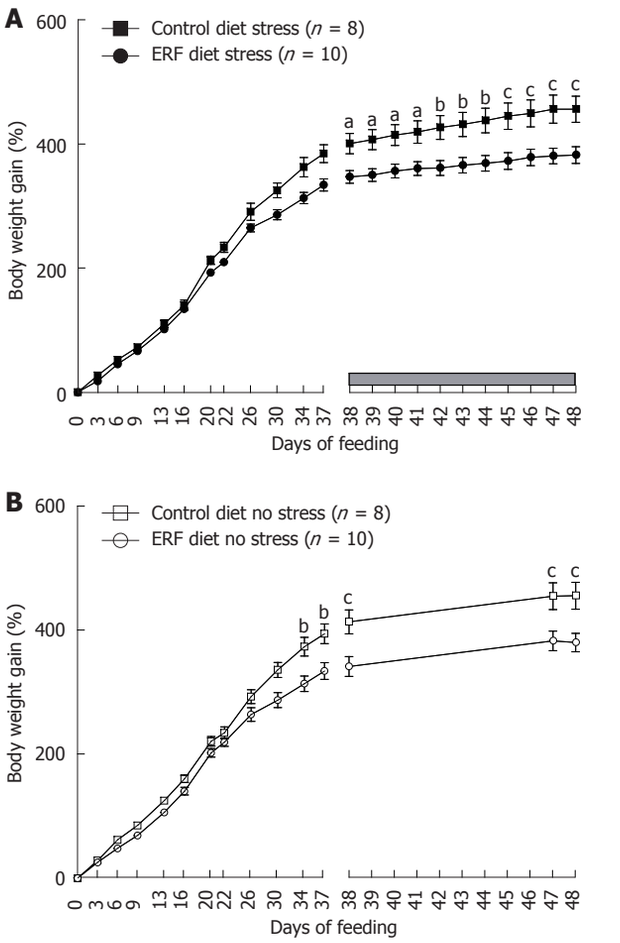

Figure 6 Body weight gain in rats fed either standard or 4% enzyme-treated rice fiber prebiotic diet: influence of repeated water avoidance stress. Rat body weight gain from weaning age (day 21) and during 37 d followed by 10 d of water avoidance stress (WAS) (A) or not (B). Data are expressed as mean ± SE, *n* as indicated in parenthesis for each group. [a]*P* < 0.05, [b]*P* < 0.01, [c]*P* < 0.001 *vs* enzyme-treated rice fiber (ERF) diet group; 2-way ANOVA followed by Bonferroni post hoc test.

ERF diet slows rat body weight gain independently of stress status

The body weights of rats at postnatal day 21 were similar in the four assigned groups before the start of treatments: standard diet + rWAS: 53.4 ± 2.4 g, standard diet + no stress: 58.0 ± 1.2 g, ERF diet + rWAS: 58.2 ± 1.2 g and ERF diet + no stress: 58.3 ± 1.1 g [$F(3.30) = 2.223$, $P = 0.10$]. During the whole experimental period (day 0-48), body weight gain expressed in % from initial body weight showed a significant interaction between diet and time in rats assigned to rWAS [$F(22.368) = 2.455$, $P = 0.0003$] (Figure 6A) or not [$F(14.210) = 2.808$, $P = 0.0007$] (Figure 6B). The percentage of body weight gain started to be significantly higher in standard diet *vs* 4% ERF diet group assigned to rWAS on day 38 (400.9% ± 16.5% *vs* 347.3% ± 10.2%, respectively, $P < 0.05$) or no stress on day 34 (373.3% ± 15.1% *vs* 313.7% ± 12.5%, respectively, $P < 0.01$). Animals on standard diet still maintained a 1.3-fold higher body weight gain than rats on ERF diet over the 10 d of rWAS or no stress (Figure 6A and B). No significant differences were detected in body weight gain between stressed and non-stressed rats in either standard [$F(14.210) = 0.1426$, $P = 0.99$] or ERF [$F(14.240) = 0.1252$, $P = 1.00$] diet groups.

DISCUSSION

In the present study, we demonstrated that exposure to a psychological stressor in the form of WAS 1 h per day once or repeated for 10 d in rats induced stress-related visceral analgesia in as much as 87.3% of the animals tested using a novel non-invasive solid-state manometric method. Additionally, we showed that chronic feeding with a prebiotic diet containing 4% ERF hemi-cellulose-rich dietary fibers, enhanced the expression of stress-induced visceral analgesia at all periods of testing, reduced by 2.2-fold the number of animals displaying stress-induced colonic microscopic alterations, decreased by 76% the isobutyrate cecal content, and slowed rats body weight gain independently of stress exposure and facilitated colonic fecal expulsion.

Over the years, a number of acute and chronic stress paradigms have been developed to mimic the alterations of visceral sensitivity seen in IBS patients[37,38]. Acute exposure to WAS is a well-characterized psychological stressor that induces the transcription of *corticotropin-releasing factor* (*CRF*) gene in the paraventricular nucleus of the hypothalamus[23,39] and consequently activates the pituitary-adrenal axis along with inducing a brain CRF receptor subtype 1-mediated stimulation of colonic motor function assessed at the end of the first hour of WAS[23,40]. In animals surgically equipped for EMG monitoring of the VMR to CRD as classically performed[24,35,41], this combined stress paradigm (surgery, post-surgical housing and rWAS 1 h daily for 10 d) induces visceral hypersensitivity in 82%-86% of the animals starting 24 h after the first exposure[41] which is maintained up to 40 d after the last stress session[24,25,42]. In the present experiment however, using a novel non-invasive solid-state manometric method of visceral sensitivity monitoring to CRD that bypassed the surgery and subsequent single housing[31,33], the majority of rats (66.7% to 85.7%) fed a normal diet exposed to rWAS exhibited a consistent visceral analgesic response. This was observed when CRD was performed within 45-50 min after either one or 10 sessions of WAS. Likewise, we recently demonstrated that rWAS for 10 d resulted in the development of visceral analgesia in up to 72.2% of mice tested non-invasively for the VMR to CRD[31]. Taken together these data provide evidence of visceral stress-induced analgesia in rodents as previously established in the somatic pain field[43].

Moreover, previous and present findings point to the possibility that underlying mechanisms of visceral hypersensitivity may involve both disturbances in descending inhibitory pathways along with sensitization of pain pathways in response to stress as demonstrated in somatic pain studies[44]. Indeed, we recently delineated that 69.2% of single housed mice equipped with EMG electrode to record VMR to CRD developed visceral hypersensitivity in response to a similar regimen of rWAS exposure when monitored 24 h after the last stress session[31]. Therefore VMR alterations reflects not only the effect of the stressor *per se* but also the basal state of the animal and conditions associated with visceral pain monitoring, namely the chronic implantation of abdominal wall electrodes, post surgery medications including antibiotics and single housing thereafter[31]. Other studies showed that skin incision correlates with the development of long-lasting visceral hyperalgesia in rats[45], and that stress becomes proalgesic in rats previously exposed to surgery[46]. To date, there is only one report using another minimally-invasive method, the abdominal withdrawal reflex (AWR), which suggests that rWAS in male Wistar rats decreases their threshold to visceral pain[47]. However, AWR monitoring requires observation of abdominal wall musculature contractions and this semi-quantitative score is subjective. Using this non-invasive ICP monitoring, we have previously demonstrated a hyperalgesic response in Wistar rats following a peripheral injection of the selective CRF_1 receptor agonist, cortagine, known to act directly on CRF_1 receptor subtype expressed in the colon[33], further indicating that the analgesia observed in response to an acute psychological stress in the present study is likely to recruit stress-related brain inhibitory mechanisms. Taken together the previous and present data indicate that this new method of VMR monitoring is valuable to unravel both analgesia and hyperalgesia without confounding factors in rats. In the field of somatic pain, the underlying mechanisms of stress-induced analgesia can be mediated by opioid or non-opioid dependent inhibitory systems[43]. Our preliminary results suggest that WAS-induced visceral analgesia in male rats is naloxone-independent[48]. Further studies are warranted to delineate the underlying components of the visceral analgesic response to rWAS in particular, the role of endocannabinoid tone recently reported to be increased by this stressor[42].

Interestingly, chronic feeding of rats with 4% ERF prebiotic diet potentiated the acute or repeated WAS-induced visceral analgesia. This was shown by the prolongation of the analgesic response to 24 h after the last rWAS session and expansion to all CRD pressure levels (20, 40 and 60 mmHg) compared to normal diet in which the visceral analgesia was mainly observed at 40 mmHg within the 45 min post WAS exposure. Additionally, ERF completely prevented the development of hyperalgesia seen in 14.3%-33% of rats fed a standard diet and undergoing CRD at 60 mmHg after WAS on day 1 or rWAS on day 10. These observations support the analgesic potential of ERF prebiotic diet on visceral sensitivity. In a previous report, 4% ERF diet reduced restraint stress-induced visceral hypersensitivity as monitored with AWR[19]. Collectively, these findings indicate that ERF prebiotic diet may be beneficial to strengthen the stress-related visceral analgesia as well as to curtail the development of visceral hypersensitivity.

Prebiotics as dietary carbohydrates are a source of carbon and energy for colonic bacteria which ferment SCFAs primarily to acetate, propionate and butyrate[13,49]. Based on the local site of action of prebiotics, we investigated the potential peripheral colonic mechanisms through which ERF diet enhances the stress-related analgesic response by first assessing changes in organic acid cecal content. The molar ratio among the three major SCFAs (constituting roughly 90% of the SCFAs in the lumen) was ~ 64:15:19 for acetate:propionate:butyrate/isobutyrate in the animals fed standard diet (stressed or non-stressed), which is close to the average ratio of ~ 60:20:20 described in mammals[49] while that of rats fed ERF diet was ~ 71:12:15. The change in butyrate/isobutyrate molar ratio observed in ERF fed rats was linked to an increase in butyrate consistent with our previous study[19] and a concomitant reduction in isobutyrate. Isobutyrate is derived from valine microbial degradation obtained by proteolysis of endogenous and dietary proteins and not from carbohydrates as are propionate and acetate[50]. As both standard and ERF diets contain identical levels of protein (Table 1), differences in diet protein content are unlikely to account for the reduction observed. Despite the observed increase in butyrate in ERF-fed animals, we

found no correlation between the visceral pain response and butyrate cecal concentration which does not support an underlying role in ERF modulatory effect on visceral sensitivity. In addition, while butyrate enemas have been shown to dose-dependently decrease visceral sensitivity in healthy volunteers[51], other reports indicate that they promotes visceral hypersensitivity in rats[52,53] and clinical studies showed that colonic butyrate levels are increased in IBS patients[54]. Interestingly however, we found a positive correlation between isobutyrate and total butyrate (isobutyrate + butyrate) cecal concentrations and visceral pain responses. Reduction in isobutyrate cecal levels associated with a concomitant increase in acetate and decrease in propionate as observed in ERF-fed rats, may have contributed to lowering the pH in the colon of those animals possibly participating to the modulation of the colonic microflora[55,56] and the reduction in visceral pain observed. Indeed, prebiotics alter the composition and balance of microflora, both in the colonic lumen and at the mucosal surface, in which beneficial bacteria such as *Bifidobacteria* and *Lactobacilli* become of greater prominence[57]. Of interest, probiotics (mainly *Lactobacilli*) have been found to exert antinociceptive effects on stress-induced visceral hypersensitivity in rodents, particularly by increasing the expression of μ-opioid and cannabinoid receptors in intestinal epithelial cells[18, 58-60].

Another possible mechanism through which ERF diet may have enhanced visceral analgesia is by preventing rWAS-induced subtle immune and structural histological changes in colonic mucosa as demonstrated by the reduction in the percentage of rats exhibiting histological alterations. Recently, in a murine colitis model, 4% ERF diet has been shown to reduce inflammation by modulating colonic environment and regulating immune cell differentiation[12]. There is also evidence that rWAS in naïve animals increases colonic permeability[27,28,30], which combined with a longer small intestinal transit time may enhance bacterial translocation[61,62], a phenomenon involved in the development of visceral hypersensitivity in rats[29]. Prebiotics and probiotics have a beneficial influence on gastrointestinal motility arising from a combination of increased bacterial mass, increased stool water as well as increased colonic tone, all resulting in accelerated transit[8,63]. Likewise in the present study the well established defecation response to rWAS[23,48] seen in the standard diet group was increased in ERF fed rats either when expressed by rat or per 100 g body weight. WAS is a mild stressor that does not induce diarrhea, the maintenance of a higher defecation in rats fed 4% ERF diet may be beneficial by facilitating fecal evacuation.

Lastly, independently of its effects on visceral sensitivity, colonic epithelial, immune and motor functions, 4% ERF diet compared to standard diet reduced the body weight gain in rats. Numerous reports have described the effect of prebiotics on the food intake regulation, fat mass and weight gain in experimental studies[64]. Although we did not precisely evaluate food intake in our experiment, these data are consistent with our observation. rWAS however, did not alter rats weight gain in the different groups, confirming previous reports[24].

In conclusion, the present findings demonstrate that under conditions of non-invasive solid-state manometry method to monitor visceral pain, a repeated mild psychological homotypic stressor such as WAS induces stress-related visceral analgesia in rats which so far has been mainly established in the somatic pain field. In addition, our data support an enhancing effect of the prebiotic ERF diet on stress-related visceral analgesia together with increased evacuation of colonic content and reduction in body weight gain. When evaluated in the context of research supporting the use of pre- or probiotics as treatment option for IBS[65], these preclinical findings point toward ERF ability to enhance the analgesic response which may be beneficial in IBS patients in whom altered descending inhibitory pathways have been described[66,67].

COMMENTS

Background

Alterations in intestinal microbiota, mucosal barrier function, and immune system have been implicated to contribute to irritable bowel syndrome (IBS) pathogenesis. Visceral hypersensitivity, a key feature of IBS, can be associated with a low grade colonic mucosal inflammation in a subset of patients. Recently, a new prebiotic, enzyme-treated rice fiber (ERF), has been shown to reduce inflammation and major symptoms in the murine dextran sodium sulfate colitis model by modulating the colonic microbiota environment and regulating immune cell differentiation. This study aimed to evaluate in rats the effects of chronic prebiotic diet on stress-related alterations of visceral sensitivity and colonic motor functions when monitored non-invasively, using repeated intermittent exposure to a psychological stressor in the form of water avoidance stress (WAS).

Research frontiers

It has been recently suggested that the basal state of the animals largely influences the visceral pain responses to a psychological stressor. Indeed, in mice the impact of repeated intermittent exposure to WAS on the visceral pain responses to colorectal distension varies in function of the basal state of the animal. WAS was reported to induce analgesic response when mice were tested using the novel non-invasive method of solid-state manometry which avoids prior surgery to implant recording electrodes and subsequent single housing. By contrast, mice tested with the traditional method of electromyography with the electrode surgically implanted on the abdominal muscles and post-surgical single housing, developed visceral hyperalgesia following exposure to the same repeated WAS.

Innovations and breakthroughs

The results of the present study demonstrate that under conditions of a non-invasive manometry method to monitor visceral pain, a repeated mild psychological homotypic stressor such as WAS induces stress-related visceral analgesia in rats as well, expanding our previous reports in mice. This opens a new field of investigation on stress-related visceral analgesia in rodents which so far has been little explored compared with the somatic pain field. In addition, the data support an enhancing effect of the prebiotic ERF diet on WAS-related visceral analgesia together with increased evacuation of colonic content and reduction in body weight gain.

Applications

In the context of a critical relationship between the composition and stability of the microbiota and gastrointestinal sensory-motor function as well as neuroimmune interactions within the brain-gut axis, these preclinical findings point toward ERF ability to enhance the analgesic response. In the view of growing reports suggesting that IBS patients present altered descending pain inhibitory pathways, the use of ERF as a prebiotic to enhance visceral analgesia is clinically relevant and could be therapeutically beneficial.

Terminology

Non-invasive method of monitoring visceromotor response (VMR) to colorectal distension (CRD) is a novel surgery-free method of solid-state manometry assessing changes in intraluminal colonic pressure (ICP) induced by abdominal muscles contractions in response to discomfort/painful colorectal distensions.

Enzyme-treated rice fiber is a new prebiotic made from rice bran containing dietary fiber and fat soluble fraction.

Peer review

It is a very well designed investigation, the topic is very interesting for the readers, the article is very well written, the results are applicable, the conclusions are valuable.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i3.237

World J Gastroenterol 2012 January 21; 18(3): 237-243
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Aging is a risk factor of nonalcoholic fatty liver disease in premenopausal women

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Supported by Young Scientists (B) (23790791) from Japan Society for the Promotion of Science
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Received: June 2, 2011 Revised: September 8, 2011
Accepted: October 28, 2011
Published online: January 21, 2012

Abstract

AIM: To clarify the relationship between age, menopause, and nonalcoholic fatty liver disease (NAFLD) in women.

METHODS: We conducted a follow-up study on nonalcoholic fatty liver disease by using abdominal ultrasonography, and investigated the relationship of age and menopause with the development of NAFLD in women. We followed 1829 women and 2572 men (response rate, 86%) selected in 2001 to represent the non-institutionalized adult population of Gifu, Japan. Data collected included self-reported medical history, lifestyle factors, and menopausal status. The postmenopausal state was defined as beginning 1 year after the cessation of menses. We diagnosed NAFLD with the aid of abdominal ultrasonography by using diagnostic criteria described previously.

RESULTS: The prevalence of NAFLD in women increases with age, but does not alter with age in men. Furthermore, the prevalence of NAFLD in premenopausal women (6%) was lower than that in men (24%) and in postmenopausal women (15%). The associations of the postmenopausal state and hormone replacement therapy with NAFLD were statistically significant in a univariate logistic regression model. At the follow-up examination, 67 women (5%) were newly diagnosed with NAFLD. The incidence of NAFLD was 3.5% (28/802) in premenopausal women, 7.5% (4/53) in menopausal women, 6.1% (24/392) in postmenopausal women, and 5.3% (11/206) in women receiving hormone replacement therapy. The weight gain in premenopausal women was equal to that in postmenopausal women. Metabolic syndrome and weight gain were independent risk factors for NAFLD in pre- and postmenopausal women, but age was an independent risk factor in premenopausal women only.

CONCLUSION: Aging is a risk factor for NAFLD in premenopausal women, independent of weight gain or influence of metabolic syndrome.

Key words: Nonalcoholic fatty liver disease; Cardiovascular disease; Risk factor; Steatohepatitis; Postmenopausal women

Peer reviewers: Kazuaki Takabe, MD, PhD, Assistant Professor of Surgery and Assistant Professor of Biochemistry and Molecular Biology, Surgical Oncology, VCU Massey Cancer Center, Virginia Commonwealth University/Medical College of Virginia, PO Box 980011, Richmond VA 23298-0011, United States; Dr. Luca Valenti, Internal Medicine, Università degli Studi di Milano, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, via Francesco Sforza 35, padiglione Granelli, Milano 20122, Italy; Dr. Nagarajan Perumal, Compliance veterinarian, Center for life science, IACUC OFFICE, National University of Singapore, 117456, Singapore

Hamaguchi M, Kojima T, Ohbora A, Takeda N, Fukui M, Kato T. Aging is a risk factor of nonalcoholic fatty liver disease in premenopausal women. *World J Gastroenterol* 2012; 18(3): 237-243

Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i3/237.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i3.237

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a common clinical condition with histological features that resemble those of alcohol-induced liver injury, but NAFLD occurs in patients who do not consume alcohol[1,2]. NAFLD encompasses a spectrum of conditions ranging from simple steatosis to steatohepatitis (NASH), advanced fibrosis and cirrhosis[1]. It is emerging as the most common chronic liver disease in Western countries and in other parts of the world[3]. The estimated prevalence of NAFLD in the general population in western countries is 20%-40%[3,4]. Frequent association with disturbances of glucose and lipid metabolism often renders NAFLD a "satellite" element of metabolic syndrome[5,6]. Metabolic syndrome is a cluster of metabolic and hemodynamic abnormalities linked with insulin resistance[7]. Metabolic syndrome is a strong predictor of NAFLD, particularly among people of Japanese descent[8]. Epidemiological studies have shown that metabolic syndrome is not rare and that it is a risk factor for cardiovascular disease (CVD) and stroke[9-11]. Previously, we reported that NAFLD was a predictor of CVD among apparently healthy Japanese men and women[12]. The association between NAFLD and future CVD events was independent of conventional cardiovascular risk factors, because vulnerable plaques tend to develop in patients with NAFLD[13].

Sex and age are conventional cardiovascular risk factors. The prevalence of NAFLD is higher in men than in women, particularly in Asians[14]; however, age is a risk factor for NAFLD in women, but not in men[8]. In this study, we performed a subanalysis within the patient group described in a previous report[8] and investigated the relationship of age and menopause with the development of NAFLD in women.

MATERIALS AND METHODS

Data sources

We accessed a database from a previously reported study[8] to evaluate the possibility that aging and menopause in women could be risk factors for NAFLD. We conducted a follow-up study on nonalcoholic fatty liver disease by using abdominal ultrasonography. All participants who were examined in health checkup programs between January and December 2001 were invited to enroll in the study and 1829 women and 2572 men that satisfied the inclusion criteria in 2001 were selected to represent the non-institutionalized adult population of Gifu, Japan. By the end of June 2003, 3876 of the 4401 participants (2271 men and 1605 women) had completed the second examination (response rate, 86%). The interval between the baseline and follow-up examinations was 414 d (SD = 128). Among the 1605 women, 1603 were available for longitudinal examinations.

We collected data from self-reported medical histories, assessments of lifestyle factors [e.g., age, sex, height, weight, smoking habits, alcohol consumption, menopause status, active or previous hormone replacement therapy (HRT)], and the results of health checkup programs (e.g., urinalysis, blood cell counts, blood chemistry, electrocardiography, chest radiography, barium examination of the upper gastrointestinal tract, and abdominal ultrasonography). The postmenopausal state was defined as beginning 1 year after the cessation of menses. Reports of smoking status included active and past smoking. Habits regarding alcohol consumption were evaluated by asking the participants about the amount and type of alcoholic beverages consumed per week and then estimating the mean ethanol intake per day. We defined an alcohol consumer as someone with a mean ethanol intake of more than 20 g/d. A light drinker was defined as someone with a mean ethanol intake of 0-20 g/d. On the questionnaire, participants reported the type, duration, and frequency of their participation in sports or recreational activities[15]. When participants performed any kind of sport regularly at least once a week, we categorized them as regular exercisers[16].

The ATP Ⅲ proposed the following 5 abnormalities to define metabolic syndrome: (1) abdominal obesity, abdominal circumference ≥ 102 cm for men and ≥ 88 cm for women; (2) elevated serum triglyceride level, ≥ 1.70 mmol/L (≥ 150 mg/dL); (3) decreased HDL cholesterol level, ≥ 1.04 mmol/L (≥ 40 mg/dL) for men and ≥ 1.30 mmol/L (≥ 50 mg/dL) for women; (4) elevated systolic and diastolic blood pressure, ≥ 130/85 mmHg; and (5) elevated fasting glucose level ≥ 6.11 mmol/L (≥ 110 mg/dL)[7]. Because waist measurements were not available for the entire study sample, we substituted a BMI of ≥ 25 kg/m^2 for all participants as an index of obesity. A BMI of ≥ 25 kg/m^2 has been proposed as a cutoff for the diagnosis of obesity in Asian people[17]. Individuals with ≥ 3 of the 5 abnormalities were considered to have metabolic syndrome.

We diagnosed NAFLD with the aid of abdominal ultrasonography by using diagnostic criteria described previously[18].

Statistical analysis

The SPSS statistical package, version 11.0.1 J (SPSS, Inc., Chicago, IL) was used for all statistical analyses, and a P value less than 0.05 was considered statistically significant. Because the incidence rate of NAFLD was unknown, a formal sample size estimate was not made a priori. Cases with and without follow-up visits were compared to determine the appropriateness of an analysis exclusively based on participants with complete data. Two groups of participants were compared using the unpaired t-test and the χ^2 test. Logistic regression was used to analyze associations between the development of NAFLD and metabolic syndrome, age, and weight gain. Adjusted odds ratios and 95% CIs were calculated. Data were expressed as means and SDs for continuous variables.

Men	21-39	40-49	50-59	60-80
NAFLD	109	254	213	58
Normal US	363	672	739	164
Total	472	926	952	222
Women	21-39	40-49	50-59	60-80
NAFLD	15	53	90	20
Normal US	369	642	554	86
Total	384	695	644	106
	21-39	40-49	50-59	60-80
Men	23.1%	27.4%	22.4%	26.1%
Women	3.9%	7.6%	14.0%	18.9%

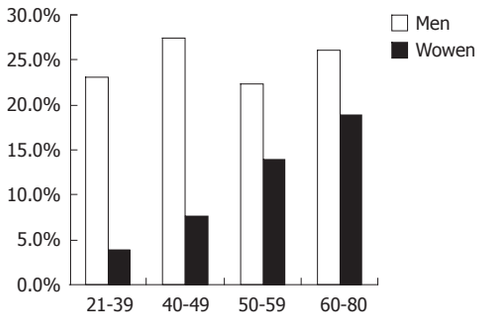

Figure 1 Age-specific prevalence of nonalcoholic fatty liver disease among 1829 women and 2572 men. US: Abdominal ultrasonography; NAFLD: Nonalcoholic fatty liver disease.

Table 1 Baseline characteristics of study participants and unadjusted associations with nonalcoholic fatty liver disease

	Women w/o NAFLD	Women with NAFLD	*P* value	Men w/o NAFLD	Men with NAFLD	*P* value
Number of subjects	1651	178		1938	634	
Age, yr (SD)	46.6 (8.8)	51.0 (7.7)	< 0.001	48.1 (9)	47.9 (8.4)	0.66
Body mass index, kg/m^2 (SD)	21.3 (2.5)	25.7 (3.7)	< 0.001	22.5 (2.5)	25.6 (2.8)	< 0.001
Fasting plasma glucose, mg/mL (SD)	92.7 (8.4)	100.7 (10.7)	< 0.001	99.6 (13.3)	106.8 (18.8)	< 0.001
Systolic blood pressure, mmHg (SD)	111.8 (15.9)	127.8 (18.2)	< 0.001	118.8 (15.8)	126.4 (15.5)	< 0.001
Diastolic blood pressure, mmHg (SD)	69.8 (10.1)	79.1 (10.1)	< 0.001	75.3 (10.1)	80.4 (9.9)	< 0.001
HDL-cholesterol, mg/dL (SD)	59.4 (13.9)	49.5 (11.8)	< 0.001	47.5 (12.2)	40.6 (9.7)	< 0.001
Triglycerides, mg/dL (SD)	73.8 (36.1)	119.6 (61.4)	< 0.001	105.5 (59.9)	156.7 (89.2)	< 0.001
LDL-cholesterol, mg/dL (SD)	131.8 (32.2)	151.1 (33.4)	< 0.001	137.2 (30.7)	145.8 (31.7)	< 0.001
AST, IU/mL (SD)	15.1 (5.2)	19.5 (7.9)	< 0.001	16.4 (7.7)	22.8 (10.9)	< 0.001
ALT, IU/mL (SD)	16.8 (6.5)	27.9 (15.7)	< 0.001	23.1 (15)	39.6 (21.4)	< 0.001
γGTP, IU/mL (SD)	11.7 (9.6)	18.9 (15.4)	< 0.001	22.8 (22.8)	31.4 (22.5)	< 0.001
Hemoglobin, g/dL (SD)	12.8 (1.2)	13.3 (1.2)	< 0.001	15 (1)	15.6 (0.9)	< 0.001
C reactive protein, mg/mL (SD)	0.1 (0.2)	0.2 (0.4)	0.113	0.1 (0.2)	0.1 (0.1)	0.242
Number (%) of light-drinkers	775 (46.9)	73 (41.0)	0.13	1513 (78)	464 (73)	0.013
Number (%) of current smokers	102 (6.2)	10 (5.6)	0.87	795 (41.0)	234 (36.9)	0.074
Number (%) of ex-smokers	184 (11.1)	12 (6.7)	0.074	555 (28.6)	213 (33.6)	0.02
Number (%) of regular exercisers	340 (20.6)	27 (15.2)	0.09	393 (20.3)	78 (12.3)	< 0.001
Number (%) of postmenopausal women	487 (29.5)	82 (46.1)	< 0.001			
Number (%) receiving hormone replacement therapy	221 (13.4)	36 (20.2)	0.017			

This table summarizes the baseline data of 1829 women and 2572 men. w/o: Without; NAFLD: Nonalcoholic fatty liver disease; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGTP: γ-glutamyl transferase. Continuous variables were compared using the unpaired *t* test, and groups were compared with the χ^2 test.

RESULTS

Baseline characteristics of the study participants

We calculated the age-specific prevalence of NAFLD among 1829 women and 2572 men (Figure 1). The prevalence of NAFLD in women increased with age, but no significant trend for age was identified in men.

We grouped women according to whether they had NAFLD at baseline, and investigated differences in age, weight, components of metabolic syndrome, serum enzymes, menopausal state, alcohol consumption, and smoking history (Table 1). More subjects with NAFLD were postmenopausal, received HRT, and met more criteria of metabolic syndrome than those without NAFLD (Table 1). The prevalence of NAFLD in premenopausal women (6%) was lower than that in men (24%), postmenopausal women (15%), and women receiving HRT (14.0 %, 36/221). In the cross-sectional study, we applied a logistic model to investigate the association of the menopausal state or HRT with NAFLD (Table 2). Univariate analysis showed that the odds ratios of the postmenopausal state and HRT were 2.73 (1.92-3.87), and 2.57 (1.66-3.97), respectively. However, they were not statistically significant after adjusting for age and metabolic syndrome.

Next, we excluded 257 subjects with a history of hysterectomy or HRT. We separated the subjects into pre- or postmenopausal groups and investigated the difference in age, components of metabolic syndrome, alcohol consumption, and smoking history (Table 3). An association of age with NAFLD was identified in premenopausal women, but not in postmenopausal women. On the other hand, an association of the components of metabolic syndrome with NAFLD was identified in both groups.

Development of nonalcoholic fatty liver disease

Of the 1603 women who completed follow up, 1453 women did not have NAFLD at baseline. Of the 1453 women who were disease-free at the baseline examina-

Table 2 Unadjusted and adjusted associations between nonalcoholic fatty liver disease and age, menopause status, hormone replacement therapy, and metabolic syndrome in 1829 women

	Unadjusted odds ratio (95% CI)	*P* values	Adjusted odds ratio (95% CI)	*P* values
Age	1.06 (1.04-1.08)	< 0.001	1.03 (1-1.06)	0.027
Postmenopausal state	2.73 (1.92-3.87)	< 0.001	1.55 (0.92-2.61)	0.096
Active hormone replacement therapy	2.57 (1.66-3.97)	< 0.001	1.54 (0.93-2.54)	0.092
Presence of metabolic syndrome at baseline	12.52 (8.03-19.51)	< 0.001	11.28 (7.04-18.06)	< 0.001

We calculated the odds ratio of each factor for nonalcoholic fatty liver disease in a univariate logistic regression model. The columns to the left indicate unadjusted odds ratio [95% confidence interval (CI)]. We selected all parameters as cofactors in a multivariate logistic regression model. The columns to the right indicate adjusted odds ratio (95% CI).

Table 3 The association of baseline characteristics with nonalcoholic fatty liver disease in pre- or postmenopausal women

	Premenopausal women (*n* = 1023)			Postmenopausal women (*n* = 549)		
	Normal US	NAFLD	*P* Value	Normal US	NAFLD	*P* Value
Number of subjects (%)	962	61 (6)		468	81 (15)	
Mean age ± SD, yr	42.2 (6.4)	44.7 (6.1)	0.003	56 (5.5)	55.9 (5.4)	0.874
Number (%) who met a criterion of metabolic syndrome						
Body mass index	75 (8)	32 (52)	< 0.001	37 (8)	43 (53)	< 0.001
Glucose	24 (2)	15 (25)	< 0.001	25 (5)	8 (10)	< 0.001
Blood pressure	94 (10)	21 (34)	< 0.001	104 (22)	35 (43)	< 0.001
HDL-cholesterol	221 (23)	31 (51)	< 0.001	128 (27)	48 (59)	< 0.001
Triglycerides	29 (3)	13 (21)	< 0.001	36 (8)	21 (26)	< 0.001
Number (%) who met ≥3 criteria of metabolic syndrome	18 (2)	17 (28)	< 0.001	26 (6)	23 (28)	< 0.001
Number (%) light-drinkers	472 (49.1)	24 (3.9)	0.15	190 (40.6)	34 (42.0)	0.81
Number (%) current smokers	48 (5.0)	4 (6.6)	0.54	34 (7.3)	5 (6.2)	0.64
Number (%) ex-smokers	103 (1.1)	5 (8.2)	0.67	50 (10.7)	5 (6.2)	0.31
Number (%) regular exercisers	181 (18.8)	8 (13.1)	0.31	105 (22.4)	15 (18.5)	0.47

This table summarizes the baseline data of 1572 women, excluding 257 subjects with hormone replacement therapy. We separated the subjects into pre- or postmenopausal groups. US: Abdominal ultrasonography; NAFLD: Nonalcoholic fatty liver disease; HDL: High-density lipoprotein.

Table 4 Unadjusted and adjusted associations between nonalcoholic fatty liver disease and age, menopause status, hormone replacement therapy, metabolic syndrome and weight gain in 1247 women

	Unadjusted odds ratio (95% CI)	*P* values	Adjusted odds ratio (95% CI)	*P* values
Age	1.05 (1.02-1.08)	0.001	1.06 (1.02-1.1)	0.004
Menopause	2.26 (0.76-6.69)	0.14	1.22 (0.38-3.99)	0.74
Postmenopausal state	1.8 (1.03-3.15)	0.039	0.72 (0.31-1.66)	0.44
Active hormone replacement therapy	1.56 (0.76-3.19)	0.22	1.32 (0.6-2.88)	0.49
Presence of metabolic syndrome at baseline	9.89 (4.67-20.94)	< 0.001	11.7 (5.02-27.25)	< 0.001
Weight gain	1.50 (1.30-1.74)	< 0.001	1.63 (1.39-1.9)	< 0.001

We calculated the odds ratio of each factor for nonalcoholic fatty liver disease in a univariate logistic regression model. The columns to the left indicate unadjusted odds ratio [95% confidence interval (CI)]. We selected all parameters as cofactors in a multivariate logistic regression model. The columns to the right indicate adjusted odds ratio (95% CI).

tion and who had the follow-up examination, 67 women (5%) received new diagnoses of NAFLD at follow-up examination. Subjects with NAFLD were older, showed more weight gain, and met more criteria for metabolic syndrome than those without NAFLD, but the 2 groups showed no differences in menopausal state, HRT, alcohol consumption, and smoking history.

Among the 1453 disease-free women at baseline, there were 855 premenopausal women and 392 postmenopausal women. Of the 855 premenopausal women, 53 women were menopausal. During the study period, 206 women received HRT. The incidence of NAFLD was 3.5% (28/802) in premenopausal women, 7.5% (4/53) in menopausal women, 6.1% (24/392) in postmenopausal women, and 5.3% (11/206) in women receiving HRT.

We applied a logistic regression model to determine the risk factors for the development of NAFLD (Table 4). Univariate analysis indicated that the postmenopausal state was a risk factor for NAFLD [odds ratio 1.8 (1.03-3.15), $P = 0.039$], but it was not statistically significant after adjusting for age, metabolic syndrome, and weight gain. The cross-sectional study at baseline revealed that the prevalence of NAFLD was higher in women receiving HRT than in those in the premenopausal state, but the logistic model did not indicate that HRT was a risk factor for the development of NAFLD.

Table 5 The relationship between menopausal status and nonalcoholic fatty liver disease in 1247 women who completed follow-up examinations and received no hormone replacement therapy

	Premenopausal women (n = 855)			Postmenopausal women (n = 392)		
	Normal US at both baseline and follow-up	Normal US at baseline and NAFLD at follow-up	*P* value	Normal US at both baseline and follow-up	Normal US at baseline and NAFLD at follow-up	*P* value
Number (% incidence of NAFLD)	823	32 (4)		368	24 (6)	
Mean age ± SD, yr (% incidence of NAFLD)	42.2 (6.3)	45.5 (6)	0.004	55.7 (5.6)	55.7 (4.7)	0.951
Number (%) who met a criterion of metabolic syndrome						
Body mass index	53 (6)	13 (41)	< 0.001	28 (8)	4 (17)	0.12
Glucose	18 (2)	5 (16)	0.001	19 (5)	3 (13)	0.14
Blood pressure	79 (10)	9 (28)	0.003	79 (21)	8 (33)	0.2
HDL-cholesterol	178 (22)	19 (59)	< 0.001	98 (27)	10 (42)	0.15
Triglycerides	18 (2)	7 (22)	< 0.001	25 (7)	4 (17)	0.091
Number (%) who met ⩾ 3 criteria of metabolic syndrome	9 (1)	7 (22)	< 0.001	17 (5)	4 (17)	0.032
Weight gain, kg (SD)	0.2 (1.7)	1.4 (1.6)	< 0.001	0 (1.6)	1.3 (1.3)	< 0.001
Number (%) light-drinkers	406 (49.3)	14 (43.8)	0.59	214 (26.0)	10 (31.3)	1
Number (%) current smokers	42 (5.1)	3 (9.4)	0.23	38 (4.6)	0 (0.0)	0.4
Number (%) ex-smokers	88 (10.7)	6 (18.8)	0.15	54 (6.6)	1 (3.1)	0.5
Number (%) regular exercisers	151 (18.3)	6 (18.8)	1	81 (22.0)	4 (16.7)	0.78

US: Abdominal ultrasonography; NAFLD: Nonalcoholic fatty liver disease; HDL: High-density lipoprotein.

Table 6 Adjusted associations between nonalcoholic fatty liver disease and age in 855 premenopausal women and 392 postmenopausal women

	Premenopausal subjects (n = 855)		Postmenopausal subjects (n = 392)	
	Adjusted odds ratio (95% CI)	*P* Value	Adjusted odds ratio (95% CI)	*P* Value
Age	1.12 (1.05–1.2)	0.001	1 (0.93–1.07)	0.91
Presence of metabolic syndrome at baseline	43.06 (12.66–146.51)	< 0.001	4.87 (1.29–18.34)	0.019
Weight gain	1.76 (1.4–2.21)	< 0.001	1.67 (1.27–2.21)	< 0.001

We applied a multivariate logistic regression model to calculate the odds ratio for age for development of nonalcoholic fatty liver disease after adjusting for weight gain and metabolic syndrome. After completing follow-up examinations, 1247 women who received no hormone replacement therapy were divided into pre- or postmenopausal groups. CI: Confidence interval.

Next, we excluded 206 women with HRT at the baseline and/or follow up. We separated the subjects into pre- or postmenopausal groups and compared parameters in subjects with NAFLD to those without (Table 5). Weight gain was equal in pre- and postmenopausal women. There was an association of the number of women who met ⩾ 3 criteria of metabolic syndrome and weight gain with the development of NAFLD in pre- and postmenopausal women, but an association of age with development of NAFLD was identified in premenopausal subjects only.

Finally, we applied a multivariate logistic model to investigate the risk of NAFLD after separating women into pre- or postmenopausal groups (Table 6). Metabolic syndrome and weight gain were dependent risk factors for NAFLD in pre- and postmenopausal women, but age was a risk factor only in premenopausal women.

DISCUSSION

Both NAFLD and NASH are reported to be more prevalent in men than in women[19,20]. Our study indicates that the prevalence of NAFLD in women increases with age, but does not alter with age in men. Furthermore, the prevalence of NAFLD in premenopausal women (6%) was lower than that in men (24%) and in postmenopausal women (15%). A univariate logistic regression model indicated that when no adjustments were made for age or metabolic syndrome, the associations of the postmenopausal state and HRT with NAFLD were statistically significant.

The present prospective study revealed that the incidence of NAFLD was highest in women in menopause (7.5%, 4/53). The incidence of NAFLD was higher in postmenopausal women (6.1%, 24/392) or in women with HRT (5.3%, 11/206) than in premenopausal women (3.5%, 28/802). Our findings are in agreement with recent cross-sectional studies, which indicate that NAFLD is more prevalent in postmenopausal women than in premenopausal women[21-23].

The univariate logistic regression model indicated that the postmenopausal state was a risk factor for NAFLD, but this was not statistically significant after adjusting for age, metabolic syndrome, and weight gain. Age, metabolic syndrome, and body weight gain were independent risk factors for NAFLD in women. However, after separating women into pre- or postmenopausal groups, age remained

the only independent risk factor in premenopausal women. These results imply that aging increases the risk for NAFLD in premenopausal women, but poses no risk after menopause; thus, the influence of aging in postmenopausal women is similar to that in men. Furthermore, the proportion of premenopausal women with NAFLD was smaller than that in men, but the proportion of postmenopausal women with NAFLD was equal to that in men.

A possible underlying factor is estrogen-related sex hormones. Estrogens lead to preferential fat accumulation in the gluteofemoral region, and the loss of estrogens during the transition of menopause is associated with an increase in central fat[22]. Gutierrez-Grobe *et al*[21] reported that women without NAFLD had significantly higher levels of serum estradiol, which is a major form of estrogen, than those with NAFLD both at pre- and postmenopausal stages. Hepatic estrogen receptors mediate estrogen action in the liver, and estradiol has a favorable role in chronic liver disease[24], which is suggestive of a protective effect of estrogens against NAFLD in women[21]. The relationship between aging in premenopausal women and the development of NAFLD may thus reflect changes in levels of estrogen-related sex hormones. Unfortunately, we did not measure the levels of estrogen-related sex hormones in this study.

In the present cross-sectional study, we found that NAFLD was more prevalent in women receiving HRT than in premenopausal women, as reported elsewhere[23]. However, the logistic model did not indicate that HRT was a risk factor for NAFLD. The protective effect of estrogen-related sex hormones might be limited to endogenous estrogens. However, since the present prospective study was observational, several background factors, including age, differed between the HRT and non-HRT groups. A recent study suggested that HRT improved the results of liver-function tests[25]. Moreover, tamoxifen, an anti-estrogen compound used in the treatment of estrogen receptor positive breast cancer, is associated with an increased risk of fatty liver and NASH[26,27]. An animal study showed that hepatic steatosis occurs spontaneously in aromatase-deficient mice who lack the ability to produce estrogen and who have impaired hepatocellular lipid β-oxidation[28]. Estradiol replacement reduces hepatic steatosis and restores the impairment in mitochondrial and peroxisomal fatty acid β-oxidation to the level found in wild-type mice[29]. Thus, a well-designed randomized controlled study is required to determine the influence of HRT on NAFLD.

Recent studies have shown that visceral fat is an independent predictor of fatty liver, even in patients with normal BMI, and is much more harmful than subcutaneous accumulation of adipose tissue[30,31]. We demonstrated that metabolic syndrome and weight gain are risk factors of NAFLD[8] and evaluated these factors in pre- and postmenopausal women. The number of women who met the criteria for metabolic syndrome was larger among postmenopausal women, but weight gain was the same in both.

We note the following limitations in our study. We assessed active and past medical history, state of menses, smoking habits, drinking habits, and physical activities by conducting interviews. A trained nurse supported the interviewer, but we cannot exclude the possibility of interviewer bias. Furthermore, information on dietary composition was lacking.

In conclusion, aging is a risk factor for NAFLD in premenopausal women, but is not a risk factor in postmenopausal women or in men. This is one of the reasons that the number of women with NAFLD increases with age, and reaches the maximum level in women in their sixties.

ACKNOWLEDGMENTS

We thank all staff members in the medical health checkup center, Murakami Memorial Hospital.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is emerging as the most common chronic liver disease. NAFLD is more common in men than women, particularly in Asians.

Research frontiers

The prevalence of NAFLD in women increases with age, but not in men. Age and menopause may be risk factors for the incidence of NAFLD. But, this has not been clarified yet.

Innovations and breakthroughs

The prevalence of NAFLD was lower in premenopausal women than in men, but in postmenopausal women, the prevalence of NAFLD was similar to that in men. The prospective study indicated that aging is a risk factor for the development of NAFLD in premenopausal women independent of weight gain or metabolic syndrome.

Applications

The study suggests the possibility that premenopausal women have a protective factor against NAFLD, which is weakened by aging.

Peer review

NAFLD was less prevalent in females than in males, but only before menopause. The prevalence of NAFLD was lower in women than in men, and increased with age in women, but a significant trend for age was not identified in men. NAFLD was independently associated with BMI and metabolic syndrome in both pre- and postmenopausal women, but with age only in premenopausal women. The results are interesting and suggest that the premenopausal state may be associated with a protective factor against NAFLD, which weakens with age.

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S- Editor Lv S **L- Editor** O'Neill M **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i3.244

World J Gastroenterol 2012 January 21; 18(3): 244-250
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

High level of urokinase plasminogen activator contributes to cholangiocarcinoma invasion and metastasis

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Supported by Grant from Thailand Research Fund and Faculty of Science, Mahidol University, Thailand (to Suthiphongchai T); Institutional Strengthening Program, Faculty of Science, Mahidol University (to Thummarati P)
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Received: June 15, 2011 Revised: August 26, 2011
Accepted: October 28, 2011
Published online: January 21, 2012

Abstract

AIM: To investigate the role of urokinase plasminogen activator (uPA) in cholangiocarcinoma (CCA) invasion and its correlation with clinicopathological parameters.

METHODS: uPA expression in CCA tissue was determined by immunohistochemistry. The level of uPA from two CCA cell lines (HuCCA-1 and KKU-M213) and a non-cancer immortalized cholangiocyte cell line (H69) was monitored by plasminogen-gelatin zymography and western blotting, whereas that of plasminogen activator inhibitor type 1 (PAI-1) protein and uPA receptor (uPAR) mRNA was monitored by western blotting and quantitative real-time reverse transcriptase polymerase chain reaction, respectively. Two independent methods were employed to suppress uPA function: a synthetic uPA inhibitor (B428) and silencing of uPA gene expression using siRNA. *In vitro* invasion of the uPA-disrupted cells was assessed by Matrigel-coated Transwell assay.

RESULTS: The immunohistochemical study showed that 75.3% (131/174) of CCA tissues expressed uPA. High uPA expression was correlated with lymphatic invasion and metastasis of CCA patients. Plasminogen-gelatin zymography of the conditioned media and cell-surface eluates showed that both CCA cell lines, but not H69, expressed both secreted and membrane-bound forms of uPA. Although the two CCA cell lines, HuCCA-1 and KKU-M213, expressed a relatively high level of uPA and uPAR, the latter exhibited a much lower degree of *in vitro* invasiveness, correlating with a high expression of PAI-1 in the latter, but not in the former. Suppressing uPA function with a specific uPA inhibitor, B428, or with siRNA against uPA reduced *in vitro* invasiveness of KKU-M213 cells, demonstrating the requirement for uPA in the invasiveness of CCA cells. Therefore, our *in vivo* and *in vitro* studies suggest that uPA is an important requirement for the invasion process of CCA.

CONCLUSION: uPA expression correlates with lymphatic invasion and metastasis *in vivo* and is required for CCA cell invasion *in vitro*, suggesting its potential as a therapeutic target.

Key words: Bile duct cancer; Cholangiocarcinoma; Cancer invasion; Urokinase plasminogen activator; Cancer metastasis

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Thummarati P, Wijitburaphat S, Prasopthum A, Menakongka A, Sripa B, Tohtong R, Suthiphongchai T. High level of urokinase plasminogen activator contributes to cholangiocarcinoma invasion and metastasis. *World J Gastroenterol* 2012; 18(3): 244-250 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i3/244.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i3.244

INTRODUCTION

Cholangiocarcinoma (CCA) is a cancer that originates from the biliary epithelium, and it is the second most common form of liver cancer. Although it is a rare malignancy, the incidence and mortality rate has increased worldwide in the past decade[1]. The highest incident rate was observed in Northeast Thailand where there is a high prevalence of liver fluke (*Opisthorchis viverrini*) infection[2], a group I carcinogen classified by International Agency for Research on Cancer[3]. CCA is considered an incurable disease due to lack of efficient diagnosis, hence most patients at presentation have developed advanced disease with high rate of invasion and metastasis, resulting in a high mortality rate.

Metastasis is a multi-step process that involves spreading of cancer cells from the primary to the secondary site. During this process, cancer cells must invade the surrounding tissue, penetrate the blood or lymphatic vessels, and form a new tumor mass at distant sites. To invade, cancer cells degrade extracellular matrix (ECM) and basement membrane to generate a space for the cells to move out of the original site. This is accomplished by secretion of a variety of matrix-degrading enzymes including matrix metalloproteinases (MMPs) and serine proteases, such as plasminogen activator.

Urokinase plasminogen activator (uPA) is a serine protease that is involved in ECM degradation, cancer invasion and metastasis by regulating the plasminogen/plasmin system. uPA is synthesized as a single-chain proenzyme which is activated by proteolytic cleavage to form the high-molecular-weight two-chain active uPA or the low-molecular-weight uPA through the action of plasmin, kallikrein, or cathepsin B[4]. Active uPA cleaves inactive plasminogen to generate active plasmin, a broad-specific serine protease, which can degrade a variety of ECM proteins. Besides, plasmin and uPA can also activate several types of MMPs which, in turn, degrade ECM. Therefore, uPA amplifies proteolytic cascades in ECM degradation which is crucial for cancer invasion. uPA exerts its effect by binding to the uPA receptor (uPAR), which localizes uPA on the cell surface, enhancing its plasminogen activation capability[5]. This activity is, in turn, negatively regulated by the plasminogen activator inhibitor type 1 and 2 (PAI-1 and -2)[5].

uPA expression has been shown to be upregulated in many cancers, where its expression has been correlated with invasion and metastasis[6]. Although uPA expression has been demonstrated in some CCA cell lines[7,8], there has been no report of uPA expression in clinical specimens, nor a linkage between its expression with the clinical symptoms of CCA patients. In this study, we investigated the possible role of uPA in CCA development *in vivo* by examining the expression pattern of uPA in clinical samples and relating those findings to the various clinicopathological parameters of CCA patients. In addition, we determined the role of uPA *in vitro* using siRNA or specific inhibitor to suppress the uPA function and assessed the phenotype of the cells with uPA downregulation in an *in vitro* Transwell assay.

MATERIALS AND METHODS

Patients and tissue samples

Archival paraffin-embedded tissue samples were obtained from 174 patients (aged 32-75 years) who underwent liver resection at Srinagarind Hospital, Khon Kaen University, Thailand during 1999-2010. These samples were used to generate the tissue microarray for this retrospective study. All patients were diagnosed with intrahepatic CCA. Vascular, lymphatic and neural or perineural invasion were defined by the presence of tumor cells in the blood vessels, lymphatic vessels and in or around the nerve fibers in the liver, respectively. The study protocol was approved by the Ethical Committee of Khon Kaen University (HE 521209).

Cell lines and cell culture

Two human CCA cell lines developed from Thai patients, HuCCA-1[9] and KKU-M213 and one human immortalized cholangiocyte cell line, H69, were used. All cell culture materials (medium, serum and antibiotics) were purchased from Gibco Invitrogen (Auckland, New Zealand). The CCA cells were cultured in HAM's F-12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L glutamine, 15 mmol/L HEPES and 14 mmol/L sodium bicarbonate, 100 U/mL penicillin G and 100 U/mL streptomycin. The cells were incubated at 37 ℃ under a humidified 5% CO_2 atmosphere. H69 was grown in Dulbecco's Modified Eagle's Medium (DMEM)/DMEM F12 (1:1) supplemented with 10% FBS, hormones and epidermal growth factor, as previously described[10].

Tissue microarray and immunohistochemical staining

Tissue microarray (TMA) was generated manually from the paraffin-embedded tissues. In brief, the region of interest from each paraffin block was identified on a hematoxylin-eosin-stained slide, after which the slide was aligned with the surface of the original paraffin block to locate the sampling area. The area of interest in the paraffin block was then punched with a 1-mm-diameter needle before each punched tissue was then manually transferred to a new recipient paraffin block to generate a TMA

block. Five-micrometer-thick sections were cut from the TMA block, mounted on a silane-coated glass slide, followed by immunohistochemical staining. The specimens were deparaffinized and dehydrated before the endogenous peroxidase activity in the tissue section was blocked with 0.5% H_2O_2 in methanol for 30 min. After washing with PBS, pH 7.4, and blocking with blocking solution containing 5% normal horse serum in PBS, pH 7.4, for 30 min, the specimens were hybridized with 0.5 μg/mL monoclonal antibody against uPA (Ab No. 3689; American Diagnostica, Stamford, CT, United States), followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (Invitrogen, Carlsbad, CA, United States). The brown color corresponding to the peroxidase activity was developed using diaminobenzidine (Sigma, St Louis, MO, United States). The specimens were counter-stained with Mayer's hematoxylin. Negative controls were performed in a similar way, omitting the primary antibody. The uPA staining was scored based on signal intensity as follows: negative, weak (+), moderate (++) and strong (+++).

Determination of plasminogen activator activity by plasminogen-gelatin zymography

uPA activity secreted from cancer cells into the conditioned medium (CM) was determined by plasminogen-gelatin zymogaphy under non-reducing conditions. Cells (3.5×10^5) were cultured in six-well plates for 2 d, washed twice, and incubated in serum-free medium for 6 h. Proteins in CM were separated by 8% SDS-PAGE containing 10 μg/mL plasminogen (Roche Diagnostics GmbH, Mannheim, Germany) and 1 mg/mL gelatin (Sigma) under non-reducing conditions. After electrophoresis, the gel was washed twice with 2.5% TritonX-100 for 1 h to remove SDS, incubated for 18 h in the reaction buffer containing 100 mmol/L Tris-HCl, pH 7.8, 150 mmol/L NaCl and 1% TritronX-100, followed by staining with 0.25% Coomassie blue and destaining with 45% methanol and 10% acetic acid. A clear band with an estimated molecular weight of 43 kDa represented the uPA activity band. To confirm its PA (not gelatinase) activity, plasminogen-free gelatin zymogram gel was run in parallel as a negative control.

To detect the bound uPA, cells were washed twice with PBS before the bound uPA was eluted by elution buffer [100 mmol/L NaCl and 50 mmol/L glycine-HCl (pH 3.0)]. The eluate was neutralized by adding 0.5 mol/L Tris-HCl, (pH 7.8), at the ratio of eluate:neutralization buffer of 4:1, and analyzed by plasminogen-gelatin zymography.

Determination of uPA and PAI-1 proteins by western blotting

uPA and PAI-1 protein levels in 40× concentrated CM were determined by western blot analysis using anti-uPA and anti-PAI-1 antibodies (American Diagnostica), respectively. Proteins were separated by 8% SDS-PAGE, transferred to a nitrocellulose membrane, probed with anti-uPA and anti-PAI-1 antibodies, before being hybridized with HRP-conjugated secondary antibodies. The uPA and PAI-1 bands were developed by enhanced chemiluminescence ECL Plus reagent (GE Healthcare, Little Chalfont, Bucks, United Kingdom) and visualized by Fluor Chem SP (Alpha Innotech**,** San Leandro, CA, United States).

Quantitative real-time reverse transcriptase polymerase chain reaction

uPA and uPAR mRNA expression was determined by real-time reverse transcriptase polymerase chain reaction (RT-PCR) using ABI 7500 (Applied Biosystems, Foster City, CA, United States). RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA, United States) following the manufacturer's protocol. Two micrograms total RNA was converted to cDNA by SuperScript™ Ⅲ Reverse Transcriptase kit (Invitrogen, Grand Island, NY, United States) using random hexamer primer, and the cDNA was amplified by real-time PCR in a 20-μL reaction volume containing 0.5 U HotStart Taq polymerase (Qiagen), 1× FastStart Universal SYBR Green Master cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and 4 pmol of each specific primer (5'-TTGCTCACCACAACGACATT-3' and 5'-ATTTTCAGCTGCTCCGGATA-3' for uPA[11], 5'-GGTGACGCCTTCAGCATGA-3' and 5'-CCCACTGCGGTACTGGACAT-3' for uPAR and 5'-GTAACCCGTTGAACCCCATT-3' and 5' -CCATCCAATCGGTAGTAGCG-3' for 18sRNA, as an internal control). The reactions were started with an initial heat activation step, followed by 40 thermal cycles. The mRNA levels among the test cells were analyzed by relative quantification $2^{-\Delta\Delta Ct}$ method.

Knock-down of uPA using siRNA against uPA

KKU-M213 cells were transiently transfected with siRNA against uPA (Santa Cruz Biotechnology, Santa Cruz, CA, United States) using siRNA transfection reagent (Santa Cruz Biotechnology) following the manufacturer's protocol with some modifications. In brief, 6 μL siRNA and 6 μL transfection reagent were separately diluted in 100 μL siRNA transfection medium. The diluted siRNA solution was mixed with the diluted transfection reagent, incubated at room temperature for 15 min, before being added to a six-well plate seeded with 2×10^5 CCA cells in 0.8 mL transfection medium. The level of uPA mRNA was accessed at 48 and 72 h after transfection. Silencer Negative Control siRNA #1 (Ambion, Austin, TX, United States), a non-targeted sequence, was used as the negative control.

Transwell in vitro invasion assay

The invasiveness of CCA cells was determined using a Matrigel-coated Transwell chamber (8-μm pore size polyvinylpyrrolidone-free polycarbonate filter with 6.5 mm diameter) (Corning Inc., Corning, NY, United States) precoated with 30 μg Matrigel (BD Biosciences, San Diego, CA, United States). One hundred thousand transfected cells at 66 h post-transfection in FBS-free media were added to the upper chamber of the Transwell, while 600 μL medium containing 10% FBS was added to the lower chamber. After incubation for 6 h at 37 ℃ in a CO_2 in-

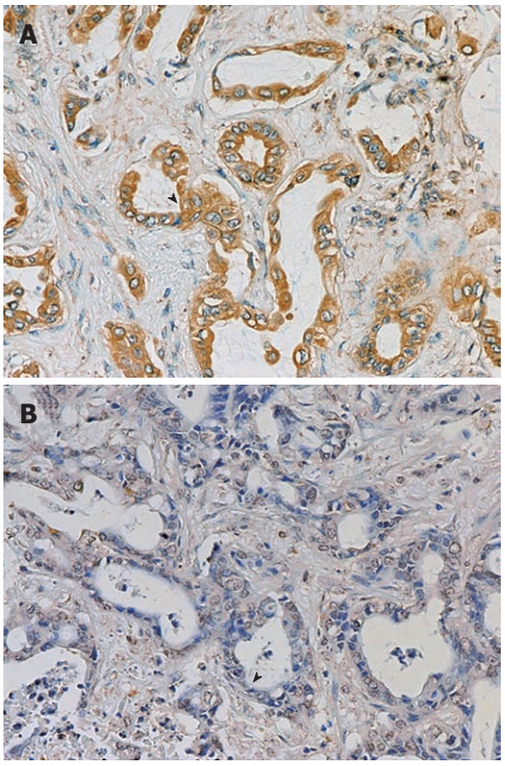

Figure 1 Immunohistochemical staining of urokinase plasminogen activator protein expression in cholangiocarcinoma tissues. Tissue microarray slide was stained with anti-urokinase plasminogen activator (uPA) antibody and counter-stained with Mayer's hematoxylin. Representative of +++ (A) and negative (B) uPA staining.

cubator, non-invaded cells were removed from the upper chamber, and invaded cells were fixed and stained for 30 min with 0.5% crystal violet in 25% methanol. The number of invaded cells in five random fields was counted under a light microscope using a 10× objective. Three independent experiments were performed, each done in duplicate.

Statistical analysis

All statistical analysis was performed using SPSS version 16.0 software. Correlation between uPA expression and clinicopathological factors was analyzed using the χ^2 test. Survival analysis was done by Kaplan-Meier and log-rank tests. mRNA and invasion data were expressed as mean ± SE from three independent experiments. Comparison of the data between groups was performed by *t* test. $P < 0.05$ was considered significant.

RESULTS

Level of uPA in CCA tissues using immunohistochemistry

We examined uPA expression in 174 CCA specimens by performing immunohistochemistry on the TMA. One hundred and thirty-one cases (75.3%) expressed uPA, which was mainly localized in cytoplasm of CCA cells (Figure 1). The low uPA expressing cells (negative and +) accounted for 50.6%, whereas those with high uPA expression (++ and +++) constituted 49.4% of the total sample number (Figure 1).

Table 1 Correlation between urokinase plasminogen activator expression and clinicopathological features

Variables	uPA		*P* value
	Low	High	
Age (yr)			
≤ 50	22	23	0.793
> 50	66	63	
Sex			
Male	61	55	0.453
Female	27	31	
Tumor size (cm)			
≤ 5	29	28	0.956
> 5	59	58	
Histotype group			
Well differentiated	33	31	0.065
Moderately differentiated	16	20	
Poorly differentiated	18	6	
Papillary	20	25	
Adenosquamous	1	4	
Gross type			
Mass forming	71	63	0.348
Periductal infiltration	15	22	
Mix type[1]	2	1	
Vascular invasion (*n* = 168)			
Absent	28	32	0.451
Present	57	51	
Nerve invasion (*n* = 166)			
Absent	55	46	0.154
Present	28	37	
Lymphatic invasion (*n* = 167)			
Absent	29	15	0.012[2]
Present	55	68	
Metastasis			
Absent	50	36	0.048[2]
Present	38	50	

[1]Classify as both mass forming and periductal or intraductal; [2]Statistically significant. uPA: Urokinase plasminogen activator.

The correlation between uPA expression and clinicopathological parameters is summarized in Table 1. There was no correlation between uPA expression level and age, sex, tumor size, histological type, vascular invasion, neural invasion (Table 1) and patients' survival (Figure 2). However, uPA expression level in the CCA tissues was positively correlated with lymphatic invasion ($P = 0.012$) and metastasis ($P = 0.048$).

Levels of secreted and cell surface-bound uPA in CCA cell lines

Expression pattern of the secreted form and the membrane-bound form of uPA was investigated using plasminogen-gelatin zymography in the CCA cell lines, HuCCA-1, and KKU-M213, compared with that of an immortalized cholangiocyte cell line, H69. Both CCA cell lines, but not the cholangiocytes, expressed a 43-kDa PA band on the plasminogen–gelatin zymogram (Figure 3A), and this was later confirmed by western blotting using anti-uPA antibody to correspond to uPA (Figure 3B). The discrepancy in the molecular weight of uPA determined by plasminogen–gelatin zymography (43 kDa) and by western blotting (50 kDa) could be accounted for by the different denaturation conditions employed by the two methods, in which

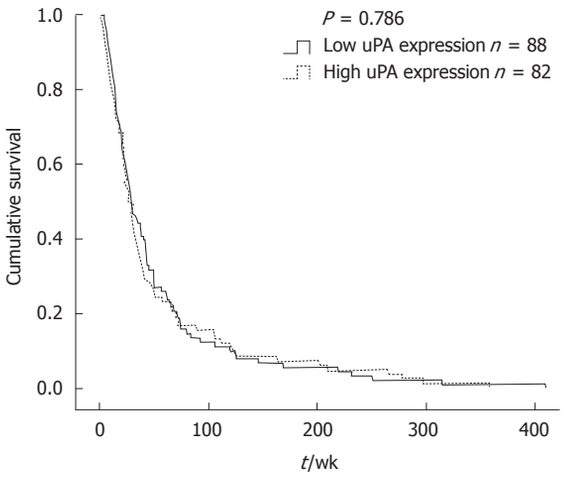

Figure 2 Correlation between urokinase plasminogen activator expression and patients' survival by Kaplan-Meier plot. Low urokinase plasminogen activator (uPA) expression was referred to as negative and +, whereas high uPA expression was ++ and +++.

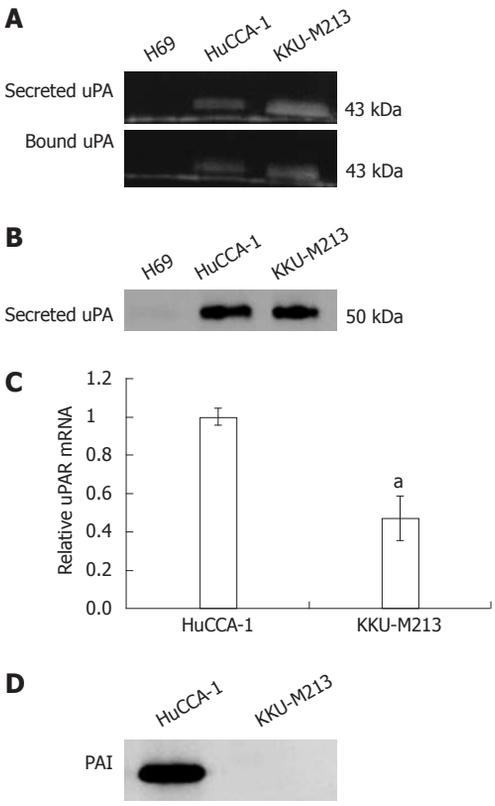

Figure 3 Levels of urokinase plasminogen activator, plasminogen activator inhibitor type 1 protein and urokinase plasminogen activator receptor expression in two cholangiocarcinoma cell lines and immortalized cholangiocytes. A: Levels of secreted urokinase plasminogen activator (uPA) in 40× concentrated conditioned medium and cell-surface-bound uPA were determined by plasminogen-gelatin zymography; B and D: Levels of secreted uPA (B); and secreted plasminogen activator inhibitor type 1 (PAI-1) (D) proteins were determined by western blotting; C: Level of uPAR mRNA was examined by SYBR Green-based qPCR and the data were calculated as relative uPAR expression compared to that of HuCCA-1, $2^{-\Delta\Delta Ct}$, and expressed as mean ± SE from three independent experiments. Significant difference is indicated by $^{a}P < 0.05$.

the protein was partially denatured by a non-reducing-unheated condition in the zymogram gel, whereas it was completely unfolded by a reducing-heated condition in the western blotting.

The uPA is under positive and negative regulation by uPAR and PAI-1, respectively. Using quantitative real-time PCR, we showed that both CCA cell lines expressed uPAR mRNA, where the expression level in HuCCA-1 was twice the level in KKU-M213 cells (Figure 3C). In addition, we showed by western blotting that PAI-1, a major uPA inhibitor, was secreted abundantly in HuCCA-1 but not in KKU-M213 cells (Figure 3D).

Effect of uPA inhibition on CCA cell invasion

Our immunohistochemical data showed that uPA expression in CCA specimens correlated with invasion and metastasis, thus, we investigated if uPA played a role *in vitro* by assaying for the invasiveness of the cancer cell line in which the uPA activity was inhibited. We showed that B428, a uPA-specific inhibitor[12], dose dependently reduced the invasiveness of KKU-M213 cells compared with controls (Figure 4). Treatment with 20 μmol/L B428 suppressed *in vitro* invasion by 32%, whereas uPA activity as determined by plasminogen-gelatin zymography, of which both gel and reaction buffer contained the inhibitor, was reduced by 64%. Suppression of *in vitro* invasion was not due to toxicity of B428, because the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay showed that B428 had no significant effect on cell survival under these conditions (Figure 4).

The significance of uPA in the invasiveness of CCA cells was confirmed by silencing of uPA using siRNA to target *uPA* gene expression in KKU-M213 cells. Using quantitative real-time RT-PCR, we showed that the uPA mRNA level of cells transfected with siuPA was suppressed by about 60% compared to that of cells transfected with siNeg (Figure 5A). *In vitro* invasion of the uPA-silenced cells was reduced by 66% ± 12% compared to that in the negative controls (Figure 5B). Suppression of uPA activity using two independent methods confirmed that uPA is an important requirement for invasiveness of CCA cells.

DISCUSSION

One of the key features required for cancer invasion and metastasis is the ability to degrade ECM and basement membrane barrier. This process is accomplished by the action of a variety of proteolytic enzymes, including serine proteases and MMPs, which work in concert[5]. uPA, a plasminogen-specific serine protease, is one of the important proteolytic enzymes contributing to this process.

uPA has been reported to be upregulated in many cancer types, such as breast[13], prostate[14], colorectal[15], gastric[16], and thyroid[17], and its expression level correlates with cancer progression. Here, we used immunohistochemical analysis to demonstrate that uPA expression could be detected in the majority (75.3%) of CCA specimens, and that high level of uPA expression correlated with lymphatic invasion and metastasis in CCA patients.

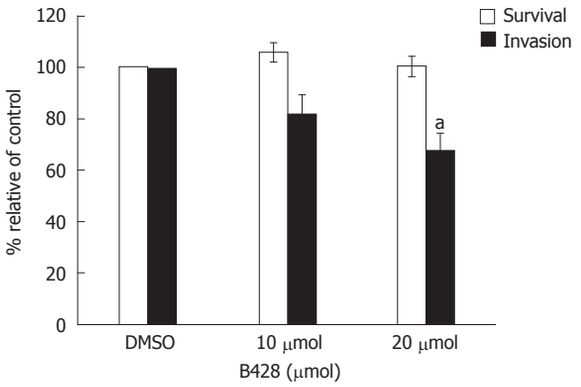

Figure 4 Effect of urokinase plasminogen activator inhibitor (B428) on KKU-M213 cell invasion. Cell suspension in medium containing B428 (10 and 20 μmol/L) and 0.1% dimethyl sulfoxide (as a control) were subjected to *in vitro* invasion assay for 6 h. Cell survival was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay after cells were incubated with the drug for 6 h. The results were presented as mean ± SE from three independent experiments. Significant difference is indicated by $^{a}P < 0.05$.

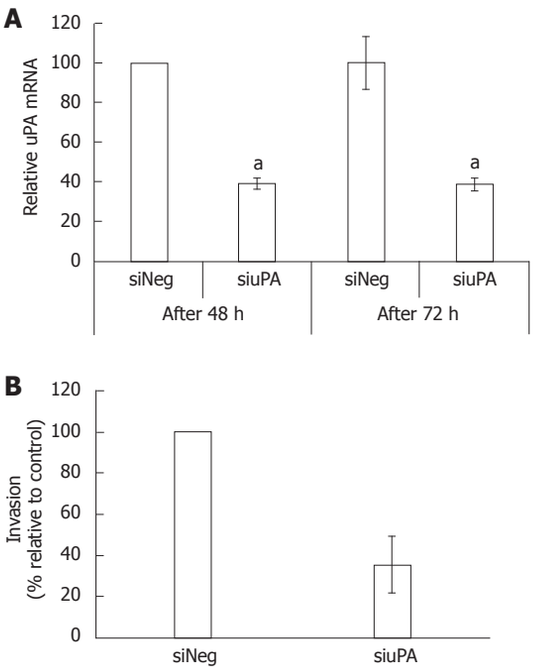

Figure 5 Effects of urokinase plasminogen activator knockdown on KKU-M213 cell invasion. A: Level of urokinase plasminogen activator (uPA) mRNA of the cells transfected with siRNA against uPA or non-targeting siRNA for 48 and 72 h were determined by real-time reverse transcriptase polymerase chain reaction. Significant difference is indicated by $^{a}P < 0.05$; B: Cells transfected with siRNA for 66 h were analyzed for cell invasiveness using Transwell *in vitro* invasion assay. The results were presented as mean ± SE from three independent experiments.

However, we found no significant correlation between uPA expression level and patients' survival rate. Several clinical features contribute to survival of intrahepatic CCA patients. Large tumor size, multifocal tumors, positive resection margin, gross appearance as mass-forming plus periductular infiltration, invasion and metastasis have been shown as clinicopathological features related to poor prognosis and short survival[18].

The role of uPA in CCA invasion was investigated *in vitro* using CCA cell lines, HuCCA-1 and KKU-M213, and compared with the non-cancer immortalized cholangiocytes, H69. Plasminogen-gelatin zymography and western blotting demonstrated that both CCA cell lines, but not the non-cancer immortalized cholangiocytes, expressed the cell-surface-bound and the secreted forms of uPA. Although the two cell lines expressed relatively high amounts of uPA, only KKU-M213, but not HuCCA-1 cells exhibited a high degree of invasiveness as previously determined by *in vitro* Transwell assay[19]. This could probably be explained by the fact that HuCCA-1, but not KKU-M213 cells expressed PAI-1, a uPA inhibitor, thereby attenuating uPA activity to promote matrix degradation and cell invasion. Expression of PAI-1 in HuCCA-1 cells may also explain why the level of cell-surface-bound uPA was similar in the two cell lines, although that of uPAR mRNA was twice as high in HuCCA-1 compared to KKU-M213 cells. Binding of PAI-1 to the uPA-uPAR complex has been reported to promote uPA-uPAR-PAI-1 complex internalization and lysosomal degradation of uPA and PAI-1[6], leading to a reduction of the bound uPA on the cell surface.

Inhibition of uPA or uPAR function by anti-uPA or anti-uPAR antibody or by non-specific serine protease inhibitor has been shown to reduce significantly *in vitro* invasiveness of papillary thyroid cancer cells[17]. Similarly, silencing of uPA and/or uPAR by siRNA has been shown to suppress *in vitro* invasion of many cancer types including those of the prostate[20] and breast[21], and glioma[22]. We showed that inhibition of uPA activity by specific uPA inhibitor, B428, or of uPA expression by siRNA efficiently suppressed *in vitro* invasion of KKU-M213 cells, consistent with a previous report in QBC939 cells, a uPA-expressing CCA cell line, whose invasiveness was markedly reduced by inhibiting plasminogen activation with tranexamic acid or 6-aminocaproic acid[7]. Observations of the importance of uPA in invasion in a variety of cancers suggest a common role of uPA in cancer invasion, including CCA. A drug inhibiting uPA is already undergoing clinical trial for some cancers[23] and if proven successful, could be applied to CCA.

In summary, we have showed by immunohistochemistry that high uPA expression in CCA tissue was correlated with lymphatic invasion and metastasis. Studies in cell lines confirmed the importance of uPA in cell invasiveness, but the presence of PAIs or uPAR can affect uPA-dependent cell invasion. The roles of these factors in modulating CCA invasion and metastasis clearly warrant future investigation.

ACKNOWLEDGMENTS

The authors thank Professor Sirisinha S (Mahidol University, Thailand) for providing us HuCCA-1 cell line and Professor Alpini G (Texas A and M University, TX, United States) and Professor Gores G (Mayo Clinic, MN, United States) for H69 cell line, and Professor Wilairat P and Dr. Kitjaroentham A for critical reading of the manuscript.

COMMENTS

Background

Cholangiocarcinoma (CCA), a cancer of the bile duct, is an aggressive cancer with high metastatic and short survival rate. One of the critical properties required for cancer metastasis is the ability to degrade extracellular matrix. Urokinase plasminogen activator (uPA), a key proteolytic enzyme involved for this process, is frequently overexpressed in many types of cancers.

Research frontiers

Although uPA has been demonstrated to play an important role in invasion of many cancer types, the correlation between uPA expression and CCA progression has not hitherto been reported.

Innovations and breakthroughs

The authors have demonstrated that high uPA expression in CCA tissue correlated with high lymphatic invasion and metastasis in patients. The importance of uPA in cell invasiveness was confirmed *in vitro* using uPA-expressing CCA cell lines. In addition, the *in vitro* study indicated that plasminogen activator inhibitors could have an important impact on uPA-mediated cancer cell invasiveness.

Applications

The requirement of uPA in CCA invasiveness highlights uPA as a potential therapeutic target.

Terminology

Urokinase plasminogen activator (uPA) is a plasminogen-specific serine protease. It converts inactive plasminogen to active plasmin, which degrades extracellular matrix and activates matrix metalloproteinases.

Peer review

This study investigated uPA expression in CCA tissues and its role in CCA cell invasion *in vitro*. The authors found that high uPA expression correlated with invasion and metastasis in CCA patients. The design of this research is good and the findings are novel and important, so it is suitable for our readers.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i3.251

World J Gastroenterol 2012 January 21; 18(3): 251-256
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Roux-en-Y reconstruction does not require gastric decompression after radical distal gastrectomy

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Received: June 13, 2011 Revised: September 2, 2011
Accepted: October 28, 2011
Published online: January 21, 2012

Abstract

AIM: To determine whether routine nasogastric (NG) decompression benefitted patients undergoing radical gastric surgery.

METHODS: Between January 1998 and December 2008, 519 patients who underwent distal gastrectomy for gastric cancer were retrospectively divided into 2 time-period cohorts; those treated with Billroth Ⅱ (BⅡ) reconstruction in the first 6 years and those with Roux-en-Y (RY) reconstruction in the last 5 years. In the latter group, the patients were further divided into 2 subgroups; with and without nasogastric decompression.

RESULTS: Postoperatively, there were no significant differences in the number of anastomotic leaks between the 3 groups. In the tubeless RY group, time to semi-liquid diet was significantly shorter than in the other 2 groups (4.4 d ± 1.4 d *vs* 7.2 d ± 1.3 d and 5.9 d ± 1.2 d, $P = 0.005$). The length of postoperative stay was significantly increased in patients with BⅡ reconstruction compared with patients with RY reconstruction with/without NG decompression (15.4 d ± 4.3 d in BⅡ group *vs* 12.6 d ± 3.1 d in decompressed RY and 11.4 d ± 3.4 d in the tubeless RY group, $P = 0.035$). The postoperative pneumonia rate was lowest in the tubeless group and highest in the BⅡ group (1.4% *vs* 4.6%, $P = 0.01$). Severe sore throat was noted in 59 (20.7%) members of the BⅡ group, 18 (17.4%) members of the decompressed RY group and 6 (4.2%) members of the tubeless RY group. Fewer patients in the tubeless group complained of severe sore throat ($P = 0.001$).

CONCLUSION: This study provides support for abandoning routine NG decompression in patients undergoing subtotal gastrectomy with Roux-en-Y gastrojejunostomy.

Key words: Nasogastric decompression; Billroth Ⅱ gastrojejunostomy; Roux-en-Y gastrojejunostomy; Radical distal gastrectomy; Gastric cancer

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Chen CJ, Liu TP, Yu JC, Hsua SD, Hsieh TY, Chu HC, Hsieh CB, Chen TW, Chan DC. Roux-en-Y reconstruction does not require gastric decompression after radical distal gastrectomy. *World J*

Gastroenterol 2012; 18(3): 251-256 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i3/251.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i3.251

INTRODUCTION

It is a commonly held belief in clinical practice that nasogastric (NG) decompression after gastric surgery is essential to prevent postoperative complications, such as postoperative ileus or anastomotic leakage. Although the necessity of NG decompression following gastric surgery has been increasingly questioned over the last 2 decades, most general surgeons have routinely used NG decompression and recommended patients fast for a period of 3-5 d after surgery. Several prospective studies have suggested that this routine practice does not provide any benefit, but could make patients feel uncomfortable[1-5]. However, these studies enrolled gastric cancer patients who underwent a variety of operations.

Recently, to prevent enterogastric reflux into the gastric remnant and decrease biliary gastritis, Roux-en-Y (RY) reconstruction has become widely used after distal gastrectomy. We have previously reported the superiority of RY reconstruction over Billroth Ⅱ (BⅡ) anastomosis[6]. Another study showed that RY reconstruction after distal gastrectomy is a safer form of anastomosis, and could prevent anastomotic leakage[7]. Therefore, the safer procedure may dispel the common belief of general surgeons that NG decompression after gastric surgery is essential.

The Tri-Service General Hospital has performed RY reconstruction in all gastric cancer patients with distal gastrectomy since January 2003. Benefits of this approach include the reduction of the amount of drained gastric remnant content *via* NG tube. Additionally, the low incidence of possible complications, including biliary gastritis, gastroesophageal reflux and aspiration pneumonia, has supported our adherence to this technique[6]. In January 2005, we therefore decided to abandon the routine use of NG tube decompression postoperatively in patients undergoing distal gastrectomy after success with early discontinuation in a few patients.

In this study, we reviewed our experience with patients undergoing distal gastrectomy to investigate the difference in complication rates between patients without postoperative NG decompression and patients with postoperative NG decompression to determine the necessity of postoperative NG decompression after distal gastrectomy.

MATERIALS AND METHODS

A retrospective study spanning an 11-year period from January 1998 to December 2008 was performed, and a total of 519 patients who underwent distal gastrectomy for carcinoma of the stomach were identified. Criteria for inclusion in this cohort study were as follows: no previous chemoradiation treatments, having R0 resection (no macroscopic or microscopic residual tumor) according to the definition of the International Union Against Cancer (UICC), and having the operative procedures described below. Approval for chart review was obtained from the institutional review boards at Tri-Service General Hospital. Each patient's clinic chart was reviewed.

Cases were divided into 2 time-period cohorts; those treated in the first 6 years (TP1, $n = 283$) and those in the last 5 years (TP2, $n = 236$). After distal gastrectomy, gastrointestinal continuity was reconstructed with BⅡ gastrojejunostomy in all patients before January 2003 (TP1). Thereafter, we adapted RY gastrojejunostomy to restore gastrointestinal continuity (TP2). All patients underwent D1 or D2 lymph node dissection. The cancer was staged according to the UICC TNM classification. Preoperatively, all patients in each group were given a normal diet, unless there was gastric outlet obstruction. Patients with symptoms of obstruction were given NG decompression and parenteral nutrition support exclusively for at least 7 d before the operation. No attempt was made to employ gastric lavage in these patients.

In TP1, all patients received insertion of a 16-French single lumen NG tube for postoperative gastric decompression until the passage of flatus and drainage amounted to less than 100 cc per day. They were allowed glucose water to drink the day after the NG tube was removed. Diet was increased in a stepwise fashion from a clear liquid to a semi-liquid diet as tolerated. In TP2, patients were divided into 2 subgroups based on whether NG tube decompression was used or not. In the decompression group (TP2A), the patients received NG decompression and resumed an oral diet in the same way as in the TP1 group. In the tubeless group (TP2B), postoperative oral intake was started with water on the second postoperative day regardless of passage of flatus. Diet was advanced in the same way as in the other groups. Routine radiographic examination using water-soluble contrast material was not done before starting oral intake. The NG tube was reinserted when clinically indicated, such as when severe vomiting or abdominal distension occurred.

The following data were recorded by the attending surgeon: intra-abdominal complications (delayed gastric emptying or Roux stasis syndrome, small mechanical bowel obstruction, gastrojejunostomy leak and duodenal stump leak) and postoperative infection (wound infection, unknown fever, intra-abdominal abscess, and pneumonia). We adopted the 1992 Centers for Disease Control definition for superficial incisional, deep incisional and organ/space surgical site infection for hospital monitoring programs and surgical audits[7]. Patients were considered to have postoperative infections if they developed intra-abdominal abscesses, surgical wound infections, unknown fever, or pulmonary infiltrates shown by radiography[8]. Postoperative unknown fever was defined as a body temperature over 38 ℃ for at least 3 d without obvious infection source. Intra-abdominal abscess was defined as an abscess that needed to be resolved by percutaneous or open drainage. Wound infection was defined as a wound

Table 1 Patients' clinicopathologic characteristics[1]

	TP1 (*n* = 283)	TP2A (*n* = 102)	TP2B (*n* = 134)	*P* value
Median age (range)	59 (26-80)	62 (30-79)	60 (35-82)	0.542
Sex (M/F)	195/88	67/35	75/59	0.23
Type of reconstruction				0.001
Billroth Ⅱ	283	0	0	
Roux-en-Y	0	102	134	
Lymphadenectomy				0.153
D1	24 (8.4)	5 (5.2)	14 (10.4)	
D2	259 (91.6)	97 (94.8)	120 (89.6)	
AJCC tumor stage				0.116
Ⅰ	41 (14.5)	12 (11.3)	20 (15.2)	
Ⅱ	80 (28.4)	31 (30.4)	45 (33.4)	
Ⅲ	133 (46.9)	51 (50.2)	58 (43.1)	
Ⅳ	29 (10.2)	8 (8.1)	11 (8.3)	
Type of pain control				0.731
Nil	18 (6.3)	5 (4.8)	11 (8.2)	
Epidural form	70 (24.9)	22 (21.3)	22 (16.5)	
Intravenous form	189 (68.8)	73 (71.9)	101 (75.3)	
Operation time	241 ± 53.8	253 ± 59.6	251 ± 63.3	0.821

[1]Data are medians with ranges in parentheses, numbers with percentages in parentheses or mean ± SD. AJCC: American Joint Committee on Cancer (6th edition).

Table 2 Postoperative clinical parameters (mean ± SD)[1]

	TP1 (*n* = 283)	TP2A (*n* = 102)	TP2B (*n* = 134)	*P* value
Persistent decompression > 7 d or reinsertion of NG tube, number of patients	19 (6.7%)	3 (2.9%)	5 (3.7%)	0.001
Duration of gastric decompression (d)[1]	5.3 ± 3.3	3.6 ± 2.2	–	0.023
Amount of gastric decompression (mL/d)	465 ± 241	58 ± 47	–	0.001
Days to passage of flatus	5.1 ± 1.7	4.4 ± 1.3	4.7 ± 1.2	0.618
Days to semi-liquid diet	7.2 ± 1.3	5.9 ± 1.2	4.4 ± 1.4	0.05
Length of postoperative hospital stay (d)	15.4 ± 4.3	12.6 ± 3.1	11.4 ± 3.4	0.035

[1]Average amount of nasogastric drainage in the first three postoperative days. NG: Nasogastric.

that needed to be laid open. Mechanical small bowel obstruction was defined as an obstruction that needed to be resolved by surgical intervention. Delayed gastric emptying or Roux stasis syndrome was defined arbitrarily as the failure to intake food orally after the 7th postoperative day. The postoperative days until the first passage of stool was observed, when semi-liquid diet was permitted, and the length of postoperative hospital stay (LOS) were also recorded.

Statistical analysis

Results were expressed as mean ± SD. Statistical analysis was performed using the one-way analysis of variance test for continuous variables and the χ^2 test for categorical variables, when appropriate, respectively. *P* values less than 0.05 were considered statistically significant. Statistical analysis was carried out with the SPSS software package, version 13.0 (SPSS, Inc., Chicago, IL).

RESULTS

Of the 519 eligible patients, 283 (54.5%) were enrolled in TP1, 102 (19.7%) were in TP2A and 134 (25.8%) in TP2B. The characteristics of the 3 groups of patients are showed in Table 1. There was no significant difference with respect to age, gender, extent of lymphadenectomy, tumor stage, analgesic use or operation time except for type of reconstruction.

Five of the 134 (3.7%) tubeless patients (TP2B) who did not receive NG tube decompression developed vomiting or abdominal distension, and needed insertion of an NG tube for decompression (Table 2). Nineteen patients (6.7%) in the decompression group with BⅡ (TP1) and 3 patients (2.9%) in the decompression group with RY (TP2A) reconstruction required persistent NG decompression or reinsertion of an NG tube for vomiting or abdominal distension. More patients in the TP1 group needed persistent NG decompression or reinsertion of an NG tube ($P = 0.001$). In the decompression groups (TP1 and TP2A), the NG tube was removed after a mean of 5.3 d and 3.6 d, and the mean amounts of NG drainage were 465 cc and 58 cc per day in the first 3 operative days, respectively. The duration of NG decompression and the amount of NG drainage in the RY group were significantly less than in the BⅡ group ($P = 0.023$ and $P = 0.001$, respectively). Time to passage of flatus was no different for the 3 groups. However, in the tubeless RY group, time to semi-liquid diet and length of postoperative hospital stay were significantly shorter than in the other 2 groups ($P = 0.001$ and $P = 0.035$). The LOS was significantly increased in patients with BII reconstruction compared with patients with RY reconstruction with/without NG decompression (15.4 ± 4.3 d in BⅡ group *vs* 12.6 d ± 3.1 d in decompressed RY and 11.4 ± 3.4 d in the tubeless RY group, $P = 0.035$). The 27 patients who required persistent NG decompression or NG tube reinsertion were carefully examined for factors that might lead to predictive criteria for postoperative NG decompression. No factors, including analgesics use, preoperative gastric outlet obstruction, and history of diabetes, could be determined to be predictive of the need for postoperative NG decompression (data not shown).

There was no significant difference in the occurrence rate of each of the classified intra-abdominal complications in the 3 groups except for delayed gastric emptying or Roux stasis syndrome (Table 3). There were 18, 3 and 4 patients who developed delayed gastric emptying or Roux stasis syndrome in the TP1, TP2A and TP2B groups, respectively. All 25 patients were able to tolerate a normal diet from the 12th to the 43rd postoperative day without surgical treatment. The reoperation rate for early postoperative mechanical small bowel obstruction was

Table 3 Comparison of postoperative complications *n* (%)

	TP1 (*n* = 283)	TP2A (*n* = 102)	TP2B (*n* = 134)	*P* value
Perioperative mortality	1[1]	0	0	NS
Intra-abdominal complications				
Delayed gastric emptying or Roux stasis syndrome	6 (2.1)	3 (2.9)	3 (2.2)	NS
Mechanical small bowel obstruction	1 (0.4)	0	1 (0.7)	NS
Gastrojejunostomy leakage	3 (1.1)	1 (1.0)	1 (0.7)	NS
Duodenal stump leakage	2 (0.7)	0	1 (0.7)	NS
Postoperative infection				
Wound infection	10 (3.5)	3 (2.9)	4 (3.0)	NS
Unknown fever	9 (3.2)	4 (3.9)	3 (2.2)	NS
Intra-abdominal abscess	1 (0.4)	0 (0)	0 (0)	NS
Pneumonia	13 (4.6)	4 (3.9)	2 (1.4)	0.01
Severe sore throat	59 (20.7)	18 (17.4)	3 (2.1)	0.001

[1]Mortality is associated with aspiration pneumonia-related sepsis. NG: Nasogastric.

0.4% in the TP1 and 0.7% in the TP2B group. Omitting NG decompression did not increase the risk of gastrojejunostomy and duodenal stump leakage. Three patients in the TP1 group leaked from a BⅡ gastrojejunostomy and one of them developed an intra-abdominal abscess, which was treated by CT-guided percutaneous drainage. Two patients in the 2 RY groups who developed gastrojejunostomy leaks recovered spontaneously without further percutaneous drainage and did not develop intra-abdominal abscesses. Even though there were no differences in major life-threatening complications among the 3 groups, 1 patient died of aspiration pneumonia-related sepsis in the TP1 group. The postoperative pneumonia rate was lowest in the tubeless group (1.4%) and highest in the BⅡ group (1.4% *vs* 4.6%, $P = 0.01$). Fewer patients in the tubeless group complained of severe sore throat ($P = 0.001$). Severe sore throat was noted in 59 (20.7%) members of the TP1 group, 18 (17.4%) members of the TP2A group and 6 (4.2%) members of the TP2B group.

DISCUSSION

For the past century, NG decompression has been commonly thought to be necessary for patients undergoing gastric operation to protect against gastric or intestinal distension with subsequent anastomotic failure[9]. Even today, most general surgeons still follow the routine procedure[10]. Our study has demonstrated that NG decompression is not routinely required postoperatively after distal gastrectomy with RY reconstruction in patients with gastric cancer. Patients can be discharged more rapidly while tolerating semi-liquid diets without increasing postoperative complications.

Concerns regarding greater risks of anastomotic leak associated with distended gastric remnant and postoperative ileus are obstacles to the abandonment of postgastrectomy NG decompression. Historically, surgeons believed that a 3-5-d gastric decompression and fast after a gastric operation could prevent anastomotic leak resulting from increased intraluminal pressure of the postoperative atonic gastric remnant and physiologic ileus of the intestine. For radical gastrectomy, it is unavoidable that most autonomic nerve fibers controlling the upper gastrointestinal tract in the abdomen are destroyed by skeletonization of the celiac axis and lesser curvature during radical lymph node dissection. This may interfere with the motility of the gastrointestinal tract postoperatively. In addition, the bowel is much more extensively manipulated in gastric cancer surgery than in lower gastrointestinal tract surgery and may be a potential risk factor for the development of functional ileus during the early postoperative period. For these reasons, prophylactic NG decompression after operations for gastric cancer seems to be reasonable and very important. Until recently, therefore, NG intubation for gastric decompression has been a routine part of perioperative care after radical gastrectomy. However, the necessity of NG decompression after gastric surgery has been increasingly questioned over the past 2 decades. Studies regarding gastric decompression after gastric cancer surgery are very rare, because surgeons are concerned that swallowed saliva and gastric secretion can make direct contact with the anastomotic wound and consequent anastomotic disruption. Anastomotic disruption is a potentially fatal complication, and may lead to severe morbidity and mortality when it happens. Four prospective studies from Taiwan[1,2] and South Korea[3,4] have suggested that it is unnecessary to decompress the gastric remnant after gastrectomy for gastric cancer. Another European multicenter prospective study has also been performed to assess the use of a nasojejunal tube after total gastrectomy and the authors recommended that no use of postoperative NG decompression decrease postoperative fever and pulmonary problems, and improved patient comfort by decreasing sore throat and nausea[5]. However, these studies enrolled gastric cancer patients undergoing a variety of operations, such as total and subtotal gastrectomy. Our study focused on distal gastrectomy with BⅡ or RY reconstruction for gastric cancer and tried to abandon the routine use of NG decompression after distal gastrectomy. It did not increase the rates of intra-abdominal morbidities such as anastomotic leakage compared with the decompression group. Moreover, in our study, 5 of the 134 patients without NG decompression required reinsertion of the NG tube due to vomiting or abdominal distension. None developed anastomotic leaks. Temporary gastric remnant distension did not seem to disrupt anastomosis in patients receiving distal gastrectomy with RY reconstruction.

Interestingly, 2 patients in the RY groups who suffered from gastrojejunostomy leaks healed spontaneously with the drains placed during the operation and did not develop intra-abdominal abscesses, which means RY reconstruction may lower the risk of intra-abdominal abscess after anastomotic leakage. This may be due to a decreased amount of leaked fluid from the gastric remnant immediately following distal gastrectomy with RY reconstruction, which reduced the amount of gastric remnant content.

The magnitude of gastric remnant secretory output immediately following distal gastrectomy is unknown. In lower digestive tract surgery, the average volume of gastric juice in patients with gastrointestinal decompression was 200 mL daily postoperatively[11]. Normally, the daily secretion of gastric juice and saliva ranges between 2300 and 3000 mL[12]. Secretion of saliva and gastric juice is under the control of the autonomic nervous system. Surgical transection of the vagus nerve during radial subtotal gastrectomy may decrease salivatory and gastric secretion postoperatively. Our results showed that after distal gastrectomy with BⅡ reconstruction and RY reconstruction, the average daily outputs of gastric remnant drainage were 465 cc and 58 cc, respectively. Therefore, with RY reconstruction, the reason for the decreased drainage amount from the gastric remnant is that not only the salivatory and gastric remnant secretions decrease, but the pancreatic and biliary secretions could be diverted from the gastric remnant[6]. This is why there is no need for NG decompression in the patients with RY reconstruction compared with those with BⅡ reconstruction.

The avoidance of complications associated with NG decompression is another potential benefit of our approach. Most patients complained of discomfort secondary to NG intubation. This discomfort included sore throat, hoarseness, dysphagia, nasal trauma, sinusitis, and psychological problems[13,14]. Use of NG decompression also increased the risk of respiratory complications. Several studies indicated that NG intubation increased the risk of atelectasis and pneumonia[15,16]. The ability of patients to cough and breathe deeply after surgical intervention is severely compromised by discomfort from an NG tube. In addition, NG intubation causes gastroesophageal reflux, increasing the risk of postoperative pneumonia[17]. In our study, the difference in postoperative pneumonia (1.4% without NG *vs* 3.9% and 4.6% with NG, $P = 0.05$) and severe sore throat (2.1% without NG *vs* 20.7% and 17.4% with NG, $P = 0.01$) reached statistical significance.

In Taiwan, the use of the NG tube to decompress the gastric remnant after distal gastrectomy is still in widespread use by most general surgeons. It is well known that changing common practice in hospitals is difficult and at all levels resistance is usually abundant. In fact, our study showed that a minimal percentage of patients with distal gastrectomy with RY reconstruction required gastric decompression for relieving gastric distension, and the vast majority of patients with RY anastomosis did not need NG decompression after distal gastrectomy and could avoid the discomfort and morbidity associated with NG intubation. The data from the present study not only confirmed that placement of an NG tube can be safely omitted in distal gastrectomy with RY anastomosis, but also demonstrated that routine NG decompression may increase postoperative complications, such as pulmonary infection and pharyngolaryngitis. Our study comprises the largest reported series of patients undergoing a single type of gastrojejunal anastomosis, which provides a large enough series to support the avoidance of NG decompression after distal gastrectomy with RY reconstruction as a safe and effective modification of standard surgical practices.

There are several limitations to this study that are inherent in the source of our data. First, the retrospective nature of this study analysis limits the ability to attribute causality. Second, the comparison of BⅡ and Roux-Y is within different time periods. There could have been several events during such long periods. Third, the comparison between the nasal decompression group and non-decompression group in the latter phase was not randomized. However, our data show that the magnitude of gastric remnant content decreases immediately following subtotal gastrectomy with RY reconstruction. NG decompression offers no benefit for patients and increases patient discomfort and potential NG intubation-related morbidity, and it can therefore be omitted as a routine procedure in gastric cancer patients with distal gastrectomy and RY anastomosis. Further larger-scale properly designed prospective studies, ideally having validated data collections, will enable us to clearly determine the risks and/or benefits in naso-decompression after gastric cancer surgery.

COMMENTS

Background

The nasogastric (NG) decompression after gastric surgery is essential to prevent postoperative complications conventionally. However the necessity of NG decompression following gastric surgery has been increasingly questioned over the last 2 decades.

Research frontiers

Several prospective studies have suggested that the routine NG decompression after gastric surgery does not provide any benefit. Roux-en-Y (RY) reconstruction has become widely used after distal gastrectomy to prevent enterogastric reflux into the gastric remnant and decrease biliary gastritis.

Innovations and breakthroughs

The authors' hospital has performed RY reconstruction in all gastric cancer patients with distal gastrectomy since January 2003. Benefits of this approach include the reduction of the amount of drained gastric remnant content *via* NG tube. In January 2005, the authors abandoned the routine use of NG tube decompression postoperatively in patients undergoing distal gastrectomy.

Applications

The data show that the magnitude of gastric remnant content decreases immediately following subtotal gastrectomy with RY reconstruction. NG decompression offers no benefit for patients and increases patient discomfort and potential NG intubation-related morbidity, and it can therefore be omitted as a routine procedure in gastric cancer patients with distal gastrectomy and RY anastomosis.

Terminology

Nasogastric decompression: placement of a tube into the stomach through the nose to remove stomach contents; Roux-en-Y gastrojejunostomy: distal limb of jejunum is brought up through the mesocolon in a retrocolic fashion, and an end-to-side gastrojejunostomy is made, using a running inner layer of 3-0 absorbable suture and an interrupted outer layer of 3-0 silk Lambert sutures.

Peer review

The manuscript on the whole is well written. This study focused on the possibility of omitting nasogastric tubes in Roux-en-Y reconstruction after gastric cancer operation. It is well organized and very practical.

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S- Editor Lv S **L- Editor** O'Neill M **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i3.257

World J Gastroenterol 2012 January 21; 18(3): 257-267
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Microencapsulated tumor assay: Evaluation of the nude mouse model of pancreatic cancer

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Supported by The Science and Technology Commission Foundation of Shanghai, No. 09140902300; and the Municipal Education Commission Foundation of Shanghai, No. 09YZ84
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Received: June 27, 2011 Revised: August 26, 2011
Accepted: September 2, 2011
Published online: January 21, 2012

Abstract

AIM: To establish a more stable and accurate nude mouse model of pancreatic cancer using cancer cell microencapsulation.

METHODS: The assay is based on microencapsulation technology, wherein human tumor cells are encapsulated in small microcapsules (approximately 420 μm in diameter) constructed of semipermeable membranes. We implemented two kinds of subcutaneous implantation models in nude mice using the injection of single tumor cells and encapsulated pancreatic tumor cells. The size of subcutaneously implanted tumors was observed on a weekly basis using two methods, and growth curves were generated from these data. The growth and metastasis of orthotopically injected single tumor cells and encapsulated pancreatic tumor cells were evaluated at four and eight weeks postimplantation by positron emission tomography-computed tomography scan and necropsy. The pancreatic tumor samples obtained from each method were then sent for pathological examination. We evaluated differences in the rates of tumor incidence and the presence of metastasis and variations in tumor volume and tumor weight in the cancer microcapsules *vs* single-cell suspensions.

RESULTS: Sequential *in vitro* observations of the microcapsules showed that the cancer cells in microcapsules proliferated well and formed spheroids at days 4 to 6. Further *in vitro* culture resulted in bursting of the membrane of the microcapsules and cells deviated outward and continued to grow in flasks. The optimum injection time was found to be 5 d after tumor encapsulation. In the subcutaneous implantation model, there were no significant differences in terms of tumor volume between the encapsulated pancreatic tumor cells and cells alone and rate of tumor incidence. There was a significant difference in the rate of successful implantation between the cancer cell microencapsulation group and the single tumor-cell suspension group (100% *vs* 71.43%, respectively, $P = 0.0489$) in the orthotropic implantation model. The former method displayed an obvious advantage in tumor mass (4th wk: 0.0461 ± 0.0399 *vs* 0.0313 ± 0.021, $t = -0.81$, $P = 0.4379$; 8th wk: 0.1284 ± 0.0284 *vs* 0.0943 ± 0.0571, $t = -2.28$, respectively, $P = 0.0457$) compared with the latter in the orthotopic implantation model.

CONCLUSION: Encapsulation of pancreatic tumor cells is a reliable method for establishing a pancreatic tumor animal model.

Key words: Nude mice; Model of pancreatic neoplasms; Encapsulation; Subcutaneous implantation model; Orthotopic implantation model

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Ma MZ, Cheng DF, Ye JH, Zhou Y, Wang JX, Shi MM, Han BS, Peng CH. Microencapsulated tumor assay: Evaluation of nude mouse model of pancreatic cancer. *World J Gastroenterol* 2012; 18(3): 257-267 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i3/257.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i3.257

INTRODUCTION

Pancreatic adenocarcinoma (PA) is an aggressive malignancy that is more common in elderly persons than in younger persons, and less than 20% of patients present with localized, potentially curable tumors. The overall 5-year survival rate among patients with pancreatic cancer is < 5%[1]. Despite advances in chemoradiation and adjuvant chemotherapy, chemotherapeutic options prolong life only minimally[2]. Therefore, animal models that allow further examination of pancreatic cancer progression are urgently needed.

The majority of *in vivo* experimental studies of human cancer have been conducted in subcutaneous, orthotopic and heterotopic implantation nude mouse models. Current animal models of PA have low rates of tumor progression and metastases[3]. A variety of techniques for inducing pancreatic cancer growth in immunodeficient mice have been described, and each is associated with potential shortcomings. We hypothesized that the delivery of tumor cells in three-dimensional microcapsules could overcome these shortcomings by providing a contained, growth-enhancing environment in which tumor cells can propagate. Therein, the tumor cells are likely to grow well or even progress to metastatic disease. Matrigel, a widely-used extracellular matrix, has severe technical limitations such as lot-to-lot variability and handling difficulties during the injection of cell suspensions. Since the introduction of alginate-poly-l-lysine-alginate membranes as an immunoisolation device by Chang *et al*[4] in 1966, further studies have been conducted on the use of microencapsulation in cancer therapies using immunodeficient animals[5]. The first stage of this study compared the growth of tumor cells in microcapsules with the growth of cells alone. Preclinical testing of novel therapeutic strategies in animal models also requires a meticulous assessment of the effects of treatment on local and systemic tumor growth. The second stage of this study evaluated local tumor progression and the systemic spread of tumor cells in microcapsules *vs* those with single tumor cells.

MATERIALS AND METHODS

Materials

Laboratory animals: In total, 82 82 nude mice (BALB/c nu/nu), between 4 and 6 wk of age, weighing 18-20 g, half males and half females, were purchased from Shanghai Laboratory Animal Co., Ltd and were kept in a specific pathogen-free laboratory. All the procedures and observations were performed in the laboratory animal center at the Shanghai JiaoTong University School of Medicine. All studies were conducted with the approval and guidance of Shanghai Jiao-Tong University Medical Animal Ethics Committees (approval ID: 2010060).

Cell lines: The undifferentiated human pancreatic cancer cell line MiaPaCa-2 was obtained from the Shanghai Institute of Digestive Surgery at the Ruijin Hospital, which is affiliated with the Shanghai JiaoTong University School of Medicine.

Encapsulation device: A high-frequency pulse microdroplet generator (Figure 1) was provided by the Biological Engineering Institute of the University of Shanghai for Science and Technology.

Animal *in-vivo* imaging system: An Inveon micro positron emission tomography-computed tomograph (PET-CT) was purchased from Siemens Ltd.

Methods

Cell culture: MiaPaCa-2s were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY). Cells were cultured at 37 ℃ in an incubator with 50 mL/L CO_2. MiaPaCa-2 cells in logarithmic growth phase were harvested with 0.25% trypsin, centrifuged at 500 *g* for 15 min, washed 3 times in phosphate buffered saline (PBS) at 4 ℃ and the cell number was adjusted to 1×10^7 cells/mL.

Tumor cell encapsulation: The encapsulation process begun with the preparation of a suspension of tumor cells in 1.8% sodium alginate with Matrigel (BD Bioscience, Bedford, MA) in a 25% (v/v) at a final concentration of 2×10^7 cells/mL. Matrigel can accelerate the proliferation of the cell line. The cell-alginate mixture is then extruded from a 31-gauge needle at 4 mL/min to yield spherical microdroplets using a high frequency pulse microdroplets generator (Mode 1, Voltage 60, Frequency 90, Pulse 06). The microdroplets are formed into discrete insoluble gel spheres by immersion in 1.1% $CaCl_2$ to the neutralize of the negative charge on the surface of the microdroplet, causing gelation. The next step involves the formation of a semipermeable membrane on the surface of the gel beads by washing them with 0.05% (w/v) poly-L-lysine. The gel beads are then overcoated with 0.1% (w/v) alginate. The interior gel is liquefied by contact with a solution of 3% (w/v) sodium citrate. This process results in microcapsules with semipermeable membranes containing viable tumor cells in liquid suspension are made[5-8] (Figure 2A). Cancer

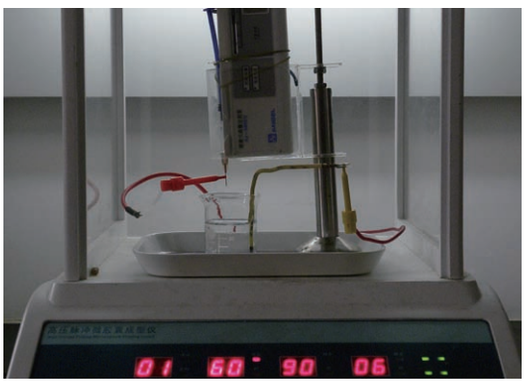

Figure 1 High frequency pulse microdroplet generator.

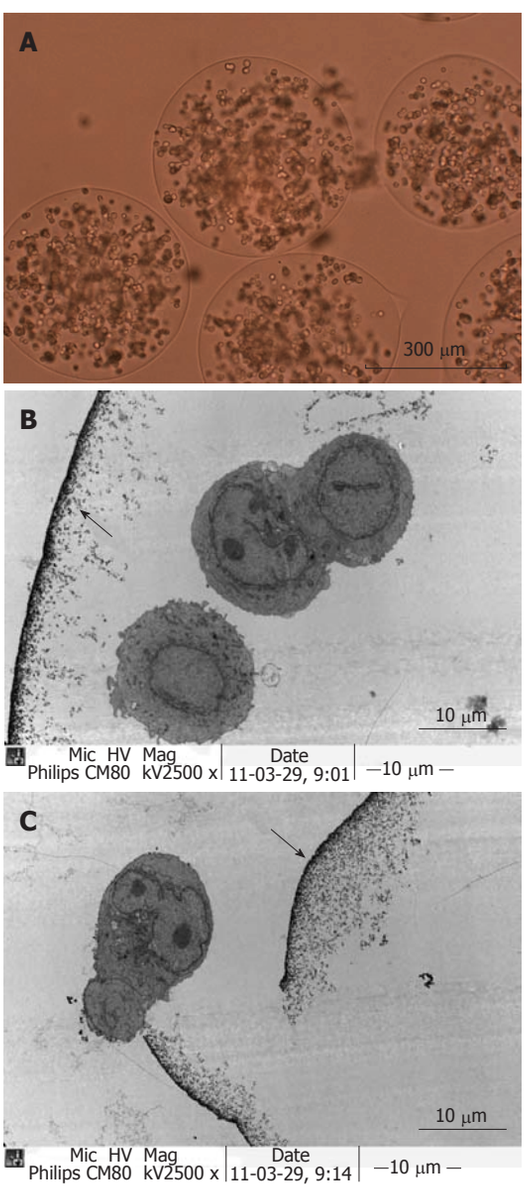

Figure 2 *In vitro* culture of cancer microcapsules after 5 to 7 d of *in vitro* incubation. A: Cancer microcapsules were filled with proliferated cells forming spheroids (× 100); B: Transmission electron microscope observation of encapsulated tumor cells (× 2500); C: Transmission electron microscope observation of encapsulated tumor cells after membrane burst (× 2500). Arrows are pointing to membrane of the microcapsule in B and C.

microcapsules were incubated in complete medium *in vitro* at 37 ℃ with 50 mL/L CO_2 to ensure that they were viable at the time of administration to the mice. The complete medium was removed and PBS was added to prepare a 50% (v/v) suspension of microcapsules.

Groups: The test groups were defined as follows, each consisting of 26 nude mice (half males and half females): The single-cell suspension group received single tumor-cell (MiaPaCa-2) suspensions. The cancer-cell microencapsulation group received encapsulated pancreatic tumor cells. The negative control group received saline instead of the tumor cells. As a positive control group, four additional mice were given intraperitoneal injections (IP) of tumor-cell suspensions to determine whether the metastases originated from cell contamination or genuine metastasis.

Establishing a subcutaneous implantation model for each group: Each of twelve nude mice was injected with 100 μL of the encapsulated tumor cell suspensions as described above, into the left flanks. Another twelve nude mice were injected with 100 μL of single tumor cell suspensions as described above, into the right flanks. Twelve nude mice were injected with saline as a negative control group.

Monitoring subcutaneous xenografts: Subcutaneous xenograft progression was measured with a vernier caliper on a weekly basis. Tumor volume was calculated according with the formula V = length × width × depth/2[9]. The growth curves were constructed accordingly.

Establishing an orthotopic implantation model for each group: Fourteen nude mice were employed for the injection of single tumor cell suspensions. For the tumor induction surgery, they were anesthetized by IP injection of 0.3% chloral hydrate (0.3 g/kg). All procedures were done under aseptic conditions in a laminar airflow workstation. The tail of the pancreas was gently exteriorized through a midline incision into the peritoneal cavity. Mice received a subcapsular injection of 50 μL of single tumor cells. A technically successfully injection was characterized by the formation of a visible bubble within the pancreatic parenchyma. The needle was slowly withdrawn to avoid macroscopic cell leakage from the injection site. The pancreas was closed with 7-0 Prolene. The abdomen was closed using interrupted 6-0 silk sutures closing both skin and muscle simultaneously.

Likewise, fourteen nude mice were injected subcapsularly with encapsulated pancreatic tumor cells 50 μL as described above, and another fourteen nude mice were additionally injected subcapsularly with saline as a negative control group. To determine whether the metastasis was seeding from tumor cell suspensions or were true metastasis originated from primary tumor development, four additional mice were given IP injections of tumor cell suspensions (1×10^7 cells/mL, 100 μL).

PET-CT scan and necropsy: Twenty-eight orthotop-

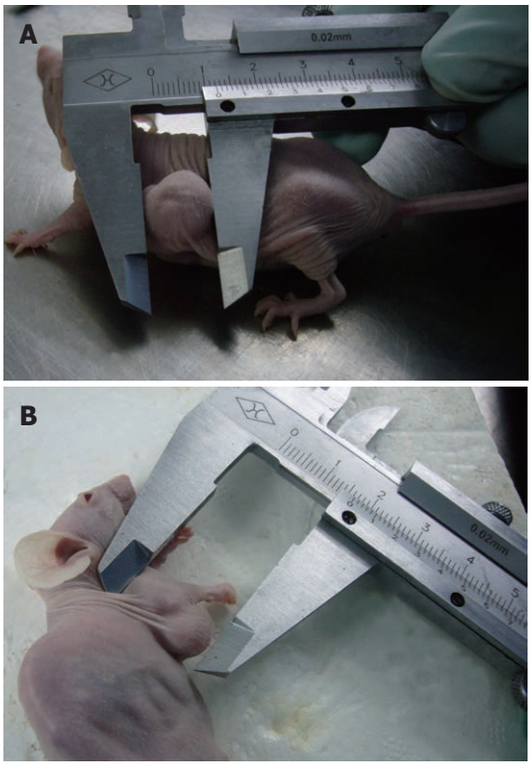

Figure 3 Progression of subcutaneous implantation tumor 8 wk after injection. A: Cancer cell microencapsulation group; B: Single cell suspensions group.

ically-injected mice, seven from the single tumor-cell suspension group and seven from the tumor-cell microencapsulation groupreceived PET-CT scans and were respectively sacrificed at at 4 and 8 wk post implantation, respectively, to ascertain the extent of metastasis and determine tumor weight. Whole-body imaging was used for primary tumor assessment *via* ^{18}fluorine-fluorinedeoxyglucose; necropsy was used for identification. Isolated tumor nodules with no anatomic connection to the primary tumor were judged as distant metastases.

To evaluate the occurrence of metastasis, organs and other areas, i.e., liver, kidney, spleen, mesentery, peritoneal cavity, injection site and lymph nodes, were carefully examined macroscopically. Any suspicious lesion was removed and subjected to histological analysis.

Ultrastructural observation: Microencapsulated tumor cells were sampled and fixed with 2.5% glutaraldehyde, 1% osmic acid, alcohol and epoxy. The samples were then sent for evaluation by transmission electron microscopy (JEM-1200EX) (Figure 2B and C).

Pathological examination: Specimens were embedded in paraffin, cut into 4-mm serial sections, stained with hematoxylin and eosin and tagged with the following immunohistochemical markers: AE1AE3, carbohydrate antigen 19-9 (CA199), CAM5.2, epidermal growth factor receptor (EGFR), MIB-1 and VIM.

Statistical analyses

Differences in the rates of tumor incidence and the presence of metastasis between the cancer-cell microencapsulation group and the single-cell suspension group were analyzed using Fisher's exact test. Variations in tumor volume and tumor weight between the cancer-cell microencapsulation group and the single-cell suspension group were analyzed using student's *t*-test. Differences in tumor weight were also analyzed with the Wilcoxon signed rank sum test. $P < 0.05$ was considered statistically significant. Statistical calculations were done with the SAS 8.0 statistical software package.

RESULTS

In vitro culture of cancer microcapsules

The size of cancer microcapsules engineered in this study were uniform in size. The average diameter of 20 randomly sampled microcapsules was 420 μm ± 24 μm.

The microcapsules are completely transparent, and cell growth within them can be readily visualized with the use of an inverted microscope. Sequential *in vitro* observations of microcapsules showed that the cancer cells within them proliferated well and formed spheroids from days 4 to 6. We observed that all microcapsules containing cancer cells proliferated in a three-dimensional manner. The microcapsule membranes *in vitro* burst with increasing culture time, and the cells deviated outward and continued to grow in the flasks. The bursting day was defined as the day on which more than 10% of the microcapsules burst. Two or three days before the bursting day was assumed to be the optimal time for injection. Typically, bursting occurred from day 6 to 8. Therefore, the microcapsules were injected at 5 d after tumor cell encapsulation. A total of 100 cancer cell microcapsules were observed in sequence. The proportion of ruptured microcapsules increased to 65% (65 of 100) at 10 d and 100% at 28 d.

Rate of tumor incidence in subcutaneous implantation model

Gray nodules growing in round or elliptical patterns were found at the injection site 3 to 5 d after implantation. All mice remained viable during 8 wk of sequential observations; two from the single tumor-cell suspension group (2/12) and one from the encapsulated tumor-cell group (1/12) presented no detectable tumors. None of the negative control group presented detectable tumors. The rate of tumor incidence was 83.3% in the single-cell suspension group and 91.67% in the cancer-cell microencapsulation group. There was no significant difference in the rate of tumor incidence between the cancer-cell microencapsulation group and the single-cell suspension group in the subcutaneous implantation model (91.67% *vs* 83.3%, respectively, $P = 0.5371$) (Figure 3A and B).

Progression of subcutaneous implantation tumor

The tumor size was measured with vernier calipers, and the tumor volume was calculated on a weekly basis (Table 1). There was no significant difference in terms of tumor volume between the encapsulated pancreatic tumor-cell

Table 1 Tumor's volume of subcutaneous implantation

At day post-implantaion (cm^3)	Tumor volume							
	4th d	11th d	18th d	25th d	32th d	39th d	46th d	53th d
Tumor volume of single cells	0.054 ± 0.038	0.092 ± 0.055	0.139 ± 0.082	0.267 ± 0.155	0.596 ± 0.296	1.188 ± 0.570	2.020 ± 0.989	2.602 ± 1.100
Tumor volume of microencapsulated cells	0.060 ± 0.070	0.083 ± 0.097	0.145 ± 0.140	0.316 ± 0.278	0.758 ± 0.655	1.316 ± 0.927	2.266 ± 1.307	2.794 ± 1.310

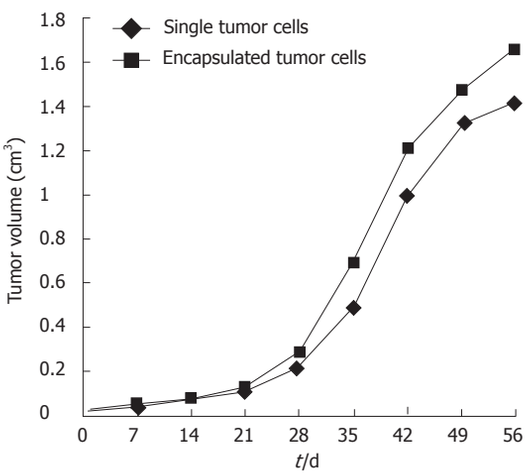

Figure 4 Growth curve of subcutaneous implantation tumor volume.

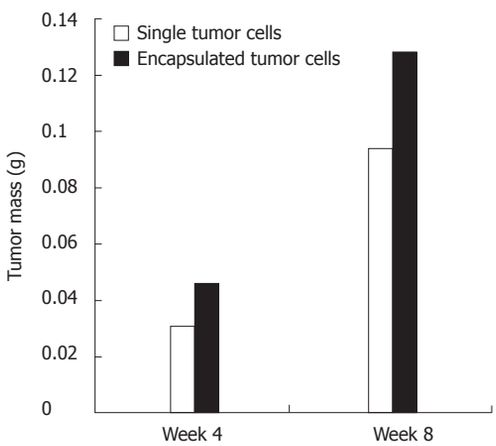

Figure 5 Tumor weights at different stages. Group of microencapsulation had statistically larger tumors at 8th wk compared with single tumor cells group yet no statistical differences at 4th wk.

group and the single tumor-cell group (6th wk: 1.316 ± 0.927 *vs* 1.188 ± 0.570, respectively, $P = 0.5348$, $P > 0.05$; 7th wk: 2.266 ± 1.307 *vs* 2.020 ± 0.989, $P = 0.5467$; 8th wk: 2.794 ± 1.310 *vs* 2.602 ± 1.100, $P = 0.5988$). The growth curve for the subcutaneous implantation tumors is shown in Figure 4.

Assessment of orthotopic implantation tumor progression

No nude mice orthotopically injected with microencapsulated cancer cells or single cancer-cell suspensions showed any signs of transplantation-related complications. No obvious changes were observed in the first four weeks postimplantation. Six weeks after implantation, the mice displayed marked abdominal distension with palpable abdominal masses. Symptoms such as obvious weight loss and anorexia were observed in nude mice injected with microcapsules or single cancer cells during the 8th wk postimplantation. As expected, no symptoms were observed in the negative control group.

It should be noted that 4 out of 14 mice from the single-cell suspension group (2 of 7 at the 4th wk and 2 of 7 at the 8th wk) in the single cell suspension group had sidewall implants. These implants were located on abdominal wall and appeared as groups of gray nodules around the site of injection; they were not counted as metastases. None of the microcapsule group had sidewall implants. The orthotopic implantation model was successfully established; the rate of successful implantation was 71.43% (10 of 14) in the single-cell suspension group and 100% (14 of 14) in the cancer-cell microencapsulation group. There was a significant difference (100% *vs* 71.43%, $P = 0.0489$) in the success rates of the cancer-cell microencapsulation and single-cell suspension groups. As a control, four additional mice were given IP injections of tumor cell suspensions (1×10^7 cells/mL 100 μL) to determine whether the metastasis was caused by seeding or represented true metastasis. These IP-injected cells resulted in tumors attached to the abdominal wall at various locations within the abdomen. However, none of the free IP injections resulted in tumor sites similar to those of the metastases from the orthotopically injected cells, such as the mesentery.

At the 4th wk postimplantation there were no statistical differences between the two groups in terms of tumor mass (0.0461 ± 0.0399 *vs* 0.0313 ± 0.021, $t = -0.81$, $P = 0.4379$). However, the microcapsules had significantly heavier tumors compared to the single tumor cells at eight weeks (0.1284 ± 0.0284 *vs* 0.0943 ± 0.0571, $t = -2.28$, $P = 0.0457$) (Figure 5). Another calculation method showed the same results (Table 2).

In terms of metastases, one mouse in the microcapsule group exhibited tumor spread to the small bowel mesentery (Figure 6C and D) at the 8th wk, which correlated with the PET-CT (Figure 7). No mice in either group had visible metastatic foci at the 4th week. At the 8th week, two mice in the microencapsulated group displayed metastatic spreading of the tumor to sites distant from the pancreas, which included the mesentery.

The histological characteristics of the primary tumors in the two groups exhibited similar morphologies at the 4th and 8th wk. Histopathological examinations confirmed that the suspected metastasis spots in the PET-CT truly originated from primary tumors. Immunohistochemical

Table 2 Number of mice related to the weight of tumor mass at different stages

Different stage (wk)	Group	No. of mice	No. of tumor mass > 500 mg	No. of tumor mass > 1000 mg	Z[1]	*P* value[1]
4th	Single cells	5	1	0	1.7618	0.0643
	Microencapsulation	7	2	3		
8th	Single cells	5	2	1	2.1971	0.0221
	Microencapsulation	7	1	6		

Data indicate the number of mice in each group that exhibited the characteristic listed. [1]Got by Wilcoxon signed rank sum test.

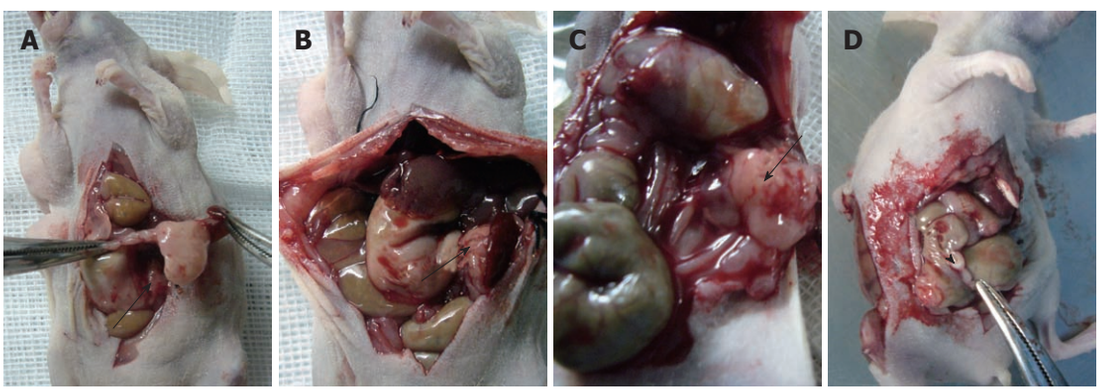

Figure 6 Orthotopic implantation tumor production. A: Single tumor cells at 4th wk post-implantation; B-D: Microencapsulated tumor cells at 8th wk post-implantation. Arrows: Primary tumor; Arrow head: Metastasis lesion.

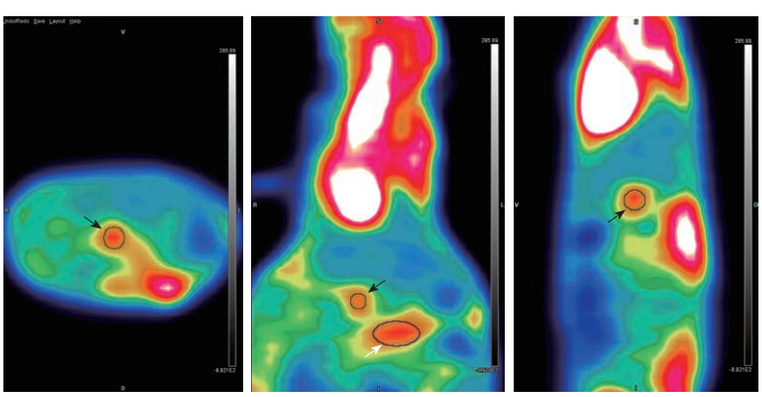

Figure 7 Positron emission tomography-computed tomography scan of mice from tumor cell microencapsulation group. White arrow points to the primary tumor, and black arrows point to metastasis lesions.

staining showed that tumors arising from both microencapsulated cells and single cells exhibited similar pathological characteristics: AE1AE3 (+), CAM 5.2 (+), EGFR (+), MIB-1 (60% +), CA199 (±), and VIM (-) (Figures 8-14).

DISCUSSION

Types of implantation nude mouse model

The majority of *in vivo* experimental studies of human cancer have been conducted *via* subcutaneous, orthotopic and heterotopic implantation nude mouse models. The subcutaneous model has the virtues of simplicity of operation, and ease of tumor assessment. Although subcutaneous xenografts generally grow well and may be phenotypically malignant locally, they tend to form pseudocapsules, resembling encapsulated benign tumors rather than infiltrative malignancies, even when derived from undifferentiated or poorly differentiated aggressive cell lines. Importantly, subcutaneously injected human cancer cells only rarely metastasize to the parenchymatous organs and do not display the signs and symptoms that may arise as a consequence of tumor growth within visceral organs[9-14]. Thus, the subcutaneous model is of limited clinical value.

Orthotopic tumor induction is regarded as being quite suitable for modeling clinical pathology because it mimics the entire process of primary tumor growth, local tumor infiltration, and subsequent distant metastatic spread. Previous studies have shown that injection of cell line suspensions[15-18] and the transplantation of tissue fragments from primary pancreatic tumors[19-21] into the pancreas of

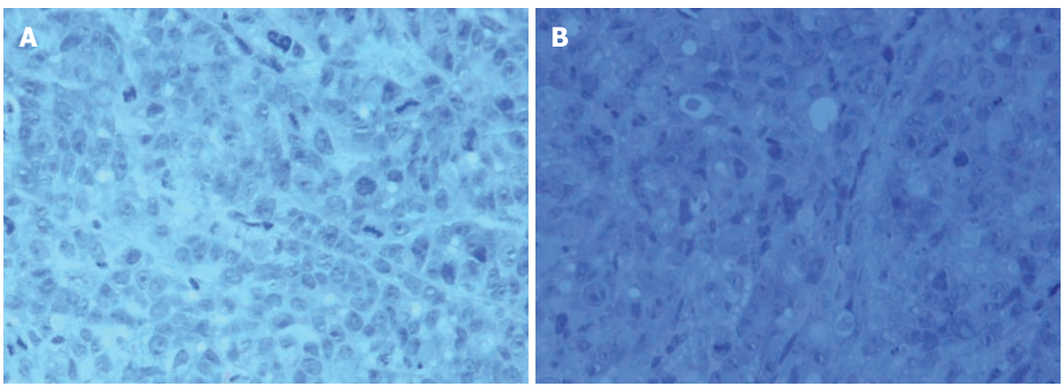

Figure 8 Hematoxylin and eosin staining of tumors. A: Single tumor cells; B: Microcapsules.

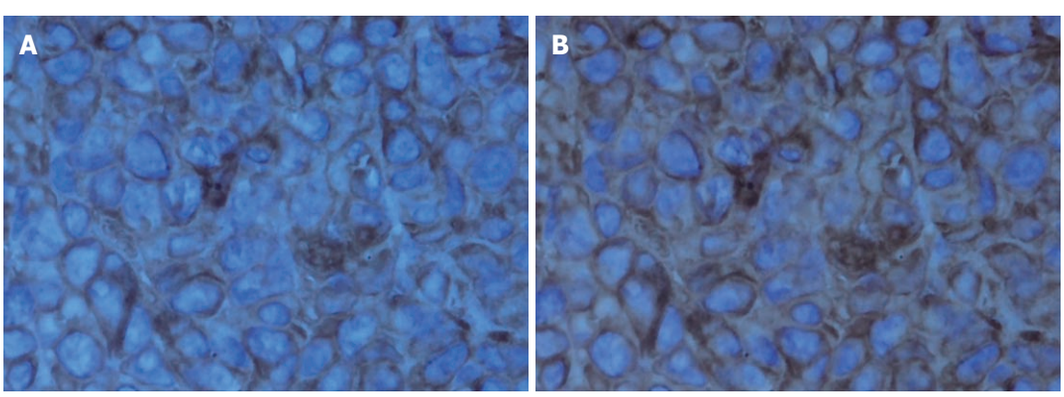

Figure 9 AE1AE3 immunohistochemical staining of tumors. A: Single tumor cells, AE1AE3 (+); B: Microcapsules, AE1AE3 (++).

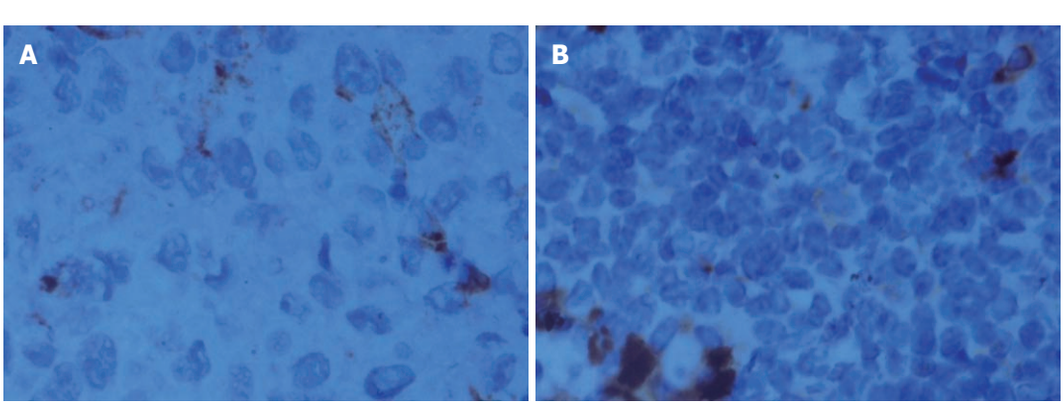

Figure 10 Carbohydrate antigen 19-9 immunohistochemical staining of tumors. A: Single tumor cells, carbohydrate antigen 19-9 (a few +); B: Microcapsules, CA199 (a few ++).

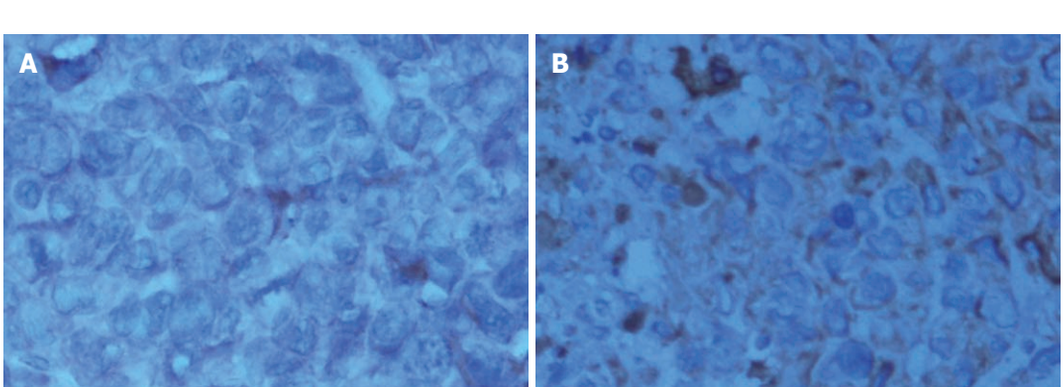

Figure 11 CAM 5.2 immunohistochemical staining of tumors. A: Single tumor cells, CAM 5.2 (+); B: Microcapsules, CAM 5.2 (+).

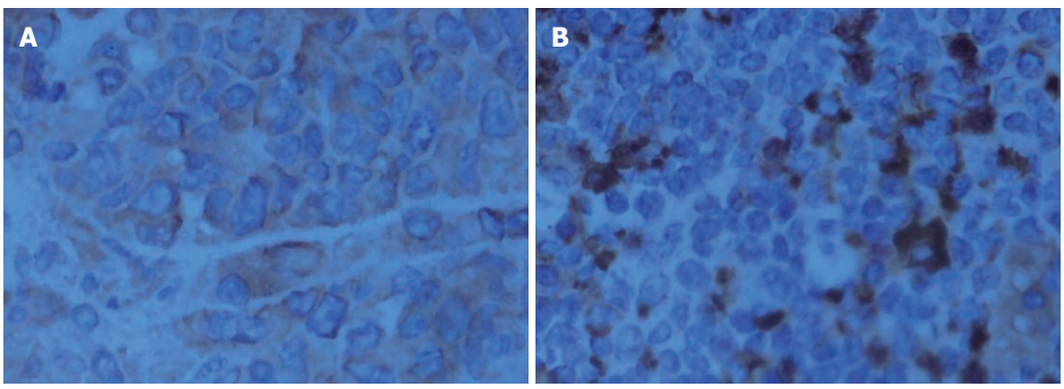

Figure 12 Epidermal growth factor receptor immunohistochemical staining of tumors. A: Single tumor cells, epidermal growth factor receptor (EGFR) (+); B: Microcapsules, EGFR (++).

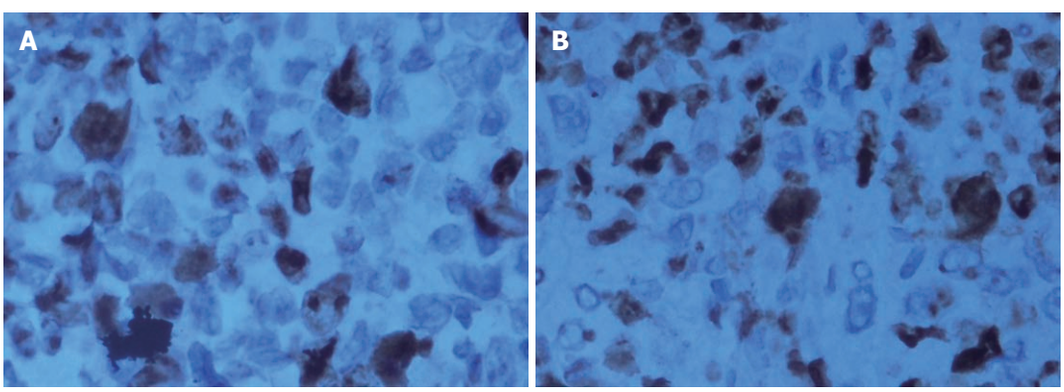

Figure 13 MIB-1 immunohistochemical staining of tumors. A: Single tumor cells, MIB-1 (60%); B: Microcapsules, MIB-1 (60%).

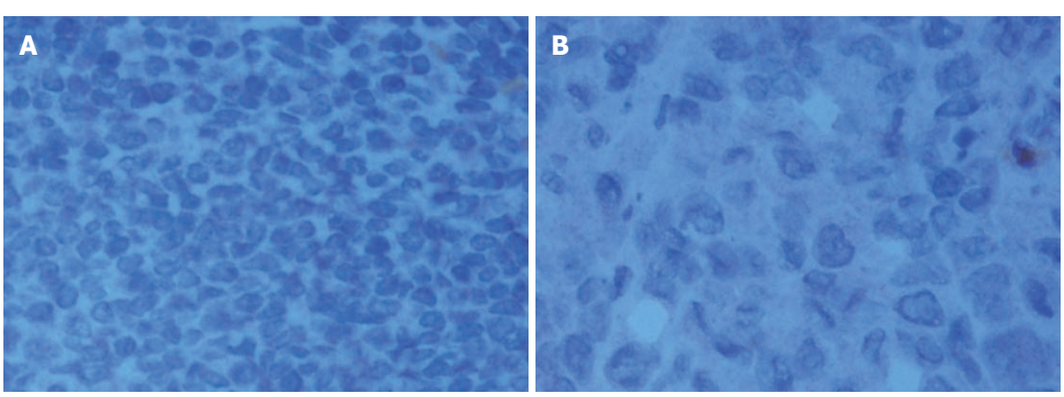

Figure 14 VIM immunohistochemical staining of tumors. A: Single tumor cells, VIM (-); B: Microcapsules, VIM (-).

nude mice or Syrian golden hamsters generally result in a high degree of tumorigenicity and the establishment of metastases, albeit at a varying rates[13,22]. Implantation of intact tumor fragments yields a higher rate of tumor incidence and avoids the artificial intraabdominal cell seeding associated with direct injection of pancreatic cancer cells[13].

In a clinical setting, patients with positive prognostic factors such as complete resection of the tumor and the absence of lymph node involvement or vascular invasion often succumb to metastatic disease, suggesting that metastasis may occur early in the course of the disease. Therefore, animal models that allow for the examination of cellular mechanisms in the early stage of pancreatic cancer progression are needed. Viable tumor implantation requires the preparation of subcutaneous donor tumors. Thus, implantation is more expensive and time consuming than the injection of cell-line suspensions. Moreover, transplantation of tissue fragments skips the early stage of tumor progression because the primary tumor does not develop orthotopically. Thus, the merits of injecting encapsulated tumor cells manifest themselves in a high rate of tumorigenicity and the establishment of metastases, a low rate of sidewall implants, and the ability to

mimic the early progression of tumor cells.

Advantages of the nude mouse model with microencapsulated cancer cells

Recently, immunoisolation technology has attracted increasing attention. Microcapsules were originally used for cell transplantation as an immunoisolation device[23]. The cell encapsulation techniques play a vital role in modern science. Microcapsules are spherical, with diameters that can be controlled in the range of 200-1500 μm, and feature a biocompatible semipermeable membrane that allows the bidirectional diffusion of nutrients, oxygen, secreted therapeutic products, and waste but prevents the passage of high-molecular-weight substances into the microcapsule, e.g., antibodies and immunocytes[24]. Immunodeficient mouse hosts were employed in our study; their immune systems were suppressed artificially. The mice did not exclude human tumor cells, which resulted in a higher rate of tumor incidence. Therefore, the immunoisolation function of microcapsules is not the key to this study. Most importantly, microcapsules in our study provide contained environments in which tumor cells grew in a three-dimensional manner and adjusted to the intravital environment of the host so that more viable tumor cells proliferated in the host and deviated to form tumor nodules.

Because nude mice are relatively expensive, delicate, and highly susceptible to infection, breeding and experimental conditions are strict, which means that nude mice cannot be used for large-scale studies. In large-scale studies with conventional mice, large quantities of tumor cells could be protected from immune eradication using tumor-cell encapsulation technology. Cancer cells would be efficiently protected by the outer layer of the microcapsule and could undergo interaction with the host in the process of initial administration and growth. Furthermore, prior studies have confirmed that encapsulated tumor cells are more viable than single cells, and the stability of tumor-associated genes is not affected[5,24].

The coupling of extracellular matrix (Matrigel) to alginate microcapsules enhanced cell proliferation by triggering a cascade of intracellular signaling events through cell-matrix interactions[25-27]. Due to the cell-matrix interactions, more membrane antigens such as AE1AE3 and ERGF may be present in the microencapsulated tumor cell membrane, as illustrated in Figures 9 and 12. Thus, more viable and adaptable cells are expected to proliferate during the primary period before bursting. Under our conditions, the cancer cells finally deviated outward and continued to grow in the flasks, unlike isolated cancer cells derived from a monolayer culture system. Thus, microencapsulated tumor cells are more likely to form tumor nodules.

Our experiments showed that in the subcutaneous implantation model, there were no statistical differences in terms of tumor volume and the rates of tumor incidence between the cancer-cell microencapsulation group and the single-cell suspension group. The former method displayed an obvious advantages in tumor mass (P = 0.0457, $P < 0.05$) and the rate of model success (P = 0.0489, $P < 0.05$) compared with the latter in the orthotopic implantation model. Additionally, it is impossible to distinguish sidewall implants from metastases using current pathological examinations. It is also very difficult to distinguish sidewall implants from metastases macroscopically in the advanced stage of pancreatic cancer. The fourth or eighth week postimplantation was not yet the terminal stage of pancreatic cancer according to the results of our experiments. Therefore, it was possible to distinguish seeding from true metastasis by the number and distribution of tumor nodules. Intraperitoneal tumor dissemination was an early event and macroscopically more extensive after injection and was most likely initiated by cells lost during the injection procedure. Thus, sidewall implants usually presented as clusters of gray nodules. For mice without distant tumors spreading to visceral organs such as the liver or mesentery at the 4th to 8th wk postimplantation, the tumor nodules described above can be seen as sidewall implants. During our experiments, we observed sidewall implants in four mice from the single-cell suspension group and did not count them as successful implantations. The primary modes for the spreading of tumors to distant sites are lymphatic and hematogeneous metastasis. Metastases usually appeared as a single nodule or dispersed nodules.

Previous studies using direct cell implantation into the peritoneal cavity, the portal vein, the spleen or the implantation of pancreatic tumor tissue grown subcutaneously in a primary animal have reported higher rates of distant tumor spread, but these models do not replicate the cell signaling and biological changes necessary for a primary tumor to develop viable secondary metastatic cell implantation and growth[3,14,22,28]. Matrigel has been reported to facilitate tumor formation and growth, but it is unknown whether such components exert similar effects on tumor metastasis[26,29]. Our experiments showed that the microencapsulated cancer-cell group was not statistically different from the single-cell suspension group with respect to metastases (2 *vs* 0, 28.57% *vs* 0%, χ^2 = 2.333, P = 0.2308, $P > 0.05$), which may be attributed to the small sample size ($n < 40$) and must be confirmed by further large-scale experiments.

The use of PET-CT in the model allowed for intravital monitoring of tumor growth and improved our evaluation of the extent of disease and metastasis at necropsy. The ability to monitor tumor progression intravitally will facilitate the evaluation of therapeutic interventions.

In summary, we have described a novel orthotopic implantation technique for human pancreatic cancer in a nude mouse model. Compared with the orthotopic implantation approach using direct tumor-cell injection, the implantation of encapsulated tumor cells yielded a comparably higher rate of tumorigenicity and avoided the typically associated artificial intra-abdominal cell seeding; it thus appears to be the favorable technical approach. It is worth attempting to apply tumor-cell encapsula-

tion technology in rats and even in conventional mice to establish an orthotopic model by exerting its advantage as an immunological barrier. By studying the pancreatic cancer model of microencapsulated tumor cells, it is possible to improve our ability to investigate the mechanism of tumor progression and metastasis. This technique provides a novel orthotopic model for anticancer drug development, screening and testing.

ACKNOWLEDGMENTS

The authors wish to thank the staff of the Shanghai Institute of Digestive Surgery for their support in this study.

COMMENTS

Background

Pancreatic adenocarcinoma (PA) is an aggressive malignancy; therefore, animal models that allow further examination of pancreatic cancer progression are urgently needed. The majority of *in vivo* experimental studies of human cancer have been conducted in subcutaneous, orthotopic and heterotopic implantation nude mouse models. Current animal models of PA have low rates of tumor progression and metastasis.Microcapsules are spherical, with diameters that can be controlled in the range of 200-1,500 μm and a biocompatible semipermeable membrane, that allows the bidirectional diffusion of nutrients, oxygen, secreted therapeutic products, and waste but prevents the passage of high-molecular-weight substances such as antibodies and immunocytes into or out of the microcapsule.

Research frontiers

Orthotopic tumor induction is regarded as being highly suitable for modeling clinical pathology because it mimics the whole process of primary tumor growth, local tumor infiltration, and subsequent distant metastatic spread. The implantation of intact tumor fragments yields a higher rate of tumor incidence and avoids the artificial intra-abdominal cell seeding associated with the direct injection of pancreatic cancer cells but skips the early stages of tumor progression. Thus, animal models that allow for the examination of cellular mechanisms during the early stages of pancreatic cancer progression are needed.

Innovations and breakthroughs

The microencapsulation technique displays advantages in the rate of successful implantation and tumorigenicity.

Applications

It is possible to improve our ability to investigate the mechanism of tumor progression and metastasis using this new assay, which can provide a novel orthotopic model for anticancer drug development, screening and testing.

Terminology

Microencapsulation: a method of encapsulating cells in small microcapsules with semipermeable membranes.

Peer review

This study evaluated the effectiveness of a mouse model using an encapsulated pancreatic tumor cell line as a PA assay in comparison with the classical model using the injection of single tumor cells. The authors found no statistically significant differences among the different models used in terms of tumor mass and tumor presence.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i3.268

World J Gastroenterol 2012 January 21; 18(3): 268-274
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Comparative quantification of human intestinal bacteria based on cPCR and LDR/LCR

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Received: January 17, 2011 Revised: March 3, 2011
Accepted: March 8, 2011
Published online: January 21, 2012

Abstract

AIM: To establish a multiple detection method based on comparative polymerase chain reaction (cPCR) and ligase detection reaction (LDR)/ligase chain reaction (LCR) to quantify the intestinal bacterial components.

METHODS: Comparative quantification of 16S rDNAs from different intestinal bacterial components was used to quantify multiple intestinal bacteria. The 16S rDNAs of different bacteria were amplified simultaneously by cPCR. The LDR/LCR was examined to actualize the genotyping and quantification. Two beneficial (*Bifidobacterium*, *Lactobacillus*) and three conditionally pathogenic bacteria (*Enterococcus*, *Enterobacterium* and *Eubacterium*) were used in this detection. With cloned standard bacterial 16S rDNAs, standard curves were prepared to validate the quantitative relations between the ratio of original concentrations of two templates and the ratio of the fluorescence signals of their final ligation products. The internal controls were added to monitor the whole detection flow. The quantity ratio between two bacteria was tested.

RESULTS: cPCR and LDR revealed obvious linear correlations with standard DNAs, but cPCR and LCR did not. In the sample test, the distributions of the quantity ratio between each two bacterial species were obtained. There were significant differences among these distributions in the total samples. But these distributions of quantity ratio of each two bacteria remained stable among groups divided by age or sex.

CONCLUSION: The detection method in this study can be used to conduct multiple intestinal bacteria genotyping and quantification, and to monitor the human intestinal health status as well.

Key words: 16s rDNA; Comparative quantification; Comparative polymerase chain reaction; Intestinal bacteria; Ligase chain reaction; Ligase detection reaction

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Tang ZR, Li K, Zhou YX, Xiao ZX, Xiao JH, Huang R, Gu GH. Comparative quantification of human intestinal bacteria based on cPCR and LDR/LCR. *World J Gastroenterol* 2012; 18(3): 268-274 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i3/268.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i3.268

INTRODUCTION

Intestinal bacteria consist of more than 500 species with the concentrations ranging from 10^2-10^9/mL[1], and play

important roles in human growth, immunity, drug metabolism, pathogenesis and health maintenance. Intestinal bacterial components can be divided into beneficial and pathogenic bacteria according to their effects on human health[2]. Previous research showed the quantity of *Enterobacterim, Bifidobacterium, Enterococcus* and *Lactobacillus* varied largely among the infants, middle/old-aged and diarrhea groups[3], which was helpful to know the status of human intestinal ecology. Quantitative assay of such species are of great value in clinical practice and scientific researches. However, most of the interested bacteria differ a lot in the amount between samples[4,5], which is a big challenge to quantitatively detect multiple intestinal bacteria simultaneously.

In this study, comparative polymerase chain reaction (cPCR) and ligase detection reaction (LDR)/ligase chain reaction (LCR) methods were first employed to quantify five intestinal bacterial species simultaneously based on their 16s rDNAs. The selected target bacteria consisted of three conditional pathogenic bacteria (*Enterococcus, Enterobacterium, Eubacterium*) and two beneficial bacteria *Bifidobacterium* and *Lactobacillus*.

cPCR has been used to quantify nucleic acids for many years[6]. In a cPCR reaction, the target and the internal reference templates have the same primer recognition sequence and length, and similar internal sequences, which guarantee the amplification efficiency between the templates[7]. Therefore, the amount of the amplified products can precisely reflect the initial concentrations of their own templates. The highly conserved sequences of the bacterial 16s rDNAs fit well with the requirement of cPCR. We applied one pair of universal primers to amplify multiple target bacterial DNAs simultaneously, each kind of bacterial 16s rDNA was a competitive template of the others.

LDR is an eminent method to genotype low-abundant DNA under high backgrounds with a high specificity[8,9]. LCR has even higher amplification efficiency than LDR, which was introduced to this study to detect the lower abundant templates. Given that the ligation efficiency of different probes kept constant among tubes under the same reaction conditions with the same running cycle number, the ratio of fluorescence signals of the LDR/LCR products could reflect their initial ratio of the template concentrations.

Standard curves were made to verify the feasibility of our method and were applied to subsequent quantification of samples. Two internal controls were added to monitor the whole detection flow. One was applied to the LDR detection, the other to the LCR detection. The internal controls were added into the mixed standard DNAs or sampled DNAs at a fixed concentration before PCR amplification.

Eighty-two fecal samples (45 from males and 37 from females) were used to test the method for specimen detection, and all target bacteria could be detected in these samples. The distributions of the quantity ratio between each two bacterial species were obtained. Samples were also divided into groups by age or gender, the distribution diversities between groups were analyzed by non-parametric test.

Table 1 Specific bacterial primers

Genus	Primer(5'→3')
Universal primer	F: CAGGATTAGATACCCTGGTAGT
	R: TTGCGCTCGTTGCGGGACTT
Enterococcus	F: CACCGGAGCTTGCTCCACCG
	R: TGGCTCCAAAAGGTTACTTC
Enterobacterium	F: AGAGCTTGCTCTCGGGTGAC
	R:TAAGCTACCTACTTCTTTTGCAA
Eubacterium	F: GCAACCCTCTCCGGAGGGAAGCG
	R: TTCACCCCCCTCACCCTCCACAC
Bifidobacterium	F: GGCTNGAGCTTGCTCCGGCT
	R: GNCTCACCTTAGACGGCTCC
Lactobacillus acidophilus	F: GAGTTTGATCCTGGCTCAGG
	R: CTGTCCCACCTTAGRCGGCT
Lactobacillus casei	F: GAGTTTGATCCTGGCTCAGG
	R: CTGTCCCACCTTAGRCGGCT

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bifidobacterium longum, *Lactobacillus acidophilus* and *Lactobacillus casei* strains were donated by Department of Medical Microbiology and Parasitology, Shanghai Jiao Tong University School of Medicine. *Bifidobacterium longum* was grown anaerobically in MRS broth with L-Cysteine hydrochloride at 37 ℃ for 48 h. *Lactobacillus* was grown anaerobically in MRS broth at 37 ℃ for 48 h.

Collection and preparation of fecal samples

Fecal samples were collected from healthy adults aged 20-86 years who had not received antibiotics or other intestinal drugs within 3 mo prior to sampling at the First Hospital of Suzhou University. Samples were collected in sterile bags, with the wet weight of 4 g. A fecal sample was added into 6 mL of sterile phosphate-buffered saline (PBS; 0.05 mmol/L, pH 7.4) and mixed by inverting and vortexed for 5-10 min. The sample was then centrifuged at 500 × *g* for 5 min to collect the upper phase. The upper phase was then centrifuged to collect the bacterial cells in the pellets. The resulting pellets were washed in 20 μL pre-cooled ethanol for three times and then stored at 4 ℃ until use.

DNA extraction from bacterial cultures and fecal samples

For cultured bacteria, genome DNA was extracted from 1 mL harvested culture using bacteria genome DNA extracting kit (HuaShun W6511, Shanghai, China). For fecal samples, 200 μL TE containing lysozyme (20 mg/mL) was added to each pretreated fecal sample, well mixed, and then incubated at room temperature for 40 min. The bacterial genome DNA was extracted using column bacterial genome DNA extraction kit (Sangon, Shanghai, China).

Preparation of bacterial standard plasmid DNA

The specific 16s rDNA of each bacterial species was am-

Table 2 Specific probes

Genus	Ligase detection reaction probes	Ligase chain reaction antisense probes	Product length (bp)
Enterococcus	F: $(T)_{20}$-TTTGACCACTCTAGAGATAG	F: $(T)_{10}$-TTGCCCCCGAAGGGGAAGCT	80
	R: P-AGCTTCCCCTTCGGGGGCAA$(T)_{20}$-FAM	R: CTATCTCTAGAGTGGTCAAA-$(T)_{10}$	
Enterobacterium	F: $(T)_{23}$-TTGGAGGTTGTGCCCTTGAG	F: CTCAAGGGCACAACCTCCAA-$(T)_{10}$	85
	R: P-GCGTGGCTTCCGGAGCTAAC$(T)_{22}$-FAM	R: $(T)_{10}$-GTTAGCTCCGGAAGCCACGC	
Eubacterium	F: $(T)_{25}$-TTGACATATGGGTGAAGCGG	F: CCGCTTCACCCATATGTCAA-$(T)_{10}$	90
	R: P-GGGAGACCCCGTGGCCGAGA$(T)_{25}$-FAM	R: $(T)_{10}$-TCTCGGCCACGGGGTCTCCC	
Bifidobacterium	F: $(T)_{28}$-GGATGTGGGGCCCGTTCCA	F: $(T)_{10}$-TAGCTCCGACACGGAACCCG	95
	R: P-CGGGTTCCGTGTCGGAGCTAT$(T)_{27}$-FAM	R: TGGAACGGGCCCCACATCCA-$(T)_{10}$	
Lactobacillus casei	F: $(T)_{30}$-CAGGTCTTGACATCTTTTGA	F: $(T)_{10}$-AAACCTGATCTCTCAGGTGA	100
	R: P-TCACCTGAGAGATCAGGTTT$(T)_{30}$-FAM	R: TCAAAAGATGTCAAGACCTG-$(T)_{10}$	
Lactobacillus acidophilus	F: $(T)_{33}$-GGTCTTGACATCTAGTGCAA	F: $(T)_{10}$-GAACTCCGTATCTCTACGGA	105
	R: P-TCCGTAGAGATACGGAGTTC$(T)_{32}$-FAM	R: TTGCACTAGATGTCAAGACC-$(T)_{10}$	
IC 1#	F: $(T)_{30}$-CACAGGGCTTTCCACCATCCGTGTC		110
	R: P-GTAGCGGCCAAGCTGCCACGACAGG$(T)_{30}$-FAM		
IC 2#	F: $(T)_{33}$-GACATTCGGCAGGCAATCACAGCCT	F: $(T)_{10}$-GTAAGGTCTTGCAAACGTTCACATC	115
	R: P-GATGTGAACGTTTGCAAGACCTTAC$(T)_{32}$-FAM	R: AGGCTGTGATTGCCTGCCGAATGTC-$(T)_{10}$	

P: 5′-phosphorylation; **FAM : 3′-ends labeled with FAM fluorescence group; IC: Internal control.**

Table 3 Design of two sets of probe mixture

Genus	Mixed probes No. 1		Mixed probes No. 2	
	LDR (pM)	LCR (pM)	LDR (pM)	LCR (pM)
Enterobacterium	5[1]		5	
Eubacterium	5			5
Bifidobacterium	5			5
Enterococcus		5		5
Lactobacillus acidophilus		5		5
Lactobacillus casei		5		5
IC 1#	5		5	
IC 2#		5		5

[1]The number is the final probe concentration after mixing. LDR: ligase detection reaction; LCR: Ligase chain reaction.

plified by genus or species specific primers (Table 1) with the PCR product about 1500 bp in length. The amplified bacterial 16s rDNA was cloned in *Escherichia coli* (*E. coli*) DH5α by the pMD 18-T Vector system (Takara, Japan). Colonies carrying the specific inserts were cultured and their plasmid DNAs were extracted using a Spin Column Plasmid DNA Minipreps Kit (Sangon, Shanghai, China). The concentration of the extracted plasmid DNA was measured by ultraviolet spectrophotometer and then diluted to a fixed concentration as genus standard plasmid DNA (equivalent to 10^9/μL).

Universal primers and specific probes

Universal primers and LDR probes were designed according to the DNA sequences of *16s rRNA* gene available at GenBank.

For each kind of bacteria, more than fifty 16s rDNA sequences were used to align together (DNAsistant 2.0), a section containing three conserved regions and two variable regions was selected as the target segment. Universal primers were complementary to the conserved sequence flanking the target region; the variable region was the target of their specific LDR/LCR probes. Therefore, all the sequences of each bacterial species could be amplified by universal primers. LDR/LCR probes were genus-specific, except for *Lactobacillus*. As there was not a specific region matching with all *Lactobacillus* species, the *Lactobacillus* genus could be divided into two groups. Each group had a specific uniform sequence, and two specific probe sets were designed for typing the two groups of *Lactobacillus* genus.

The GenBank program Basic Local Alignment Search Tool was used to ensure that the proposed primers and probes were target specific. Ligation probes are listed in Table 2. Two sets of probe mixture for multiplex ligation reactions are shown in Table 1. Universal primers: up (5'-CAGGATTAGATACCCTGGTAGT-3'), down (5'-TTGCGCTCGTTGCGGGACTT-3').

Construction of internal control

Two oligonucleotides of internal control were synthesized to monitor the whole detecting flow. These oligonucleotides were approximately 310 bp in length, comparative to that of the bacterial 16s rDNA aplicon amplified with universal primers. Sequences at both ends were completely complementary to the universal primers, but the middle part was random. The internal control DNA was inserted into a plasmid and the concentration of internal control plasmid DNA was fixed to the same as bacterial standard plasmid DNA (equivalent to 10^9/μL).

Preparation of standard curve

Standard bacterial and internal control plasmid DNA was used to simulate the sample detection and verify the feasibility of the quantitative genotyping method. Each standard DNA was diluted to five series of concentrations. Six standard DNAs and two internal control DNAs were mixed together according to the symmetrically distributing test design[10] (Tables 2 and 3). The mixed templates were then applied to cPCR and LDR/LCR by two sets of mixed probes, and the fluorescence signal was collected by the ABI 377 sequencing system. Data of each two

Table 4 Experiment design

Trial	Factor							
	Enterobacterium c	*Eubacterium*	*Bifidobacterium*	*Enterococcus*	*Lactobacillus casei*	*Lactobacillus acidophilus*	IC 1#	IC 2#
Detected with probe mixture No. 1								
1	1	0.5	0.0625	0.01	0.005	0.000625	0.25	0.0025
2	0.5	0.0625	0.125	0.005	0.000625	0.00125	0.0625	0.000625
3	0.25	0.25	0.25	0.0025	0.0025	0.0025	1	0.01
4	0.125	1	0.5	0.00125	0.01	0.005	0.125	0.00125
5	0.0625	0.125	1	0.000625	0.00125	0.1	0.5	0.005
Detected with probe mixture No. 2								
1	1	0.005	0.000625	0.01	0.005	0.000625	0.25	0.0025
2	0.5	0.000625	0.00125	0.005	0.000625	0.00125	0.0625	0.000625
3	0.25	0.0025	0.0025	0.0025	0.0025	0.0025	1	0.01
4	0.125	0.01	0.005	0.00125	0.01	0.005	0.125	0.00125
5	0.0625	0.00125	0.01	0.000625	0.00125	0.1	0.5	0.005

"1" is a fixed concentration unit equivalent to 10^5/μL which was diluted by 10^4 from standard DNA concentration. IC: internal control.

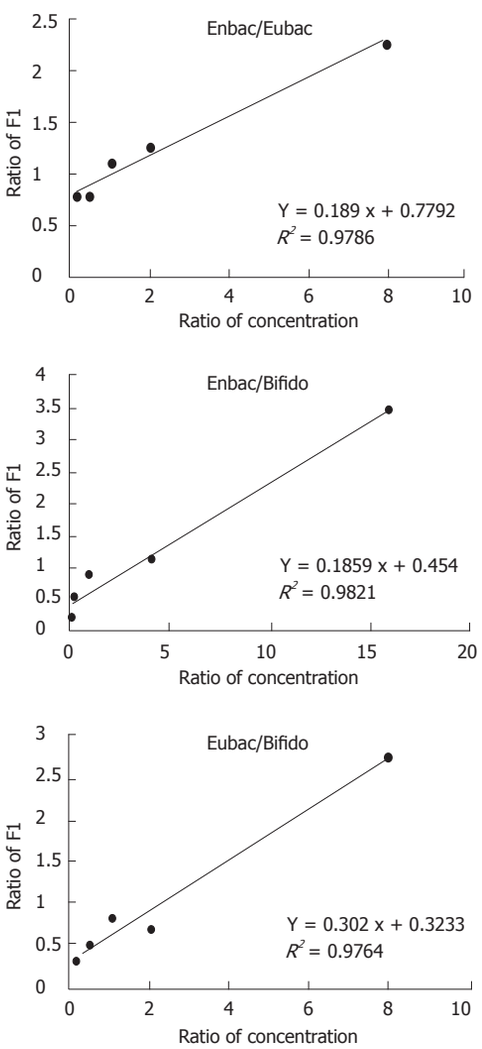

Figure 1 Linear relationship between the initial template concentrations and fluorescence signal intensity of ligase detection reaction/ligase chain reaction in standard curve test. The results of *Enterobacterium* (Enbac)/*Eubacterium* (Eubac), *Enbac/Bifidobacterium* (Bifido) and Eubac/Bifido tested by ligase detection reaction probes were presented. The linearity analyses were done twice, and results had a high reproducibility (data not shown).

kinds of standard DNAs were used to obtain the linear equation. The ratio of two collected fluorescence signals of two templates and the ratio of their concentrations were used to simulate the linearity. The standard curves were prepared by combining all the equations.

cPCR and LDR/LCR

The mixed standard DNA and sample DNA were processed as the same protocol. The cPCR reaction mixture consisted of 1 × PCR Buffer, 2 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleoside triphosphate, 0.5 mmol/L universal primers, 1.0 U *Taq* DNA polymerase, and 1 μL of template DNA in a final volume of 10 μL. The PCR thermocycling program was: 95 ℃ for 15 min; 40 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 60 s; and 72 ℃ for 7 min. The following LDR/LCR reaction mixture consisted of 1 × LDR Buffer, 0.1 mmol/L probe mixture No. 1 or No. 2 (Table 3), 4.0 U *Taq* ligase, and 1 μL of PCR products in a final volume of 10 μL. The LDR/LCR thermocycling program was: 94 ℃ for 2 min; 25 cycles of 94 ℃ for 30 s, and 60 ℃ for 2 min.

Sample detection

Before the detection, 10^5 copies of internal control template were added to each sample. The mixed samples were subject to cPCR and LDR (with probe mixture No. 1) along with designed mixed standard DNAs, 377 sequencer was used to collect the signals. If the fluorescence signal of any specific probe was not obtained by LDR, the PCR product was subject to LCR by probe mixture No. 2.

RESULTS

Linearity between template initial concentrations and their LDR/LCR fluorescence signals

Standard DNAs were mixed at different concentrations (Table 4) and applied to LDR and LCR test simultaneously. The results showed that the fluorescence intensity of each two templates was correlated with their initial concentrations in good linearity by LDR probes (R^2 > 0.97) (Figure 1) while the LCR probes could not contribute to a satisfactory linearity (R^2 ranged from 0.57 to 0.96).

Table 5 Statistics of sample detection

Bacterium	*Enterococcus*	*Enterobacterium*	*Eubacterium*	*Bifidobacterium*		*Lactobacillus casei*	*Lactobacillus acidophilus*
Probe	LCR	LDR	LDR	LDR	LCR	LCR	LCR
No. of detected sample	29	77	42	46	23	80	77
Detection rate(%)	35.37	93.90	51.22	84.15		97.56	93.90

LDR: Ligase detection reaction; LCR: Ligase chain reaction.

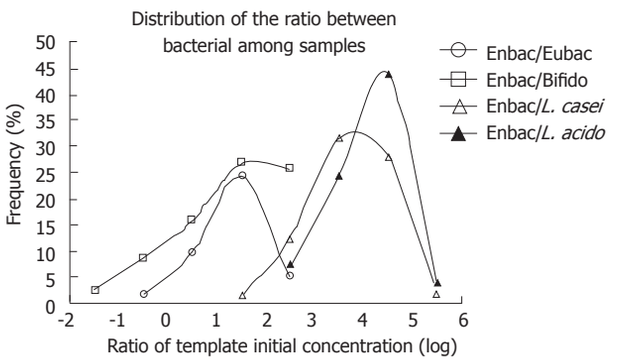

Figure 2 Distributions of ratio between each two bacteria. Enterobacterium was used to compare with other four species, respectively. The abscissa is the ratio of template concentration, divided into a 10-fold section. The ordinate is the frequency of each ratio section between two species. Enbac: *Enterobacterium*; Eubac: *Eubacterium*; Bifido: *Bifidobacterium; L. casei: Lactobacillus casei; L. acido: Lactobacillus acidophilus*.

Sample detection

Eighty-two samples were detected for the six bacterial strains. The detected rates ranked as: *Lactobacillus casei* > *Enterobacterium* = *Lactobacillus acidophilus* > *Bifidobacterium* > *Eubacterium* > *Enterococcus* (Table 5). *Eubacterium* and *Bifidobacterium* had the largest quantities among the six bacteria, which could be detected by LDR probes. In *Bifidobacterium,* 46 of the 82 samples could be detected by LDR probe, the other 23 samples were detected by LCR probe. *Lactobacillus* and *Enterococcus* were able to be detected only by LCR probes, while the detection rate of the former was much higher than the latter (> 90% *vs* 35.37%).

The ratio of the initial template concentrations between two bacteria were calculated by combining the LDR/LCR fluorescence signals and the standard curves. *Eubacterium* was selected as the comparing standard because of its highest detection rate and relatively steady quantity. The other bacteria were compared with *Eubacterium* except for *Enterococcus* because of its insufficient statistic data.

The ratio of two bacteria templates ranged from < 0.01 to > 10^4 deduced from the fluorescence signals and standard curves. In order to compensate the unsatisfactory linearity of the LCR products, the ratio of the initial template concentration was divided into several sections with 10-fold each. Frequencies of ratios in each section among all the samples were determined. The distributions of the quantity ratio between each two bacteria were plotted (Figure 2). Variations between distributions were analyzed by nonparametric test. There was on significant difference in the distributions between *Enterobacterium*/*Eubacterium* and *Enterobacterium*/*Bifidobacterium* (P = 0.605), which indicated that the quantity of *Bifidobacterium* and *Enterobacterium* kept constant in all the samples, while the distributions varied significantly among the other samples ($P < 0.05$).

These samples were divided into different groups by gender or age (young, 20-39 years; middle, 40-59; and old, above 60), and the bacterial ratio distribution was compared among groups through nonparametric test, without significant difference.

Detection sensitivity for single template

The *Bifidobacterium* standard DNA was selected and was serially diluted by 10-fold. The LDR and LCR detection could both obtain signals at a concentration of 10^{-9} of the original products (1-10 copies/μL) after 40 cycles of PCR and 25 cycles of LDR.

Detection limits for multiple templates

There is a detection limit of the concentration range of templates because of the amplification limit of PCR and LDR and the signal detection range of 377 sequencer.

The detection limit of LDR probes was tested by mixing standard DNAs of *Eubacterium* and internal control 1# at a serial ratio. The concentrations and detection results of the two components are shown in Table 6. The results showed that signals could be obtained by LDR probes simultaneously at the concentration varying between the two templates within 30 times.

The detection limit between LDR and LCR probes was tested by *Bifidobacterium* and internal control 1# standard DNAs. The concentrations and detection results of the two components are listed in Table 6. The results showed that both LDR and LCR signals could be detected simultaneously, even at template concentrations varying within 10 000 times. The detection limit between LCR probes was tested by *Bifidobacterium* and *Lactobacillus.casei* standard DNAs. The concentrations and detection results of the two components are shown in Table 6.

DISCUSSION

LDR and LCR were both introduced to this study to genotype and comparatively quantify the final cPCR products, which extended the quantitative detection range significantly. LDR is a linear amplification process, while LCR is an exponential one. The templates of high quantity samples were detected by LDR probes, while those of low quantity by LCR probes. Therefore, the ligation

Table 6 Detection limits of mixed samples with ligase detection reaction probes

Eubac	LDR	Dilution times	64	32	16	8	4	2	1[1]	1	1	1	1	1	1	
		Detection result	-	+	+	+	+	+	+	+	+	+	+	+	+	
IC 1#	LDR	Dilution times	1	1	1	1	1	1	1	2	4	8	16	32	64	
		Detection result	+	+	+	+	+	+	+	+	+	+	+	+	+	
Bifido	LCR	Dilution times	100	200	400	800	1600	3200	6400	12 800	25 600					
		Detection result	+	+	+	+	+	+	+	+	-					
IC 1#	LDR	Dilution times	1[1]	1	1	1	1	1	1	1	1					
		Detection result	+	+	+	+	+	+	+	+	+					
Bifido	LCR	Detecion result	128	64	32	16	8	4	2	1[2]	1	1	1	1	1	1	1
		Dilution times	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L. casei	LCR	Detection result	1	1	1	1	1	1	1	1	2	4	8	16	32	64	128
		Dilution times	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

[1]The standard concentration of standard DNA equivalent to 10^9/μL; [2]The 100 times dilution of the standard concentration.+: the positive result; -: the negative result. LDR: Ligase detection reaction; LCR: Ligase chain reaction; Bifido: *Bifidobacterium*; *L. casei*: *Lactobacillus casei*.

products of different templates could be detected on one platform simultaneously.

The universality of universal primers was validated as they could direct the amplification of all the investigated bacterial standard DNAs. By mixed LDR/LCR probes, only one fluorescence signal of the target product was obtained after the LDR/LCR reaction templated by all bacterial standard PCR products. PCR products of other bacterial 16s rDNA including *E. coli*, *Clostridium coccoides*, *Bacteroides* and *Clostridium leptum* were not able to produce fluorescence signals (data not shown).

According to the standard curves, the ratio of LDR products and that of the initial template concentrations have perfect linear relationship because of the stable ligation efficiency in the linear amplification process. However, the relationship between the ratio of signals from LCR probes and that of the initial template concentrations did not show good linearity, which implied that the efficiency of the exponentially amplifying process could not keep stable in every cycle and between different reaction tubes. Nevertheless, on a large scale (10-fold), the amount of the LCR product was able to reflect the concentration of the templates, because of the limited number of reaction cycles (25 cycles) and the same reaction condition.

The quantity differences were shown between bacterial species, while the distribution of quantity ratio among species showed no difference in healthy human population whether grouped by sex or by age.

In this study, a precise genotyping and a stable comparative quantification were achieved by combining cPCR and LDR, and the detection throughout could be developed to tens of target bacteria at one time. Although the template concentration range of quantification needs to be extended and optimized, the cPCR and LDR/LCR method has shown its potential in intestinal bacteria detection in both basic research and clinical practice.

ACKNOWLEDGMENTS

We would like to thank our colleagues from the Department of Medical Microbiology and Parasitology, Shanghai Jiao Tong University School of Medicine for donating the bacterial strains and those from the First Hospital of Suzhou University for their help in sample collection, and Chen B for her experimental assistance and cooperation.

COMMENTS

Background

Increasing researches suggested that the human health status was tightly correlated with the quantitative variation of intestinal bacterial components. However, it is a big challenge to quantify multiple intestinal bacterial components simultaneously, for the significantly quantitative variations between different components and individual samples.

Research frontiers

Comparative quantification polymerase chain reaction (cPCR) has been used to quantify nucleic acids for many years. Due to the similarity of 16S rDNAs of different intestinal bacteria, it is hard to quantify multiple intestinal bacteria by cPCR alone. ligase detection reaction/ ligase chain reaction (LDR/LCR) is an eminent method to genotype low-abundant DNA under high backgrounds and its merit of high specificity fits for multiple detections. In this study, the authors tried to combine the advantages of both cPCR and LDR/LCR to fulfill multiple quantitative detections.

Innovations and breakthroughs

Using the universal primer target at the 16S rDNA of intestinal bacteria, cPCR could get amplifying product of nearly all species with similar efficiency. LDR could achieve precise genotyping and quantification of different bacteria simultaneously. This study demonstrated the possibility to realize accurate multiple quantification of intestinal bacteria by the combined cPCR and LDR/LCR.

Applications

By the detection method used in this study, the authors could quantify tens of intestinal bacteria simultaneously after test optimization. And it could be used to monitor the intestinal health status of human as well.

Peer review

To quantify multiple intestinal bacterial components simultaneously, the authors described a multiple detection method for comparative quantification of 16S rDNA from different intestinal bacterial components based on cPCR and LDR/LCR methods. The results show differences between bacterial species, while the distribution of quantity ratio among species made no difference in healthy human population. This study indicates potential combination of cPCR and LDR/LCR method in measuring multiple intestinal bacterial components simultaneously.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i3.275

World J Gastroenterol 2012 January 21; 18(3): 275-278
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Efficacy of intraductal ultrasonography in the diagnosis of non-opaque choledocholith

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Author contributions: Wan R and Lu J contributed equally to this work; Wan R, Lu J, Guo CY, Xu XF and Wang XP designed the research; Wan R and Lu J performed the research; Xu XF and Guo CY performed the analysis of the data; Lu J and Wan R wrote the first draft of paper; all authors contributed to the interpretation of the study and to the modification of further drafts of the paper; Wan R is the guarantor.

Supported by Shanghai Natural Science Foundation, No. 08411962900

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Received: April 26, 2011 Revised: August 18, 2011
Accepted: August 27, 2011
Published online: January 21, 2012

Abstract

AIM: To evaluate the efficacy of intraductal ultrasonography (IDUS) in the diagnosis of non-opaque, common bile duct stones.

METHODS: A total of 183 patients (102 males, mean age 73 years; 81 females, mean age 70 years) with suspected common bile duct stones diagnosed through abdominal computed tomography (CT), magnetic resonance imaging (MRI), and abdominal Type-B ultrasound were included in the study. The diagnosis was confirmed through endoscopic retrograde cholangiopancreatography (ERCP) followed by IDUS.

RESULTS: A total of 183 patients with suspected common bile duct (CBD) stones were included in the study as follows: 36 patients with high-density CBD stones, 68 patients with sand-like stones, 44 patients with low-density stones, 21 patients with ampullary cancer, and 14 patients with pancreatic cancer. Conventional imaging revealed 124 cases of choledochectasia, and only 36 cases of suspected CBD stones; ERCP revealed 145 cases of CBD stones with three missed diagnoses. IDUS revealed 148 cases of CBD stones, 21 cases of ampullary tumors, and 14 cases of pancreatic cancer.

CONCLUSION: IDUS was more effective in the diagnosis of bile duct stones than ERCP, upper abdominal CT or upper abdominal MRI.

Key words: Biliary intraductal ultrasonography; Endoscopic retrograde cholangiopancreatography; Common bile duct stones; Non-opaque stones; Sand-like stones

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Lu J, Guo CY, Xu XF, Wang XP, Wan R. Efficacy of intraductal ultrasonography in the diagnosis of non-opaque choledocholith. *World J Gastroenterol* 2012; 18(3): 275-278 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i3/275.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i3.275

INTRODUCTION

Common bile duct (CBD) stones can cause a range of serious complications such as obstructive jaundice, biliary tract infection, and pancreatitis. These stones may even cause severe septic shock and lead to life-threatening conditions[1]. With the continuous development of endoscopic technology, endoscopic treatment has now become a standard diagnostic procedure for bile duct stones. Endoscopic retrograde cholangiopancreatography (ERCP) has long been considered the gold standard for the diag-

nosis of CBD stones because it can accurately image the pancreatic duct system. With diagnostic accuracy higher than 90%, ECRP is significantly more effective in the diagnosis of extra-hepatic bile duct stones than computed tomography (CT), or magnetic resonance imaging (MRI). However, misdiagnoses and missed diagnoses still occur due to operator error and variations in the contrast agent concentration and stone density[2]. Biliary intraductal ultrasonography (IDUS), which has emerged as a safe and effective new method for the diagnosis of CBD stones in recent years, combines high-resolution ultrasound imaging with endoscopy, thereby preventing imaging of the pancreatic duct system from being affected by intestinal gas. It is accurate in the diagnosis of smaller non-opaque stones and low-density stones, i.e., stones with density similar to that of the contrast agent. Through the IDUS examination of ERCP surgery patients between January 2009 and August 2010, in this study we investigated the efficacy of IDUS combined with ERCP in the diagnosis of bile duct stones.

MATERIALS AND METHODS

Materials

A total of 183 ERCP surgery patients treated between January 2009 and August 2010 were retrospectively analyzed. The patient population was as follows: 102 males, mean age 69 years; 81 females, mean age 71 years. All patients exhibited upper abdominal pain, nausea, vomiting, chills, fever and jaundice. The preoperative imaging studies included abdominal CT or upper abdominal enhanced MRI + magnetic resonance cholangiopancreatography (slice thickness of 7 mm, plane scanning with layer gap of 3 mm, cross section T1W1/T2W1), and these examinations were done at the Radiology Department of Shanghai Tenth Hospital, revealing 124 patients with suspected choledochectasia, 36 patients with suspected common bile duct stones, and 23 patients with negative imaging but suspicious clinical presentation and laboratory test results.

Instruments and methods

IDUS surgery was performed in all cases. First, we conducted preoperative preparations for conventional ERCP. Next, ERCP was carried out with inclusion of the following three procedures: cholangiography after successful wire-guided bile duct intubation; a partial intraoperative duodenal papillotomy based on the specific circumstances; and biliary tract imaging to determine whether a stone or a stenosis was the cause of the clinical findings. This was followed by the insertion of an ultrasound scanning probe along the guide wire to observe the bile ducts. The ultrasound probe was removed after the IDUS examination was completed, and the subsequent stages of the ECRP procedure, which included biliary stent placement and nasal biliary drainage, were determined from the specifics of the clinical situation, e.g., the presence of bile duct stones. The criteria for IDUS diagnosis of bile duct stones were evaluated by experts who were experienced in IDUS imaging.

Statistical analysis

The sensitivity, specificity, negative predictive value, positive predictive value, and diagnostic accuracy of the comparisons were calculated. The SPSS13 statistical package was used for the statistical analysis. The χ^2 test was used to compare rates, and the Scheffé method was used for pairwise comparisons among groups. P values < 0.05 were considered statistically significant.

RESULTS

Case information

A total of 183 patients with suspected CBD stones were included in the study as follows: 36 patients with high-density CBD stones, 68 patients with sand-like stones, 44 patients with low-density stones, 21 patients with ampullary cancer, and 14 patients with pancreatic cancer.

Conventional imaging in the diagnosis of common bile duct stones

Conventional imaging revealed 124 cases of choledochectasia, and only 36 cases of suspected CBD stones in the 148 confirmed cases; this represents a diagnosis rate of 24.3%, sensitivity of 24.3%, specificity of 100%, and diagnostic accuracy of 38.3%.

Endoscopic retrograde cholangiopancreatography in the diagnosis of common bile duct stones

ERCP revealed 145 cases of CBD stones with three missed diagnoses, which resulted in a diagnostic accuracy of 98%. The remaining 35 cases that did not involve CBD stones comprised 21 cases of ampullary tumors and 14 cases of pancreatic cancer; all were diagnosed with an accuracy of 100%. The sensitivity, specificity, and accuracy of ERCP were 98%, 100% and 98.4%, respectively.

Intraductal ultrasonography in the diagnosis of common bile duct stones

IDUS revealed 148 cases of CBD stones, 21 cases of ampullary tumors (Figure 1), and 14 cases of pancreatic cancer (Figure 2), which resulted in a specificity of 100%, sensitivity of 100%, and accuracy of 100%. Of the 148 cases of CBD stones, 27 patients underwent cholecystectomy and CBD stones were intraoperatively confirmed in all cases.

Complications

Of the 183 cases included in the study, 3 patients showed pancreatitis after ERCP, which represents an incidence rate of 1.64% (3/183). The pancreatitis complications improved after conservative treatment that included fasting, acid-suppression, inhibition of enzymes, and anti-inflammatory drugs. No other complications such as bleeding or perforation were observed (Table 1).

DISCUSSION

CBD stones are commonly encountered in clinical settings, and jaundice is usually observed as the first symp-

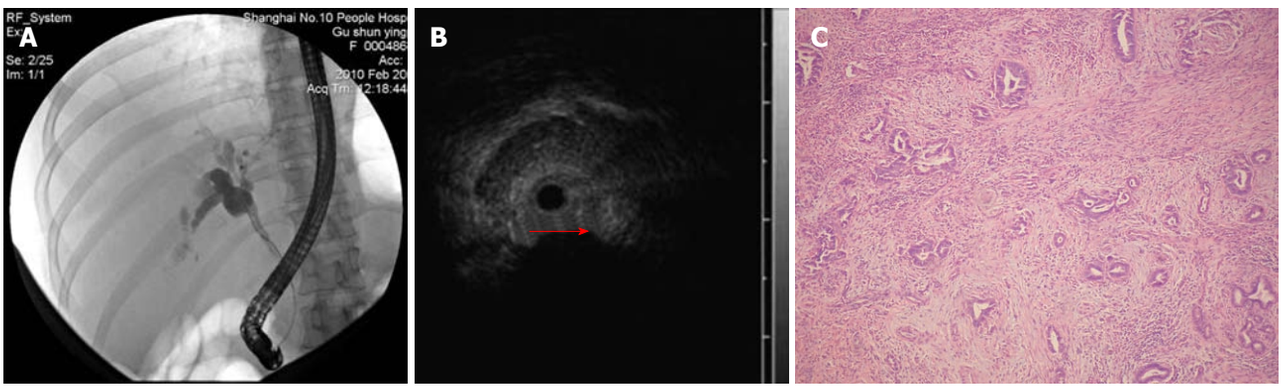

Figure 1 Ampullary tumor. A: Presentation in endoscopic retrograde cholangiopancreatography; B: Presentation in intraductal ultrasonography, red tag displays the focus; C: Histopathology change.

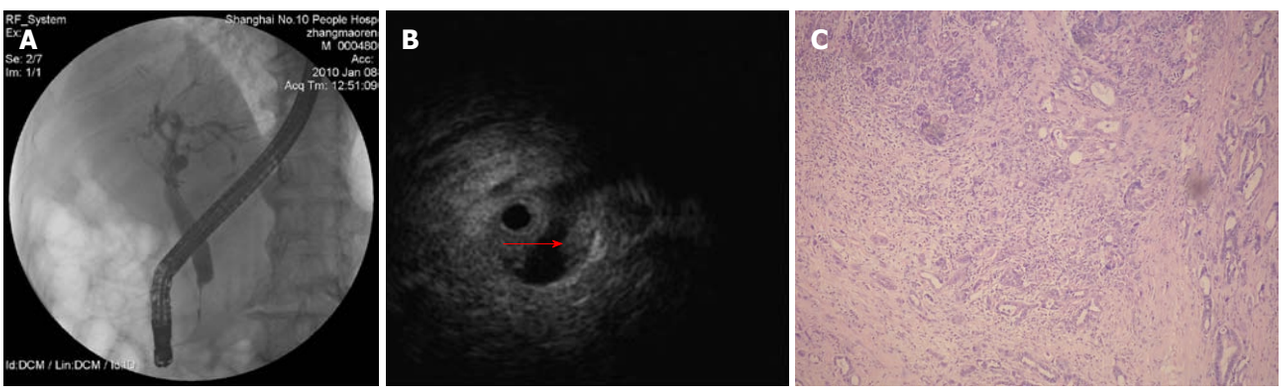

Figure 2 Pancreatic cancer. A: Presentation in endoscopic retrograde cholangiopancreatography; B: Presentation in intraductal ultrasonography, red tag displays the focus; C: Histopathology change.

Table 1 Comparison of the diagnostic accuracy of common imaging procedures with that of endoscopic retrograde cholangiopancreatography and intraductal ultrasonography + endoscopic retrograde cholangiopancreatography for common bile duct stones

	Diagnosis	Missed diagnosis	Accuracy (%)
Common imaging (n = 148)	36	112	24.3
ERCP (n = 148)	145	3	98
ERCP + IDUS (n = 148)	148	0	100

ERCP: Endoscopic retrograde cholangiopancreatography; IDUS: Intraductal ultrasonography.

tom[3]. Although the diagnosis of such a disease using surface ultrasonography, CT and MRI has been greatly improved, their diagnostic accuracy is still not ideal[4]. In this study, conventional imaging showed an accurate preoperative diagnosis rate of 24.3%.

In ERCP images, opaque CBD stones clearly appear as round or crescent shaped objects. Although cholangiectasis is also typically observed, other low-density and sand-like stones are not visible[5]. ERCP, as a direct biliary imaging method, exhibits high resolution, which not only helps in further clarifying the location and number of stones, but also facilitates further interventions. Therefore, since the 1970s, ERCP has been the gold standard for the diagnosis of extrahepatic bile duct stones[6]. However, the evaluation of biliary pathological changes through ERCP is evaluated by contrast agent and image shapes of X-ray. Therefore, several factors may contribute to misdiagnosis and missed diagnoses using ERCP, such as the nature and size of the stones, air bubbles in the bile duct, variations in contrast agent concentration, and the injection rate[7]. The diagnostic accuracy of ERCP in this study was 98%. Endo-ultrasonography (EUS), which is a technological combination of ultrasonography and direct endoscopic visual inspection, was introduced in the 1980s. After years of technological development, EUS now plays an important role in the diagnosis and treatment of digestive diseases[8]. Miniature ultrasonic probes exhibit small diameters and high frequencies. In this manner, IDUS can be used to explore the pancreatic duct *via* the guide wire during ERCP. When used in the bile duct, IDUS repeatedly scans the involved anatomy and generates cross-sectional images of the bile duct wall and the lesion, which clearly show the inner structures with reduced interference. Lesions that are at least 0.5 cm in diameter, as well as the structure of adjacent organs such as the liver, portal vein, pancreas and portions of the pancreatic duct, can be clearly observed. Determining the specific causes of obstructive jaundice and evaluating the severity of such lesions is valuable[9]. In this study, based on the previously reported criteria for the diagnosis

of CBD stones, IDUS showed a diagnostic accuracy of 100%, which is significantly higher than that of ERCP and other imaging methods. For the diagnosis of CBD malignant strictures, IDUS is also more accurate than surface imaging and ERCP. In this study, no malignant lesions were diagnosed prior to the ERCP procedures; however, with the use of IDUS, 21 cases of ampullary cancer and 14 cases of pancreatic cancer were found. Therefore, IDUS is useful in the early diagnosis of bile duct or ampullary tumors[10].

Bleeding, perforation, and acute pancreatitis are major complications of ERCP[11]. In this study, severe pancreatitis and gastrointestinal perforation did not occur in any of the cases; however, there were three cases of pancreatitis after ERCP with minimal edema that improved after conservative treatment such as enzyme inhibition, acid-suppression, and anti-inflammatory drug administration. In conclusion, the use of IDUS in combination with ERCP is a safe and reliable method of increasing the diagnostic accuracy of ERCP.

COMMENTS

Background

The incidence of choledocholith is currently rising with the improvement of living standards and changes in food habits. Common bile duct (CBD) stones can cause a range of serious complications such as obstructive jaundice, biliary tract infection, and pancreatitis. A safe and accurate method to diagnosis CBD stones is urgently needed.

Research frontiers

Endoscopic retrograde cholangiopancreatography (ERCP) is a significant method in the diagnosis of CBD stones. However, misdiagnoses still occur. IDUS is a new, safe method for the diagnosis of bile duct pathological changes.

Innovations and breakthroughs

This is the first research to report the outcome of intraductal ultrasonography (IDUS) in the diagnosis of non-opaque stones. IDUS showed a diagnostic accuracy of 100%.

Applications

IDUS is useful in the diagnosis of non-opaque, common bile duct stones and early diagnosis of bile duct or ampullary tumors.

Peer review

The paper evaluates the efficacy of intraductal ultrasonography (IDUS) in the diagnosis of non-opaque, common bile duct stones. The study is very interesting.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i3.279

World J Gastroenterol 2012 January 21; 18(3): 279-284
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Present and future of prophylactic antibiotics for severe acute pancreatitis

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Supported by The National Natural Science Foundation of China, No. 81072910
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Received: February 22, 2011 Revised: August 12, 2011
Accepted: October 14, 2011
Published online: January 21, 2012

Abstract

AIM: To investigate the role of prophylactic antibiotics in the reduction of mortality of severe acute pancreatitis (SAP) patients, which is highly questioned by more and more randomized controlled trials (RCTs) and meta-analyses.

METHODS: An updated meta-analysis was performed. RCTs comparing prophylactic antibiotics for SAP with control or placebo were included for meta-analysis. The mortality outcomes were pooled for estimation, and re-pooled estimation was performed by the sensitivity analysis of an ideal large-scale RCT.

RESULTS: Currently available 11 RCTs were included. Subgroup analysis showed that there was significant reduction of mortality rate in the period before 2000, while no significant reduction in the period from 2000 [Risk Ratio, (*RR*) = 1.01, *P* = 0.98]. Funnel plot indicated that there might be apparent publication bias in the period before 2000. Sensitivity analysis showed that the *RR* of mortality rate ranged from 0.77 to 1.00 with a relatively narrow confidence interval ($P < 0.05$). However, the number needed to treat having a minor lower limit of the range (7-5096 patients) implied that certain SAP patients could still potentially prevent death by antibiotic prophylaxis.

CONCLUSION: Current evidences do not support prophylactic antibiotics as a routine treatment for SAP, but the potentially benefited sub-population requires further investigations.

Key words: Severe acute pancreatitis; Prophylactic antibiotics; Mortality; Meta-analysis

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Jiang K, Huang W, Yang XN, Xia Q. Present and future of prophylactic antibiotics for severe acute pancreatitis. *World J Gastroenterol* 2012; 18(3): 279-284 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i3/279.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i3.279

INTRODUCTION

Acute pancreatitis (AP) is still a common pancreatic disease, with an increasing incidence rate during the past two decades[1]. In the United States, AP accounts for more than 220 000 hospital admissions annually[2]. Severe acute pancreatitis (SAP) composes about 20% of AP, with a high mortality rate around 20%[3]. For several decades, the administration of prophylactic antibiotics has been one of the great controversies worldwide about the management of SAP.

Totally, the mortality rate for SAP is 10% with sterile and increased to 25% with infected pancreatic necrosis[4]. Hospitalization for patients with SAP may frequently extend beyond 2 wk and often involves an intensive care unit stay and increased infection rate[4]. Up to the late period of last century, complications of infection account for 80% of deaths from AP[5]. Currently, 30%-50% of the dead cases were due to infectious complications for 2 wk from onset[6]. Therefore, theoretically, no antibiotics are indicated in mild cases, but antibiotics were considered to play an important role in either therapeutic or prophylactic intention for SAP. The recognition and exploration of antibiotic prophylaxis for SAP experienced more than a half of a century. However, there was still a gap between theory and truth, and the proper role of antibiotics in SAP remains controversial[7].

Why pancreatologists keep dwelling on this controversy? In this review, we critically estimated the currently available evidence to find out the gap between theory and clinical practice. Moreover, through our hypothesis and calculation, we predicted what would occur in antibiotic prophylaxis for SAP if robust evidence was available in the future.

MATERIALS AND METHODS

Search strategy

We searched the electronic databases of PubMed up to 2009. The reference lists from relevant articles, containing meta-analysis, systematic reviews or clinical trials, were screened for potential eligible studies. There was no limitation of publication date and language. The following strings were used in the search strategy for PubMed: "pancreatitis" (MeSH Terms) or "pancreatitis" (All Fields); "antibacterial agents" (MeSH Terms) or "anti-bacterial" (All Fields); "agents" (All Fields) or "anti-bacterial agents" (All Fields) or "antibiotics" (All Fields) or "anti-bacterial agents" (Pharmacological Action); "randomized controlled trial" (Publication Type) or "randomized controlled trials as topic" (MeSH Terms) or "randomized controlled trials" (All Fields) or "clinical trial" (Publication Type) or "clinical trials" (MeSH Terms) or "clinical trial" (All Fields); "meta-analysis" (Publication Type) or "meta-analysis" (MeSH Terms) or "meta-analysis" (All Fields); and "review literature" (MeSH Terms) or "systematic review" (All Fields).

Inclusion and exclusion criteria

The currently available meta-analyses and randomized controlled trials were analyzed by meta-analysis. The patients were all diagnosed as having SAP. The intervention group received prophylactic antibiotics. The control group received placebo or none-treatment. All potentially eligible meta-analyses or trials should report the mortality rate of each group. There were no limitations for race, age or gender. If any conditions did not conform to the above criteria or the essential data could not be extracted, the meta-analyses of trials were excluded.

Selection and data collection

All procedures were reviewed by two independent reviewers: (1) for descriptive review of available meta-analyses, the publication year, the number of included trials, and the effect sizes of mortality or infected necrosis were extracted. The effect sizes involved risk ratio (*RR*), odds ratio (OR), absolute risk reduction (ARR) and their 95% confidence interval (CI); (2) for updating meta-analysis, the general information including publication year, sample size, study design, general characteristics of patients, and intervention details were extracted. The dichotomous data for the mortality were extracted, including total number of participants and events for each group. The number of events was calculated by the reported percentage if possible; and (3) For the predication of the future meta-analysis, the synthesized mortality rate of each group was extracted by our updated meta-analysis.

Statistics analysis

Meta-analysis: Outcomes of eligible studies were statistically synthesized by Reviewer Manager 5.0 (The Nordic Cochrane Center, Cochrane Collaboration, Copenhagen, Denmark). The statistical method was referred to the Cochrane Handbook for Systematic Review of Intervention. The pooled statistics were calculated using a fixed effects model initially. The *RR* was reported for dichotomous data. The 95% CI was also calculated. The Mantel-Haenszel method was used to test significance of dichotomous data. *P* value less than 0.05 was considered statistically significant. Heterogeneity between comparable studies was tested in all analyses using a standard χ^2 test for between-study statistical heterogeneity and considered significant at $P < 0.1$. If heterogeneity existed, the random effects model was used for analysis.

Sample size calculation: The format for equivalency estimate of rates between two arms is shown below.

Expected sample size in each group = $(\mu_\alpha + \mu_\beta)^2[p_1(1 - p_1) + p_2(1-p_2)]/(\Delta - |p_1-p_2|)^2$; Limitation: $\Delta > |p_1-p_2|$

(Δ: threshold of difference value; α: possibility of type I error; β: possibility of type Ⅱ error; μ_α: critical value corresponding to α; μ_β: critical value corresponding to β; p_1: possibility of mortality in prophylactic antibiotics group; p_2: possibility of mortality in placebo or blank control group; $|p_1-p_2|$: absolute value of difference between two groups.)

Hypothesis test: SPSS 13.0 (SPSS, Chicago, IL, United States) was used for statistical analysis. For dichotomous data, the χ^2 test was used to compare frequencies of mortality. Linear correlation between accumulated sample size and time (year) was analyzed by the Pearson correlation test. *P* value of less than 0.05 (two-sided) was considered significant.

RESULTS

Current evidences

Published meta-analysis: Recently, there have been several RCTs on prophylactic antibiotic for SAP[8-14], and therefore several meta-analyses on this topic have already published (Table 1, Figure 1). For the primary outcome

Table 1 Recent meta-analyses on the outcomes of prophylactic antibiotics for severe acute pancreatitis

Meta-analysis	Year	No. of RCTs	Mortality		Infected necrosis		Recommendation
			Effect size	95% CI	Effect size	95% CI	
Jafri *et al*[15]	2009	8	RR = 0.76	(0.49, 1.16)	RR = 0.79	(0.56, 1.11)	Unfavorable
Xu *et al*[16]	2008	8	RR = 0.76	(0.50, 1.18)	RR = 0.69	(0.50, 0.95)[a]	Pending
Bai *et al*[17]	2008	7	RR = 0.70	(0.42, 1.17)	RR = 0.81	(0.54, 1.22)	Unfavorable
Hart *et al*[18]	2008	7	OR = 0.71	(0.41, 1.23)	OR = 0.72	(0.45, 1.16)	Unfavorable
de Vries *et al*[19]	2007	6	ARR = 0.058	(-0.017, 0.134)	ARR = 0.055	(-0.084, 0.194)	Pending
Dambrauskas *et al*[20]	2007	10	RR = 0.76	(0.586, 0.976)	RR = 0.57	(0.418, 0.784)[a]	Pending
Villatoro *et al*[21]	2006	5	OR = 0.37	(0.17, 0.83)[a]	OR = 0.62	(0.35, 1.09)	Pending
Mazaki *et al*[22]	2006	6	RR = 0.78	(0.44, 1.39)	RR = 0.77	(0.54, 1.12)	Unfavorable
Xiong *et al*[23]	2006	6	RR = 0.54	(0.28, 1.04)	RR = 0.77	(0.48, 1.24)	Unfavorable

[a]$P < 0.05$ antibiotic prophylaxis *vs* placebo/none; RCT: Randomized controlled trial; CI: Confidence interval; RR: Risk ratio; OR: Odds ratio; ARR:Absolute risk reduction.

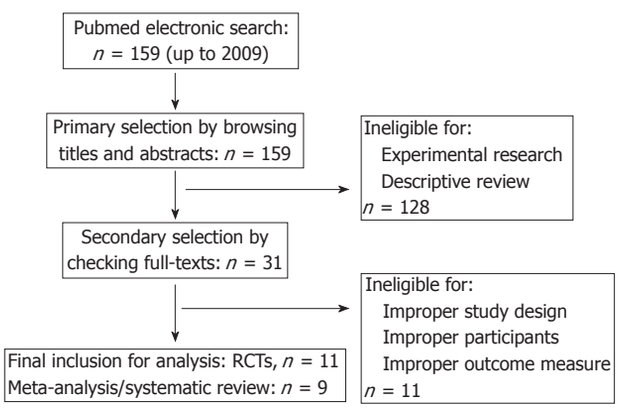

Figure 1 The flow chart of literature search and selection in PubMed.

in mortality rate, only one meta-analysis showed a significant reduction by antibiotic prophylaxis. Moreover, only two other meta-analyses showed a significant preventive effect on infected necrosis for SAP by antibiotic prophylaxis. More than a half of the meta-analyses did not recommend the administration of antibiotic prophylaxis, while the others suggested that the effectiveness of antibiotic prophylaxis was still controversial.

Update on meta-analysis: To improve the robustness of evidence, the meta-analysis was updated in present review. We comprehensively searched the PubMed database to identify available RCTs on the comparison between prophylactic antibiotics and placebo/none-treatment for SAP. There were 11 eligible RCTs (Figure 1)[8-14,24-27], which were re-pooled to update the meta-analysis (Figure 2). There were two newly published RCTs in 2009[13,14], which was different from previous meta-analysis[15].

Interestingly, we found that before 2000 the pooling estimate of 4 RCTs (183 patients) showed a significant benefit to reduce the mortality of SAP (*RR* = 0.31, 95% CI: 0.12-0.79, $P = 0.01$)[24-27]. The mortality rates were 5.26% (5/95) and 18.18% (16/88) in prophylactic antibiotics and placebo/none-treatment groups, respectively. The number needed to treat (NNT) was one of 8 treated patients potentially being benefited to prevent death by prophylactic antibiotics (Table 2).

However, since 2000, seven RCTs (439 patients) have been identified and pooled to meta-analysis[8-14]. Differently, the result indicated that there was no benefit of preventing death in the prophylactic antibiotics group (*RR* = 1.01, 95% CI: 0.65-1.56, $P = 0.98$). The mortality rates were 15.00% (33/220) and 15.07% (33/219) in the prophylactic antibiotics and placebo/none-treatment groups, respectively. Sadly, the NNT was one of 1429 treated patients potentially being benefited (Table 2), and it was indeed a negative evidence to support administration of prophylactic antibiotics.

In the funnel plot, the asymmetric distribution of RCTs before 2000 implied that there might be apparent publication bias (Figure 3). Moreover, the distribution of RCTs from 2000 was relatively symmetric.

How long a way to end the controversy?

What a scale does a trial require? To the best of our knowledge, the key to end the controversy is to conduct a large-scale RCT so as to control the random sampling error. To perform an ideal RCT, we have to calculate the required sample size by statistical approach. The mortality rates were calculated based on the pooling estimate from 2000, and the results of calculation are shown in Table 3.

If the difference between mortality rates was no more than 10% as an acceptable threshold of equivalence, at least 544 patients would be required for a single robust trial (Table 3), while the difference was limited to no more than 5%, 3230 patients would be demanded in a single trial (Table 3).

In practice, it is hard to conduct a single-center randomized trial of such large scale in the study of SAP treatment. A multi-center trial might be a way out of this corner, but difficulties in quality control and possible performance bias might occur. Among the included RCTs in the above updated meta-analysis, the absolute value of difference between mortality rates $|p_1-p_2|$ was 6.7% ± 5.9% (range, 2.0%-19.3%), and the mean was more than 5%. Therefore, the threshold of 10% and the sample size of 544 patients could be rational. It would take more than two years to complete a large-scale multi-center RCT.

The above meta-analysis showed a minor difference between mortality rates, $|p_1-p_2| = 0.0007$. Thus, we have to choose the lower threshold 5% to calculate the required accumulated sample size, and the calculation result

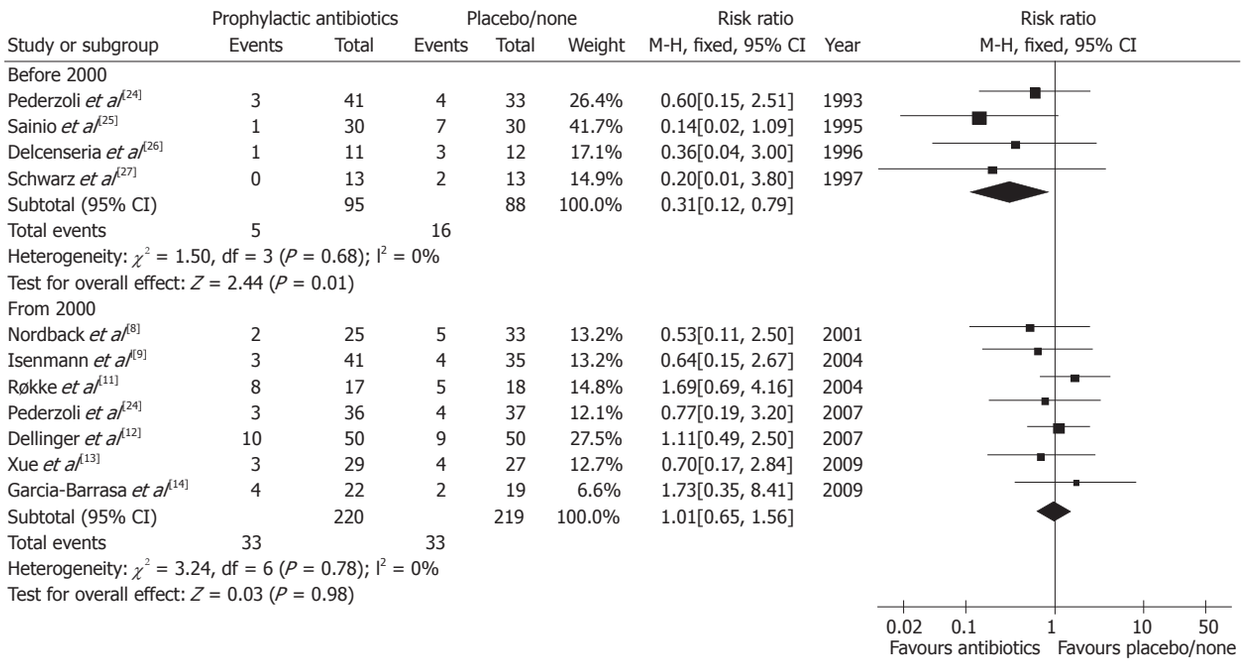

Figure 2 Updated meta-analysis of antibiotic prophylaxis *vs* placebo/none-treatment. The meta-analysis was stratified into two periods, i.e., before 2000 and from 2000. In the later period, no benefit was obtained from prophylactic antibiotics (RR = 1.01, *P*=0.98; Mantel-Haenszel test, fixed effect model, two-sided).

Table 2 Mortality rates and related parameters between different periods

Period	Antibiotics[1] (%)	Control[2] (%)	ARR (%)	RRR (%)	NNT
Before 2000	5.26	18.18	12.92	71.07	8
From 2000	15.00	15.07	0.07	0.46	1429

[1]The mortality rate of the subgroup before 2000 was significantly lower than the subgroup from 2000 ($P = 0.019$; by SPSS 13.0, Pearson χ^2 test, two-sided); [2]No significant difference of mortality rate between two periods ($P = 0.571$; by SPSS 13.0, Pearson χ^2 test, two-sided). ARR: Absolute risk reduction; RRR: Relative risk reduction; NNT: Number needed to treat.

Table 3 Estimate of required sample size based on the pooling estimate from 2000

Δ	α	β	μα	μβ	p1	p2	|p1-p2|	Group	Study
0.10	0.05	0.10	1.9600	1.2816	0.1500	0.1507	0.0007	272	544
0.05	0.05	0.05	1.9600	1.9600	0.1500	0.1507	0.0007	1615	3230

Δ: Threshold of difference value; α: Possibility of type I error; β: Possibility of type Ⅱ error; μα: Critical value corresponding to α; μβ: Critical value corresponding to β; p1: Possibility of mortality in prophylactic antibiotics group; p2: Possibility of mortality in placebo or blank control group; |p1-p2|: Absolute value of difference between two groups.

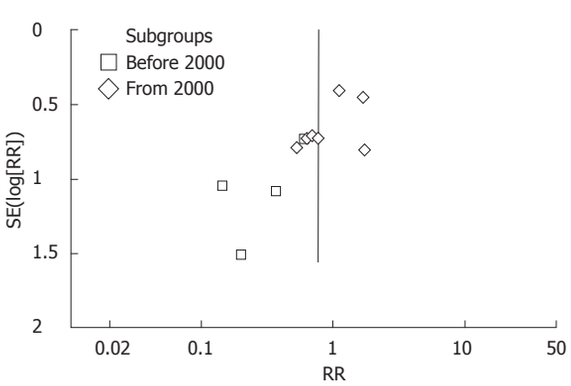

Figure 3 Funnel plot of the meta-analysis. Apparently asymmetrical distribution was found in the period before 2000.

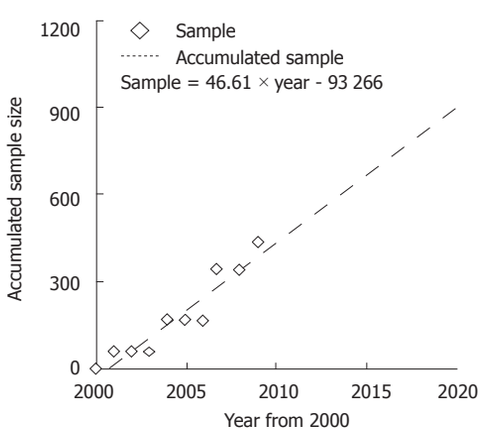

Figure 4 Linear correlation of accumulated sample size from 2000. There is highly positive correlation between the accumulated sample size and the year from 2000 ($r = 0.954$, $P = 0.000$; Pearson correlation test, two-sided).

indicated that the overall accumulated sample size need 3230 patients. The linear regression test showed the accumulated sample size is positively correlated with the time period (year by year from 2000) (Figure 4). If there were no larger-scale RCTs, it would take a long time to achieve the statistical goal, and the controversy would continue.

An assumed large-scale trial (sensitivity analysis): If years later a large-scale RCT is completed, how will it contribute to addressing the controversy on prophylactic antibiotics for SAP? Thus, we carried out a sensitivity analysis

Table 4 Sensitivity analysis by adding a large-scaled randomized controlled trial

	Antibiotics		Placebo/none-treatment	
	Mortality rate	No. of events	Mortality rate	No. of events
By best/worst data[1]	7.32%	40/544	27.78%	151/544
Meta-analysis[3]	*RR* = 0.76, 95% CI: 0.38-1.53, *P* = 0.44 (random effect model)			
By pooling data[2]	15.00%	82/544	15.07%	82/544
Meta-analysis[3]	*RR*=1.00, 95% CI: 0.79-1.27, *P* = 0.99 (fixed effect model)			

[1]The mortality rate of antibiotic prophylaxis was obtained from the best outcome in a randomized controlled trial (RCT) after 2000, while that of placebo/none-treatment was from the worst outcome in another RCT after 2000; [2]The mortality rates were from the pooling data of the subgroup from 2000 in the updated meta-analysis; [3]The meta-analysis included both the 7 RCTs published from 2000 and an assumed large-scale RCT (by Cochrane RevMan 5.0, Mantel-Haenszel test, two-sided).

to estimate the margin of potential benefit from prophylactic antibiotics (Table 4). It was performed by re-pooling both the 7 RCTs from 2000 and an assumed large-scale RCT containing 544 patients for meta-analysis[8-14]. The theoretical mortality rates of assumed RCT were evaluated as follows.

Given there was obvious random sampling error, the best-worst method was used to calculate the best marginal benefit (Table 4). Since there was between-study heterogeneity ($P = 0.0003$), the meta-analysis was performed by random effect model. The result of sensitivity analysis showed no significant benefit of antibiotic prophylaxis for SAP ($RR = 0.76$, $P = 0.44$), which was similar with the previous meta-analyses (forest plot not shown).

If there was no random sampling error, the mortality rates would be equal to the pooling data from above updated meta-analysis (Table 4). The heterogeneity of repooling estimate was not significant ($P = 0.86$), so fixed effect model was used. The result of sensitivity analysis showed nearly equivalent efficacy between antibiotic prophylaxis and non-antibiotic treatment ($RR = 1.00$, $P = 0.99$) (forest plot not shown).

Therefore, we can assume that even if a large-scale RCT was completed, the *RR* of the mortality rate would only range from 0.77 to 1.00 with a relatively narrow confidence interval ($P < 0.05$). It means, as a whole, that antibiotic prophylaxis is not effective for SAP patients. Moreover, by the sensitivity analysis, the NNT ranged from 7 to 5069 patients.

DISCUSSION

As the mortality of SAP is obviously associated with the complications of infection, prophylactic antibiotics have been administrated for SAP patients for several decades, which seemed to play an important role in the treatment of SAP. However, currently, its role is highly questioned by more and more RCTs and meta-analyses, and the controversies continue due to insufficient evidence.

Among the existing meta-analyses, only one meta-analysis showed a significant reduction in the mortality rate by antibiotic prophylaxis, and most of the meta-analyses did not recommend the administration of antibiotic prophylaxis. Therefore, the current academic opinion obviously trends to be unfavorable for antibiotic prophylaxis for SAP. However, antibiotic prophylaxis has not been given up in clinical practice in the treatment of SAP. Why do physicians often go reversely in aspect of the decision-making on antibiotic prophylaxis for SAP?

Practice of evidence-based medicine is a procedure of integrating the best available external clinical evidence with clinical expertise and patient needs[28]. There should be a balance among these three aspects. If the power of current available evidence is not robust enough, the influence of clinical expertise will be inevitably enhanced. Thus, physicians are quite cautious to the available evidence due to the weakness in the meta-analyses. Firstly, the eligible RCTs are fairly small-sized and the accumulated sample size is also limited. Secondly, the validity of some RCTs in earlier period is affected by the absence of blinding method.

In the funnel plot of our updated meta-analysis, the asymmetric distribution of RCTs before 2000 implied that there might be apparent publication bias. At that period, the positive results of trials tended to be accepted for publication more easily. Therefore, most of the scholars believed that the evidence before 2000 would be weak to validate. Another critical reason is that blinding method was not used in these trials, which may result in performance and observation biases. Thus, positive results might be more easily to reach under that condition. Since the year of 2000, the improved methodology of RCTs has made the pooling estimate non-significant.

By now, physicians have become more conservative and suspicious about the administration of prophylactic antibiotics for SAP. The effectiveness of prophylactic antibiotics seemed to be equal to the placebo or blank control. Whether the evidence obtained in the current decade is robust enough to make a mandatory recommendation to quit the administration of prophylactic antibiotics for SAP? As small-sized RCTs inevitably result in the random sampling error. Thus, there must be a long way to go to answer this question.

Through our hypothesis and calculation, we predict that antibiotic prophylaxis would not be effective as a whole in reducing the mortality of SAP patients, even if a large enough RCT was completed. However, the minor lower limit of NNT range implies that certain SAP patients might potentially be benefited by antibiotic prophylaxis. Therefore, if possible, the individual patient data analysis will be meaningful to identify potential candidates who can gain survival benefit from antibiotic prophylaxis.

COMMENTS

Background

The mortality of severe acute pancreatitis (SAP) is obviously associated with the infectious complications, so prophylactic antibiotics have been administrated for SAP patients for several decades. However, the role of prophylactic antibiotics in reduction of mortality of SAP patients has been highly questioned by more and more randomized controlled trials (RCTs) and meta-analyses.

Research frontiers

Evidence-based medicine is frequently used in clinical practice. Although there have been several meta-analyses on the administration of prophylactic antibiotics for SAP, the conclusion is still not confirmed. By now, some new reports on this topic have become available for updated review and analysis.

Innovations and breakthroughs

An updated meta-analysis on the mortality of SAP patients was performed. Prophylactic antibiotics for SAP was compared with control or placebo. Subgroup analysis showed that there was significant reduction of mortality rate in the period before 2000, while no significant reduction in the period from 2000. Sensitivity analysis by assuming an ideal large-scale RCT was performed to prove the results, and found that SAP patients did benefit from prophylactic antibiotics. In addition, current evidences do not support administration of prophylactic antibiotics as a routine treatment for SAP.

Applications

Although the administration of prophylactic antibiotics for SAP in general practice is controversial, there is still a potentially high-risk sub-population who could benefit from prophylactic antibiotics.

Terminology

Prophylactic antibiotics, is administrated to the sterile SAP patients to prevent the potential and even fatal complications, involving both peri-pancreatic and systematic infection.

Peer review

The paper investigates the role of prophylactic antibiotics on mortality of severe acute pancreatitis. The statistical analysis used in the study is appropriate and the results are reliable.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i3.285

World J Gastroenterol 2012 January 21; 18(3): 285-290
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

^{1}H NMR-based serum metabolic profiling in compensated and decompensated cirrhosis

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Received: April 26, 2011 Revised: June 13, 2011
Accepted: June 20, 2011
Published online: January 21, 2012

Abstract

AIM: To study the metabolic profiling of serum samples from compensated and decompensated cirrhosis patients.

METHODS: A pilot metabolic profiling study was conducted using three groups: compensated cirrhosis patients (n = 30), decompensated cirrhosis patients (n = 30) and healthy controls (n = 30). A ^{1}H nuclear magnetic resonance (NMR)-based metabonomics approach was used to obtain the serum metabolic profiles of the samples. The acquired data were processed by multivariate principal component analysis and orthogonal partial least-squares discriminant analysis (OPLS-DA).

RESULTS: The OPLS-DA model was capable of distinguishing between decompensated and compensated cirrhosis patients, with an R^2Y of 0.784 and a Q^2Y of 0.598. Twelve metabolites, such as pyruvate, phenylalanine and succinate, were identified as the most influential factors for the difference between the two groups. The validation of the diagnosis prediction showed that the accuracy of the OPLS-DA model was 85% (17/20).

CONCLUSION: ^{1}H NMR spectra combined with pattern recognition analysis techniques offer a new way to diagnose compensated and decompensated cirrhosis in the future.

Key words: Liver cirrhosis; Metabolic profiling; Orthogonal partial least-squares discriminant analysis

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Qi SW, Tu ZG, Peng WJ, Wang LX, Ouyang X, Cai AJ, Dai Y. ^{1}H NMR-based serum metabolic profiling in compensated and decompensated cirrhosis. *World J Gastroenterol* 2012; 18(3): 285-290 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i3/285.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i3.285

INTRODUCTION

Liver cirrhosis (LC) and its associated complications are a major cause of morbidity and mortality worldwide[1,2]. During early cirrhosis, the liver is able to compensate the changes resulting from bridging fibrosis, and most patients do not show specific symptoms until they enter the stage of decompensated cirrhosis[3]. Patients often miss the best opportunity for therapy because the hepatic reserve in the decompensated stage is unable to compensate for hepatocyte loss and structural distortions.

The diagnostic confirmation of cirrhosis is based on a histological examination or the combined results of clinical and imaging examinations[1]. However, the proposed

methods cannot be satisfactorily applied to a clinical diagnosis. Firstly, histological examination requires the puncturing of liver tissue and causes substantial pain. Secondly, the clinical results derived from a series of laboratory assays are costly and require long periods of time. Finally, imaging studies cannot provide sensitive diagnoses and are vulnerable to the influence of subjective factors. Therefore, establishing a simple and specific strategy to diagnose cirrhosis and distinguish decompensated LC from compensated LC is important for patient care and treatment decisions.

Metabonomics is a comprehensive and fully quantitative analysis of low molecular weight molecules within a particular biological sample. ^{1}H nuclear magnetic resonance (NMR) spectra combined with principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) are frequently conducted in metabolic profiling studies[4-6].

The incidence of acute and chronic viral hepatitis in China is very high and hepatitis B virus (HBV) contributes to 80% of liver cirrhosis[7]. Thus, in the present study, we employed quantitative ^{1}H NMR to analyze the serum metabolic profiling in HBV-infected cirrhosis patients. Our purpose was to establish a diagnostic method for decompensated and compensated cirrhosis and to discover metabolomic biomarkers related to the cirrhosis conditions.

MATERIALS AND METHODS

Patients and controls

This study enrolled 60 patients (30 compensated and 30 decompensated cirrhosis patients) from the Department of Gastroenterology and Infectious Diseases of Shenzhen People's Hospital from November 2009 to July 2010. We excluded patients with past or current hepatocellular carcinoma, alcoholic cirrhosis, diabetes, cardiovascular and cerebrovascular disease, kidney disease and any other viral co-infection, including human immunodeficiency virus, hepatitis delta or hepatitis C virus. Thirty healthy volunteers served as controls. The baseline clinical characteristics of the cirrhotic patients and controls are summarized in Table 1. This study was performed according to the guidelines of Chongqing Medical University, which abides by the Declaration of Helsinki on ethical principles for medical research involving human subjects.

Clinical criteria

All patients were positive for hepatitis B surface antigen for at least 1 year before screening. Cirrhosis patients were diagnosed according to the results of histological examination or the combined results of clinical and imaging examinations. Decompensated cirrhosis was defined as the presence of at least two of the following five criteria: ascites, hyperbilirubinemia, peripheral edema of noncardiac or renal origin, hypoalbuminemia and an INR (clotting times, as reflected by the international normalized ratio) > 1.3[8]. The severity of liver disease was calculated according to the model for end-stage liver disease[9].

Table 1 Clinical and biochemical characteristics of the cirrhotic patients and controls

Parameter	Control (n = 30)	Liver cirrhosis	
		Compensated LC (n = 30)	Decompensated LC (n = 30)
Age (yr)	48.8 ± 10.5	56.3 ± 12.9	58.7 ± 14.5
Gender (M:F)	12:18	14:16	15:15
ALT (U/L)	20.4 ± 10.2	110.4 ± 20.2[a]	85.4 ± 32.6[a]
AST (U/L)	15.3 ± 11.6	187.5 ± 100.5[a]	142.4 ± 52.9[a]
TP (g/L)	65.4 ± 5.6	61.5 ± 10.2	60.4 ± 5.7[a]
ALB (g/L)	50.4 ± 10.2	48.6 ± 6.9	35.8 ± 11.2[a,b]
T-BIL (μmol/L)	11.4 ± 3.6	35.8 ± 30.9[a]	45.6 ± 20.2[a]
D-BIL (μmol/L)	5.4 ± 1.2	15.4 ± 8.3[a]	23.4 ± 10.2[a,b]
GLU (mmol/L)	5.31 ± 1.6	5.48 ± 1.23	5.47 ± 0.98
CRE (μmol/L)	113.6 ± 56.3	140.3 ± 50.8	152.6 ± 34.2[a]
INR	1.0 ± 0.05	1.1 ± 0.06	1.2 ± 0.12
MELD score	-	12.7 ± 3.5	14.2 ± 6.3

[a]P < 0.05 *vs* control, [b]P < 0.05 *vs* compensated group. Data are the number of patients or the mean ± SD.

Metabolomic and clinical chemistry analysis

Approximately 5 mL of peripheral venous blood from fasted healthy volunteers and LC patients was collected. The blood was allowed to clot for 30 min at room temperature before being centrifuged at 2000 *g* for 10 min at 25 ℃; the serum was separated and stored at -80 ℃. Prior to NMR analysis, serum samples were thawed and 400 μL aliquots were mixed with 150 μL of deuterium oxide. The serum samples were centrifuged at 12 000 *g* for 10 min at 4 ℃, and 500 μL aliquots of the resulting supernatants were placed into 5 mm NMR tubes. All NMR spectra were recorded at 25 ℃ on a Varian Unity INOVA 600 NMR spectrometer. One-dimensional spectra were recorded using the Carr-Purcell-Meiboom-Gill sequence[10,11] with a spin-spin relaxation delay of 120 ms and a spectral width of 8000 Hz. All spectra were carefully phase- and baseline-corrected and referenced to the internal lactic acid CH3 resonance at 1.33 ppm. Spectra were segmented into 0.005-ppm chemical shift "bins" between 0.5 and 9.0 ppm, and the spectral area within each bin was integrated. Bins between 4.7 and 5.2 ppm containing residual water were removed. The free induction decay was zero-filled to 64 K and multiplied by an exponential line-broadening function of 0.3 Hz prior to Fourier transformation.

Multivariate analysis

Multivariate statistics, including unsupervised PCA and supervised OPLS-DA, were performed using SIMCA-P 11.0 software (Umetrics, Umea, Sweden). Analysis of the metabolite signals in the ^{1}H NMR serum profiles was first performed using unsupervised PCA, which displays the internal structure of datasets in an unbiased way and decreases the dimensionality of data[12-14]. After an initial overview of the PCA analysis, we obtained a more sophisticated OPLS-DA model with the specific discriminant information between the different groups[15,16]. The differences in the metabolites between groups were shown as coefficient of variation plots. Using a significance level of

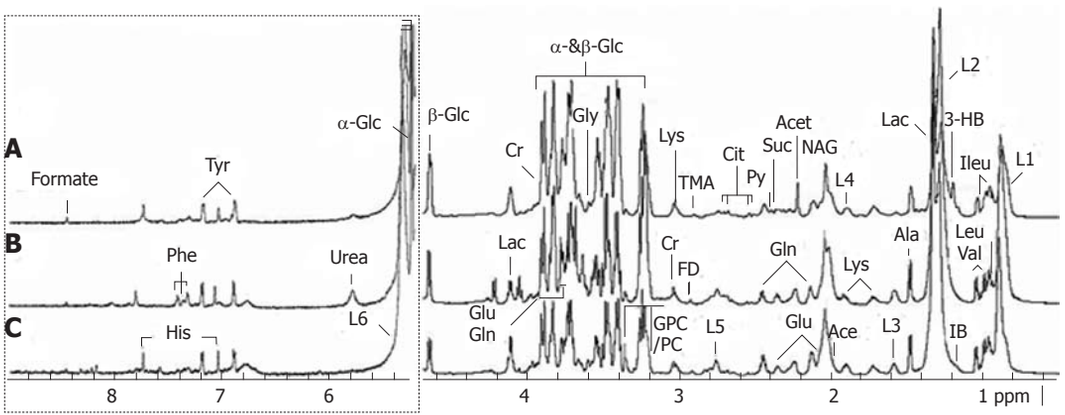


Figure 1 600 MHz 1H NMR spectra (δ0.5-4.7 and δ5.2-9.0) of serum obtained from (C) control, (B) compensated liver cirrhosis and (A) decompensated liver cirrhosis patients. The region of δ5.2-9.0 (in the dashed box) is magnified 8 times compared to the corresponding region of δ0.5-4.7 for the purpose of clarity. 3-HB: 3-Hydroxybutyrate; Ace: Acetate; Acet: Acetone; Ala: Alanine; Cit: Citrate; Cr: Creatine; FD: Formaldehyde; Glu: Glutamate; Gln: Glutamine; Gly: Glycine; GPC: Glycerophosphocholine; His: Histidine; Ile: Isoleucine; IB: Isobutyrate; Lac: Lactate; Leu: Leucine; L1 Lipid: CH_3-$(CH_2)n$-(LDL&VLDL); L2 Lipid: CH_3-$(CH_2)n$-(LDL&VLDL); L3 Lipid: -CH_2-CH_2-C=O; L4 Lipid: -CH_2-CH=CH-; L5 Lipid: =CH-CH_2-CH=; L6 Lipid: -CH=CH-; Lys: Lysine; NAG: N-acetyl glycoprotein signals; PC: Phosphocholine; Phe: Phenylalanine; Py: Pyruvate; Suc: Succinate; TMA: Trimethylamine; Tyr: Tyrosine; Val: Valine; α-Glc: α-Glucose; β-Glc: β-Glucose.

0.05, we employed a correlation coefficient of ± 0.355 as the threshold to choose the variables that best correlated with the OPLS-DA discriminative scores. To further test whether the metabolic profiling can effectively distinguish decompensated cirrhosis patients from compensated cirrhosis ones, we randomly selected 20 cirrhotic patients (10 compensated and 10 decompensated cirrhosis patients) to validate the discriminatory power of the OPLS-DA model.

Statistical analyses were performed using SPSS 11.5 software (SPSS Company, Chicago, IL, United States). The threshold *P* value was set at 0.05 throughout the study.

RESULTS

^{1}H NMR spectroscopy of serum samples

The ^{1}H-NMR signals of all common metabolites, such as amino-acids, organic acids and carbohydrates, were assigned according to previous publications[10]. Examples of typical serum spectra from control, compensated and decompensated cirrhosis groups are shown in Figure 1. The 600 MHz ^{1}H NMR spectra demonstrated resonances arising from glucose, glutamine, acetate, leucine, glycerophosphocholine, histidine, isoleucine, citrate, etc. The useful extracted information was subsequently analyzed using multivariate statistics including PCA and OPLS-DA.

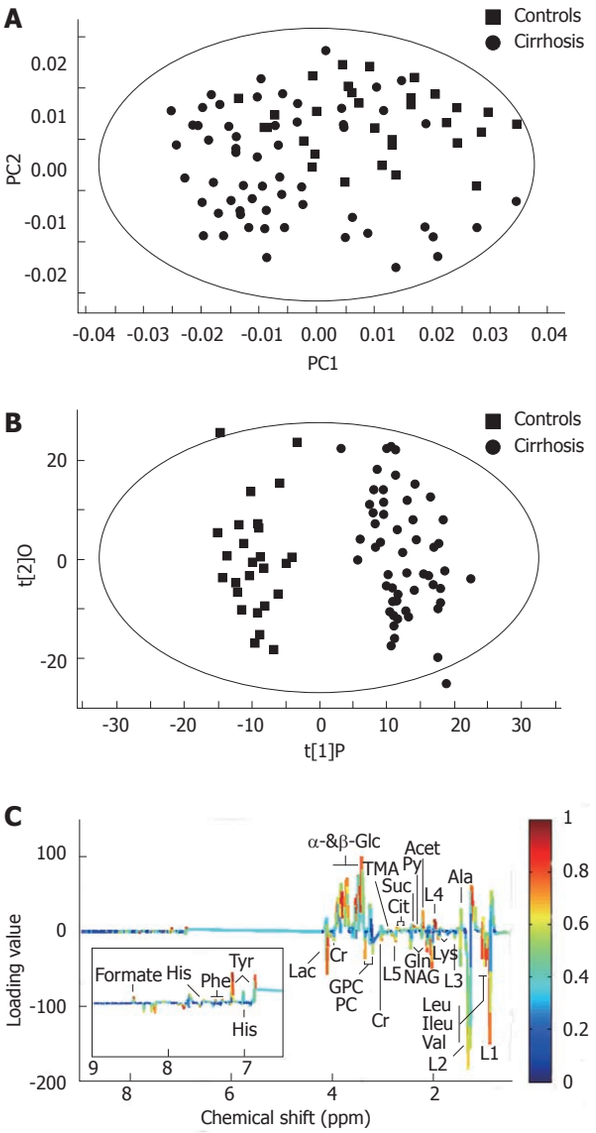
Multivariate statistics

As a proof of principle, we first evaluated whether a metabonomics approach would be capable of distinguishing cirrhotic patients (included compensated and decompensated cirrhosis) from healthy controls.

At first, we analyzed the serum metabolic profiles using unsupervised PCA. Figure 2A displays the PCA score plots of cirrhotic patients and healthy controls, with an R^2X of 0.812 and a Q^2Y of 786. The PCA was followed by OPLS-DA, which is more focused on discriminatory variations. Excellent separation with negligible overlapping was observed in OPLS-DA score plots between controls and cirrhosis patients. This model showed very good fit and predictability values with an R^2Y of 0.941 and a Q^2Y of 0.836 (Figure 2B). The loading plots (Figure 2C) revealed that the responsible variables were those corresponding to β-glucose, α-glucose, low-density lipoproteins (LDL), very low-density lipoproteins (VLDL), valine, tyrosine, succinate, lipid, isobutyrate, glutamine, glutamate, *etc*.

A similar procedure was performed to assess the utility of metabonomics to distinguish decompensated LC from compensated LC patients. A PCA score plot (Figure 3A) could not reveal an obvious separation between compensated and decompensated LC, with an R^2X of 0.795 and a Q^2Y of 0.736. The overlap in PCA score plots suggested that there was some metabolic variation between the two classes not related to the disease state. OPLS-DA revealed a clear separation between decompensated and compensated LC patients (Figure 3B), with an R^2Y of 0.784 and a Q^2Y of 0.598. The majority of the disease-related variance could be explained by the first component of the model. The OPLS-DA loading plot (Figure 3C) revealed that the significant variables were those corresponding to pyruvate, phenylalanine, succinate, lysine, *etc*. The variables meeting the cutoff value (i.e., |correlation coefficient| > 0.355) are summarized in Table 2.

The serum metabolic profiles of 20 cirrhotic patients were overlaid in the OPLS-DA model to evaluate the diagnostic performance (Figure 3D). Of these, eight compensated cirrhosis patients were clustered together in the left section of the OPLS-DA model (compensated group), nine decompensated cirrhosis patients were clustered together in the right section of the OPLS-DA model (decompensated group), and the three remaining cirrhosis patients were situated in an intermediate area between the decompensated and compensated cirrhosis patients.



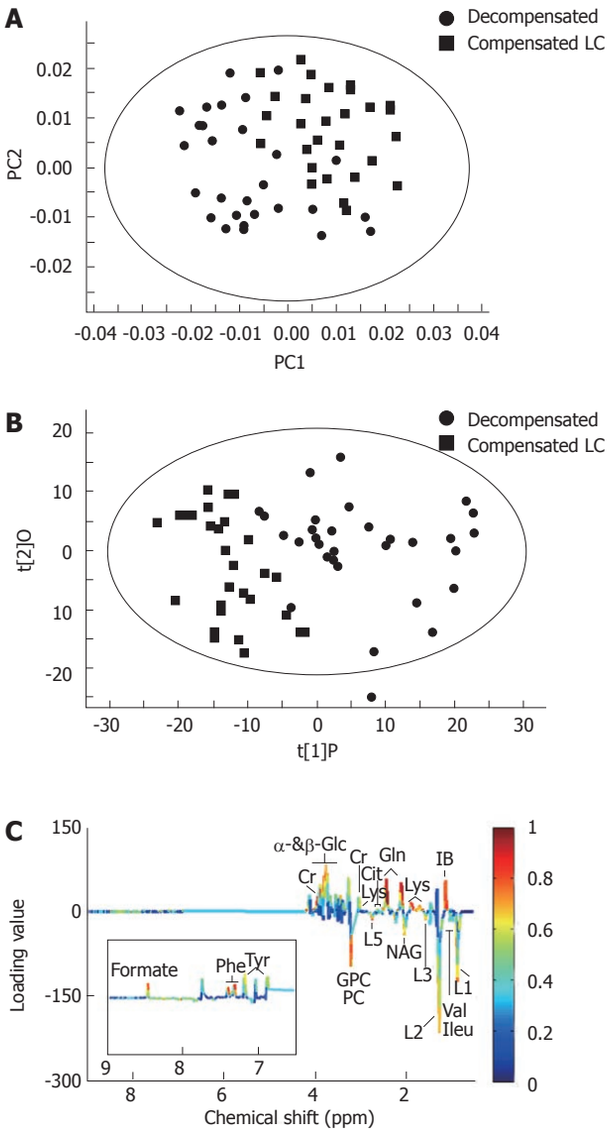


Figure 2 Multivariate modeling of control and cirrhotic subjects. A: Principal component analysis score plot of the serum ^{1}H nuclear magnetic resonance spectra from controls (squares) and cirrhotic patients (dots); B: Orthogonal partial least-squares discriminant analysis (OPLS-DA) score plot of controls (squares) and cirrhotic patients (dots); C: Corresponding coefficient loading plots for the discrimination of the OPLS-DA model.

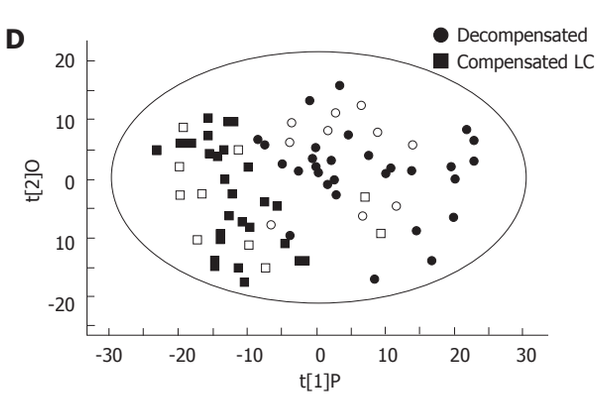


Figure 3 Multivariate modeling of compensated and decompensated liver cirrhosis patients. A: Principal component analysis score plot of the serum ^{1}H nuclear magnetic resonance spectra from compensated liver cirrhosis (LC) (squares) and decompensated LC (black dots) patients. B: Orthogonal partial least-squares discriminant analysis (OPLS-DA) score plot of compensated LC (squares) and decompensated LC (dots) patients; C: Corresponding coefficient loading plots for the discrimination of the OPLS-DA model; D: Validation tests of the discriminatory power using the OPLS-DA model. Twenty cirrhotic patients were selected randomly and included 10 compensated LC (empty squares) and 10 decompensated LC (empty dots) patients.

DISCUSSION

Previous studies have shown that the transition from compensated to decompensated cirrhosis is so insidious that many patients miss the best opportunity for treatment[8,17]. In the present study, we applied NMR-based metabolic profiling to examine the serum from decompensated and compensated LC patients and identified low molecular weight biomarkers for the diseases.

The findings of this pilot study showed that the ^{1}H NMR serum metabolic profiling of cirrhotic patients and control subjects was clearly separated. Compared with healthy controls, the serum metabolic profiling of cirrhotic patients showed increased levels of glucose and lactate and decreased levels of lipids and choline. In

Table 2 Orthogonal partial least-squares discriminant analysis coefficients derived from the nuclear magnetic resonance data of metabolites in serum obtained from compensated and decompensated cirrhosis patients

Metabolites	δ^1H	Correlation coefficients	
		Compensated LC	Decompensated LC
β-Glucose	3.24, 4.65	0.654	0.722
α-Glucose	3.425.24	0.602	0.806
CH_3-(CH_2)n-(LDL&VLDL)	1.28	0.748	-0.693
Valine	0.99, 1.04	-0.903	-0.910
Tyrosine	6.89, 7.19	0.675	0.805
Succinate	2.40	-	0.603
Pyruvate	2.37	-	0.585
Phenylalanine	7.32, 7.41	-	0.749
NAG:N-acetyl glycoprotein	2.04	-0.583	-0.774
Lysine	1.72, 3.01, 3.78	-0.602	-
Lipid, H_3-(CH_2)n-(LDL&VLDL)	0.86	0.805	-0.793
Leucine	0.93	-0.876	-0.727
Lactate	1.33, 4.12	-0.671	-0.769
Isoleucine	0.96, 1.01	-0.916	-0.859
Histidine	7.06, 7.75	-	0.616
GPC/PC	3.21, 3.35	-0.732	-0.712
Glutamine	2.14, 2.45, 3.78	-0.659	0.706
Glutamate	2.03, 2.35, 3.78	-	0.554
Creatine	3.04, 3.93	-0.709	0.555
Citrate	2.53, 2.67	0.657	0.604
Alanine	1.48	-	0.571
Acetone	2.23	0.729	-

The positive and negative signs indicate a positive and negative correlation in the concentrations, respectively. "-": The |correlation coefficient| is less than 0.355.

this context, our results are consistent with the studies of Gao *et al*[18], which showed that ^{1}H NMR metabonomics analysis of serum is helpful for differentiating cirrhotic patients from healthy subjects.

However, little work has been done to discriminate patients with compensated and compensated cirrhosis through metabonomics. Corbin *et al*[8] used phosphorus-31 magnetic resonance spectroscopy to document the differences in the hepatic metabolite concentrations among patients with compensated and decompensated cirrhosis. Although this study provided a useful and noninvasive diagnostic tool, the researchers only studied five metabolites and did not investigate all metabolites related to cirrhosis.

In contrast, NMR has the advantage of full quantitative analysis and minimal requirements for sample preparation[5]. In the present study, we obtained a 'metabolic fingerprint' from the pathological process of cirrhosis. Compared with compensated LC patients, the decompensated LC patients displayed higher levels of pyruvate, phenylalanine, succinate, lysine, histidine, alanine, glutamate, glutamine, creatine and lower levels of LDL, VLDL and acetone.

The increased concentration of pyruvate and succinate in decompensated LC serum is possibly due to the reduced utilization of pyruvate and succinate into the tricarboxylic acid cycle[19]. Phenylalanine belongs to the aromatic group of amino acids, and it is converted to tyrosine by the catalysis of phenylalanine hydroxylase, which is a liver-specific enzyme[20]. The increase in phenylalanine suggests that the BCAA/AAA ratio has changed in patients with decompensated LC. Glutamate is at the very center of hepatic amino acid metabolism. Glutamine, histidine, arginine, ornithine, proline, and glutamate comprise the 'glutamate family' of amino acids[21]. The elevated levels of glutamate, glutamine and histidine reflect the abnormal metabolism of amino acids with cirrhosis progression.

Our study showed that the levels of VLDL and LDL were reduced in the decompensated LC group compared to the compensated LC group. Because liver tissue loses some degree of its lipid synthesizing ability in the later stage of cirrhosis, the blood lipid level decreases in the serum of decompensated LC patients[22].

The remaining metabolite changes include increased lysine and alanine and decreased acetone; altered levels of these metabolites in decompensated LC patients may be the result of the impairment of hepatocytes, but may also be the result of liver perfusion.

Taken together, these results imply that hypermetabolism of the liver was prevalent when compensated cirrhosis developed into the decompensated stage. Hence, these metabolites may serve as biomarkers that can be used to monitor the changes in LC patients.

Regarding predictive tests of the discriminatory power, the results showed that the accuracy of the OPLS-DA was 85% (17/20). Three patients located in the middle area of the OPLS-DA model were observed carefully in the following two months. One compensated LC patient developed decompensated cirrhosis, and the remaining two LC patients did not exhibit distinct changes. It is conceivable that the metabolic changes revealed by the NMR spectra could be more sensitive than other clinical symptom and lab tests.

Serum metabolic analysis bears the potential to be a useful and convenient method to diagnose LC patients. However, there are some potential limitations of the present study that require consideration. Firstly, a limited number of samples prevented us from drawing a more reliable conclusion about the predictive power of this model. Larger numbers of patients and controls will be crucial to validate this model. Secondly, the specificity of the biomarkers indentified by OPLS-DA requires a longitudinal study to determine their validity.

In summary, the metabolic profiling obtained from ^{1}H NMR-based metabonomics analysis of serum may be a simple and reliable way to diagnose compensated and decompensated LC. The metabolic differences between the two groups also facilitate a better understanding of the metabolic changes associated with cirrhosis progression.

ACKNOWLEDGMENTS

The authors would like to thank the patients and healthy volunteers who participated in this study.

COMMENTS

Background

The diagnostic confirmation of cirrhosis is based on histological examination or the combined results of clinical and imaging examinations. However, the proposed methods cannot be satisfactorily applied in clinical diagnosis.

Research frontiers

^{1}H nuclear magnetic resonance (NMR) is an ideal instrumental platform for metabolic analysis of biofluids, and NMR analysis has been reported as successfully applied in the diagnosis and prognosis of some human diseases.

Innovations and breakthroughs

The authors compared the serum metabolic profiling of compensated and decompensated cirrhosis patients. The orthogonal partial least-squares discriminant analysis (OPLS-DA) model was successful in distinguishing decompensated cirrhosis from compensated cirrhosis. Twelve metabolites were identified as the most influential biomarkers for the difference between the two groups.

Applications

Serum metabolic analysis bears the potential to be a useful and convenient method to diagnose compensated and decompensated cirrhosis.

Terminology

Principal component analysis (PCA): PCA is a mathematical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of uncorrelated variables called principal components; orthogonal partial least-squares discriminant analysis (OPLS-DA): OPLS-DA is a method of discriminating between two or more groups. The variables responsible for the differences may be identified.

Peer review

In this manuscript, the authors employed metabolomics to compare serum metabolic profiles among control, compensated and decompensated cirrhosis patients. Rationale and results are sound.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i3.291

World J Gastroenterol 2012 January 21; 18(3): 291-294
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Endoscopic submucosal dissection for large laterally spreading tumors involving the ileocecal valve and terminal ileum

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Received: October 11, 2010 Revised: May 27, 2011
Accepted: June 4, 2011
Published online: January 21, 2012

Abstract

Endoscopic submucosal dissection is a challenging technique that enables *en-bloc* resection for large colorectal tumors, as laterally spreading tumors, particularly difficult, if the ileocecal valve and terminal ileum is involved. Herein, we report on one of 4 cases. The procedures, using a bipolar needle knife (B-Knife) to reduce the perforation risk and carbon dioxide instead of conventional air insufflation for patient comfort, achieved curative resections without any complications.

Key words: Ileocecal valve; Colorectal neoplasms; Laterally spreading tumor; Endoscopic mucosal resection; Endoscopic submucosal dissection; Bipolar current needle knife; B-Knife; IT-Knife

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Kishimoto G, Saito Y, Takisawa H, Suzuki H, Sakamoto T, Nakajima T, Matsuda T. Endoscopic submucosal dissection for large laterally spreading tumors involving the ileocecal valve and terminal ileum. *World J Gastroenterol* 2012; 18(3): 291-294 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i3/291.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i3.291

INTRODUCTION

Laterally spreading tumors (LSTs)[1] are mainly located in the cecum and rectum and endoscopic mucosal resection (EMR) is the therapeutic choice[2,3] because of their lower incidence of submucosal (sm) invasion. Involvement of the ileocecal valve with possible spreading to the terminal ileum, however, complicates the EMR and resulting in piecemeal resection in which persistent recurrence leads to surgery even for adenomas and intramucosal cancers[4]. In addition, the thin wall of the narrow ileum increases the risk of perforation during EMR[5].

Our experience suggests that the well-established endoscopic submucosal dissection (ESD) also produces good results in colorectal cases as in early gastric cancer treatment[6]. Unlike the stomach, colonic ESD presents high risk of complications[7] and increased patient discomfort. These factors motivated us to develop better techniques to achieve successful ESDs such as using a bipolar needle knife (B-Knife® Zeon Medical Co. Tokyo, Japan)[8] to reduce the risk of perforation and carbon dioxide (CO_2) instead of conventional air insufflation for patient comfort[9].

In the present report, we describe our experience and the utility of using ESD on one of 4 complete resections of large LSTs (70 mm in diameter) involving the ileocecal valve and terminal ileum.

CASE REPORT

A 76-year-old woman was referred to our hospital for endoscopic treatment of a neoplastic lesion located at the

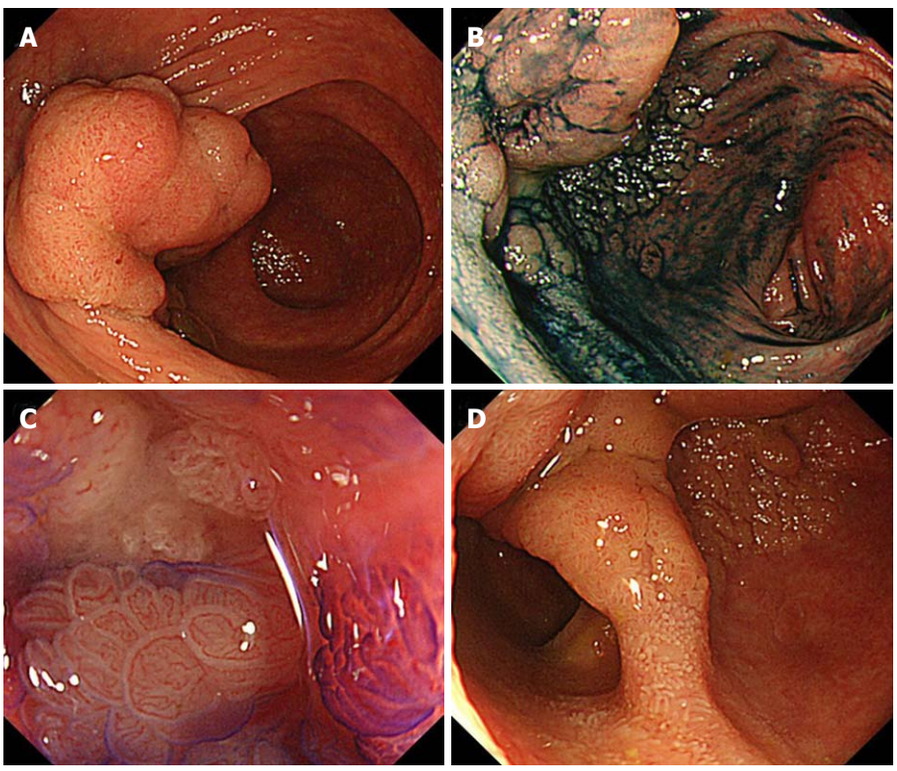

Figure 1 Pre-treatment endoscopic evaluation. A: Close view of the cecum revealed a 70 mm Ⅰs+Ⅱa, LST granular type (LST-G) lesion; B: Clearly delineated margin of the LST-G lesion after 0.4% indigo-carmine dye spraying; C: Magnification view of the Is component of the Ⅰs+Ⅱa (LST-G); D: Spreading confirmation of the tumor through the ileocecal valve to the terminal ileum.

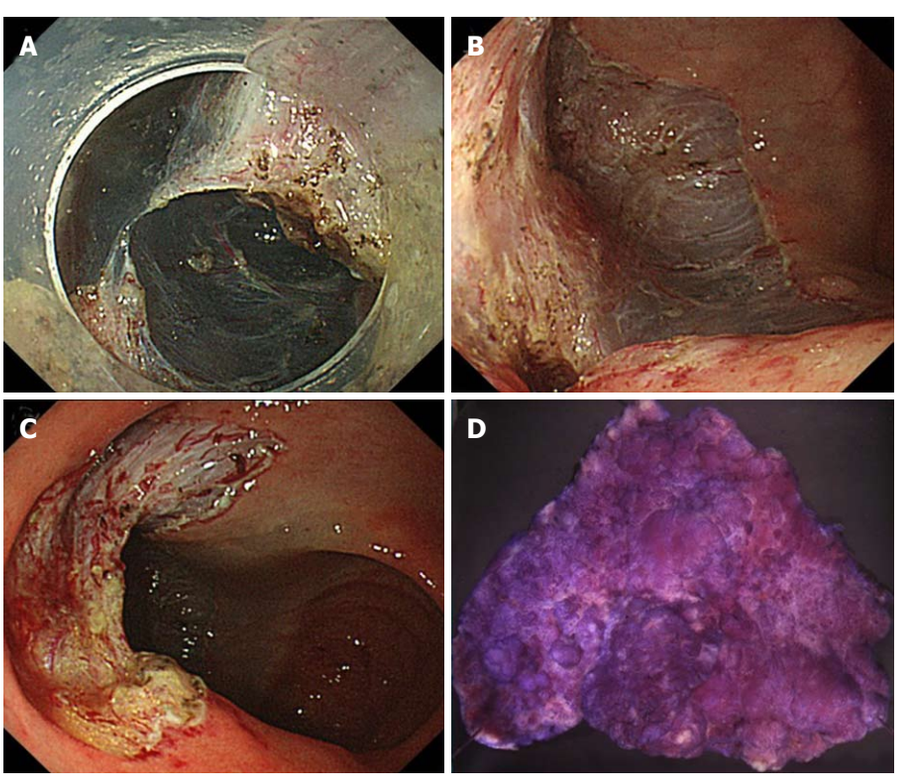

Figure 2 Procedure. A: Endoscopic view through the distal attachment showing dissection with insulation-tip knife; B: Carefully check for bleeding throughout the ileocecal region; C: The ulcer bed of ileum after *en-bloc* endoscopic submucosal dissection; D: Stereomicroscopic view presenting the resected specimen, which pathology reported as a Ⅰs+Ⅱa intramucosal cancer with tumor-free margins of 70 mm in diameter.

cecum. Conventional colonoscopy revealed a broad base, flat tumor. After 0.4% indigo-carmine dye spraying, the margin of the 70 mm-lesion was clearly delineated (Figure 1A and B). High-magnification colonoscopy (PCF-Q240ZI; Olympus Optical Co. Ltd, Tokyo, Japan) disclosed a non-invasive pit pattern[10-12] indicating an intramucosal cancer despite the lesion's large size (Figure 1C). Extension onto the terminal ileum until 1.5 cm from the ileocecal valve was also observed (Figure 1D). After diagnosing a Ⅰs+Ⅱa, LST granular type (LST-G), we performed ESD using B-Knife and insulation-tip knife (IT-Knife) (Olympus Optical Co., Tokyo, Japan) (Figure 2A). During the procedure, we used CO_2 instead of air insufflation to reduce patient's intraoperative abdominal discomfort. This is a safe and effective technique suitable in lengthy colonic endoscopic procedures with the patient under

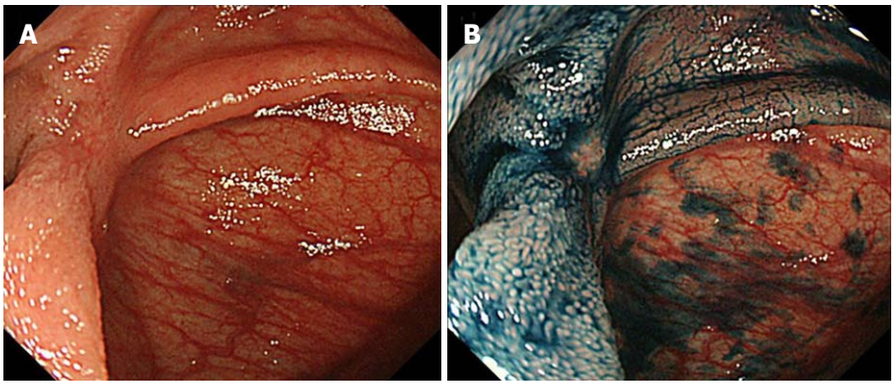

Figure 3 Post-endoscopic submucosal dissection follow-up endoscopic view of the cecum. A: After 6 mo, it shows mildly deformed ileocecal valve due to post-operative scar; B: Following indigo-carmine spraying, no recurrence can be seen.

conscious sedation[13]. Following the injection of glycerol and sodium hyaluronate solution into the sm layer[14,15], a circumferential incision was made using a B-Knife. The thickened sm layer was then dissected (oral to anal) across the ileocecal valve using both the B-Knife and IT-Knife. Finally, hemostasis was carefully checked throughout the ileocecal region (Figure 2B and C). The procedure took 150 min and neither perforation nor delayed bleeding was recognized. Hospitalization lasted four days with no further complications. Histopathology disclosed that the 70 mm resected specimen was an intramucosal cancer with tumor-free margins (Figure 2D). Although some retraction of the ileocecal valve could be observed, follow-up examinations after 6 mo revealed no residual tumor or recurrence (Figure 3A and B).

DISCUSSION

In the present report, en-bloc resection was successfully achieved by ESD using B-Knife and IT-Knife, despite the difficult location involving the ileocecal valve and terminal ileum and the large size of the lesion. IT-Knife, a developed insulation-tipped monopolar electrosurgical knife for removing large gastric lesions en-bloc, is not widely accepted in the colorectum because of its technical difficulty and the risk of complications, such as perforation and bleeding. On the other hand, a bipolar current minimizes the damage to deeper tissues. Thus, the current flow characteristics of the B-Knife reduce the vertical damage and risk of perforation demonstrating its utility for ileocecal ESDs[9,10].

Another important consideration was patient discomfort with air insufflation in long procedures. The supply of air can easily flow into the ileum causing painful distension even in EMRs for cecal lesions. In an earlier study aimed at reducing abdominal discomfort using CO_2 in colorectal ESDs, we demonstrated the advantages and safety of CO_2 compared to conventional air[14]. This factor was evident in the present case. Although a large amount of CO_2 was supplied to the ileum, only a small amount of midazolam (4 mg in both cases) was required for intra-operative sedation.

Considering the indications for colorectal ESD, we generally recommend ESD for LST-non granular type lesions > 20 mm and planned piecemeal EMR for LST-Gs M < 40 mm[3]. In these four cases, we decided to perform ESD because of the LST-Gs large size, their location at the ileocecal valve and terminal ileum spreading, the probability of sm infiltration, and an increased likelihood of incomplete resections and recurrence.

Limitations

In our institution, we have performed colorectal ESD using a B-Knife and an IT-Knife in 500 cases. Among of these 500 ESDs, large LST involving the ileocecal valve were only 4 cases, including the presented case. Based on our experience, lesions should be limited at most to 1 or 2 cm into the ileum and not circumferential. If the extension is more than 2 cm or circumferential, ESD would be very difficult and hazardous, so laparoscopy-assisted colectomy should be recommended. The reported case extended 1.5 cm into the ileum, making the most challenging one. Compared with conventional EMR[16], however, the longer procedure time for colorectal ESDs is still a problem. Nevertheless, we are improving our learning curve and using newly developed devices to reduce the length of the procedure and associated complications in order to increase the widespread use of colorectal ESD.

In conclusion, we successfully performed ESD in large LST-G involving the ileocecal valve and terminal ileum using a B-Knife and an IT-Knife with CO_2 insufflation.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2012 January 21; 18(3): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2012 January 21; 18(3): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

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Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

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The Liver Meeting
San Francisco, CA 94101, United States

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American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel Diseases
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World J Gastroenterol 2012 January 21; 18(3): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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Name of journal

World Journal of Gastroenterology

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

Editorial office
World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
Ocean International Center,
No. 62 Dongsihuan Zhonglu,
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-59080039
Fax: +86-10-85381893

Indexed and abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifer, and Directory of Open Access Journals. ISI, Thomson Reuters, 2010 Impact Factor: 2.240 (35/71 Gastroenterology and Hepatology).

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World Journal of
Gastroenterology ®
Volume 18 Number 4
January 28, 2012

2011
中国精品科技期刊
OUTSTANDING S&T JOURNALS OF CHINA

百世登
Baishideng®

Published by Baishideng Publishing Group Co., Limited
Room 1701, 17/F, Henan Building,
No. 90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-31158812
Telephone: +852-58042046
E-mail: bpg@baishideng.com
http://www.wjgnet.com

ISSN 1007-9327
0 4>

ISSN 1007-9327 CN 14-1219/R Local Post Offices Code No. 82-261

World Journal of Gastroenterology
www.wjgnet.com
Volume 18 Number 4 Jan 28 2012

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

World Journal of *Gastroenterology*

World J Gastroenterol 2012 January 28; 18(4): 295-392

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World Journal of Gastroenterology

Contents

Weekly Volume 18 Number 4 January 28, 2012

World Journal of Gastroenterology
Volume 18 Number 4 January 28, 2012

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NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

RESPONSIBLE INSTITUTION
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SPONSOR
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PUBLISHER
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Telephone: +852-58042046
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PRINT SUBSCRIPTION
RMB 300 Yuan for each issue, RMB 14400 Yuan for one year.

PUBLICATION DATE
January 28, 2012

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

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Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i4.295

World J Gastroenterol 2012 January 28; 18(4): 295-301
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Physiological and pathological role of local and immigrating colonic stem cells

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Received: May 4, 2011 Revised: June 27, 2011
Accepted: July 4, 2011
Published online: January 28, 2012

Abstract

The latest avenue of research is revealing the existence of and role for the colonic stem cells in the physiological renewal of the mucosa and in pathological circumstances where they have both positive and negative effects. In the case of human colon, different levels of stem cell compartments exist. First, the crypt epithelial stem cells, which have a role in the normal crypt epithelial cell dynamics and in colorectal carcinogenesis. Close to the crypts, the second layer of stem cells can be found; the local subepithelial stem cell niche, including the pericryptic subepithelial myofibroblasts that regulate the epithelial cell differentiation and have a crucial role in cancer progression and chronic inflammation-related fibrosis. The third level of stem cell compartment is the immigrating bone-marrow-derived stem cells, which have an important role in wound healing after severe mucosal inflammation, but are also involved in cancer invasion. This paper focuses on stem cell biology in the context of physiological and pathological processes in the human colon.

Key words: Colon; Mesencyhmal stem cells; Bone marrow; Myofibroblast; Fibrosis; Colorectal cancer; Parathyroid hormone; Transforming growth factor-β

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Sipos F, Valcz G, Molnár B. Physiological and pathological role of local and immigrating colonic stem cells. *World J Gastroenterol* 2012; 18(4): 295-301 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i4/295.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i4.295

INTRODUCTION

Beside water and electrolyte absorption, barrier function is a major physiological function of the colonic mucosa, which is the basis of the defense against the luminal pathogens and toxins. Keeping the integrity of the epithelial layer therefore is of great importance, in which stem cells as the key elements of the mucosal regeneration have an important role.

The epithelial layer of the colon consists of a single sheet of columnar epithelial cells folded into finger-like invaginations that are surrounded by the lamina propria to form a functional unit, called Lieberkühn's crypt. It has been estimated that there are about 20 million of these crypts in the human colon[1]. There are four epithelial cell lineages within the crypt. Enterocytes, goblet cells and endocrine cells are terminally differentiated cells, which are found in the upper third of the crypt, and are derived from multipotent stem cells located at the bottom of the crypt. During asymmetric division, these epithelial stem cells undergo self-renewal and generate a population of transit amplifying cells that, upon migration upward

through the crypt, proliferate and differentiate into one of the epithelial cell types of the crypt wall. The fourth type is the Paneth cell, which differentiates during a downward migration to the base of the crypt, where they reside below the crypt epithelial stem cell population[2].

Extensive experimental evidence has demonstrated that Wnt/β-catenin signaling plays a central role in maintaining the intestinal stem-cell niche and regulating normal crypt dynamics[3-7].

The origin of crypt epithelial stem cells is probably dual. Regarding their origin, there are two major kinds of stem cells in the subepithelial layer of the colonic mucosa: the local mesenchymal stem cells (MSCs) and the immigrating bone-marrow-derived stem cells[8,9] (Figures 1 and 2). In the case of mild to moderate mucosal injury, the local subepithelial stem cell niche is enough for differentiating to the epithelial lineage[8]. In the adult gut, both the number and differentiation capacity of the local stem cells are low. In the case of serious tissue injury [i.e., graft-versus-host disease, inflammatory bowel disease (IBD)] the regenerative capacity of local stem cells is not enough to complete tissue healing. In this case, bone-marrow-derived MSCs (BM-MSCs) migrate into the gastrointestinal wall, where they may contribute to the repair progress[9-11] as differentiated mesenchymal cells (e.g., myofibroblasts)[12,13]. Intestinal subepithelial myofibroblasts have a key role in the support of the epithelial stem cell compartment. They can originate from local subepithelial fibroblasts[14], and from immigrating bone marrow derived cells[15]. The bone marrow origin of these cells may be supposed by such observations in which co-expression of epithelial and hematopoietic lineage markers on them were found in inflamed mucosa adjacent to subepithelial lymphoid aggregates[16-20]. Both processes are regulated by transforming growth β-1 (TGF-β1), which plays an important role in intestinal mucosal healing[21].

Although our knowledge is expanding about the origin and behavior of subepithelial colonic stem cells, data about their effect in pathological circumstances like inflammation, fibrosis or cancer development are scattered throughout the literature. A better understanding of the regulation of their differentiation may help to establish new therapeutic strategies.

LOCAL SUBEPITHELIAL STEM CELL COMPARTMENT OF THE COLON

The local MSCs, namely the pericryptal myofibroblasts, which form the epithelial stem cell niche and also regulate epithelial cell differentiation are important cells orchestrating many diverse functions in the intestine, and are involved in growth and repair, tumorigenesis, inflammation, and fibrosis[22]. Adult myofibroblasts are derived or replenished after injury or in response to neoplastic transformation from several sources: differentiation or activation of resident fibroblasts, dedifferentiation from perivascular smooth muscle cells and adipocytes, epithelial-to-mesenchymal transition of epithelial and endothelial cells, and bone-marrow-derived stem cells. MSCs or hematopoietic stem cells, *via* $CD14^+$ monocytes, transdifferentiate into circulating $CD34^+$ fibrocytes, which become resident $CD34^+$ fibrocytes[7,12].

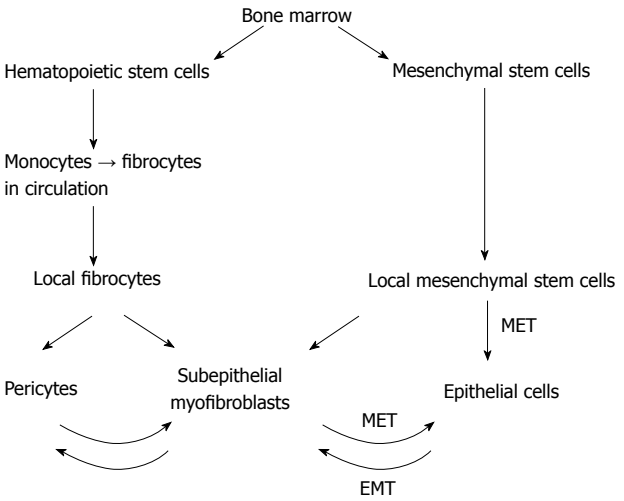

Figure 1 Possible origin of the colonic mesenchymal and epithelial cells. Arrows represents possible stem cell origin and routes for derivation of colonic mesenchymal and epithelial cells. MET: Mesenchymal-to-epithelial transition; EMT: Epithelial-to-mesenchymal transition.

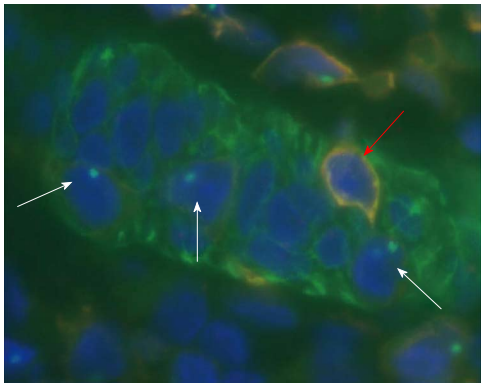

Figure 2 $CD45^-$/cytokeratin$^+$ epithelial cells (green cytoplasmic immunoreaction) containing Y chromosomes (green nuclear dots; white arrows) in the germinative zone of a colonic crypt are well differentiating from the $CD45^+$ intraepithelial lymphocyte (red arrow) (cytokeratin-fluorescein isothiocyanate; CD45-rhodamine; Y-chromosome fluorescence in situ hybridization in a biopsy sample of male bone marrow recipient female patient with moderate non-specific colitis; 100 × magnification).

Migration of colonic fibroblasts into and through the extracellular matrix during the initial phase of mucosal healing appears to be a fundamental component of wound contraction. In recent studies, it has been shown that colonic lamina propria fibroblast-conditioned media induce migration of primary human colonic fibroblasts in the modified Boyden chamber, and fibronectin is mainly responsible for this autocrine migration induction[23]. The differentiation of fibroblasts into myofibroblasts is an important step in tissue repair. It has been described that TGF-β1 potently stimulates the production of smooth muscle actin and stress fiber formation in fibroblasts and therefore their differentiation into myofibroblasts[24]; moreover, it also regulates their migration[14]. Only fibroblasts expressing Thy-1 (CD90), a cell-surface glycoprotein of T-cells, can differentiate into myofibroblasts

Table 1 Summary of the characteristics of the different types of stem cells in the human colon

	Crypt epithelial stem cells	Subepithelial (pericryptal) myofibroblasts-local stem cell niche	BM-MSCs
Origin	Pericryptal myofibroblasts	Subepithelial CD34+ fibroblasts, perivascular smooth muscle cells, adipocytes, endothelial- and epithelial cells (*via* EMT), BM-MSCs	Bone marrow
Daughter cells	Enterocytes, goblet cells, endocrine cells, paneth cells	Follicular dendritic cells, crypt epithelial stem cells, pericytes	Subepithelial myofibroblasts, fibrocytes, pericytes, adipocytes, crypt epithelial stem cells
Main regulator pathways of differentiation and/or homing	Wnt/β-catenin, Lgr-5, integrins, growth factor receptors (EGFR, HGFR, I1GFR)	TGF-β1, cytokines (IL-1, -6, -10, TNFα), growth factors (TGFα, GM-CSF, PDGF-AA, -BB, bFGF, KGF, HGF), chemokines (IL-8, MCP1, MIP-1α,-2), inflammatory mediators (PGE2, PAF, PGI2)	Chemokine receptors (CCR-1,-2,-7,-8,-9; CXCR-1,-2,-4,-5,-6), cell adhesion molecules involved in extravasation (VCAM, ICAM, selectins), proinflammatory cytokines (TNFα, IL-8), von Willebrand factor
Physiological function	Epithelial renewal	Growth, repair	Growth, repair, wound healing
Pathological function	Tumorigenesis, ulcer development	Tumorigenesis, cancer progression, inflammation, fibrosis	Tumorigenesis, inflammation, fibrosis
Cell type specific markers	Positive markers: Lgr-5, Musashi-1, CDX-2	Negative markers: smoothelin, caldesmon, desmin Positive markers: α-SMA, vimentin, SMM, prolyl 4-hydroxylase, CD90	Negative markers: CD13, -14, -45, c-Kit, MHC class Ⅰ and Ⅱ Positive markers: CD54,-90,-133,-146,-166, Flk-1, Sca-1, stage-specific antigen Ⅰ, musashi-1, HLA class Ⅰ
References	1-6, 35, 50	12, 15, 25, 37, 48, 49	8, 9, 11, 20, 26, 29, 35, 37, 41, 58

Lgr-5: Leucine-rich repeat-containing G-protein coupled receptor 5; EGFR: Epithelial growth factor receptor; HGFR: Hepatocyte growth factor receptor; I1GFR: Insulin-like growth factor receptor-1; CDX2: Caudal type homeobox transcription factor 2; EMT: Epithelial-to-mesenchymal transition; TGF: Transforming growth factor; IL: Interleukin; TNF: Tumor necrosis factor; GM-CSF: Granulocyte macrophage colony-stimulating factor; PDGF: Platelet-derived growth factor; bFGF: Basic fibroblast growth factor; KGF: Keratinocyte growth factor; HGF: Hepatocyte growth factor; MCP1: Monocyte chemotactic protein 1; MIP: Macrophage inflammatory protein; PGE2: Prostaglandin E2; PAF: Platelet-activating factor; PGI2: Prostacyclin; SMA: Smooth muscle actin; SMM: Smooth muscle myosin; CCR: Chemokine receptor; CXCR: Chemokine CXC motif receptor; VCAM: Vascular cell adhesion molecule; ICAM: Intercellular cell adhesion molecule; MHC: Major histocompatibility complex; Flk-1: Fetal liver kinase-1; Sca-1: Stem cell antigen-1; HLA: Human leukocyte antigen.

after treatment with TGF-β, whereas only Thy-1$^-$ fibroblasts differentiate into lipofibroblasts upon exposure to 15-deoxy-δ-prostaglandin J2[25]. After differentiation, subepithelial myofibroblasts form pericryptal fibroblast sheet adjacent to the basal lamina of colonic crypts[10,26]. Intestinal subepithelial myofibroblasts contribute to the coordination of tissue regeneration by producing TGF-β, epidermal growth factor, basic fibroblast growth factor, proinflammatory cytokines, and the formation of new basement membrane[27].

Brown *et al*[28] have reported a cyclooxygenase-2-expressing stromal cell that moves in response to Toll-like receptor (TLR) signals from a position in the upper aspect of the lamina propria to a position adjacent to the pericryptal myofibroblasts in the base of the crypts, where the crypt epithelial stem cells reside. This relocation and the prostaglandin secretion appear critical to colonic epithelial repair in a dextran sodium sulfate colitis model. Their study has not ruled out a role for prostaglandin production or TLR responses by the conventional myofibroblasts or pericytes that are also present.

COLONIC BM-MSCs

The number of myofibroblasts originating from the bone marrow significantly increased in the lamina propria of severe colitis compared to the healthy colon[9,13]. This homing process is driven by chemokines and adhesion molecules[29] (Table 1). Based on the former results[30-32], emerging evidence suggests that bone-marrow-derived stem cells contribute to tissue regeneration in the colon partly by promoting neovascularization or arteriogenesis. After human hematopoietic cell transplantation, epithelial tissue chimerism appears[33-35]. The bone marrow origin of subepithelial stem cells may be supposed by observations in which epithelial cell markers and leukocyte markers show that double positive cells are found in inflamed mucosa adjacent to lymphoid aggregates[18,19,36]. The presence of cytokeratin, epithelial growth factor receptor, hepatocyte-derived growth factor receptor or CDX2 co-expression in CD45$^+$ cells of subepithelial lymphoid aggregates may support the mesenchymal origin of epithelial stem cells.

Presumably, intestinal subepithelial myofibroblasts originated from BM-MSCs and may create a local microenvironment for the immigrated BM-MSCs that are committed to the epithelial lineage. The high percentages of intraepithelial cells of bone marrow origin are immune cells, such as CD45$^+$ leukocytes. In bone-marrow-transplanted patients, the numbers of CD45$^+$ and Y-FISH$^+$ (male donor origin) double-positive, intraepithelial lymphocytes were significantly higher number in regenerating

colonic epithelium than in the normal samples[35].

Previous studies using animal models of IBD have shown that transplanted bone marrow cells contribute to tissue repair by forming epithelial cells, activated myofibroblasts, and can also contribute to neovasculogenesis in the inflamed colon *via* the formation of entire new blood vessels[37]. It has also been shown that myofibroblasts of bone marrow origin are functional in their production of pro-collagen α1 mRNA[38]. These studies showing bone marrow contribution to tissue regeneration in IBD are now supported by the results of Khalil *et al*[39], who have further shown that regeneration can occur from a defined subpopulation of $CD34^-$ stem cells, present in both the bone marrow and peripheral blood, and moreover, that these stem cells can significantly enhance tissue regeneration in IBD without the need for prior ablation of the recipient's immune system by irradiation.

REGULATION OF STEM CELL HOMING IN THE COLON

The homing of BM-MSCs to colonic mucosa has been poorly revealed to date. BM-MSCs migrate *via* the bloodstream to the sites of colonic mucosal damage, which have been certified in several *in vivo* experiments[11,35,40]. Regulation of BM-MSC migration may happen as an effect of chemical signals, which are upregulated during injury.

Systemically delivered or natively circulating MSCs accumulate in injured tissues. During homing, MSCs adhere to endothelial cells and infiltrate underlying tissue. Previously, it has been shown that adhesiveness of endothelial cells for MSCs correlates with the inhibition of mitochondrial function of endothelial cells and secretion of von Willebrand factor (vWF)[41]. Potapova *et al*[42] have demonstrated that the treatment of endothelial cells with vWF stimulates MSC adhesion in a time- and concentration-dependent manner. MSCs do not adhere to immobilized vWF and do not express receptors for vWF, suggesting that the stimulation of MSC adhesion is a result of endothelial cell activation with vWF. In cell culture experiments, it has also been shown that normal colonic endothelial cells highly express vWF[43]. Based on these results, vWF seems to be an auto/paracrine regulator of colonic endothelial cells. Activation of p38 mitogen-activate protein kinase (MAPK) in endothelial cells by vWF may be responsible for the regulation of endothelial cell adhesiveness for MSCs in the colon.

CXC chemokine receptor (CXCR)4 has also a pivotal role in stem cell homing. It has recently been shown that CXC chemokine ligand 12 and CXCR4 are constitutively expressed on intestinal epithelial cells, lamina propria T cells, and the expression is increased in those of ulcerative colitis patients[44,45]. Induction of CXCR4 is associated with upregulation of two genes encoding transcription factors previously shown to control CXCR4 expression (hypoxia-inducible factor-2α and achaete scute complex like protein 2) and maintenance of crypt epithelial stem cells[46,47].

Subepithelial isolated lymphoid follicles and lymphoid aggregates of the colon are supposed to be the central organizer elements of stem cell homing and the mesenchymal-to-epithelial transition by producing an ideal cytokine, chemokine and cellular milieu[48,49].

PATHOLOGICAL ROLE OF COLONIC STEM CELLS

Epithelial cancer stem cell development

Cancer stem cells are defined as cells that are endowed with both self-renewal and multilineage differentiation potential and, as such, are believed to expand clonally and repopulate the various types of differentiation lineages present within the tumor[50,51].

Crypt epithelial stem cells appear to be the cell of origin of colorectal cancer, based on their existence throughout the lifetime of an individual, and thus, their capacity to acquire multiple genetic mutations leads to carcinogenesis. Direct evidence for crypt epithelial stem cells as the source of intestinal tumors has come from a study of tissue-specific expression of Cre recombinases to inactivate a conditional Apc allele[52]. Although deletion of Apc in epithelial cells results in adenomas at very low frequency and with long latency, inactivation of Apc within epithelial stem cells leads to formation of macroscopic adenomas within 36 d. Moreover, these adenomas retain a small percentage of cells expressing Lgr5, an intestinal stem cell marker. These data support the view that crypt epithelial stem cells are the target of the origin of colorectal cancer. Alteration of crypt epithelial stem cell number or proliferation state may increase the probability of intestinal tumorigenesis.

Múnera *et al*[53] have tested the epithelial cell autonomous function of Ets2, a member of the Ets family of transcription factors, which is located on human chromosome 21, and has been identified as a Wnt target in colorectal cancer cells and crypt epithelial stem cells[54], during chemical carcinogenesis of the colon by using a conditional Ets2 allele and a transgene expressing Cre recombinase only in intestinal epithelial cells. Their results indicate that, although Ets2 is a Wnt pathway target gene within crypt epithelial stem cells, its loss provides a competitive advantage for crypt epithelial stem cells to colonize crypts, increase basal crypt cell proliferation, and increase crypt fission. Ets2 loss may increase the number or sensitivity of colon stem cells for tumor initiation.

The results of Deka *et al*[55] have revealed the essential role for Bcl9/Bcl9l in regulating a subset of Wnt target genes involved in controlling epithelial-to-mesenchymal transition and stem-cell-related features and suggest that targeting the Bcl9/Bcl9l arm of Wnt signaling in Wnt-activated cancers might attenuate these traits, which are associated with tumor invasion, metastasis, and resistance to therapy.

Role of subepithelial stem cells in carcinogenesis and cancer progression

The interaction between cancer cells and non-trans-

formed cells in the tumor microenvironment is essential for tumor growth and progression[56]. The tumor stroma includes several non-transformed cell types, such as endothelial cells, immune cells, and fibroblastic stromal cells (cancer-associated fibroblasts). This latter cell type plays an important role in cancer progression by promoting angiogenesis, epithelial-to-mesenchymal transition, and genetic instability[57-59]. Although tumor stromal fibroblasts are mainly recruited from local tissue fibroblasts, it has been proposed that BM-MSCs are recruited into the stroma of developing tumors[57,60]. Several studies have demonstrated that BM-MSCs can selectively migrate to sites of mucosal damage and wound healing including colorectal cancers, where a number of tumor-related inflammatory reactions and abnormal tissue regeneration phenomena take place actively. It also has been shown that cancer cells release specific factors that induce BM-MSC mobilization and recruitment to the tumor stroma where they eventually contribute to the formation of a tumor-supportive microenvironment[57].

The cause of metastasis remains elusive despite a vast amount of information on cancer cells. According to recent research, cancer cell fusion with macrophages or immigrating BM-MSCs provides an explanation[49,61,62]. BM-MSCs fused with tumor cells are present not just in animal tumor xenografts where they are associated with metastases, but in human carcinomas, including colon cancer. BM-MSC-tumor cell fusion explains the epithelial-to-mesenchymal transition in cancer since BM-MSCs express mesodermal traits and epithelial-to-mesenchymal transition regulators like Twist and SPARC (secreted protein acidic and rich in cysteine). If bone-marrow-derived-tumor cell fusion underlies invasion and metastasis in human cancer, new therapeutic strategies would be mandated.

A new association between parathyroid hormone (PTH) and cancer development has been revealed recently. Based on recent results, PTH can stimulate the phosphoinositide 3-kinase/MAPK-mediated proliferation of rat enterocytes, and primary hyperparathyroidism in humans is associated with an increased incidence of colon cancer[63]. It has been shown in a large cohort[64] that high serum PTH levels may be associated with incident, sporadic colorectal cancer in Western European populations, and in particular among men. PTH has its effect on MSCs/progenitors as they express PTH receptor[65]. Taking together these data, further investigations on PTH and colorectal carcinogenesis would be of great clinical importance.

Stem cells in chronic inflammation-related intestinal fibrosis

Intestinal fibrosis is among the most common complications of IBD, especially Crohn's disease (CD), resulting in stricture formation in the small intestine and colon. About 75% of CD patients will undergo surgery at least once over the course of their disease, and fibrotic strictures represent the main indication for surgery and the first cause of hospitalization and costs for CD patients[66]. Fibrosis in CD is the result of transmural chronic inflammation with repeated episodes of immune-mediated damage and repair[67]. Key factors for intestinal fibrosis are excessive deposition of extracellular matrix, proliferation of profibrogenic mesenchymal cells in the colon, thickening of all layers of the bowel wall, overgrowth of muscular layers of the intestine, enhanced local Th-1 type immune response, and overexpression of profibrogenic cytokines and growth factors[68]. As mentioned above, stromal cells derived from MSCs, fibrocytes, or BM-MSCs may also home to sites of inflammation and, in the presence of ongoing inflammation, become activated myofibroblasts/fibroblasts and contribute to tissue fibrosis[22]. Specific inhibition of the TGF-β signaling pathway, the key regulator of this pathologic process may be a promising therapeutic strategy to reduce the number of profibrogenic mesenchymal cells in chronic intestinal fibrosis[69].

CONCLUSION

The local and immigrating stem cells of the human colon are of major clinical importance because they are all involved in the regeneration of the damaged mucosa (Table 1). The results of the first attempts of MSC therapy in IBD are promising[70-72]. The regulation of the homing and differentiation of stem cells also provides new, individual and disease-specific therapeutic targets in the case of colorectal cancer. Inhibition of the TGF-β signaling pathway may be a promising therapeutic strategy in chronic inflammation-related colon fibrosis. The expected results of the ongoing and forthcoming studies hopefully will open the door to the development of new cures for old diseases.

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S- Editor Sun H **L- Editor** Kerr C **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i4.302

World J Gastroenterol 2012 January 28; 18(4): 302-308
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

8-Hydroxydeoxyguanosine: Not mere biomarker for oxidative stress, but remedy for oxidative stress-implicated gastrointestinal diseases

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Supported by A grant from the Ministry of Education and Science Technology, South Korea, No. 2010-0002052
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Received: May 16, 2011 Revised: August 26, 2011
Accepted: September 2, 2011
Published online: January 28, 2012

Abstract

Reactive oxygen species (ROS) attack guanine bases in DNA easily and form 8-hydroxydeoxyguanosine (8-OHdG), which can bind to thymidine rather than cytosine, based on which, the level of 8-OHdG is generally regarded as a biomarker of mutagenesis consequent to oxidative stress. For example, higher levels of 8-OHdG are noted in *Helicobacter pylori*-associated chronic atrophic gastritis as well as gastric cancer. However, we have found that exogenous 8-OHdG can paradoxically reduce ROS production, attenuate the nuclear factor-κB signaling pathway, and ameliorate the expression of proinflammatory mediators such as interleukin (IL)-1, IL-6, cyclo-oxygenase-2, and inducible nitric oxide synthase in addition to expression of nicotinamide adenine dinucleotide phosphate oxidase (NOX)-1, NOX organizer-1 and NOX activator-1 in various conditions of inflammation-based gastrointestinal (GI) diseases including gastritis, inflammatory bowel disease, pancreatitis, and even colitis-associated carcinogenesis. Our recent finding that exogenous 8-OHdG was very effective in either inflammation-based or oxidative-stress-associated diseases of stress-related mucosal damage has inspired the hope that synthetic 8-OHdG can be a potential candidate for the treatment of inflammation-based GI diseases, as well as the prevention of inflammation-associated GI cancer. In this editorial review, the novel fact that exogenous 8-OHdG can be a functional molecule regulating oxidative-stress-induced gastritis through either antagonizing Rac-guanosine triphosphate binding or blocking the signals responsible for gastric inflammatory cascade is introduced.

Key words: 8-hydroxydeoxyguanosine; Oxidative stress; Inflammation; Carcinogenesis; Prevention

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Ock CY, Kim EH, Choi DJ, Lee HJ, Hahm KB, Chung MH. 8-Hydroxydeoxyguanosine: Not mere biomarker for oxidative stress, but remedy for oxidative stress-implicated gastrointestinal diseases. *World J Gastroenterol* 2012; 18(4): 302-308 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i4/302.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i4.302

INTRODUCTION

When we consume a soft drink or eat a meal, the gastrointestinal (GI) mucosa is continuously stressed with various antigens that we have ingested because the GI lumen is actually outside the body[1]. As far as the interaction between materials from outside and the physiological barriers of the GI tract is concerned, everything existing outside the GI lumen can cause stressful reactions at the cellular level through mechanisms including antigen challenge, concurring oxidative stress, and some inflammatory assaults. The GI lumen can come under attack by many factors, including solid food, *Helicobacter pylori* (*H. pylori*) and other commensal bacteria, nonsteroidal anti-inflammatory drugs, and gastric acid[2-4]. In particular, one important means of attack is mediated by oxygen, which is also the most important metabolic substance in the body. In spite of being essential for life, oxygen can cause various cellular stresses called oxidative stress by generating reactive oxygen species (ROS)[5]. The definition of oxidative stress is a disturbance of oxidant-antioxidant homeostasis, leading to potential cellular damage. Even though the existence of ROS and their pathological implications were discovered < 50 years ago, it is surprising that many diseases can be explained by oxidative stress and its subsequent dysregulation. Although ROS are a crucial regulator of cellular signal transduction and energy transmission, disturbance of the balance between generating and scavenging capability of ROS might lead to cell damage. ROS can react with cellular proteins or lipids, transforming them into oxidized forms, or bind with nucleic acids, turning them into mutated forms. It is particularly interesting that oxidative stress is closely associated with carcinogenesis.

Fortunately, to cope with these harmful effects of oxidative stress, cells may endeavor to enhance defensive factors of various types. The common examples of defense factors are reduced glutathione/oxidized glutathione, superoxide dismutase, catalase, heme oxygenase 1, G protein gamma-like, and nicotinamide adenine dinucleotide phosphate (NADPH) dehydrogenase, quinine 1[6-8]. However, disease can occur if there are insufficient defense factors or overwhelming offensive factors. Therefore, if we set up a sensitive marker that predicts the degree of oxidative stress, we can prevent the initiation or progression of disease by measuring this marker[9]. Moreover, if the level of this marker reflects the severity of disease, appropriate levels of scavenging agents can prevent complications of the disease, because the extreme is organ dysfunction or cancer. Several biomarkers to estimate oxidative stress have been suggested, but most of them have failed to reach clinical significance. One successful discovery of the late 1980s was the level of 8-hydroxydeoxyguanosine (8-oxo-7, 8-dihydroguanosine, 8-OHdG), because it has been proven to be increased in serum or urine of patients who have oxidative-stress-associated disease[10].

Table 1 Application of 8-hydroxydeoxyguanosine as a potential biomarker for various clinical diseases

Diseases	References
Helicobacter pylori infection	Hahm *et al*[58], Baik *et al*[59]
Colorectal tumor	Sato *et al*[60], Gushima *et al*[61]
Breast cancer	Matsui *et al*[62], Djuric *et al*[63], Musarrat *et al*[64]
Bladder/prostate cancer	Chiou *et al*[65]
Lung cancer	Erhola *et al*[66]
Atherosclerosis	Martinet *et al*[67]
Diabetes	Kanauchi *et al*[68]
Smoking	Asami *et al*[69], Kiyosawa *et al*[70]

8-OHdG: GENERATION AND SIGNIFICANCE AS A BIOMARKER FOR OXIDATIVE STRESS

Generation and metabolism of 8-OHdG

When DNA is attacked by oxidative stress such as ROS, ultraviolet light, or genotoxic agents, guanine is easily oxidized into 8-oxo-7,8-dihydroguanine (8-oxo-Gua)[11]. The existence of this oxidized guanine in genomic DNA can cause transversion mutation such as G-T or G-A binding, accumulation of which can lead to detrimental consequences[12-14]. Fortunately, mammalian cells have multiple repair systems such as base excision repair enzymes or nucleotide excision repair (NER) enzymes, which counteract the hazardous effects of 8-oxo-Gua. Consequently, 8-OHdG, a nucleoside form of 8-oxo-Gua, is generated from either damaged oligomer which contains 8-oxo-Gua by NER or from cytoplasmic oxidized nucleotides like 8-hydroxy-deoxyguanosine triphosphate (8-hydroxy-dGTP). Fortunately, exogenously administered 8-OHdG cannot reincorporate into genomic DNA because the activity of deoxynucleotide kinase which converts 8-OHdG into 8-hydroxy-dGTP is very low, although wild deoxyguanosine can be actively converted to deoxyguanosine triphosphate which can be used as a substrate of DNA polymerase[15-22].

Biological significance of 8-OHdG

8-OHdG can cross the cell membrane unlike any other species that contains oxidized guanine, thus, it is usually detected in the urine or serum of patients who have diseases associated with oxidative stress[23]. Examples of application of 8-OHdG as a disease-associated clinical marker are summarized in Table 1.

8-OHdG: NEW FOCUS ON PARADOXICALLY RELIEVING ACTION OF OXIDATIVE STRESS

Anti-oxidative and anti-inflammatory actions of 8-OHdG

Although many studies have shown increased levels of 8-OHdG in oxidative-stress-associated diseases, the exact biological role of 8-OHdG has not been investigated. Oxidized deoxyguanosine is notorious for inducing mutagenesis, therefore, most researchers have felt that 8-OHdG might have mutagenic or at least harmful effects in cells, and that is why mammalian physiology tries hard to excrete this oxidized guanosine. However, under the innovative hypothesis that the generation of this molecule can be one of the defense mechanisms of cells against oxidative-stress-induced inflammation, we have tried to obtain evidence that oxidized guanosine can interact with the GTPase family, which is broadly involved in cytoskeleton modification, triggering inflammation, regulating apoptosis, and carcinogenesis[24-27]. Interestingly, genetically modified oxidized GTP, 8-oxo-GTPγS, seems to interact with the small GTPase family such as Ras, Rho, Rac and cdc42[28]. Among these, we have focused on the role of Rac1 in inflammatory cascades[29,30] because Rac1 activation is crucial for aggregating NADPH oxidase (NOX) complex and subsequent ROS production[31]. As a result, we have concluded that inhibition of Rac1 by exogenous 8-OHdG, which is a transmittable form of oxidized guanosine, can significantly block ROS-mediated inflammation. Compared with other nucleoside products, 8-OHdG has a potent anti-inflammatory effect by inhibiting the activity of Rac1 on lipopolysaccharide (LPS)-stimulated microglial cells, chemokine-activated neutrophils, and inflammatory mediator-stimulated macrophages[21,32-34]. Moreover, since endogenously produced 8-OHdG is much lower than exogenously treated concentrations of 8-OHdG, we propose the biological role of the antioxidative and anti-inflammatory actions of 8-OHdG, implying that 8-OHdG formation can be a defense mechanism against oxidative stress, and enrichment with exogenous 8-OHdG can be a strategy to prevent the initiation or progression of inflammatory disease, backed up with additional fact that only pretreatment or earlier administration of 8-OHdH is effective. To clarify and compare the cellular effect of 8-OHdG with existing anti-inflammatory agents, we have also investigated several animal models of acute inflammation. Intraperitoneal LPS injection to mice causes severe inflammation in lung tissues by inducing tumor necrosis factor (TNF)-α, interleukins, and myeloperoxidase activity and recruiting neutrophils. Simultaneous treatment with 8-OHdG significantly decreases the level of the above markers, and the efficacy of 8-OHdG is even more potent than that of aspirin; a conventional anti-inflammatory agent[35]. We also have investigated the antiallergic effects of 8-OHdG in ovalbumin-sensitized mice[36,37]. One of the major phenomena of oxidative stress is stress-related mucosal disease (SRMD), the mechanism of which is mucosal damage induced by ROS involved in reperfusion injury after local ischemia. We established a water-immersion restraint stress model, which mimics SRMD, causing severe ulceration and hemorrhagic lesions in gastric mucosa. Treatment with 8-OHdG reduces the pathological lesions, as well as other angiogenesis mediators such as TNF-α and vascular endothelial growth factor[21]. Recently, we have established an animal model of *H. pylori*-infected gastric inflammation by infecting four times with bacteria, followed by ingestion of a high-salt diet for > 16 wk. The degree of gastric inflammation induced by *H. pylori* is significantly decreased by continuous ingestion of 8-OHdG-dissolved water ad libitum. Moreover, dysplastic and precancerous lesions are observed in this animal model with chronic *H. pylori* infection, but curiously, the carcinogenesis that results from chronic *H. pylori* infection is apparently ameliorated with exogenous administration of 8-OHdG (Figure 1).

Molecular mechanisms to impose anti-inflammatory action of 8-OHdG

Although the possible mechanism to explain how 8-OHdG inhibits the activity of Rac1 has not been clearly documented yet, we hypothesize that 8-OHdG can interfere with the GTP-binding pocket of Rac1, because GTP and 8-OHdG share a similar conformation with a guanine base. 8-OHdG does not affect the activity of phosphoinositide 3-kinase/AKT or that of Rac1-guanosine exchange factor, which is an upstream pathway of Rac1 activation by exchanging Rac1-bound-GDP into GTP, turning inactive Rac1 into the active form[38-40]. However, treatment with 8-OHdG dramatically decreases the portion of Rac1-GTP, implying the specific molecular target of 8-OHdG might be Rac1 inactivation[21]. Rac1 is a crucial mediator of activating NOX complex[41,42], therefore, inactivating Rac1 could decrease the generation of ROS, and block the redox-sensitive nuclear factor (NF)-κB pathway. Rac1 also directly binds to signal transducer and activator of transcription (STAT)3 and regulates its activity[43], therefore, the ratio of phosphorylated to total STAT3 would be decreased by treatment with 8-OHdG[32]. The regulation of Rac1-mediated ROS, NF-κB and STAT3, which are the important mediators of inflammatory cascades, is the latest possibility of how 8-OHdG exerts an anti-inflammatory action.

Antitumorigenic action of 8-OHdG based on an efficient anti-inflammatory action

8-oxoguanine DNA glycosylase 1 (OGG1) is an endogenous DNA repair enzyme that repairs 8-OH-Gua in genomic DNA, cutting it into 8-OHdG (Figure 2). Although 8-OHdG does not reincorporate into genomic DNA as explained above, exogenously administered 8-OHdG can increase the ratio of 8-OH-Gua in genomic DNA by increasing error-prone DNA polymerase in the specific types of cells that have mutated forms of OGG1[19,44]. Although wild-type OGG1 can correct and repair 8-OH-Gua, OGG1 mutation cannot remove this harmful oxidized guanine, causing mutation and apoptosis. Treatment with 8-OHdG induces G1 arrest and

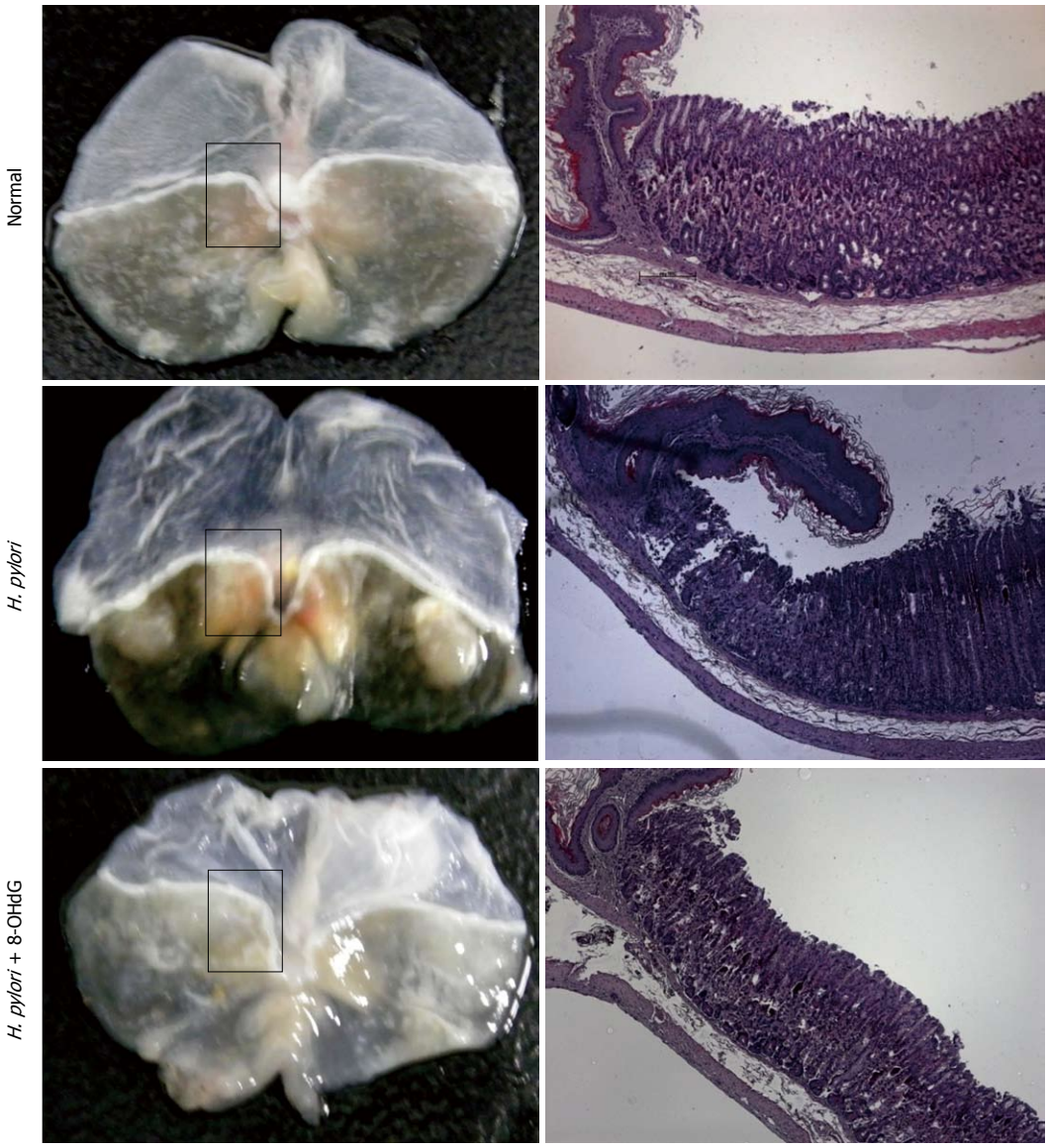

Figure 1 Antitumorigenic action of exogenous 8-hydroxydeoxyguanosine on *Helicobacter pylori*-induced gastric tumorigenesis. *Helicobacter pylori* (*H. pylori*) infection with additional administration of high-salt diet resulted in gastric tumorigenesis in interleukin-10 knockout mice. Continuous administration of exogenous 8-hydroxydeoxyguanosine (8-OHdG) led to significant prevention of *H. pylori*-induced gastric tumorigenesis as well as amelioration of gastritis. *H. pylori* infection followed with high-salt diet resulted in significant tumorigenesis as well as atrophic changes in gastric mucosa, whereas the gastric mucosa co-treated with 8-OHdG was free from tumorigenesis or atrophic changes.

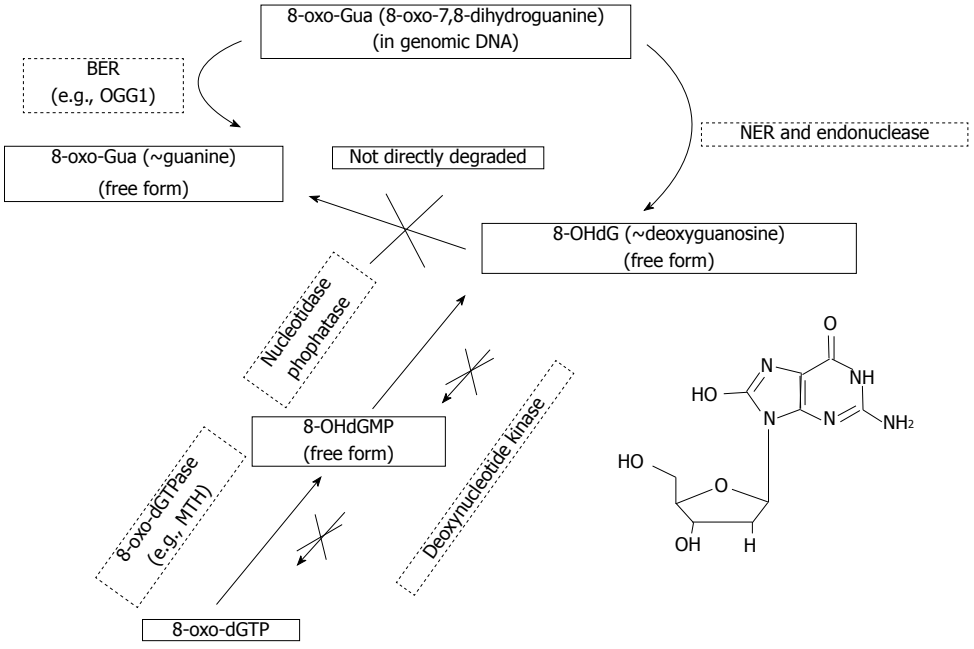

Figure 2 Generation and metabolism of 8-hydroxydeoxyguanosine. 8-oxoguanine DNA glycosylase 1 (OGG1) is a DNA glycosylase enzyme involved in base excision repair (BER), and is the primary enzyme responsible for excision of 7,7-dihydro-8-oxoguanine that occurs as a result of exposure to reactive oxygen species (ROS). OGG1 is a bifunctional glycosylase, because it is able to both cleave the glycosidic bond of the mutagenic lesion, and cause strand breakage in the DNA backbone. Nucleotide excision repair (NER) is a DNA repair mechanism, which is a particularly important mechanism by which the cell can prevent unwanted mutations by removing the vast majority of ROS-induced DNA damage.

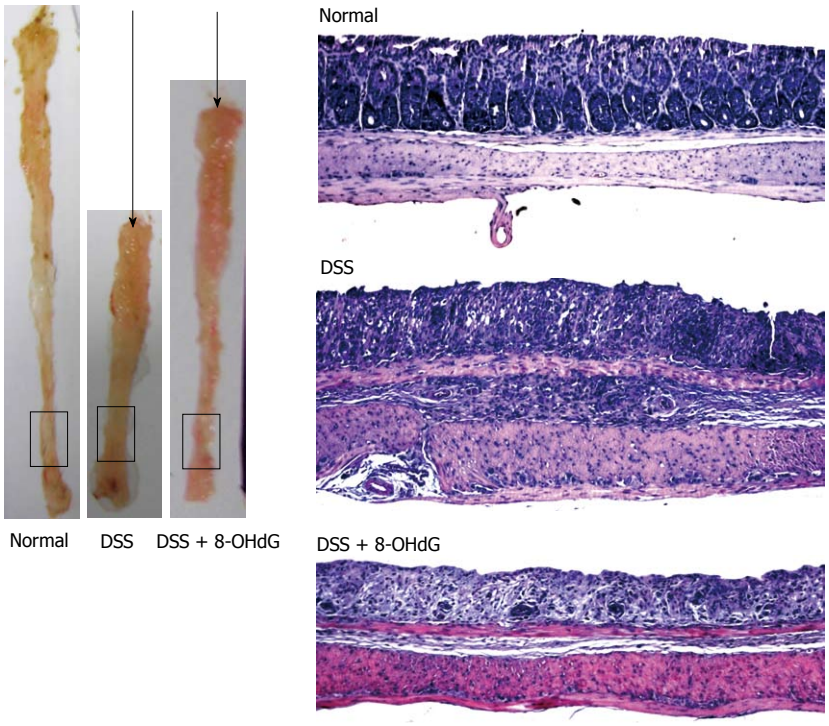

Figure 3 Ameliorating effect of exogenous 8-hydroxydeoxyguanosine against dextran sodium sulfate-induced colitis. Dextran sodium sulfate (DSS) administration resulted in moderate to severe colitis manifested as significantly shortened colon length. However, colon length was significantly preserved by exogenous administration of 8-hydroxydeoxyguanosine (8-OHdG) (see arrows to compare the colon length), as shown by the pathological observation that the degree of DSS-induced colitis was apparently improved, suggesting that exogenous 8-OHdG had a significant preventive effect against DSS-induced colitis[47]. Eighteen mice were divided into three groups of six: a non-treated control group (Normal group); 5% DSS in tap water ingestion for 1 wk (DSS group); and DSS with daily injection of 8-OHdG (DSS + 8-OHdG group). 8-OHdG powder was dissolved in PBS and the Normal and DSS groups were treated as a negative control. Clinical phenotypes such as hematochezia and rectal prolapse were investigated and charted daily. There was no mortality observed in any of the groups. After 7 d DSS ingestion, all mice were killed and colons were removed, opened longitudinally, and rinsed with phosphate buffer solution. The lengths of colon were measured, and isolated tissues were subjected to histological examination.

apoptosis in KG-1 leukemia cells, which have an OGG1 mutation, but not in U937 leukemia cells, which have wild-type OGG1[45-48]. The wide profile of OGG1 mutations in human cancer is now actively under investigation[49-54], therefore, treatment of cancer that has OGG1 mutations with 8-OHdG might be developed as a new model of targeted chemotherapy. For example, we have investigated models of colitis induced by dextran sodium sulfate (DSS) and colitis-associated cancer induced by DSS combined with azoxymethane (AOM + DSS)[55-57], and determined whether exogenous 8-OHdG has a preventive effect on colitis and colitis-associated cancer[57]. Daily injection of 8-OHdG significantly inhibits recruitment of inflammatory cells in DSS-induced colitis, and chronic ingestion of diet containing 8-OHdG exerts a chemopreventive role in AOM + DSS-induced colitis-associated cancer (Figure 3)[47].

CONCLUSION

There is extensive experimental evidence that oxidative damage permanently occurs to lipids of cellular membranes, proteins and DNA. In nuclear and mitochondrial DNA, 8-OHdG or 8-oxodG is one of the predominant agents of free-radical-induced oxidative lesions. This is why 8-OHdG has been used widely in many studies as a biomarker for the measurement of endogenous oxidative DNA damage, and as a risk factor for many diseases including cancer, because urinary 8-OHdG is a good biomarker for risk assessment of various cancers and other degenerative diseases. Several lines of evidence[7,29-32,47] show that exogenous 8-OHdG can: inhibit allergy-induced inflammation; remodel airway and lung tissues through Rac inactivation; regulate oxidative-stress-induced gastritis through antagonizing Rac-GTP binding or blocking the signals responsible for gastric inflammatory cascade; play a role in anti-inflammatory actions *via* suppression of intercellular adhesion molecule-1 gene expression by blockade of the Toll-like receptor 4/STAT3 signal cascade in inflammation-enhanced brain microglia; and prevent colitis-associated carcinogenesis based on efficient TNF-α inhibition or *H. pylori*-associated gastric carcinogenesis based on inhibition of cytokine generation. All this clearly suggests that 8-OHdG could be used as a potential tool to modulate GI tract inflammation as well as allergy-related bronchial disease, and is especially applicable to diverse inflammation-based diseases including gastritis, colitis, and esophagitis, as well as GI cancer, including esophageal, gastric and colon cancer, for which more extensive and well-designed clinical trials are required.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i4.309

World J Gastroenterol 2012 January 28; 18(4): 309-322
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Embryonic hepatocyte transplantation for hepatic cirrhosis: Efficacy and mechanism of action

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Received: April 21, 2011 Revised: July 14, 2011
Accepted: July 21, 2011
Published online: January 28, 2012

Abstract

AIM: To investigate the efficacy and mechanism of action of allogeneic embryonic hepatocyte transplantation for the treatment of hepatic cirrhosis.

METHODS: Rat embryonic hepatocytes were characterized by examining cell markers. Wistar rats with CCl_4-induced cirrhosis were randomly divided into two groups: a model group receiving continuous CCl_4, and a cell transplantation group receiving continuous CCl_4 and transplanted with embryonic fluorescent-labeled hepatocytes. In addition, a normal control group was composed of healthy rats. All rats were sacrificed after 2 wk following the initiation of the cell transplant. Ultrasound, pathological analyses and serum biochemical tests were used to evaluate the efficacy of embryonic hepatocyte transplantation. To analyze the recovery status of cirrhotic hepatocytes and the signaling pathways influenced by embryonic hepatocyte transplantation, real-time polymerase chain reaction was performed to examine the mRNA expression of stellate activation-associated protein (STAP), c-myb, α smooth muscle actin (α-SMA) and endothelin-1 (ET-1). Western blotting and immunohistochemistry were employed to detect α-SMA and ET-1 protein expression in hepatic tissues.

RESULTS: Gross morphological, ultrasound and histopathological examinations, serum biochemical tests and radioimmunoassays demonstrated that hepatic cirrhosis was successfully established in the Wistar rats. Stem cell factor receptor (c-kit), hepatocyte growth factor receptor (c-Met), Nestin, α fetal protein, albumin and cytokeratin19 markers were observed in the rat embryonic hepatocytes. Following embryonic hepatocyte transplantation, there was a significant reversal in the gross appearance, ultrasound findings, histopathological properties, and serum biochemical parameters of the rat liver. In addition, after the activation of hepatic stellate cells and STAP signaling, α-SMA, c-myb and ET-1 mRNA levels became significantly lower than in the untreated cirrhotic group ($P < 0.05$). These levels, however, were not statistically different from those of the normal healthy group. Immunohistochemical staining and Western blot analyses revealed that α-SMA and ET-1 protein expression levels in the transplantation group were significantly lower than in the untreated cirrhotic group, but being not statistically different from the normal group.

CONCLUSION: Transplantation of embryonic hepatocytes in rats has therapeutic effects on cirrhosis. The described treatment may significantly reduce the expression of STAP and ET-1.

Key words: Embryonic hepatocytes; Cirrhosis; Stellate activation-associated protein; Endothelin-1; Cell transplantation

Peer reviewer: Shashi Bala, PhD, Post Doctoral Associate, Department of Medicine, LRB 270L, 364 Plantation street, UMass Medical School, Worcester, MA 01605, United States

Bin WT, Ma LM, Xu Q, Shi XL. Embryonic hepatocyte transplantation for hepatic cirrhosis: Efficacy and mechanism of action. *World J Gastroenterol* 2012; 18(4): 309-322 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i4/309.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i4.309

INTRODUCTION

Clinical studies of liver cell transplantation are primarily performed with adult hepatocytes and liver stem cells[1]. Adult hepatocytes are highly differentiated cells and cannot produce sufficient and sustained biological effects[2,3]. However, *in vitro* proliferation of liver stem cells allows for the generation of a sufficient number of cells to be used in clinical practice[4]. Following transplantation, liver stem cells continue to proliferate and may play an important role in liver regeneration[5,6].

There are currently several types of liver stem cells used in experimental research to treat cirrhosis, including adult liver stem/progenitor cells from the liver portal canal, which are activated and differentiate into hepatic oval cells following severe liver damage[7,8]. Other types of liver stem cells include embryonic hepatocytes[9,10], bone marrow/hematopoietic stem cells[11-16], and embryonic stem cells[17-19].

Early embryonic hepatocytes are self-renewing progenitor cells with the capacity to differentiate into adult hepatocytes and bile duct cells[20]. On embryonic days (E) 16 and 17, rat hepatic stem cells begin to differentiate. On E16, the gene expression profiles of rat embryonic liver epithelial cells change abruptly and become similar to those of mature cells; at this point, the bi-differentiation potential of these cells is significantly reduced[21]. On E9.5-15 in rats, most hepatocytes are liver stem cells, which possess potent proliferative capacity as compared with adult hepatocytes. After transplanted into injured livers, these cells can improve liver function and reduce animal mortality[22,23]. To investigate the effects of allogeneic embryonic hepatocyte transplantation on hepatic cirrhosis and the associated mechanisms of action, we utilized E15 rat hepatocytes as seed cells. The results presented here provide a theoretical and experimental framework for future studies of embryonic liver stem cell transplantation for the treatment of hepatic cirrhosis.

MATERIALS AND METHODS

Materials

Experimental animals: One hundred adult female Wistar rats and 6 male rats were purchased from the Specific Pathogen Free Grade Animal Department, Peking Union Medical College.

Primary reagents: Rabbit anti-human albumin (ALB) antibody and rabbit anti-rat cytokeratin 19 (CK19) antibodies were purchased from Beijing Gene Biology (Beijing, China). The rabbit anti-human α-fetoprotein (AFP) polyclonal antibody, the PV-6001 immunohistochemistry kit, PV-9003 immunohistochemistry kit and the chromogenic kit were purchased from Beijing Zhongshan Biology (Beijing, China). The rabbit anti-human/mouse/rat stem cell factor receptor (c-kit) antibody, the rabbit anti-human/mouse/rat hepatocyte growth factor receptor (c-Met) antibody, rabbit anti-human/mouse/rat Nestin antibodies were purchased from Wuhan Boster Biology (Wuhan, China). Percoll centrifugal liquid was purchased from Beijing Kehaijunzhou Bitechnology (Beijing, China). Collagenase type Ⅳ was purchased from Beijing Tianlai Biotech. Co. (Beijing, China). The rabbit anti-human/mouse/rat α smooth muscle actin (α-SMA) polyclonal antibody (ab66133) was purchased from Abcam (Cambridge, United Kingdom), the goat anti-human/mouse/rat endothelin-1 (ET-1) polyclonal antibody was purchased from Santa Cruz Biotechnology (California, United States), the biotinylated goat anti-rabbit immunoglobulin G (IgG) and anti-goat IgG antibodies were purchased from Beijing Zhongshan Biology (Beijing, China). β-actin was purchased from Santa Cruz Biotechnology (California, United States). The Power SYBR Green polymerase chain reaction (PCR) Master mix was purchased from ABI (California, United States), the M-MLV, dNTP Mix and Oligo (dT15) were purchased from Promega Corporation (Wisconsin, United States), and the Taq enzyme was purchased from TIANGEN (Beijing, China). Primers for stellate activation-associated protein (STAP), c-myb, α-SMA and ET-1 were purchased from Invitrogen Corporation (California, United States) and were synthesized by Beijing GENELIFE Biotech Co., Ltd (Beijing, China).

Experimental methods

Experimental animals: Females and male rats (2 females/1 male) were housed together overnight. Female vaginal smears were examined the following morning. The day when the sperm was detected was defined as day 0 of the pregnancy. Pregnant female rats at E15 were used to isolate embryonic hepatocytes.

Isolation of embryonic hepatocytes: The hepatocyte suspension was prepared using the enzymatic digestion method, and the hepatocytes were separated using Percoll gradient centrifugation. Percoll and a 9% NaCl solution were mixed at a ratio of 9:1. This solution was then diluted with D-F12 culture medium to 50%, 70% and 90% gradient centrifugation solutions. Each dilution of Percoll solution (5 mL) was added sequentially into a centrifuge tube, and the cell suspension was placed on the top of these solutions. The tube was centrifuged at 4330 *g* for 30 min at 4 ℃. The cells between the 50% and 70% Percoll layers were carefully removed and washed twice with Hank's balanced salt solution by centrifuging at 719 *g* and 4 ℃. The supernatant was discarded, and culture medium was added to the tube to a final volume of 1 mL. Cells

were counted using trypan blue after being re-suspended in the medium. Following calculation of the cell density, 2 × 10^6/mL cells were cultured in a 25-mm^2 flask.

***In vitro* culture of embryonic hepatocytes:** The flask was placed in a 37 ℃ incubator filled with 50 mL/L carbon dioxide. The D-F12 culture solution contained 15% fetal bovine serum (FBS), and the medium was replaced every 24 h. Cells were microscopically analyzed for growth and proliferation status, and the number of colonies was recorded.

Identification of colonies: Colonies were marked with a variety of liver stem cell markers using the two-step immunoperoxidase method. The analyzed markers included ALB (1:2000), AFP, c-kit, c-Met, Nestin and CK19 (all 1:1000).

Experimental groups: The Wistar rats were randomly divided into a cirrhotic group (n = 50) and a normal control group (n = 10). In the normal control group, olive oil was injected subcutaneously into the abdomen. In the cirrhotic group, 50% CCl_4 in olive oil was injected subcutaneously into the abdomen; the first four and final four doses were 0.5 mL/100 g body weight, and the other doses were 0.3 mL/100 g body weight. The 50% CCl_4 solution was injected every four days for a total of 15 injections. A 10% ethanol solution, which was prepared with white wine and distilled water, constituted the only liquid drank by the rats in the cirrhotic group.

After 63 d, the 24 surviving rats in the cirrhotic group (16 rats died during the induction of cirrhosis) were randomly divided into two groups: a control cirrhotic group (n = 12) and a cell transplantation group (n = 12). Rats in the control cirrhotic group received continuous CCl_4. In the cell transplantation group, rats were transplanted with E15 rat hepatocytes labeled with carboxyfluorescein diacetate and succinimidyl ester (CFSE) fluorescent molecules. CCl_4 was also continuously administered in this group. The rats were sacrificed 2 wk after the beginning of the transplantation treatment, and their serum was collected. A portion of liver tissue from the rats was used to prepare frozen and paraffin sections, and the remaining hepatic tissue was stored in liquid nitrogen for Western blotting and real time-PCR.

B-ultrasound image analysis: A solution of 0.5 mL/100 g chloral hydrate was used to anesthetize rats, and hair removal agents were used for skin preparation. Rats were immobilized on the operating table prior to the procedure. A small animal, high-resolution ultrasound system (Vevo 770TM) was used for image collection and analysis, and a PEF-704LA laparoscopic linear probe was used for abdominal exploration. The following ultrasound parameters were included: 2-D measurement of the portal vein and hepatic vein, Doppler imaging measurement of portal vein velocity (PVV) and hepatic vein velocity (HVV), echo intensity, the liver morphology and angle of the liver's blunt edge. SPSS13.0 software was used for statistical analyses following a completely randomized single-factor F test and paired t test.

Fluorescent labeling and tracking of E15 hepatocytes: CFSE was dissolved in dimethyl sulfoxide, and a 1-mmol/L CFSE solution was stored at -20 ℃. The stock solution was diluted to a 5 μmol/L working solution with phosphate buffer solution (PBS). Following a 20-min incubation with the CFSE working solution, fluorescence of the E15 hepatocytes was confirmed by excitation using a wavelength of 488 nm. The cells were digested with 0.25% trypsin, counted with trypan blue staining and diluted to 2 × 10^7 cells/0.5 mL.

Orthotopic liver transplantation of fluorescently-labeled embryonic hepatocytes: The rats were anesthetized with 0.5 mL/100 g chloral hydrate and immobilized on a surgical table. A 1.5-cm vertical incision on the right side of the abdomen was made under the xiphoid process to expose the liver. Using a 1-mL syringe, 0.5 mL of the labeled E15 hepatocyte suspension was slowly injected into the liver parenchyma. The abdomen was closed, and the rats were placed at ≥ 26 ℃ overnight.

Serum assay: Blood was taken from the abdominal aorta, and the following indicators were analyzed: total protein (TP), ALB, total bilirubin (TBil), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). The levels of collagen type Ⅲ, AFP and ET-1 were determined by radioimmunoassays. SPSS13.0 software was used for statistical analysis using a completely randomized single-factor F test.

Histopathology: The rat livers were fixed in formalin for 48 h, paraffin embedded and sectioned into 4-μm thick slices for hematoxylin and eosin (HE) and van gieson (VG) staining.

Immunohistochemistry: Immunohistochemical staining of α-SMA, ET-1. Marker expression was examined using the two-step immunoperoxidase method. Briefly, after tissue sections were deparaffinized and rehydrated, sections were heated in a microwave for 10 min to enhance antigen retrieval. Slides were then incubated for 10 min with 3% H_2O_2 to inactivate endogenous peroxidase activity. Primary antibodies (rabbit anti-human/mouse/rat α-SMA polyclonal antibody, 1:50; goat anti-human/mouse/rat ET-1 polyclonal antibody, 1:50) were applied and incubated overnight at 4 ℃. The appropriate secondary antibody (biotinylated goat anti-rabbit IgG, 1:200; anti-goat IgG antibody, 1:200) was applied for 40 min, and 3,3'-diaminobenzidine was used as a chromogen. Negative controls were performed for each antibody using PBS instead of the primary antibody.

Immunohistochemical staining analysis: Immunohistochemical staining of paraffin-embedded liver sections was analyzed with a Leica Q500 IW Imaging Worksta-

Table 1 Primer sequences used for real-time polymerase chain reaction

Gene	Forward primer	Reverse primer	Segment length
STAP	AGTCCTCAGCTGCGGAAACA	AGCGCGAGCACAGAGGATAC	100 bp
α-SMA	CGGGCTTTGCTGGTGATG	GCTGTCCTTTTGGCCCATT	100 bp
C-myb	CCATCCAGAGACATTATAACGATGA	CTGTCCCTTCAGTTCGTTCTCTGT	100 bp
ET-1	GACCACAGACCAAGGGAACAG	TGGCATGGCCGAACTCAT	100 bp
β-actin	CATTGCTGACAGGATGCAGAAG	GAGCCACCAATCCACACAGAGT	100 bp

STAP: Stellate activation-associated protein; SMA: Smooth muscle actin; ET: Endothelin.

tion. The average optical density was calculated in image analysis. For quantitative analyses, several fields were selected in each slice using a micro-camera (Magnification: 10 × 10), and the images were transferred to a computer. The position of positive staining was first observed under low magnification, and the optical density values were measured using a high power (Magnification: 10 × 20). Five high-power fields of each specimen were randomly selected, and their average optical density value was calculated. SPSS13.0 software was used for the statistical analysis following a completely randomized single-factor *F* test.

Western blotting: Liver tissue samples and cells were lysed in ice-cold RIPA buffer supplemented with protease inhibitors. Whole cell lysates were obtained by subsequent centrifugation at 14 000 r/min for 10 min at 4 ℃. Protein concentrations were determined using the Bradford protein assay with bovine serum albumin as a standard. Twenty-five micrograms of protein extracts were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to a nitrocellulose membrane. Membranes were blocked with a 7.5% solution of nonfat dry milk dissolved in a Tris-HCl-buffered solution (TBS, pH 7.5) and were then probed with a primary antibody (rabbit anti-human/mouse/rat α-SMA polyclonal antibody, 1:50; goat anti-human/mouse/rat ET-1 polyclonal antibody, 1:50). Membranes were subsequently washed with TBST (TBS, 0.1% Tween 20) and exposed for 45 min at room temperature to the appropriate secondary antibody (anti-goat IgG antibody, 1:5000; anti-rabbit IgG antibody, 1:5000). Staining was detected using chemiluminescence followed by autoradiographic and densitometric analyses. Experiments were performed at least 3 times, and similar results were obtained for each replicate.

Real-time PCR: RNA from liver tissue was extracted using TRIzol. First-strand cDNA was reversely transcribed (RT) from 1 μg of total RNA using Oligo (dT) 15 primers and a M-MLV reverse transcriptase. The primers were designed based on gene sequence data from GenBank. Primer sequences used for real-time PCR are shown in Table 1. Real-time PCR was performed using an ABI Prism 7900HT Sequence Detection System (SDS). Triplicate reactions of each sample were performed in a 96-well plate using SDS instrumentation for 40 cycles. Real-time PCR amplifications were carried out using 2 μL cDNA, 12 μL 2 × SYBR Green PCR Master Mix, 0.3 μL primers (forward/reverse, 15 pmol/μL), and 10.4 μL H_2O for a final reaction volume of 25 μL. Real-time PCR cycling parameters were 50 ℃ for 2 min, 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 s, 60 ℃ for 1 min, 95 ℃ for 15 s, 60 ℃ for 15 s and 95 ℃ for 15 s.

Data were analyzed using SDS2.2 software. The relative quantities of the target genes were normalized against the expression of β-actin[24]. The following formulas were used for RT-PCR data analysis: △Ct = Ct value of the target gene - Ct value of the normalization gene (the reference gene); quantity of the experimental group = 2-△Ct of the experimental group; quantity of the control group = 2-△Ct of the control group; quantity (relative expression levels) = quantity of the experimental group /quantity of the control group. After the expression of each gene in the normal control group was normalized to 1, the relative 2-ΔΔCt values for each gene in the untreated cirrhotic and the cell transplantation groups were calculated.

RESULTS

In vitro culture and identification of isolated cells

The primary fetal hepatocytes became adherent approximately 5 h into the culture period, and spheres formed on day 2 (Figure 1A). Spheres had a round or oval homogeneous morphology, and round or oval nuclei. Microscopic analysis revealed that the adherent cells grew in a monolayer and had a clear cell boundary (Figure 1B). Cells were round or polygonal and were arranged in neat rows. In addition, the cells had abundant cytoplasm and round nuclei. However, a small number of fibroblasts were observed within the cultures. Following a few days in culture, approximately half of the clones gradually grew into sheets and merged with one another or gradually differentiated (Figure 1C). In a 25-mm^2 flask, the peak number of cell colonies (> 4000 colonies) appeared on day 2 into the culture period. Most cells isolated from the 50% Percoll sample were not adherent, and a few gradually died over the course of the culture period.

Immunohistochemical staining revealed that most liver stem cell-specific markers, including c-kit (Figure 2A), c-Met (Figure 2B), Nestin (Figure 2C), AFP (Figure 2D), ALB (Figure 2E) and CK19 (Figure 2F), were expressed in the cell colonies on day 2 of culture.

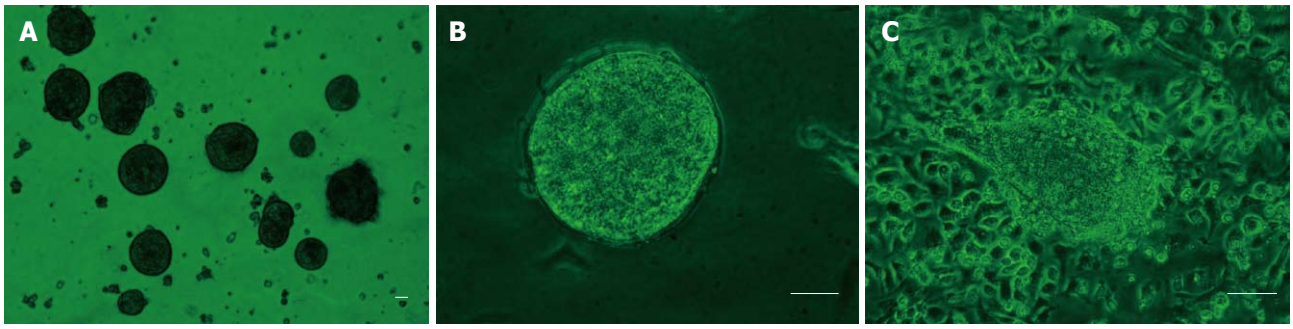

Figure 1 E15 rat hepatocytes *in vitro*. A: E15 rat hepatocytes 2 d after cell inoculation; B: E15 rat hepatocytes 2 d following cell inoculation; C: Differentiation of rat embryonic hepatocytes. Scale bars: 20 μm.

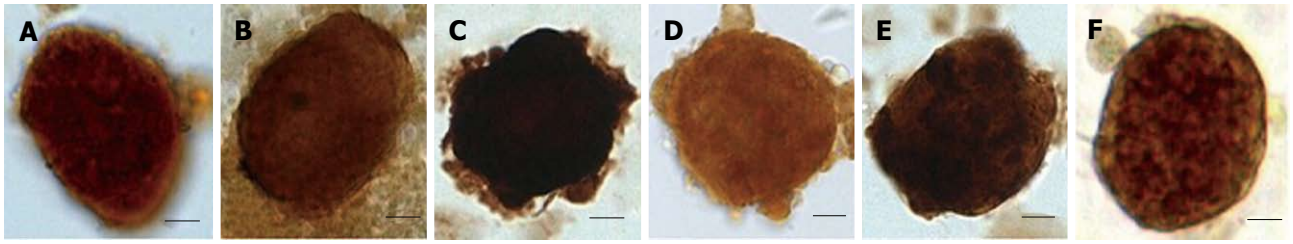

Figure 2 Immunohistochemistry staining of E15 rat hepatocytes. A: c-kit-positive colony; B: c-Met-positive colony; C: Nestin-positive colony; D: α-fetoprotein-positive colony; E: Albumin-positive colony; F: Cytokeratin 19-positive colony. Scale bars: 20 μm.

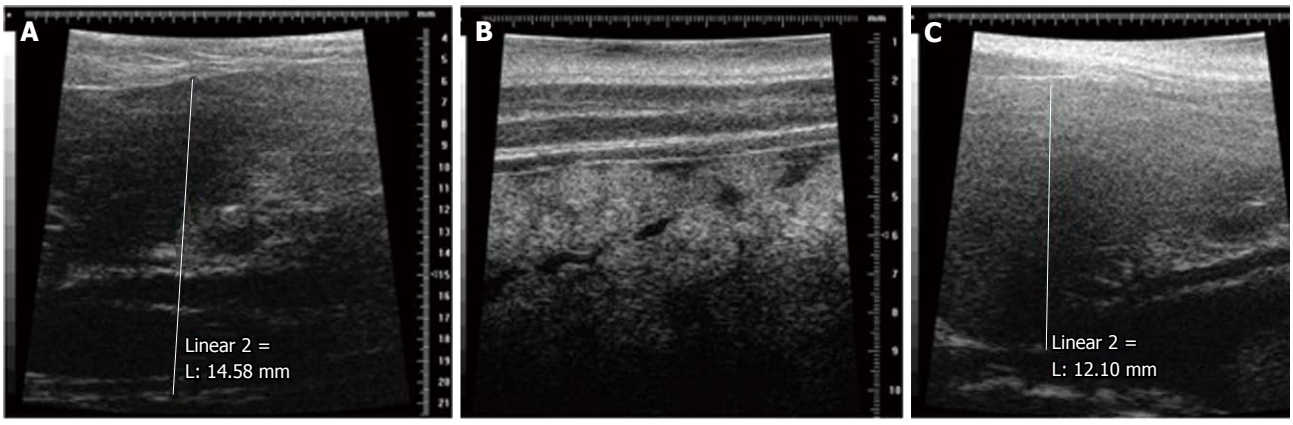

Figure 3 Parameters of ultrasound examination. A: Livers exhibit a smooth capsule, sharp edges, clear vascular texture and uniform echoes in the normal control group; B: Livers from rats of the untreated cirrhotic group exhibit uneven surfaces, blunt edges, unclear vascular texture and serrated and enhanced echoes with uneven distribution; C: In the transplantation group, rat livers exhibit a smooth capsule, smooth edges, clear vascular texture and even echo patterns.

Ultrasound examination

As shown in Figure 3A, ultrasound examination revealed that the livers of normal control rats had a smooth capsule, sharp edges, clear vascular texture and a uniform echo. In rats of the untreated cirrhotic group, however, livers had an uneven surface, blunt edges, unclear vascular texture, and serrated and enhanced echoes with uneven distributions (Figure 3B). No specific liver parenchymal lesions were observed in the untreated cirrhotic rats. Compared with the normal control group, the portal and hepatic veins in the untreated cirrhotic group were significantly widened ($P < 0.01$), and both PVV and HVV were significantly decreased ($P < 0.01$). Visible signs of ascites were observed in the untreated cirrhotic group. Ultrasound examination, therefore, revealed that livers of untreated cirrhotic rats were characterized by cirrhosis, portal hypertension, and the presence of ascites.

In the cell transplantation group, rat livers had a smooth capsule, smooth edges, clear vascular texture and even echo patterns (Figure 3C). Compared with the normal control group, the portal and hepatic veins were not obviously enlarged ($P > 0.05$, Table 2). The portal and hepatic veins were significantly narrower than those of rats in the untreated cirrhotic group ($P < 0.01$). PVV and HVV were decreased compared with the velocity in the normal control group ($P = 0.03$ and $P < 0.01$, respectively), but being higher than those observed in the untreated cirrhotic group (both $P < 0.01$). Findings and

Table 2 Results of ultrasound examination (mean ± SD, *n* = 10)

	Diameter of the major trunk of the portal vein (mm)	Hepatic vein diameter (mm)	PVV (mm/s)	HVV (mm/s)
Normal control group	1.545 ± 0.095	0.775 ± 0.058	117.319 ± 15.306	122.00 ± 14.553
Untreated cirrhotic group	2.518 ± 0.138[b]	1.378 ± 0.128[b]	53.663 ± 9.730[b]	56.817 ± 17.855[b]
Transplantation group	1.565 ± 0.135[d]	0.804 ± 0.096[d]	104.535 ± 11.654[a,d]	98.561 ± 15.955[b,d]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* the normal control group; [d]$P < 0.01$ *vs* the untreated cirrhotic group. PVV: Portal vein velocity; HVV: Hepatic vein velocity.

Table 3 Effects of hepatocyte transplantation on ultrasound measurement (mean ± SD, *n* = 8)

	Diameter of the major trunk of the portal vein (mm)	Hepatic vein diameter (mm)	PVV(mm/s)	HVV (mm/s)
Before hepatocyte transplantation	2.571 ± 0.343	1.361 ± 0.136	67.441 ± 6.666	54.679 ± 17.453
After hepatocyte transplantation	1.575 ± 0.146	0.790 ± 0.137	100.968 ± 9.796	94.423 ± 5.207

PVV: Portal vein velocity; HVV: Hepatic vein velocity.

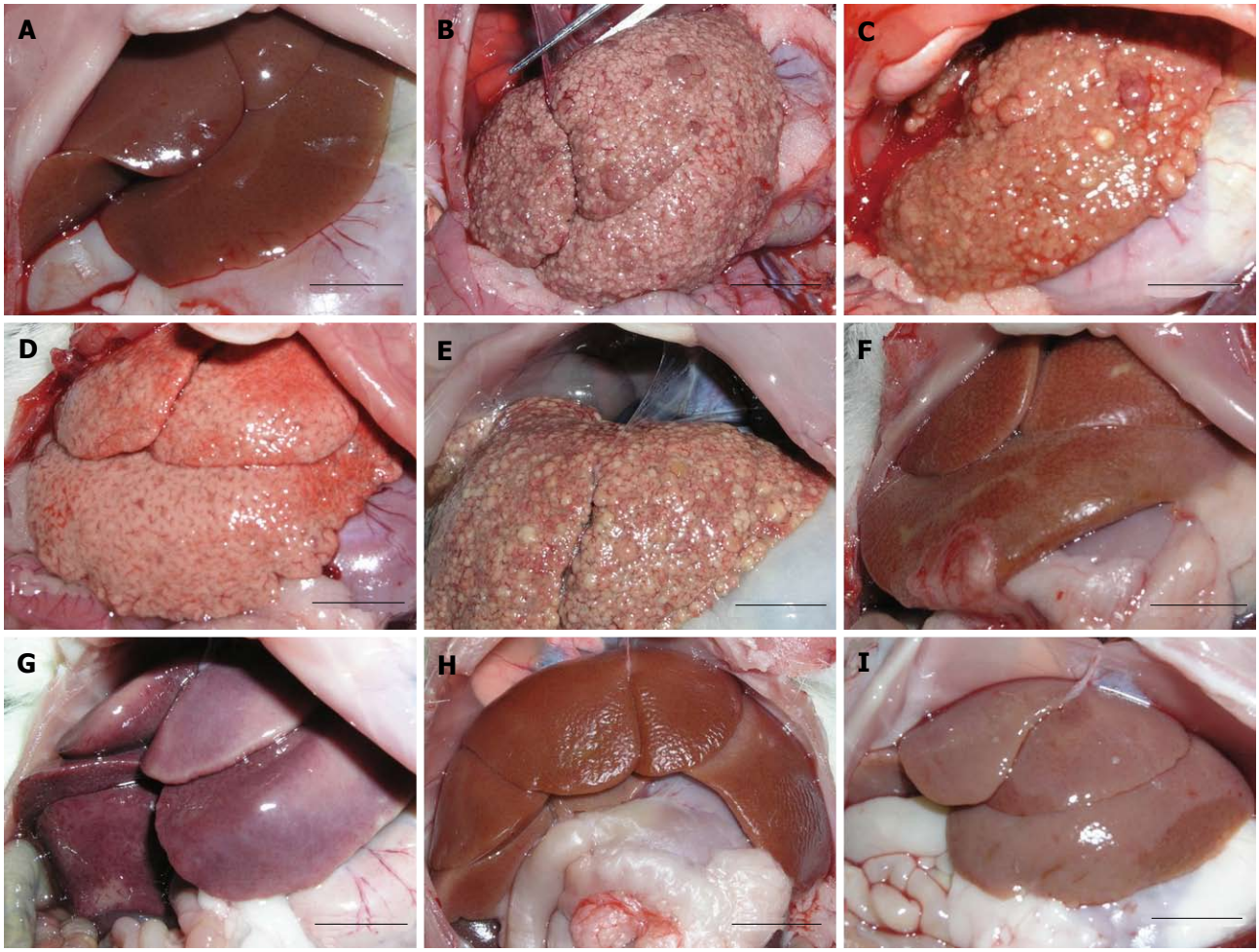

Figure 4 The appearance of rat livers. A: Normal control group; B-E: Untreated cirrhotic group; F-I: Transplantation group. Scale bars: 1 cm.

statistical results for each parameter of the ultrasound examination before and after liver transplantation (paired *t* test, $P < 0.01$) are shown in Table 3.

Pathological changes in rats with hepatic cirrhosis

The livers of normal control rats were dark red, elastic with a smooth surface, sharp edges and a soft texture (Figure 4A). However, the livers of rats in the untreated cirrhotic group had a hard texture, a brown or gray-brown appearance, diffuse surface nodules of variable sizes, blunt edges, uneven surfaces and were adhered to the adjacent organs (Figures 4B-E). The livers of rats in

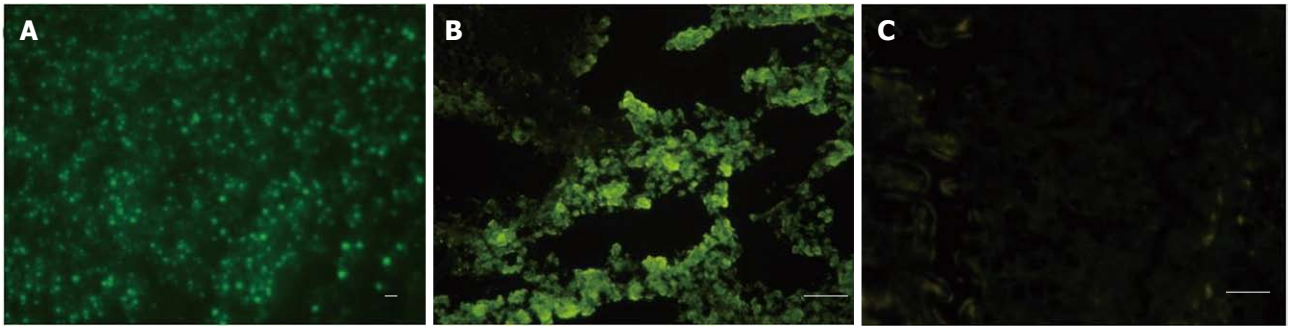

Figure 5 Tracking E15 hepatocytes. A: Using fluorescence microscopy, *in vitro* E15 hepatocytes demonstrated strong green fluorescence 2 d into the culture period with the carboxyfluorescein diacetate and succinimidyl ester fluorescent label; B: Cells in frozen liver tissue sections (2 wk following transplantation); scattered green fluorescent markers were observed; C: No fluorescent cells were observed in the frozen liver tissue sections of the untreated cirrhotic group. Scale bars: 20 μm.

Table 4 Effects of hepatocyte transplantation on serum biochemical tests (mean ± SD, *n* = 10)

	ALT (μmol/L)	AST (μmol/L)	TP (μmol/L)	ALB (μmol/L)	TBil (μmol/L)
Normal control group	22.297 ± 2.445	52.393 ± 32.356	61.984 ± 2.489	33.860 ± 1.102	2.015 ± 0.451
Untreated cirrhotic group	627.264 ± 113.580[b]	432.771 ± 100.330[b]	45.697 ± 2.646[b]	26.030 ± 1.282[b]	35.355 ± 9.587[b]
Transplantation group	86.623 ± 38.953[a,d]	158.284 ± 94.099[b,d]	56.174 ± 2.780[b,d]	31.330 ± 1.222[b,d]	1.957 ± 0.723[d]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* the normal control group; [d]$P < 0.01$ *vs* the untreated cirrhotic group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TP: Total protein; ALB: Albumin; TBil: Total bilirubin.

Table 5 Effects of hepatocyte transplantation on serum marker levels (mean ± SD, *n* = 10)

	PⅢ (μg/L)	ET-1 (pg/mL)	AFP (ng/mL)
Normal control group	48.096 ± 5.010	45.459 ± 12.626	12.150 ± 11.430
Untreated cirrhotic group	92.954 ± 4.481[b]	132.192 ± 8.906[b]	12.099 ± 8.529
Transplantation group	54.023 ± 4.535[b,d]	42.383 ± 11.701[d]	61.315 ± 20.973[b,d]

[b]$P < 0.01$ *vs* the normal control group; [d]$P < 0.01$ *vs* the untreated cirrhotic group. ET: Endothelin; AFP: α-fetoprotein.

the transplantation group had a partially red appearance, soft texture, elasticity, sharp edges and smooth facets, similar to the normal control livers (Figures 4F-I).

Tracking E15 hepatocytes

Under fluorescence microscopy, E15 hepatocytes labeled *in vitro* with CFSE showed strong green fluorescence (Figure 5A). CFSE-labeled cells were transplanted into cirrhotic rat livers, and the livers were removed 2 wk after the transplantation. Scattered green fluorescent markers were observed in frozen tissue sections from these livers (Figure 5B); however, no fluorescent cells were observed in sections from the normal control or untreated cirrhotic rats (Figure 5C). These results suggest that embryonic hepatocytes labeled with CFSE *in vitro* are able to survive in the livers of cirrhotic rats.

Effects of hepatocyte transplantation on serum indicators

As shown in Table 4, ALT, AST and TBil levels in rats in the untreated cirrhotic group were much higher than in the normal control and cell transplantation groups. ALT and AST levels in the cell transplantation group were also slightly higher than in the normal control group ($P < 0.01$); however, no statistical differences in TBil levels were observed between the cell transplantation and normal control groups ($P > 0.05$). The TP and ALB levels of the model rats were lower than those in the cell transplantation group, which were also lower than the TP and ALB levels in the control group ($P < 0.01$).

PⅢ and ET-1 levels of rats in the untreated cirrhotic group were much higher than in the normal control or cell transplantation groups ($P < 0.01$, Table 5). PⅢ levels in rats with transplanted cells were higher than those observed in normal control rats ($P < 0.01$); however, no statistical difference in ET-1 levels was observed between the cell transplantation and normal control groups ($P > 0.05$). AFP levels in the cell transplantation group were much higher than in the control and untreated cirrhotic groups ($P < 0.01$); no statistical difference in AFP levels were observed between the control and untreated cirrhotic groups ($P > 0.05$).

Effects of hepatocyte transplantation on histopathology

Results of HE staining revealed that normal control rats exhibited typical liver lobules and orderly hepatic cords (Figure 6A). In contrast, livers from cirrhotic rats exhibited swollen hepatocytes, severe diffuse hydropic degeneration, and fatty degeneration. Some necrotic hepatocytes and pyknotic or dissolute nuclei were observed in the cirrhotic livers. The following abnormalities were also noted in the cirrhotic livers: remarkable expansions of the hepatic sinus and central vein; disorganization of the hepatic cord; apparent focal lymphocyte infiltration in the portal area; hyperplasia of the bile duct epithelia; brown pigmentation; diffuse hyperplasia of interstitial

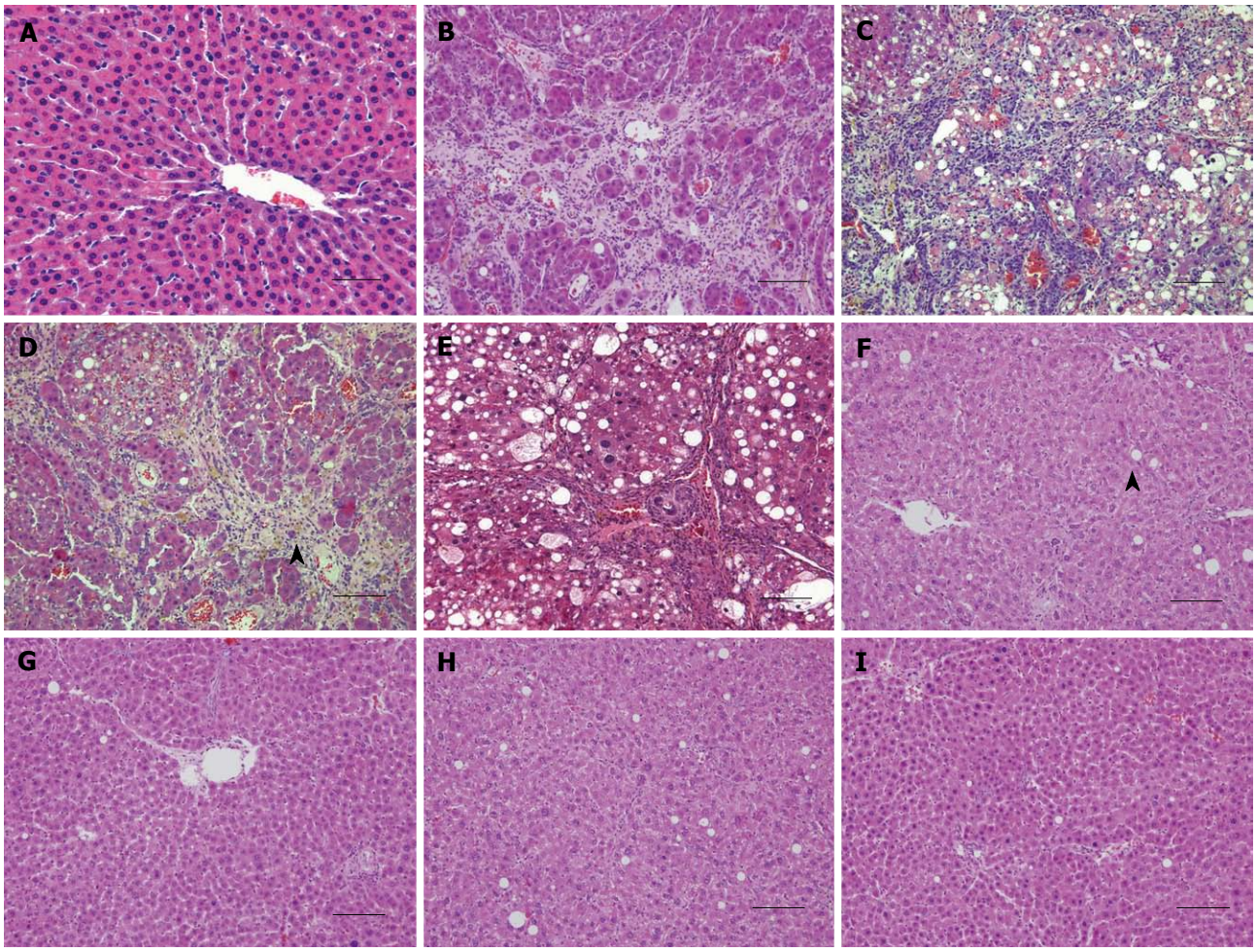

Figure 6 Hematoxylin and eosin staining of tissue slices from rat livers in each group. A: Normal liver lobules from the normal control group; B-E: Pseudo-lobules, fatty degeneration, necrotic hepatocytes, brown pigmentation (arrow) in livers from the untreated cirrhotic group; F-I: Sections from the cell transplantation group; a few cells have vacuoles (arrow), and liver lobules are present. Scale bars: 20 μm.

Table 6 Average optical density values (mean ± SD, *n* = 10)

	α-SMA	ET-1
Normal control group	0.140 ± 0.023	0.161 ± 0.022
Untreated cirrhotic group	0.602 ± 0.060[b]	0.523 ± 0.063[b]
Transplantation group	0.150 ± 0.031[d]	0.172 ± 0.021[d]

[b]$P < 0.01$ *vs* the normal control group; [d]$P < 0.01$ *vs* the untreated cirrhotic group. SMA: Smooth muscle actin; ET: Endothelin.

cells; noticeable fibrosis with septation; and formation of pseudo-lobules (Figures 6B-E). In the cell transplantation group, no pseudo-lobules or disorganized hepatic cords were detected, and the hepatocytes were similar to those in normal control rats. Although a small number of cells had vacuoles, most hepatocytes of the transplantation group had abundant cytoplasm (Figures 6F-I).

Depositions of collagen fibers were examined in VG-stained rat liver tissues. In the normal control group, rats had normal hepatic lobules. Hepatocytes were radially arranged around the central vein and no hyperplasia of the fibrous tissue was observed (Figure 7A). In the cirrhotic rats, the hepatic lobule was destroyed, and pseudo-lobules were surrounded by fibrous tissues (Figures 7B-E). The cell transplantation group exhibited noticeably less hyperplasia of the collagen fibers when compared with the untreated cirrhotic group (Figures 7F-I).

Immunohistochemical analysis

α-SMA expression was only observed in the vascular wall of normal rats; no significant expression was observed at other sites (Figure 8A). In untreated cirrhotic rats, α-SMA was primarily expressed in the periportal areas, fibrous septa, pseudo-lobules and hyperplastic fibers (Figure 8B). α-SMA expression in the cell transplantation group was observed in the periportal vascular wall while it was rarely detected in the periportal areas or fibrous septa (Figure 8C). The optical density (OD) values of α-SMA in the untreated cirrhotic group were much higher than in normal control or cell transplantation groups ($P < 0.01$, Table 6). No statistical difference was observed in the α-SMA OD values between the cell transplantation and normal control groups ($P > 0.05$).

Weak ET-1 expression was detected in a small number of sinusoid endothelial cells of the normal control rats (Figure 8D). In contrast, clear expression was ob-

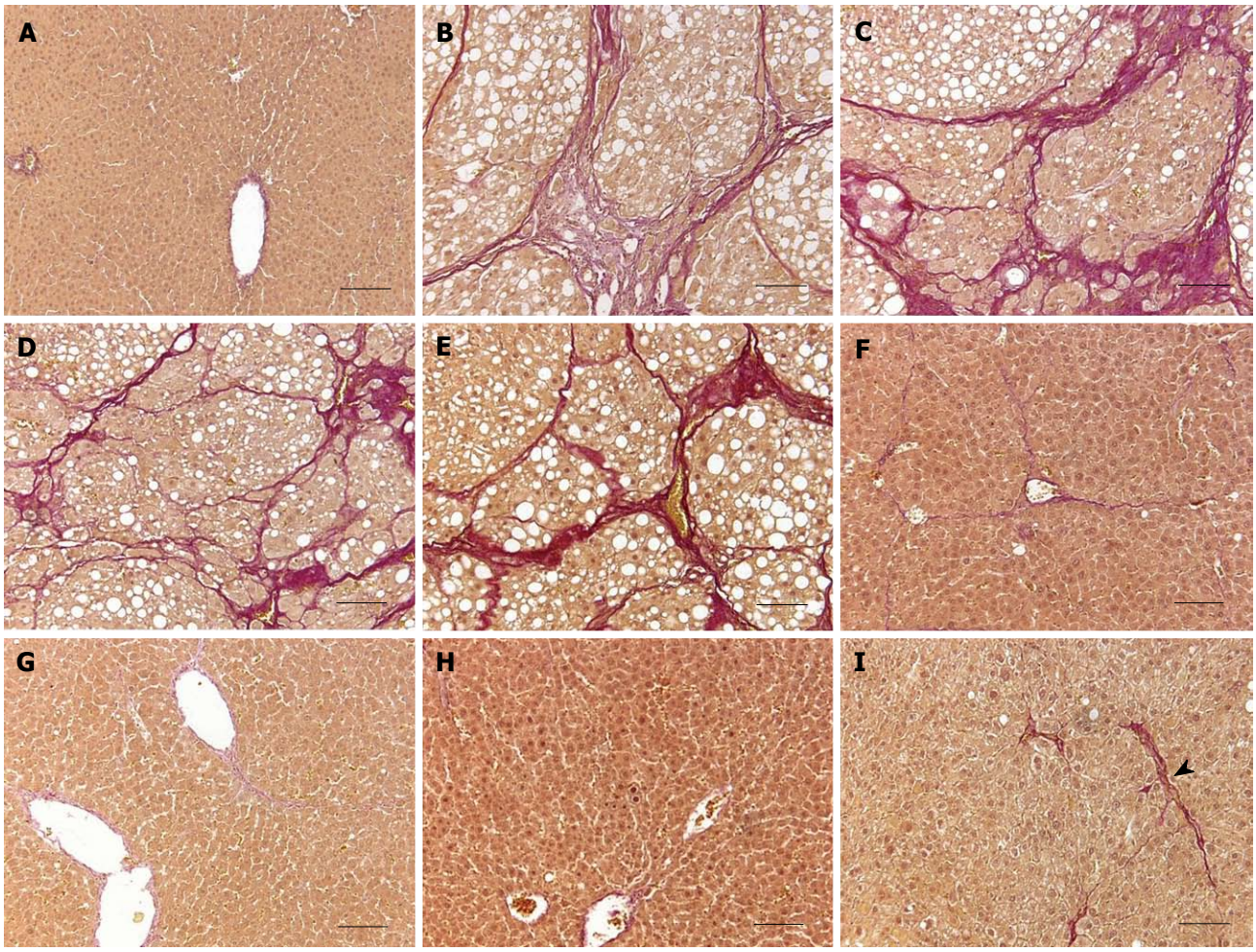

Figure 7 Van gieson staining of tissue slices from rat livers in each group. A: No hyperplasia of the fibrous tissue was observed in the normal control group; B-E: Pseudo-lobules were surrounded by fibrous tissues in livers from the untreated cirrhotic group; F-I: A few collagen fibers (arrow) were observed in livers from the transplantation group. Scale bars: 20 μm.

Table 7 Optical density values of Western blotting protein bands (mean ± SD, *n* = 3)

	α-SMA	ET-1
Normal control group	0.457 ± 0.055	0.593 ± 0.006
Untreated cirrhotic group	0.929 ± 0.036[b]	1.034 ± 0.112[b]
Transplantation group	0.502 ± 0.066[d]	0.607 ± 0.012[d]

[b]$P < 0.01$ *vs* the normal control group; [d]$P < 0.01$ *vs* the untreated cirrhotic group. SMA: Smooth muscle actin; ET: Endothelin.

served in the pseudo-lobules (Figure 8E) and fibrous septa (Figure 8F) of the untreated cirrhotic rats. In the cell transplantation group, ET-1 expression was only observed in the sinusoid endothelial cells (Figure 8G). The OD values of the untreated cirrhotic group were much higher than those of the normal control and cell transplantation groups ($P < 0.01$, Table 6). There was no significant difference in these values between the cell transplantation and control groups ($P > 0.05$).

Western blotting analysis

α-SMA and ET-1 expression levels in normal rat livers were extremely low (Figure 9). α-SMA and ET-1 protein was more highly expressed in the liver tissues of the untreated cirrhotic group than in the normal group. Additionally, the expression of α-SMA and ET-1 in rats treated with cell transplantation was significantly lower than that of the untreated cirrhotic group ($P < 0.01$). There was no statistically significant difference between the cell transplantation group and the control group ($P > 0.05$). The Western blotting optical density ratios are shown in Table 7.

Real-time PCR

After the expression of each gene in the normal control group was normalized to 1, the relative $2^{-\Delta\Delta Ct}$ value of each gene in the untreated cirrhotic and cell transplantation groups was calculated. Compared with the control group, the expression levels of STAP, c-myb, α-SMA and ET-1 were increased in the untreated cirrhotic group ($P < 0.01$). However, the expression levels of these mRNAs in rats of the cell transplantation group were significantly reduced when compared with the untreated cirrhotic group ($P < 0.01$) (Table 8). There was no statistically significant difference between the cell transplantation and

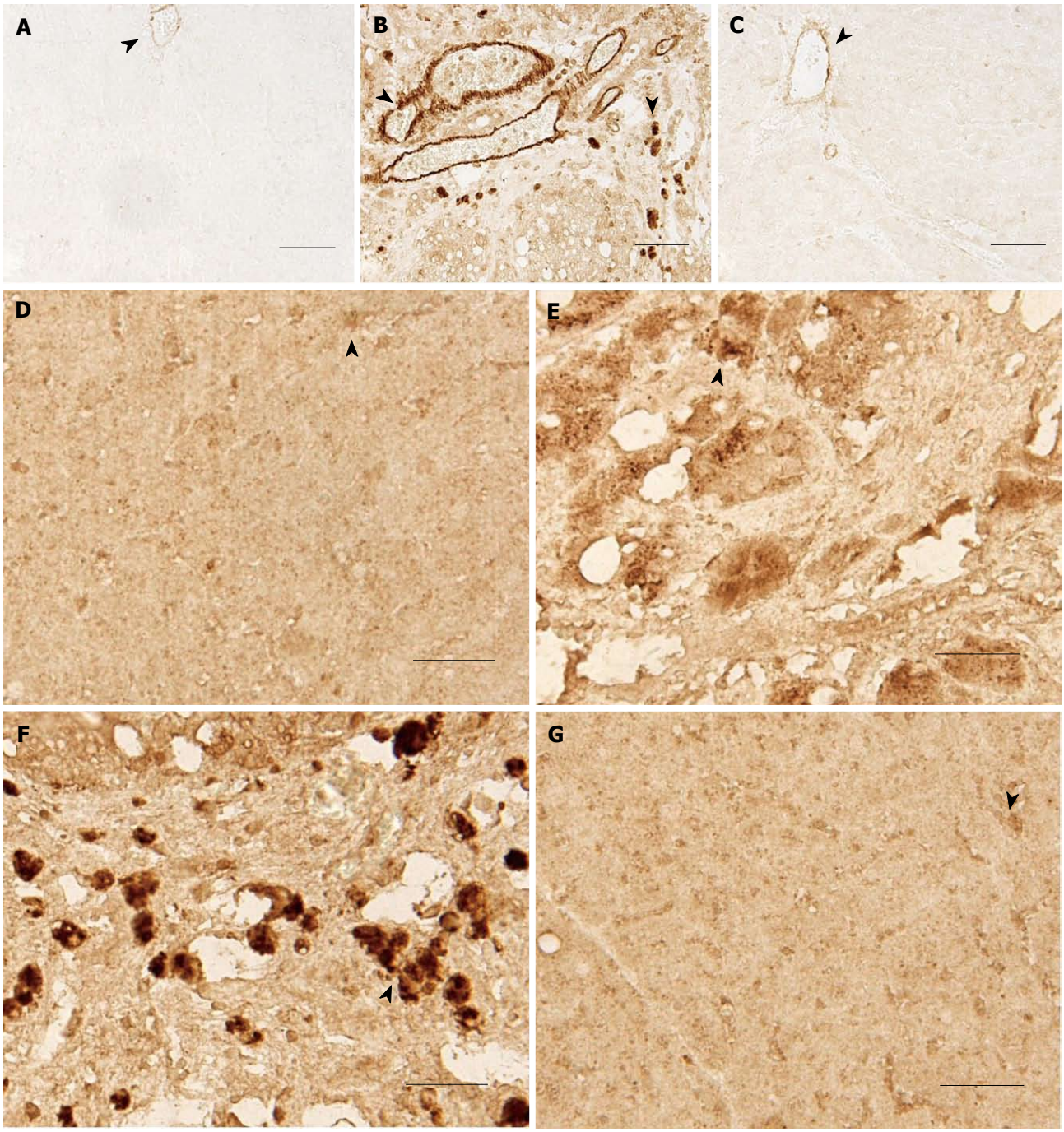

Figure 8 Immunohistochemical staining of rat livers in each group. A: α smooth muscle actin (α-SMA) expression (arrow) was only observed in the vascular wall of the normal group; B: α-SMA (arrow) was highly expressed in the periportal areas and fibrous septa of livers from the untreated cirrhotic group; C: The cell transplantation group exhibited noticeably weaker α-SMA (arrow) expression than the untreated cirrhotic group; D: Weak endothelin-1 (ET-1) (arrow) expression was detected in a few sinusoid endothelial cells of livers from the normal control group; E: Clear ET-1 expression (arrow) was observed in the pseudo-lobules of livers from the untreated cirrhotic group; F: Clear ET-1 (arrow) expression in the fibrous septa of livers from the untreated cirrhotic group; G: In the cell transplantation group, ET-1 (arrow) expression was only observed in the sinusoid endothelial cells. Scale bars: 20 μm.

control groups ($P > 0.05$).

DISCUSSION

There is no precise or unified definition for hepatic stem cells. In 1956, Farber *et al*[25] observed hepatic oval cells with a diameter of approximately 10 μm. These cells were shown to be able to differentiate into either hepatocytes or bile duct cells and were considered as liver stem cells[8,26,27]. It is well known that hepatic stem cells have unlimited proliferative capacity and the potential to differentiate into hepatocytes.

The morphology, phenotype and molecular expression profile of hepatic stem cells change dynamically in their degree of differentiation[28]. Due to the lack of specific markers, hepatic stem cells are principally identified

Table 8 Effects of embryonic hepatocyte transplantation in rats on different mRNA expression (mean ± SD, *n* = 9)

	STAP	c-myb	α-SMA	ET-1
Normal control group	1.000 ± 0.2667	1.000 ± 0.250	1.000 ± 0.2446	1.000 ± 0.3191
Untreated cirrhotic group	44.97 ± 19.40[b]	22.32 ± 5.536[b]	45.65 ± 11.98[b]	8.021 ± 1.191[b]
Transplantation group	1.133 ± 0.2222[d]	0.7143 ± 0.5714[d]	1.094 ± 0.1974[d]	1.010 ± 0.3298[d]

[b]$P < 0.01$ *vs* the normal control group; [d]$P < 0.01$ *vs* the untreated cirrhotic group. STAP: Stellate activation-associated protein; SMA: Smooth muscle actin; ET: Endothelin.

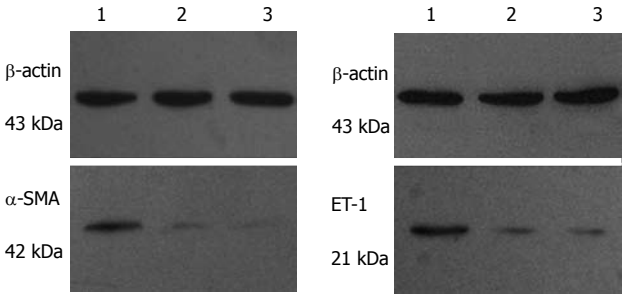

Figure 9 Western blotting analysis of α smooth muscle actin and endothelin-1 expression in liver tissues. Lane 1: The untreated cirrhotic group; Lane 2: The cell transplantation group; Lane 3: The normal control group. SMA: Smooth muscle actin. ET-1: Endothelin-1.

using their differentiation potentials. Hepatic stem cells with bi-potentiality can differentiate into hepatocytes and biliary duct epithelial cells and can be identified using antibodies for specific hepatocyte and biliary epithelial cell markers. Pack *et al*[29] reported that ALB specifically marks mature hepatocytes and that CK19 specifically marks bile duct epithelial cells[30]. Although AFP can be expressed in both embryonic hepatocytes and hepatic oval cells, AFP synthesis ceases upon differentiation and maturation of hepatocytes[31]. c-Met and Nestin are stem cell markers, and c-kit is a common epitope for hepatic and blood stem cells[32-35].

In this study, E15 hepatocytes were cultured *in vitro* and grew in a similar manner as stem cells, exhibiting colony-like growth with clear boundaries for each clone. Molecular characterization revealed that c-kit, c-Met, Nestin, AFP, ALB and CK19 were expressed in the majority of colony cells, suggesting that the majority of rat E15 hepatocytes were undifferentiated and bipotential. Because embryonic hepatocytes have a higher proliferative capacity, multiple differentiation potentials, and low immunogenicity, there is greater clinical value for embryonic hepatocytes than for mature hepatocytes in the treatment of liver injury due to chronic cirrhosis.

The use of CCl_4 and alcohol to induce and model cirrhosis has a high success rate, which is the reason why this technique is chosen for this study. Following the completion of this protocol, ultrasound examination and HE and VG staining of paraffin-embedded specimens revealed pathological changes indicative of hepatic cirrhosis. In addition, serum biochemical indicators were also abnormal, suggesting that hepatic cirrhosis was effectively induced.

Two weeks after E15 hepatocytes were transplanted into the cirrhotic rats, serum levels of ALT, TBil, type-Ⅲ procollagen and ET-1 were significantly reduced. However, serum ALB and TP were significantly increased, indicating that embryonic liver hepatocyte transplantation significantly improved the hepatic function of cirrhotic rats. This conclusion is particularly exemplified by the observed normalization of total bilirubin levels in treated cirrhotic rats. Serum AFP levels in the cell transplantation group were significantly higher than in the untreated cirrhotic group, suggesting that the biological activities of transplanted embryonic hepatocytes induced liver regeneration in cirrhotic rats. There was no significant difference in the ultrasound results between the cell transplantation and normal control groups; neither high portal vein pressure nor ascites were observed. Pathological results revealed that collagen fiber hyperplasia was significantly reduced in rats treated with liver cell transplantation. A small number of collagen fibers around the lobules were observed in treated livers, and the majority of hepatocytes in the lobules were morphologically normal. All these results demonstrate that rat embryonic hepatocyte transplantation significantly improves liver morphology and function in rats with hepatic cirrhosis. These cells also effectively inhibit hepatic necrosis and fibrosis and promote normal hepatocyte proliferation and function.

CCl_4 is a hepatotoxic compound that damages the liver *via* lipid peroxidation[36,37] and leads to intracellular calcium overload, resulting in fatty degeneration of hepatocytes. Continuous administration of CCl_4 in rats leads to hepatic necrosis. Moreover, CCl_4 may cause hepatocyte damage *via* its metabolism into CCl_3 radicals by cytochrome P450, resulting in the oxidation of unsaturated fatty acids and the production of lipid peroxides[38]. Lipid peroxidation can directly activate hepatic stellate cells (HSCs) and facilitate hepatocyte conversion to myofibroblasts. The latter cell type subsequently synthesizes extracellular matrix (ECM) proteins, leading to liver fibrosis[39].

The metabolites of alcohol, such as acetaldehyde, lactic acid, and reactive oxygen species (ROS), can stimulate oxidative stress and cytokine-mediated damage. ROS generated by alcohol metabolism in hepatocytes is also known to activate HSCs and to result in the production of large amounts of collagen[40,41]. Alcohol-induced hepatocyte apoptotic bodies can be phagocytosed by HSCs and Kupffer cells, resulting in HSC activation and ECM synthesis.

The combination of CCl_4 and alcohol induced hepat-

ic cirrhosis in rats in the current study. Although these two agents induced cirrhogenesis *via* distinct mechanisms, they ultimately converged on the key step of HSC activation. Under normal circumstances, HSCs are in a resting state. Following liver damage, however, HSCs are activated and transformed into myofibroblast-like cells, a transition characterized by the loss of cytoplasmic lipid droplets, morphological changes, and the expression of α-SMA and many cytokines and their receptors. These myofibroblast-like cells can proliferate and synthesize a variety of ECM proteins, primarily type- I and type-Ⅲ collagens. HSC activation plays an essential role in liver fibrosis and can cause excessive accumulations of ECM proteins in the liver, resulting in liver fibrosis and cirrhosis. In 2001, Kawada *et al*[42] described a novel protein in rat HSCs that is highly associated with HSC activation, namely STAP [also referred to as cytoglobin/STAP (Cygb/STAP)]. STAP is a cytoplasmic protein in HSCs and myofibroblasts, and both protein and mRNA levels are significantly up-regulated during HSC activation[42,43]. Kang *et al*[44] used the increased expression of STAP as an indicator of cirrhosis in the rat model of cirrhosis.

Our results demonstrate that STAP mRNA expression in the untreated cirrhotic rats was significantly higher than in control rats, indicating the HSC activation and the development of hepatic cirrhosis in untreated cirrhotic rats. CCl_4 and other pathogenic factors can lead to lipid peroxidation, the up-regulation of STAP-related signaling pathways, and HSC activation. Due to the peroxidative activities of STAP on hydrogen peroxide and lipid peroxides, increased expression of this gene can lower the peroxide levels and prevent hepatic fibrosis[42]. In the present study, STAP mRNA levels were much lower in the transplantation group than in the untreated cirrhotic group and were comparable to the normal control group. This result suggests that HSCs in the cell transplantation group were in a resting state and that STAP-related signaling pathways were inactive. Alternatively, transplanted embryonic hepatocytes may selectively induce activated HSCs to undergo apoptosis or inactivate HSCs. Therefore, cell transplantation treatment maintains HSCs in a resting state[45] and reverses hepatic cirrhosis in rats.

There are no definitive reports with respect to upstream regulators of STAP. *In vitro* experiments found that α-SMA can induce increased expression of STAP, but STAP is not regulated by IFN-γ, TGF-β, PDGF/BB, cAMP or cGME[42]. In the initial stages of HSC activation, the transcription factor c-myb is up-regulated. c-myb is a proto-oncogene, and its expression is growth-dependent; it is expressed at low levels in quiescent cells, and its expression is rapidly increased in exponentially dividing cells. c-myb can interact with α-SMA promoter regulatory elements and alter α-SMA expression. α-SMA is a microfilament protein with contractile properties and is widely distributed in muscle cells. It is also the cytoskeletal marker of HSC activation, and its increased expression may up-regulate STAP expression. In the present study, mRNA levels of c-myb, α-SMA and STAP were significantly increased in the untreated cirrhotic group but were much lower in the cell transplantation group. However, there was no significant difference between the normal control and the cell transplantation group. Similar results were observed in α-SMA protein expression levels. These findings suggest that the observed successful treatment of cirrhosis with embryonic hepatocyte transplantation is related to the activity of STAP-related signaling.

ET is the most powerful vasoconstrictor peptide in humans; it is also a damage-inducible factor produced during ischemia and hypoxia[46]. The occurrence and development of many chronic liver diseases, such as hepatic cirrhosis, are related to ET plasma and tissue levels. ET-1 is the most important member of the ET family and is one of the primary inducers of portal hypertension[47]. ET-1 catalyzes the phosphorylation of amino acid residues in many kinases through the G-protein complex-phospholipase C-protein kinase C signaling pathway. ET-1 thereby regulates gene expression and promotes HSC mitosis and the synthesis of collagen and matrix proteins[48]. After ET-1 binds to its receptor, endothelin receptor, it activates voltage-dependent calcium channels, promoting calcium influx and leading to vasoconstriction. HSC is considered one of the main sources of ET-1, which is involved in increased portal vein pressure and plays an important role in the regulation of blood flow in the liver. In the normal liver, ET-1 is primarily expressed in liver sinusoid endothelial cells; upon liver injury, however, ET-1 is primarily expressed in activated HSCs.

Contraction of the portal vein and sinusoid as a result of EF-1-induced HSC activation may be a significant event in cirrhogenesis, resulting in increased portal vein obstruction following liver fibrosis[49]. In the current study, serum and liver ET-1 levels were significantly higher in the untreated cirrhotic group when compared with the normal control and cell transplantation groups. Ultrasound results demonstrated that the untreated cirrhotic rats had a wider portal vein and reduced PVV. Rats in the cell transplantation group showed significant remission, suggesting that treatment of hepatic cirrhosis with embryonic hepatocytes is able to reduce ET-1 expression and modify various parameters of this pathway. The reduction in the portal vein pressure of the cell transplantation-treated cirrhotic group may indicate a remission of the portal hypertension caused by cirrhosis.

Significant therapeutic effects were observed in the treatment of hepatic cirrhosis using embryonic hepatocyte transplantation in rats. Transplanted embryonic hepatocytes significantly reduce c-myb, α-SMA and STAP expression, all of which are involved in HSC activation. This treatment also down-regulates the expression of ET-1, a vasoactive substance.

COMMENTS

Background

Hepatic cirrhosis is a terminal illness that can only be cured by a liver trans-

plant. Stem cells are capable of indefinite self-renewal and are able to generate other cell types *via* differentiation. Stem cell transplantation is the most promising therapy for hepatic cirrhosis. Embryonic hepatocytes are stem cells that have the capacity to proliferate and to differentiate into hepatocytes. These cells have a more promising clinical potential than adult hepatocytes for the treatment of liver injury due to chronic cirrhosis.

Research frontiers

Stem cell transplantation for the treatment of hepatic cirrhosis is far from application. The identification of the most effective stem cells and probe into their mechanisms of action have recently become a popular research topic.

Innovations and breakthroughs

The authors confirm the efficacy of embryonic hepatocyte transplantation for hepatic cirrhosis in rats by demonstrating reduced expression level of stellate activation-associated protein (STAP), α smooth muscle actin and endothelin-1. In addition, they initially revealed the mechanism of this therapy, which may be of significance for the treatment of hepatic cirrhosis.

Applications

Although it is far from clinical application, it is possible that embryonic hepatocyte transplantation will become a curative treatment for hepatic cirrhosis and other severe liver diseases. This study has laid a foundation for further experimental researches and clinical studies, and provided useful data for the treatment of cirrhosis.

Terminology

STAP is a protein expressed in hepatic stellate cells. Hepatocyte damage leads to increased STAP expression; therefore, STAP can be used as an indicator of cirrhosis in the rat cirrhosis model.

Peer review

This is a well-performed and comprehensive study. The authors show the effectiveness of embryonic stem cells to treat cirrhosis. Furthermore, the mechanism by which this protective effect is mediated has been elucidated.

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S- Editor Sun H **L- Editor** Ma JY **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i4.323

World J Gastroenterol 2012 January 28; 18(4): 323-330
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Liver cold preservation induce lung surfactant changes and acute lung injury in rat liver transplantation

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Supported by Grants from the National Natural Science Foundation of China, No. 30200269; Science and technology projects in Shannxi Province, No. 2006k12-G3 (6); Science and technology projects in Xi'an city, No. SF1025 (3)
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Received: April 2, 2011 Revised: May 28, 2011
Accepted: June 5, 2011
Published online: January 28, 2012

Abstract

AIM: To investigate the relationship between donor liver cold preservation, lung surfactant (LS) changes and acute lung injury (ALI) after liver transplantation.

METHODS: Liver transplantation models were established using male Wistar rats. Donor livers were preserved in University of Wisconsin solution at 4 ℃ for different lengths of time. The effect of ammonium pyrrolidinedithiocarbamate (PDTC) on ALI was also detected. All samples were harvested after 3 h reperfusion. The severity of ALI was evaluated by lung weight/body weight ratio, lung histopathological score, serum nitric oxide (NO) and endothelin (ET)-1 levels, lung tumor necrosis factor (TNF)-α and interleukin (IL)-1β levels. Lung surfactants (LSs) were determined by micellar electrokinetic capillary chromatography.

RESULTS: With extended donor liver cold preservation time (CPT), lung histopathological scores, serum ET-1 levels, lung weight/body weight ratio and the level of TNF-α and IL-1β in lung were increased significantly in the 180-min group compared with the sham group (3.16 ± 0.28 *vs* 1.12 ± 0.21, $P < 0.001$; 343.59 ± 53.97 *vs* 141.53 ± 48.48, $P < 0.001$; 0.00687 ± 0.00037 *vs* 0.00557 ± 0.00056, $P < 0.001$; 17.5 ± 3.0 *vs* 1.3 ± 0.3, $P < 0.001$; 10.8 ± 2.3 *vs* 1.8 ± 0.4, $P < 0.001$), but serum NO levels decreased remarkably (74.67 ± 10.01 *vs* 24.97 ± 3.18, $P < 0.001$). The expression of lung phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) increased when CPT was < 120 min, and decreased when CPT was > 180 min (PC: 1318.89 ± 54.79 *vs* 1011.18 ± 59.99, $P < 0.001$; PE: 1504.45 ± 119.96 *vs* 1340.80 ± 76.39, $P = 0.0019$; PI: 201.23 ± 34.82 *vs* 185.88 ± 17.04, $P = 0.2265$; PS: 300.43 ± 32.95 *vs* 286.55 ± 55.55, $P = 0.5054$). All these ALI-associated indexes could be partially reversed by PDTC treatment.

CONCLUSION: Prolonged CPT could induce or inhibit the expression of LSs at the compensation or decompensation stage, and some antioxidants (e.g., PDTC) may reverse the pathological process partially.

Key words: Liver transplantation; Acute lung injury; Organ preservation; Lung surfactants

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Jiang A, Liu C, Liu F, Song YL, Li QY, Yu L, Lv Y. Liver cold preservation induce lung surfactant changes and acute lung injury in rat liver transplantation. *World J Gastroenterol* 2012; 18(4): 323-330 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i4/323.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i4.323

INTRODUCTION

Acute lung injury (ALI) is a common complication of liver transplantation, which may develop into its severest form, acute respiratory distress syndrome (ARDS), and play a pivotal role in the death of patients post-transplantation[1]. It has been reported that the incidence of lung complications after liver transplantation is 60%-80%; the morbidity rate of ARDS is 4.5%-18%, and the case-fatality rate is 50%-70%[2,3]. Thus, prevention of the complications is important in reducing in-hospital mortality after liver transplantation, and investigation of the etiological factors of liver-transplantation-related ALI is therefore of great importance.

Lung surfactant (LS) is synthesized primarily by alveolar type Ⅱ cells and is stored in lamellar bodies. In response to some stimuli, LS is secreted to supply the surface-active monolayer. LS is a complex mix of phospholipids, neutral lipids and proteins that lines the gas/liquid interface. LS is essential for normal breathing and the severity of ALI correlates with surfactant dysfunction and abnormalities in surfactant composition[4].

In our clinical practice, we have observed that ALI often occurs in the patients transplanted with long-persevered donor livers. Therefore, we raised a hypothesis that prolonged donor liver cold preservation time (CPT) might induce lung damage. Therefore, we conducted the present study to investigate the change of LS in liver-transplantation-induced ALI along with changes in donor liver CPT, aiming at uncovering the association between prolonged CPT and ALI.

MATERIALS AND METHODS

Experimental design

Male Wistar rats (n = 110) were used as donors and recipients to establish orthotopic liver transplantation models. The rats were randomly divided into six groups (n = 10 in each group, n = 10 for donor in liver transplantation groups). Donor livers from each group were preserved in 4 ℃ University of Wisconsin solution respectively for 0 min (sham operation), 45 min, 90 min, 120 min, 180 min, and 180 min plus intravenous injection of ammonium pyrrolidinedithiocarbamate (PDTC) at a dosage of 100 mg/kg immediately after the onset of liver reperfusion. All recipients were sacrificed at 180 min after liver transplantation.

Animal preparation and sample collection

A total of 110 adult male Wistar rats (272 g ± 31 g; Tongji Medical Center, Central China University of Science and Technology) were used as donors and recipients. Prior to the experiment, the rats were fasted for 12 h and allowed free access to water. Liver harvesting and liver transplantation were performed under anesthesia with intraperitoneal injection of ketamine hydrochloride (80 mg/kg) and by using the method described by Kamada *et al*[5] and Hori *et al*[6]. Protocols for animal care and experimental management were approved by the ethics committee.

Blood samples were collected from the suprahepatic vena cava to determine the serum levels of nitric oxide (NO) and endothelin (ET)-1. Body weight and lung weight were measured and part of left lower lobe of lung was fixed with polyoxymethylene for histological examination. Lung tissue (200 mg) was harvested for the extraction of lung phospholipids.

Lung histopathological assessment

Left lower lobe of lung was harvested, fixed in 10% formalin, and embedded in paraffin. Tissue sections (4 μm) were stained with hematoxylin and eosin for light microscopy, and evaluated blindly by an independent consultant pathologist. Damage to the lung tissue was graded by the pathologist on a scale of 1 (no injury) to 4 (worst), as described previously[7].

Serum ET-1 measurement

Serum ET-1 was quantified with a radioimmunoassay kit (Radioimmunity Institute of PLA General Hospital, China) by Wizard gamma counter (PerkinElmer, United States) according to the manufacturer's recommendations.

Serum NO analysis

Serum NO was measured by an NO assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. The absorbance of the reaction mixture was read at 550 nm in a BioTek ELX 808 microplate reader (Bio-Tek Instruments, Winooski, Vermont, United States).

Lung weight/body weight ratio

All recipients' body weight and lung weight were measured by electronic balance.

Measurement of tumor necrosis factor (TNF)-α and interleukin (IL)-1β in lung by immunohistochemistry

Histological sections (4 μm) of lung were cut on a rotary microtome and stained to detect intragraft expression of TNF-α and IL-1β. Paraffin sections were spread on a slide, and rabbit anti-rat TNF-α, IL-1β polyclone antibodies (diluted 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, United States) were used to detect the intragraft expression of TNF-α, IL-1β respectively. Biotin-labeled goat anti-rabbit secondary antibody (Santa Cruz Biotechnology), horseradish-peroxidase-labeled anti-

biotin, and 3,3-diaminobenzidine were used to visualize positive expression. We counted 10 randomly chosen fields per section using a light microscope at high power (400 × magnification; Leica Q550CW, Germany) and the results were expressed as absorbance units (*A*).

Lung phospholipid extraction

Fresh lung tissue (200 mg) was homogenized with 2 mL PBS on ice, and 1 mL tissue homogenate was dissolved in 2 mL chloroform/methanol (2:1, v/v). After thermal agitation and 30 min standing at room temperature, the solution was centrifuged at 2500 rpm at 4 ℃ for 5 min. The supernatant was transferred to a freezing tube. The above procedures were repeated, and then all the supernatants obtained were evaporated and frozen at -80 ℃.

Lung phospholipid analysis

Chemicals and reagents used were of analytical reagent grade. Phospholipid standard solutions (Sigma, United States) included: phosphatidylcholine (PC), phosphatidylethanolaraine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS). Micellar electrokinetic capillary chromatography (MECC) was performed in an Agilent G1600AX Capillary Electrophoresis (Agilent, Santa Clara, CA, United States). The electrophoresis buffer for MECC was composed of 35 mmol/L sodium deoxycholate, 6 mmol/L disodium tetraborate, 10 mmol/L disodium hydrogen phosphate, and 30% 1-propanol. The buffer pH value was adjusted to 8.5 with 1 mol/L HCl. MECC was conducted at a temperature of 40 ℃ and a voltage of 18 kV. The total length of capillary was 570 mm and the detection region was 500 mm away from the injection end. Vacuura injection was performed at 50 mbr for 5 s, and ultraviolet detection was performed at 200 nm[8].

Statistical analysis

All data were expressed as mean ± SEM, and analyzed with one-way ANOVA and Student's *t* test. All the statistical analyses were carried out using SPSS version 13.0. $P < 0.05$ was considered to represent statistical significance.

RESULTS

General condition of the animals

The duration of donor operation was 45.6 ± 13.8 min, the time for donor liver preparation was 20.3 ± 6.7 min, and warm ischemia was avoided. The receptor operation took 26.7 ± 5.5 min, and anhepatic phase lasted for 16.1 ± 2.5 min. No significant difference was seen in portal clamping time among groups. No animal died before sample harvest.

Lung histopathological investigation

The representative lung injuries in different groups are shown in Figure 1. Lung microscopic examination revealed alveolar, perivascular and interstitial edema, neutrophil infiltration, atelectasis, disruption of alveolar and bronchiolar epithelial cells, and local hemorrhage in severe cases with prolonged CPT. Consistent with these histopathological observations, the lung injury scores in the 180-min group were significantly higher than those in the sham group and 45-min group (3.16 ± 0.28 *vs* 1.12 ± 0.21, $P < 0.001$; 3.16 ± 0.28 *vs* 2.05 ± 0.24, $P < 0.001$), and were also higher than those in PDTC group although the difference was not significant (3.16 ± 0.28 *vs* 2.95 ± 0.16, $P = 0.054$).

Serum NO and ET-1 assaying

The experiment results showed that with donor liver CPT prolonged, the serum NO level in the 180-min group were significantly lower than those in the sham group and 45-min group (24.97 ± 3.18 *vs* 74.67 ± 10.01, $P < 0.001$; 24.97 ± 3.18 *vs* 69.05 ± 2.74, $P < 0.001$) and the decline could not be reversed by PDTC (24.97 ± 3.18 *vs* 7.67 ± 3.79, $P < 0.001$; Figure 2A).

With longer liver CPT, serum ET-1 levels increased significantly in the 180-min group compared with the sham group (343.59 ± 53.97 *vs* 141.53 ± 48.48, $P < 0.001$). There was a significant difference between each of the operation groups and the control group ($P < 0.05$). The expression of ET-1 was inhibited significantly by PDTC (343.59 ± 53.97 *vs* 217.80 ± 11.32, $P < 0.001$; Figure 2B).

Lung weight/body weight ratio

Lung weight/body weight ratio increased with prolonged CPT. It peaked in the 180-min group compared with the sham group (0.00687 ± 0.00037 *vs* 0.00557 ± 0.00056, $P < 0.001$), but greatly decreased after PDTC was used 0.00687 ± 0.00037 *vs* 0.00576 ± 0.00016, $P = 0.001$; Figure 2C).

Lung TNF-α and IL-1β measurement

Figure 3 shows a significant increase in TNF-α and IL-1β in lung with prolonged CPT, which peaked in the 180-min group compared with the sham group (TNF-α: 17.5 ± 3.0 *vs* 1.3 ± 0.3, $P < 0.001$; IL-1β: 10.8 ± 2.3 *vs* 1.8 ± 0.4, $P < 0.001$). After administration of PDTC, TNF-α and IL-1β production was significantly attenuated (TNF-α: 17.5 ± 3.0 *vs* 9.8 ± 2.3, $P < 0.001$; IL-1β: 10.8 ± 2.3 *vs* 7.1 ± 2.0, $P = 0.0012$). We suggest that the prolonged CPT induced some inflammatory factors expressed in lung.

Lung phospholipid composition determination

The area under curve (AUC) represents the composition of lung phospholipids (Figures 4 and 5). With the extension of CPT, PC levels increased significantly, reaching a maximum in the 120-min group compared with the sham group (1318.89 ± 54.79 *vs* 406.79 ± 56.49, $P < 0.001$), and then declined in notably the 180-min group (1318.89 ± 54.79 *vs* 1011.18 ± 59.99, $P < 0.001$). No significant difference was observed between the 180-min group and PDTC group (1011.18 ± 59.99 *vs* 1062.58 ± 78.49, $P = 0.1173$).

PE changed similarly with PC, reaching a maximum in the 120-min group compared with the sham group

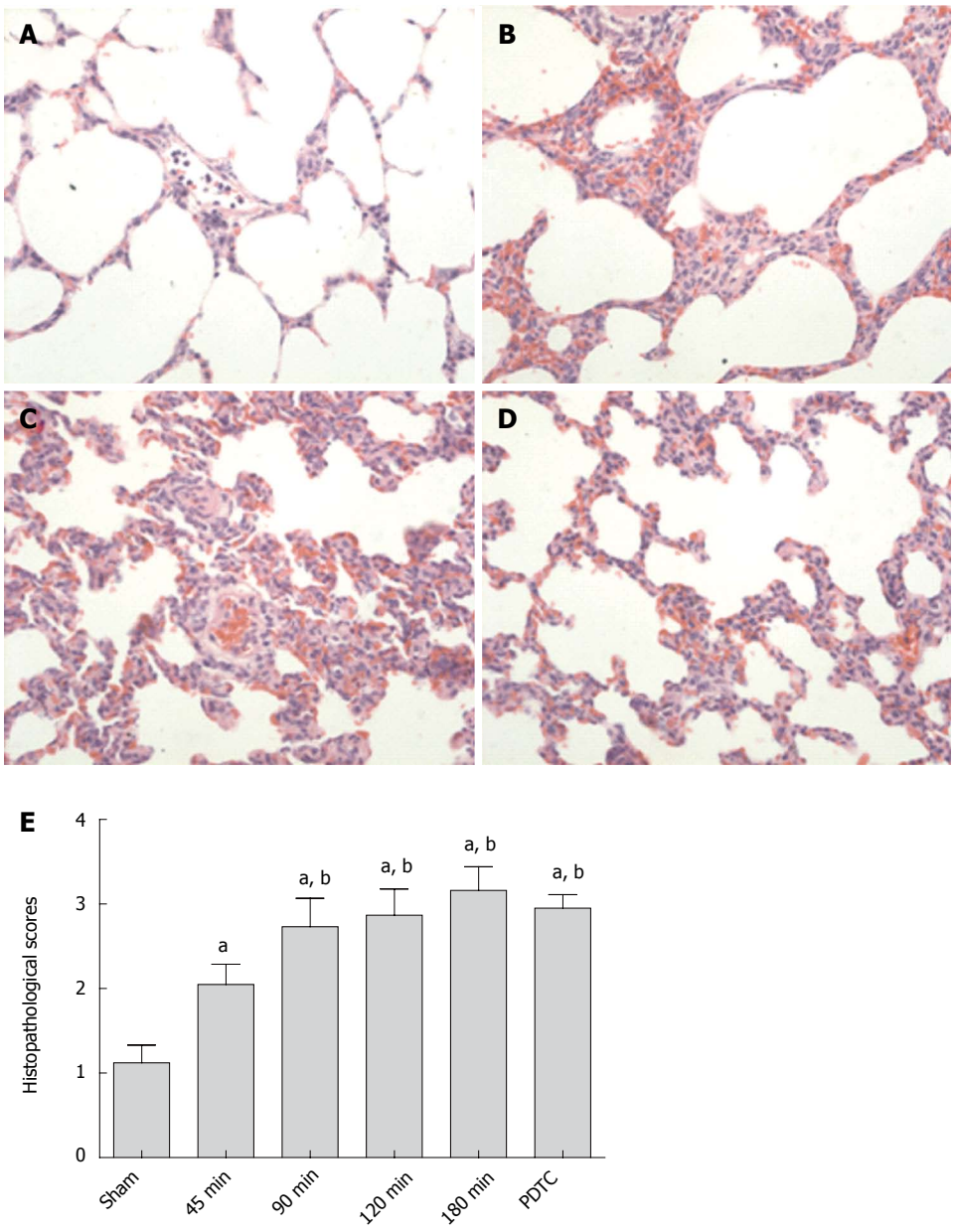

Figure 1 Representative lung tissue sections and histopathological scores. A: Sham group; B: 90-min group; C: 180-min group; D: PDTC group. Alveolar, perivascular and interstitial edema, neutrophil infiltration, atelectasis, disruption of alveolar and bronchiolar epithelial cells, and local hemorrhage aggravated with prolonged CPT; E: Lung histopathological scores in different groups. The scores in the CPT groups were significantly higher than that in the sham group, but lower than in the PDTC group. $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* 45-min group. PDTC: Pyrrolidinedithiocarbamate; CPT: Cold preservation time.

(1504.45 ± 119.96 *vs* 430.38 ± 57.91, $P < 0.001$), and decreased significantly in the 180-min group (1504.45 ± 119.96 *vs* 1340.80 ± 76.39, $P = 0.0019$). PE in the PDTC group decreased significantly compared with the 180-min group (1340.80 ± 76.39 *vs* 1222.18 ± 100.48, $P = 0.0082$).

PI kept rising gradually, and reached a peak in the 120-min group compared with the sham group (201.23 ± 34.82 *vs* 55.12 ± 10.14, $P < 0.001$), but no significant difference was found between the 120-min, 180-min and PDTC groups (201.23 ± 34.82 *vs* 185.88 ± 17.04, $P = 0.2265$; 185.88 ± 17.04 *vs* 190.10 ± 41.75, $P = 0.7707$).

PS reached a maximum in the 120-min group compared with the sham group (300.43 ± 32.95 *vs* 51.29 ± 13.89, $P < 0.001$), and decreased in the 180-min group (300.43 ± 32.95 *vs* 286.55 ± 55.55, $P = 0.5054$), but dropped significantly in the PDTC group (286.55 ± 55.55 *vs* 132.60 ± 40.27, $P < 0.001$).

DISCUSSION

The incidence of ALI post-liver transplantation has been reported to be 60%-80%[3]. Despite intense research and diverse therapeutic trials, there is no effective prevention or treatment for ALI at present[1]. Recent studies have shown that the pathogenesis of ALI involves disorders of oxidants/antioxidants, inflammation/anti-inflammation, and upregulation of inflammatory factors[9,10]. It has been suggested that the antioxidant PDTC can inhibit some oxidants and inflammatory damage[1].

In our study, levels of serum NO and ET-1 were used

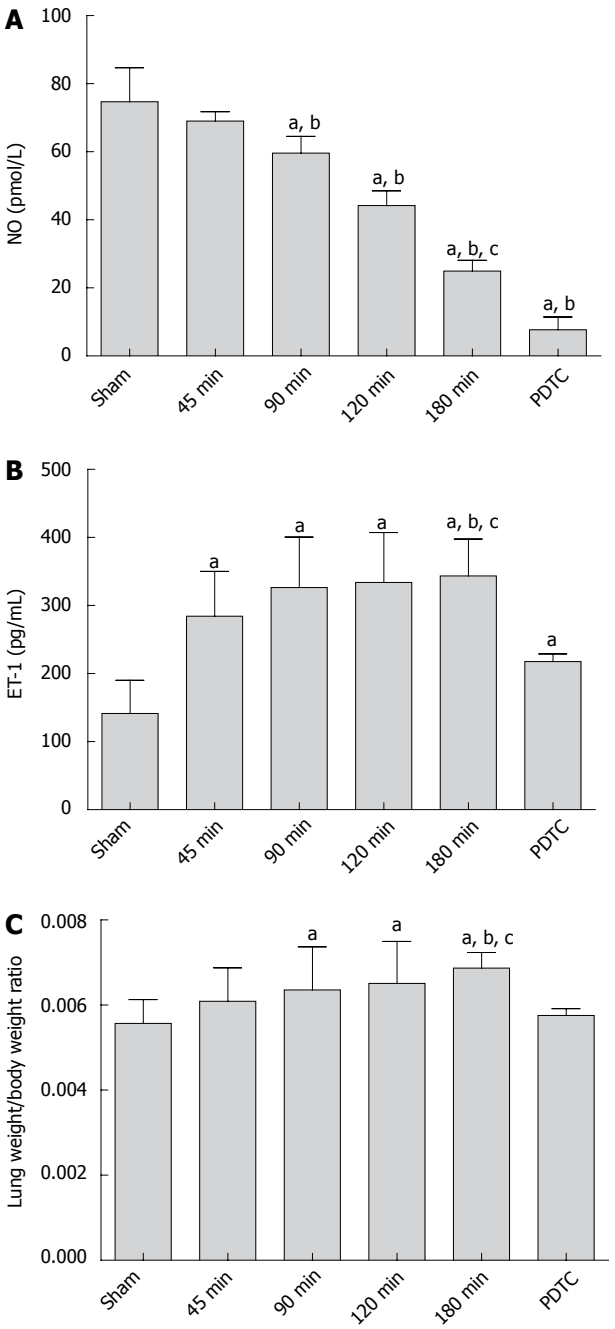

Figure 2 Serum NO, ET-1 level and lung weight/body weight ratio. A: NO levels in different groups. With CPT prolonged, NO level decreased significantly, and became even lower with PDTC injection post-reperfusion; B: ET-1 level in different groups. With CPT prolonged, ET-1 level increased significantly compared with the sham group. The PDTC group had a significantly lower ET-1 level than 180-min group; C: Lung weight/body weight ratios in different groups. The ratios increased compared with the sham group. Significant difference was observed between the 180-min group and PDTC group. $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* 45-min group; $^{c}P < 0.05$ *vs* PDTC group. PDTC: Pyrrolidinedithiocarbamate; CPT: Cold preservation time; NO: Nitric oxide; ET: Endothelin.

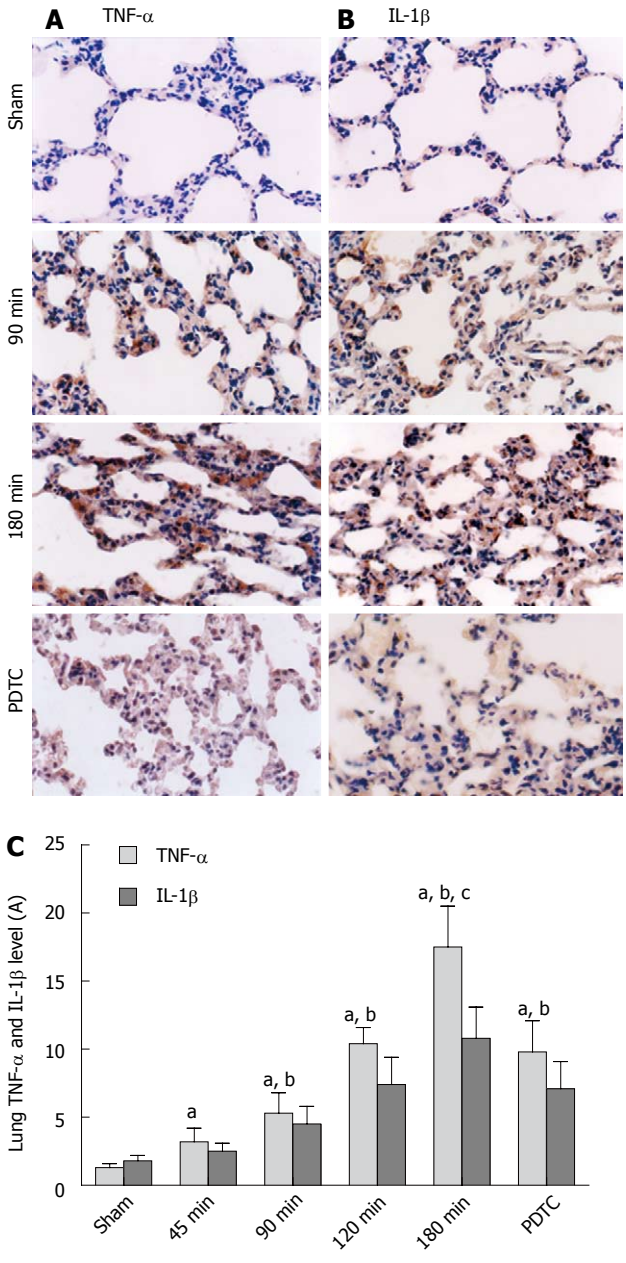

Figure 3 Immunohistochemical sections of tumor necrosis factor-α and interleukin-1β in lung (× 400). A: Immunohistochemistry sections of TNF-α with different CPT; B: Immunohistochemical staining of IL-1β with different CPT; C: Analysis of TNF-α and IL-1β levels. The results were expressed as absorbance unit (A). $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* 45-min group; $^{c}P < 0.05$ *vs* PDTC group. PDTC: Pyrrolidinedithiocarbamate; CPT: Cold preservation time; TNF: Tumor necrosis factor; IL: Interleukin.

to evaluate the severity of ALI. NO and ET-1 are secreted by endothelial cells and are two important vasoactive substances that regulate mini-circulation. NO can expand blood vessels, prevent platelet aggregation, and therefore improve microcirculation. After liver transplantation, the implanted graft liberates high amounts of arginase and causes L-arginine deficiency. L-arginine is the substrate of NO synthesis reaction. Its depletion influences NO synthesis after liver transplantation[11]. This might be the reason why serum NO level drops after liver transplantation. An increase in NO level by L-arginine could attenuate LS depletion, and therefore, ameliorate postoperative pulmonary dysfunction[12]. PDTC could inhibits cytokine-induced NO production[13], as our study showed.

ET-1, as the most powerful vasoactive substance, can cause microcirculation disturbance and induce pulmonary injury[14]. The slowdown of liver blood flow can concen-

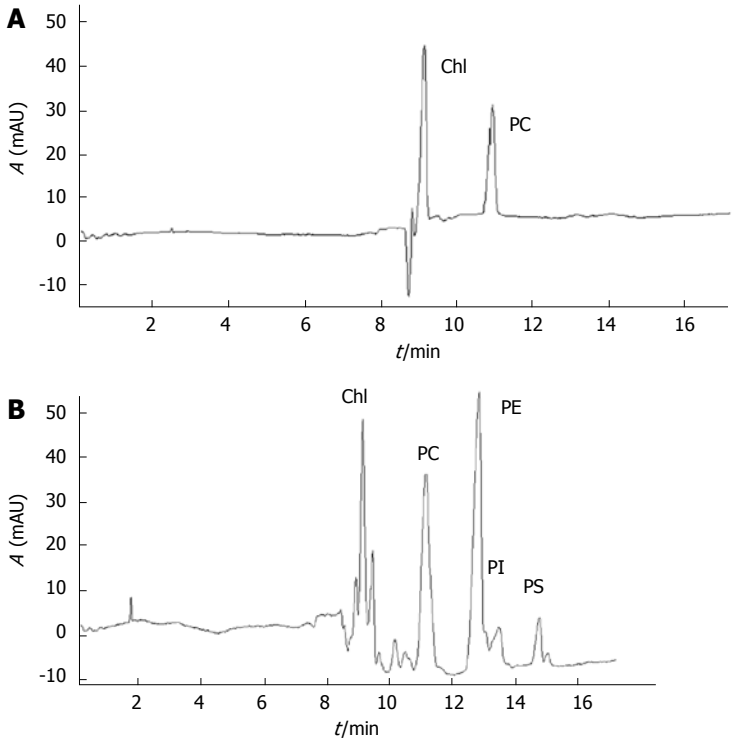

Figure 4 Pulmonary phospholipids separated by micellar electrokinetic capillary chromatography. A: Standard PC electropherogram; B: Pulmonary phospholipid extracts electropherogram. Chl: Chloroform. AUC of different chromatographic peaks represent the relative quantities of different pulmonary phospholipid components. PC: Phosphatidylcholine; AUC: Area under curve; PE: Phosphatidylethanolamine; PI: Phosphatidylinositol; PS: Phosphatidylserine.

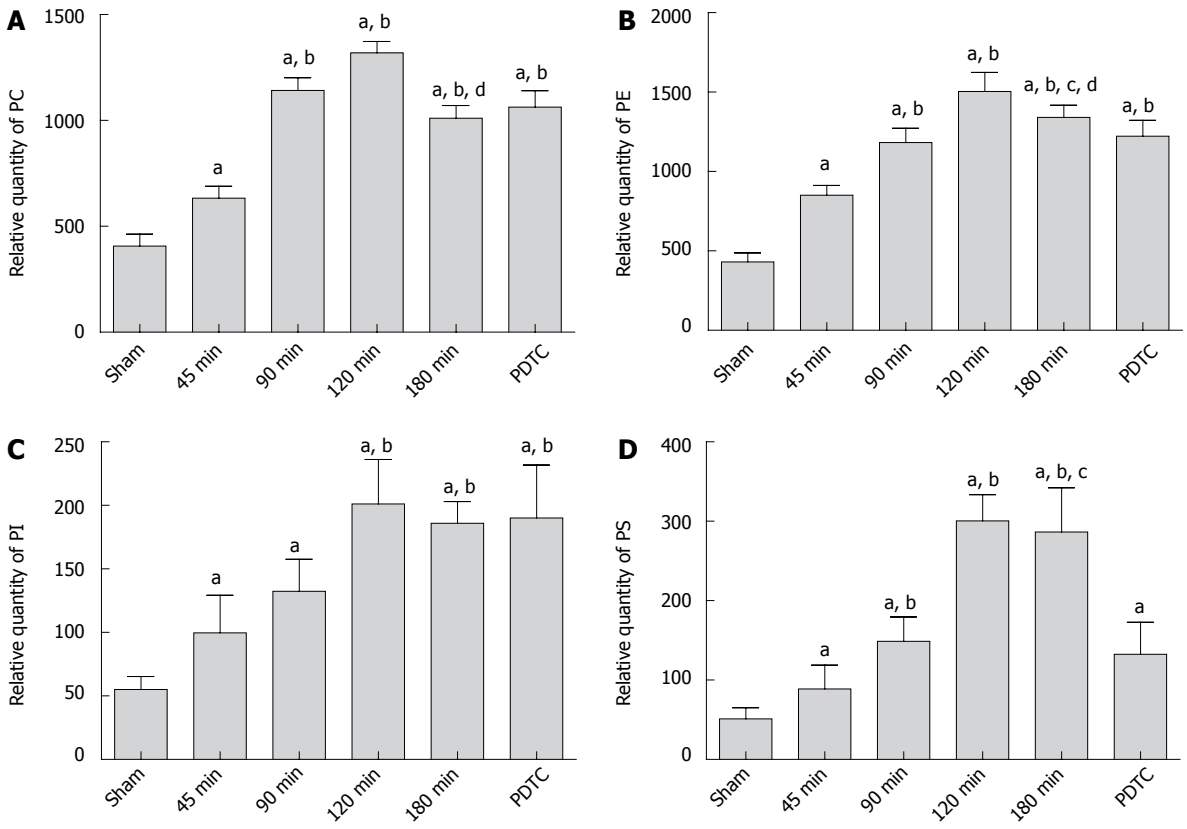

Figure 5 Relative quantities of pulmonary phospholipids. A: Relative quantity of PC; B: Relative quantity of PE; C: Relative quantity of PI; D: Relative quantity of PS. With CPT prolonged, the levels of pulmonary phospholipids increased significantly, reaching a peak in the 120-min group, and then declined in the 180-min group. The use of PDTC could inhibit the expression of PE and PS, but could not observe significant effect in PC and PI. [a]$P < 0.05$ *vs* sham group; [b]$P < 0.05$ *vs* 45-min group; [c]$P < 0.05$ *vs* PDTC group; [d]$P < 0.05$ *vs* 120-min group. PDTC: Pyrrolidinedithiocarbamate; CPT: Cold preservation time; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PI: Phosphatidylinositol; PS: Phosphatidylserine.

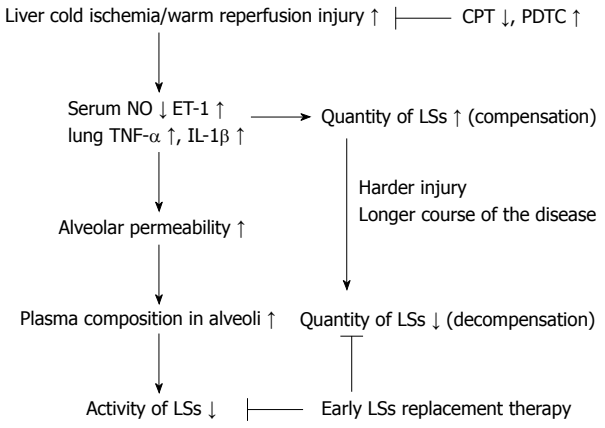

Figure 6 A schematic diagram for the mechanism of lung surfactants in liver-transplantation-related acute lung injury. With prolonged CPT of donor liver, the cold ischemia/warm reperfusion injury increased, inducing an imbalance of serum NO/ET-1 levels and production of TNF-α and IL-1β in lungs. Consequently, alveolar permeability increased, and the plasma composition in the alveoli inactivated the lung surfactants (LSs). In the early stage of ALI, LSs increased in compensation, but in the decompensation stage, the level of LSs decreased, and ALI was aggravated. Shorter CPT or using PDTC may reduce ALI, and surfactant replacement therapy at an earlier stage may be useful in the treatment of ALI. PDTC: Pyrrolidinedithiocarbamate; CPT: Cold preservation time; TNF: Tumor necrosis factor; IL: Interleukin; ALI: Acute lung injury; NO: Nitric oxide; ET: Endothelin.

trate ET-1 in the serum and liver. We observed that the balance between NO and ET-1 was disrupted after liver transplantation.

Natural lung surfactants (LSs) are a mixture of phospholipids and specific proteins, and are produced by type Ⅱ alveolar epithelial cells, stored in Golgi bodies and secreted into the alveolar space. They are important in maintaining alveolar expansion during breathing and physiological gas exchange. The pathological condition of ALI is often characterized with metabolic anomalies of LSs and disrupted lung function[15].

We found that with prolonged CPT of donor liver, that cold ischemia/warm reperfusion injury increased. The imbalanced levels of NO and ET-1 induced capillary endothelial dysfunction. Together with TNF-α and IL-1β produced in the lungs, the permeability of the air-blood barrier increases, just as shown by lung weight/body weight ratio and lung histopathological scores. Then, a variety of plasma compositions, such as serum, serum proteins, hemoglobin and proteases migrate into the alveolar space and inactivate LSs[16]. At the same time, ET-1 and some inflammatory factors, such as TNF-α and IL-1β, stimulate the production of cAMP, which can induce type Ⅱ alveolar epithelial cells to secrete LSs[17]. Because of the anti-inflammatory properties of LSs[18], an increase in their concentration in the lungs indicates a strong compensatory ability in the defense against ALI[19]. Our study showed that, when CPT was < 2 h, the expression of LSs increased with longer CPT, however, when CPT was 3 h, more severe injury of type Ⅱ alveolar epithelial cells inhibited secretion of LSs. This agrees with the results of Shu *et al*[20].

In contrast, the change in LSs after ALI is time-dependent, with early increases and late decreases. Therefore, surfactant replacement therapy should be administered at an early stage of ALI, the compensation stage, to alleviate injury[21]. If applied in the decompensation stage, the therapy may not provide satisfactory effects. This may be the reason why surfactant therapy has only limited success in ARDS[22,23]. Furthermore, as an antioxidant, PDTC can relieve cold ischemia/warm reperfusion injury of donor liver, and partly reverse the disturbance. Thus, shortening CPT or using PDTC may be useful for alleviation of ALI post-liver transplantation. A proposed scheme of the mechanism is given in Figure 6, summarizing how LS affects liver-transplantation-associated ALI.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Ke-Wei Meng and Jian Zhang for assistance with the experiments, and Qi Chen for polishing the manuscript.

COMMENTS

Background

Acute lung injury (ALI) and its severe subset acute respiratory distress syndrome (ARDS) have relatively high morbidity and mortality in post-liver transplantation patients. The pathogenesis and prevention of liver-transplantation-related ALI are not very clear. Lung surfactants (LSs) are a complicated mixture of approximately 90% lipids and 10% proteins. They are produced by type Ⅱ alveolar epithelial cells and secreted into the alveolar space to maintain normal respiratory mechanics by reducing alveolar surface tension to near-zero values.

Research frontiers

Recent studies have shown that the pathogenesis of ALI involves disorders of oxidants/antioxidants, inflammation/anti-inflammation, and upregulation of inflammatory factors. Many antioxidants can inhibit some oxidants and inflammatory damage. The severity of ALI correlates with abnormalities in surfactant composition. Supplementing exogenous surfactant to newborns suffering from respiratory distress syndrome has a satisfactory therapeutic effect. Surfactant therapy has also been used in ALI/ARDS, but with only limited success.

Innovations and breakthroughs

We observed that ALI often occurred in patients transplanted with long-preserved donor livers. Therefore, we raised a hypothesis that cold preservation/warm reperfusion injury of donor liver may induce ALI after liver transplantation. To validate the hypothesis, we transplanted donor liver after different cold preservation times in rats. Prolonged cold preservation time had a positive correlation with the severity of ALI, and could induce or inhibit the expression of LSs in different time phases, and some antioxidants (e.g., ammonium pyrrolidinedithiocarbamate) may reverse the pathological process partially.

Applications

Shorter cold preservation time or some antioxidants may reduce liver-transplantation-related ALI, and surfactant replacement therapy should be useful in the early stage of ALI to achieve better results.

Terminology

Cold ischemia/warm reperfusion injury, different from warm ischemia/reperfusion injury, is a characteristic injury in organ transplantation. It depends on the length of cold storage. Liver is one of the organs that are sensitive to ischemia/reperfusion injury. Emerging evidence suggests that the early stage of the injury can be regarded as part of the immune response to injury stress and sinusoidal endothelial cells are the targets of cold ischemia, whereas hepatocytes appear to be relatively unscathed.

Peer review

This study suggests that liver transplantation in rats and the length of cold ischemia induce surfactant changes and ALI. The study was well designed and conducted.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i4.331

World J Gastroenterol 2012 January 28; 18(4): 331-339
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Nitroglycerine effects on portal vein mechanics and oxidative stress in portal hypertension

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Supported by The Grant from the Ministry of Science and Technical Development of the Republic of Serbia, No. 175043
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Received: May 28, 2011 Revised: September 2, 2011
Accepted: November 9, 2011
Published online: January 28, 2012

Abstract

AIM: To examine the effects of nitroglycerine on portal vein haemodynamics and oxidative stress in patients with portal hypertension.

METHODS: Thirty healthy controls and 39 patients with clinically verified portal hypertension and increased vascular resistance participated in the study. Liver diameters, portal diameters and portal flow velocities were recorded using color flow imaging/pulsed Doppler detection. Cross-section area, portal flow and index of vascular resistance were calculated. In collected blood samples, superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), index of lipid peroxidation (measured as TBARS) and nitric oxide (NO) as a marker of endothelial response (measured as nitrite-NO_2^-) were determined. Time-dependent analysis was performed at basal state and in 10th and 15th min after nitroglycerine (sublingual 0.5 mg) administration.

RESULTS: Oxidative stress parameters changed significantly during the study. H_2O_2 decreased at the end of study, probably *via* O_2^- mediated disassembling in Haber Weiss and Fenton reaction; O_2^- increased significantly probably due to increased diameter and tension and decreased shear rate level. Consequently O_2^- and H_2O_2 degradation products, like hydroxyl radical, initiated lipid peroxidation. Increased blood flow was to some extent lower in patients than in controls due to double paradoxes, flow velocity decreased, shear rate decreased significantly indicating non Newtonian characteristics of portal blood flow.

CONCLUSION: This pilot study could be a starting point for further investigation and possible implementation of some antioxidants in the treatment of portal hypertension.

Key words: Portal hypertension; Endothelium; Nitroglycerine; Oxidative stress; Portal vein haemodynamics

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Vujanac A, Jakovljevic V, Djordjevic D, Zivkovic V, Stojkovic M, Celikovic D, Andjelkovic N, Jurisic Skevin A, Djuric D. Nitroglycerine effects on portal vein mechanics and oxidative stress in portal hypertension. *World J Gastroenterol* 2012; 18(4): 331-339 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i4/331.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i4.331

INTRODUCTION

Oxidative stress is a condition in which the delicate balance that exists between prooxidant (free radicals) production and their subsequent amelioration *via* the antioxidant defense system (ADS) becomes skewed in favor of free radical expression[1]. An increasing body of evidence suggests that oxidative stress is involved in the pathogenesis of many cardiovascular diseases, including hypertension, hypercholesterolaemia, atherosclerosis, diabetes and heart failure[2-5]. The existence of an interference of increased oxidative stress with the vasodilatating properties of veins is now a well known fact. The term endothelium dysfunction is usually used to refer to an impairment of endothelium-dependent vasorelaxation caused by a loss of nitric oxide (NO) bioactivity in the vessel wall, which is, in part, induced by accelerated NO degradation by reactive oxygen species (ROS)[2-8]. Endothelial function is most commonly assessed as a vasodilatatory response to various pharmacological agonists or mechanical stimuli that induce endothelium-dependent or endothelium-independent vasodilatation[9]. One such agonist is nitroglycerine (GTN)[9]. The aim of our study was to determine how sublingual administration of GTN might be used for the evaluation of portal endothelium-independent vasodilatation through estimating parameters of portal vascular mechanics and oxidative stress in patients suffering from portal hypertension. We hypothesized that the endothelium independent vasodilatation of the portal vein induced by sublingual GTN administration would lead to increased oxidative stress and significant changes in portal haemodynamics. The precise mechanism of increased oxidative stress is to be determined; we favor the importance of Haber-Weiss and Fenton's mechanism. Also, increased blood flow through the portal vein and reduced shear rate may be involved in the previously mentioned process of enhanced oxidative stress. Indeed, portal blood flow is known to be affected by factors and circumstances of chronic liver diseases, and its changes evolved from basic haemodynamic laws[10-15].

MATERIALS AND METHODS

Patients

This research was performed with a group of 39 patients with chronic liver disease and 30 healthy controls. Patients with chronic liver disease were recruited from the Department of Gastroenterology, Internal Clinic, Clinical Centre Kragujevac, while controls were recruited from the medical staff. The only obligatory inclusion criterion for the participants of the experimental group referred to the existence of previously clinically confirmed serious chronic liver disease (by ultrasonographical assessments, patient's anamnesis and biochemical parameters). Thirty eight patients had previously confirmed hepatic cirrhosis and one patient was recruited with clinical diagnosis of Hepatitis B. Patients were defined as preascitic if they had never been diagnosed with ascites according to clinical and ultrasonographical examinations. None of the subjects took any medication known to affect vascular tonus or blood flow. Written informed consent was obtained from all patients and the study protocol was approved by the local Ethics Committee prior to the onset of the study. The investigation was conducted in accordance with the principles outlined in the Declaration of Helsinki (Last updated in 2005) and principles of Good Clinical Practice (GCP).

Protocol

The examinations were performed in a quiet, air-conditioned, temperature controlled room (22-24 ℃). The antecubital vein was cannulated by using a 19-gauge polyethylene catheter for taking blood samples. Blood samples were taken at rest and in the 10th and 15th min after endothelium-independent vasodilatation induced by sublingual GTN administration (0.3 mg). Parameters of portal vascular mechanics were recorded in the 10th and 15th min after endothelium-independent vasodilatation by using a Doppler 2D machine. All ultrasonographical measurements were performed by 3 independent physicians and mean value was used for the final calculations.

Biochemical assays

Blood samples were taken from the antecubital veins into a Vacutainer test tube containing sodium citrate anticoagulant. Blood was centrifuged to separate plasma and red blood cells (RBCs). Biochemical parameters were measured spectrophotometrically.

Nitrite determination

Nitric oxide was assessed as nitrite and quantified by a spectrophotometric method using Griess reagent. 0.5 mL of perfusate was precipitated with 200 μL of 30% sulfosalicylic acid, vortexed for 30 min and centrifuged at 3000 × *g*. Equal volumes of the extracted plasma and Griess's reagent, containing 1% sulfanilamide in 5% phosphoric acid/0.1% napthalene ethylenediamine-dihydrochloride was added and incubated for 10 min in the dark and read at 543 nmol/L. The nitrite levels were calculated by using sodium nitrite as a standard[16].

Superoxide determination

The level of superoxide anion radical (O_2) was measured using Nitro Blue Tetrazolium (NBT) reaction in TRIS-buffer with plasma and read at 530 nm. Bidistilled water

was used as a blank probe[17].

Hydrogen peroxide determination

The level of hydrogen peroxide (H_2O_2) was measured using Phenol Red reaction in TRIS-buffer with plasma and read at 230 nm. Bidistilled water was used as a blank probe[18].

Index of lipid peroxidation (thiobarbituric acid reactive substances)

The degree of lipid peroxidation in coronary venous effluent was estimated by measuring of thiobarbituric acid reactive substances (TBARS) using 1% thiobarbituric acid (TBA) in 0.05 NaOH incubated with plasma TCA extracts (using 28% Trichloracetic acid-TCA) at 100 ℃ for 15 min and read at 530 nm. Bidistilled water solution was used as a blank probe[19].

Calculations

The following haemodynamic and biomechanical parameters were calculated: (1) Portal flow-blood flow rate (l/min) through portal vein; (2) Shear rate-the velocity gradient between the moving planes, $\Delta V/\Delta X$ (s^{-1}); (3) Portal cross sectional area-area normal to flow direction (cm^2); (4) Resistance index-relative maximal velocity gradient; (5) Inlet length-point where a constant flow regime is established (cm); (6) Expected (ideal) portal vein flow ratio-ideal flow ratio with regard to Poiseuille's equation; (7) Pressure ratio-estimated pressure changes derived from Poiseuille`s equation; and (8) Portal vein flow ratio-experimentally obtained value quotient flow.

The portal flow (Q) was calculated by using equation 1.1a, where D is portal diameter and V_{mean} is average blood flow velocity:

$$Q = \frac{D^2\pi}{4} V_{mean} \qquad (1.1a)$$

It is of interest to emphasize that portal blood flow calculated by equation 1.1a has to be in concordance with theoretical consideration of venous blood return and modified Poiseuille's law (equation 1.1b and 1.1c). MCP is mean circulatory pressure, RAP is right atrial pressure, R_v and R_a are vein and arterial resistance, η is viscosity, ΔP is pressure difference, P is pressure, L is length, E is incremental elastic modulus, h is wall thickness.

$$Q = \frac{MCP - RAP}{Rv + \frac{Ra}{19}} \qquad (1.1b)$$

$$Q = \frac{R_v^4\pi}{8\eta}\frac{\Delta P}{L}\frac{1}{(1-\frac{R_v P}{Eh})^4} \qquad (1.1c)$$

Shear rate (Sr) was calculated according to equation 1.2, where Q is portal flow and r is portal radius:

$$Sr = \frac{4Q}{r^3\pi} \qquad (1.2)$$

Portal cross sectional area (CSA) was calculated with regard to equation 1.3, where D is portal diameter:

$$CSA = \frac{D^2\pi}{4} \qquad (1.3)$$

RI represent portal resistance index and it was estimated by using maximal and minimal blood flow velocity (V_{max} and V_{min}) according to the formula 1.4:

$$RI = \frac{V_{max} - V_{min}}{V_{max}} \qquad (1.4)$$

The inlet length (L) was taken as:

$$L = 4,2\frac{V_{mean}D^2}{4} \qquad (1.5)$$

Expected (ideal) portal vein flow ratio (F_{ideal}) with regard to Poiseuille's equation was calculated by using equation 1.6. We made the assumption that α is 1. α is ratio of the viscosity, D_t is diameter in 10th or 15th min of test and D_0 represents initial diameter.

$$F_{ideal} = \frac{D_t^4}{D_0^4}\alpha \qquad (1.6)$$

Pressure ratio (ΔP_{ratio}) was calculated according to equation 1.7. Q_t is portal blood flow in the 10th or 15th min of test, Q_0 is basal portal flow, while D_t and D_0 are portal vein diameters in the same time intervals apparently.

$$\Delta P_{ratio} = \frac{Q_t D_0^4}{Q_0 D_t^4} \qquad (1.7)$$

We used equation 1.8 to approximately assess portal vein pressure difference between 10th min of the test and basal pressure with regard to Bernoulli's rule. In the following equation V_{mean0} and V_{meant} are the initial and 10th or 15th min mean blood flow velocity. ρ is the density of blood with assumption that it's value is 1060 kg/m^3.

$$\Delta P = \frac{1}{2}\rho(V_{meant}^2 - V_{mean0}^2) \qquad (1.8)$$

Portal vein flow ratio (F) was calculated with regard to experimentally obtained portal vein flow values:

$$Q_{10}/Q_0 \text{ and } Q_{15}/Q_0 \qquad (1.9)$$

Statistical analysis

Descriptive data were expressed as means ± SEM. The significance of difference between the two groups was assessed by Student's *t*-test, while differences between parameters in different time measurements were assessed by analysis of variance test with repeated measures and paired samples *t*-test as post-hoc. Statistical analysis of interobserver agreement for quantitative variables (D, V_{max}, V_{min}) was performed using the intraclass correlation coefficient. Results were interpreted as poor (< 0.04), regular (0.41-0.75) or excellent (> 0.76). *P* values < 0.05 were considered significant. All statistical analysis was performed using the SPSS (version 15).

Table 1 Demographic and clinical characteristics of investigated groups *n* (%)

Clinical parameters	Patients (*n* = 39)	Control (*n* = 30)	*P* value
Average age (yr) (mean ± SE)	54.8 ± 1.3	42.5 ± 0.9	< 0.01
Gender			
Male	34 (87.1)	17 (56.7)	< 0.01
Female	5 (12.9)	13 (43.3)	< 0.01
Body mass (kg ± SE)	72.5 ± 2.0	72.8 ± 2.6	NS
Height (cm ± SEM)	170.4 ± 1.4	171.1 ± 2.1	NS
Body mass index (mean ± SE)	41.9 ± 1.2	42.3 ± 1.3	NS
Diagnosis			< 0.01
Cirrhosis hepatis	38 (97.4)	0 (0)	
Hepatitis B	1 (2.6)	0 (0)	
Diameter liver (mm ± SE)	168.4 ± 3.1	147.3 ± 2.2	< 0.01
Ascites			< 0.01
Yes	4 (10.3)	0 (0)	
No	35 (89.7)	30 (100)	
Varices esophagi			< 0.01
Yes (Grade Ⅲ)	3 (7.7)	0 (0)	
No	36 (92.3)	30 (100)	
AST (U/l ± SEM)	90.5 ± 15.5	21.4 ± 1.4	< 0.01
ALT (U/l ± SEM)	45.3 ± 8.6	24.1 ± 1.5	< 0.01
GGT (U/l ± SEM)	267.5 ± 41.6	35.6 ± 1.6	< 0.01
AST			< 0.01
Increased	25 (64.1)	0 (0)	
Normal	14 (35.9)	30 (100)	
ALT			< 0.01
Increased	12 (30.7)	0 (0)	
Normal	27 (69.3)	30 (100)	
GGT			< 0.01
Increased	22 (56.4)	0 (0)	
Normal	17 (43.6)	30 (100)	
Total bilirubin (μmol/L ± SE)	43.3 ± 8.0	18.6 ± 0.8	< 0.01
Direct bilirubin (μmol/L ± SE)	18.2 ± 3.9	2.9 ± 0.2	< 0.01
Total bilirubin			< 0.01
Increased	25 (64.1)	0 (0)	
Normal	14 (35.9)	30 (100)	
Direct bilirubin			< 0.01
Increased	29 (74.4)	0 (0)	
Normal	10 (25.6)	30 (100)	

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma glutamyltransferase; NS: Not significant.

RESULTS

Subjects' characteristics and portal vein parameters

Demographic and clinical characteristics of the study population are presented in Table 1.

Investigated haemodynamic and biomechanical parameters of patients and controls before and after GTN administration are summarized in Table 2.

Groups significantly differed in the majority of investigated parameters in all three times of measurement.

Following GTN administration, the majority of portal vein vascular mechanic and hemodynamic parameters reached a maximum after 10 min in both groups and then slightly changed in the opposite direction during the last 5 min of the performed test. Figure 1 illustrates the effects of GTN administration on mean portal flow.

Vasodilatation after GTN administration was associated with a significant difference between obtained and ideal portal flow rate ratio in patients both in 10th and 15th min of the test. However, there was no significant difference in F between the controls and patients.

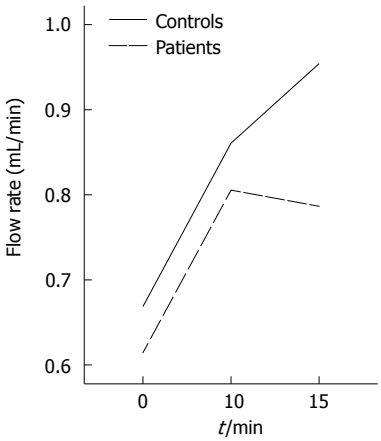

Figure 1 Portal blood flow rate in controls (full line) and patients (dotted line).

In the 15th min of the test, the decrease of the pressure ratio in controls resulted in significant differences in its values between controls and patients and this phenomenon is in concordance with the decrease in hydrodynamic pressure in controls but still significantly higher hydrodynamic pressure in controls. Bernoulli's equation was used with regard to significantly shorter portal vein (about 8 cm) when compared to the inlet length and therefore flat velocity profile. Shear rate observation could be of interest with regard to study of the boundary level and served to assess flow velocity profile. During our study velocity profile was flat, with a very thin boundary level. Table 3 shows data (diameter, maximal and minimal blood flow velocity) measured by three independent observers. Interobserver agreement evaluated by the intraclass correlation coefficient showed excellent and regular results for the quantitative variables as shown in Table 3.

Oxidative stress parameters

The dynamics of oxidative stress parameters in patients and controls can be seen in Figures 2A-D. At rest, significant differences between patients and controls were observed in levels of O_2^- (12.88 ± 2.24 nmol/mL *vs* 6.57 ± 1.07 nmol/mL, $P < 0.05$) and NO (4.85 ± 1.01 nmol/mL *vs* 15.06 ± 1.11 nmol/mL, $P < 0.01$). The same situation was observed 10 min after GTN administration, while in 15th min of the test patients experienced a significant rise in the level of the index of lipid peroxidation (2.37 ± 0.47 μmol/L *vs* 1.26 ± 0.08 μmol/L, $P < 0.05$), so groups now differed in three oxidative stress parameters.

GTN administration induced a significant rise in NO levels only in controls (NO levels were increased in the 10th min), but their NO levels returned to the basal values 15 min after GTN administration. The responses of O_2^- and TBARS to GTN administration were similar: 10 min after GTN administration levels of O_2^- and TBARS were elevated in both groups. In the 15th min of the test, controls' O_2^- and TBARS levels decreased to the levels similar to that in rest, while patients experienced further increases in the level of the index of lipid peroxidation and their

Table 2 Haemodynamic and biomechanical parameters of portal vein before and after nitroglycerine administration

Parameters	Groups	0 min	*P* value	10 min	*P* value	15 min	*P* value
D (mm)	Patients	11.60 ± 0.32	< 0.01	13.81 ± 0.31[b]	< 0.01	13.63 ± 0.33[b]	< 0.01
	Controls	9.19 ± 0.33		11.02 ± 0.34[b]		11.72 ± 0.37[b]	
CSA (cm^2)	Patients	1.08 ± 0.06	< 0.01	1.52 ± 0.69[b]	< 0.01	1.49 ± 0.70[b]	< 0.01
	Controls	0.68 ± 0.05		1.09 ± 0.06[b]		1.11 ± 0.06[b]	
Q (l/min)	Patients	0.61 ± 0.05	NS	0.80 ± 0.07[b]	NS	0.78 ± 0.71[b]	< 0.05
	Controls	0.66 ± 0.06		0.86 ± 0.05[b]		0.95 ± 0.08[b]	
Sr (1/s)	Patients	66.50 ± 3.91	< 0.01	49.85 ± 3.07[b]	< 0.01	51.71 ± 3.30[b]	< 0.01
	Controls	139.87 ± 5.25		111.29 ± 5.57[b]		83.83 ± 5.58[b]	
Vmean (cm/s)	Patients	9.39 ± 0.50	< 0.01	8.55 ± 0.55[a]	< 0.01	8.69 ± 0.57	< 0.01
	Controls	15.63 ± 0.48		13.13 ± 0.44[b]		14.34 ± 0.54[b]	
V_{max} (cm/s)	Patients	10.60 ± 0.57	< 0.01	9.77 ± 0.65[b]	< 0.01	9.84 ± 0.62[b]	< 0.01
	Controls	19.31 ± 0.56		16.51 ± 0.51[b]		18.03 ± 0.69[b]	
V_{min} (cm/s)	Patients	8.18 ± 0.43	< 0.01	7.34 ± 0.48[b]	< 0.01	7.53 ± 0.53[b]	< 0.01
	Controls	11.96 ± 0.48		9.75 ± 0.46[b]		10.65 ± 0.47[b]	
RI	Patients	0.22 ± 0.01	< 0.01	0.24 ± 0.01	< 0.01	0.24 ± 0.01	< 0.01
	Controls	0.37 ± 0.02		0.41 ± 0.02		0.40 ± 0.01	
L (cm)	Patients	13.69 ± 1.12	NS	17.95 ± 1.68[b]	NS	17.52 ± 1.58[b]	NS
	Controls	15.08 ± 1.56		17.00 ± 1.56[a]		21.26 ± 1.78[b]	
F_{ideal}	Patients	/	/	2.32 ± 0.21[d]	NS	2.11 ± 0.17[d]	< 0.05
	Controls	/		2.54 ± 0.34[d]		3.38 ± 0.51[d]	
ΔP_{ratio}	Patients	/	/	0.66 ± 0.03	NS	0.68 ± 0.03	< 0.05
	Controls	/		0.68 ± 0.04		0.49 ± 0.04	
F	Patients	/	/	1.34 ± 0.07	NS	1.31 ± 0.06	NS
	Controls	/		1.45 ± 0.10		1.48 ± 0.08	
ΔP (mmHg)	Patients	/	/	0.004 ± 0.003	< 0.01	0.005 ± 0.003	< 0.05
	Controls	/		0.029 ± 0.006		0.014 ± 0.003[e]	

Values are expressed as mean ± SE; *P* in colon 3, 5 and 7 relates to the existance of the difference between groups, while a and b represent the existance of difference between the initial measurement and the measurement in 10th or 15th min. d represents the existance of difference between F_{ideal} and F. e represents the existance of difference between the measurements in 10th and 15th min. [a]$P < 0.05$ *vs* 0 min, [b]$P < 0.01$ *vs* 0 min, [d]$P < 0.01$ *vs* F, [e]$P < 0.05$ *vs* 10 min. D: Portal diameter; CSA: Cross sectional area; Q: ; Sr: Shear rate; RI: Portal resistance index; L: Length; F_{ideal}: Expected (ideal) portal vein flow ratio; F: Portal vein flow ratio; ΔP_{ratio}: Pressure ratio; ΔP: Pressure difference; NS: Not significant.

Table 3 Parameters measured by three independent observers (Ⅰ, Ⅱ and Ⅲ)

Parameters	Groups	0 min				10 min				15 min			
		Ⅰ	Ⅱ	Ⅲ	IA	Ⅰ	Ⅱ	Ⅲ	IA	Ⅰ	Ⅱ	Ⅲ	IA
D (mm)	Patients	11.47 ± 0.31	11.70 ± 0.33	11.59 ± 0.31	0.84	13.79 ± 0.31	13.86 ± 0.32	13.80 ± 0.31	0.89	13.61 ± 0.33	13.65 ± 0.35	13.64 ± 0.32	0.95
	Controls	9.22 ± 0.32	9.19 ± 0.34	9.22 ± 0.32	0.96	11.07 ± 0.35	10.91 ± 0.29	11.00 ± 0.35	0.84	11.80 ± 0.43	11.77 ± 0.39	11.55 ± 0.34	0.9
V_{max}	Patients	10.55 ± 0.53	10.99 ± 0.64	10.17 ± 0.50	0.83	9.80 ± 0.63	10.84 ± 0.72	8.94 ± 0.52	0.73	9.91 ± 0.64	10.01 ± 0.62	9.52 ± 0.59	0.79
(cm/s)	Controls	19.74 ± 0.56	19.11 ± 0.52	19.07 ± 0.61	0.80	17.11 ± 0.53	16.44 ± 0.48	16.02 ± 0.50	0.79	18.11 ± 0.72	18.77 ± 0.81	17.43 ± 0.55	0.75
V_{min}	Patients	8.11 ± 0.40	8.59 ± 0.51	7.73 ± 0.36	0.76	7.48 ± 0.51	7.22 ± 0.50	7.32 ± 0.44	0.83	7.68 ± 0.50	7.90 ± 0.59	7.01 ± 0.51	0.79
(cm/s)	Controls	12.12 ± 0.55	12.26 ± 0.60	11.46 ± 0.34	0.79	9.86 ± 0.48	9.89 ± 0.48	9.55 ± 0.42	0.84	10.89 ± 0.55	10.22 ± 0.43	10.78 ± 0.40	0.81

Interobserver agreement for quantitative parameters (portal vein diameter, maximal and minimal blood flow velocity) calculated from the comparison of data from all three observers. D: Portal diameter; IA: Interobserver agreement.

O_2^- levels remained elevated. Hydrogen peroxide did not change significantly throughout the study.

DISCUSSION

Haemodynamics

Endothelium-independent vasodilatation observed after GTN administration was caused by smooth muscle relaxation of the portal vein. Portal vein diameter rose significantly throughout the study and concomitantly statistically significant enhancement in portal blood flow (15th min) appear to be in concordance with equation 1.1b. With regard to equation 1.1b it is clear that small changes in vein resistance lead to huge blood flow changes, whereas the influence of arterial resistance is dampened due to high value of capacitance. However, there was no statistically significant difference in portal flow rate between the groups in the first ten minutes of the test, moreover flow rate was higher in controls compared to patients. In controls portal flow rate rose continuously compared to a decreasing pattern in patients after the 10th min of the test. One possible explanation for the lower flow rate in patients *vs* controls is increased incremental elastic modulus (with regard to equation 1.1c) and consequent leftward shift of the pressure-volume curve. However, in patients we could expect a parallel shift of the pressure-volume curve. The latter does not imply changes in compliance. It is of interest to emphasize hyperdynamic circulation in

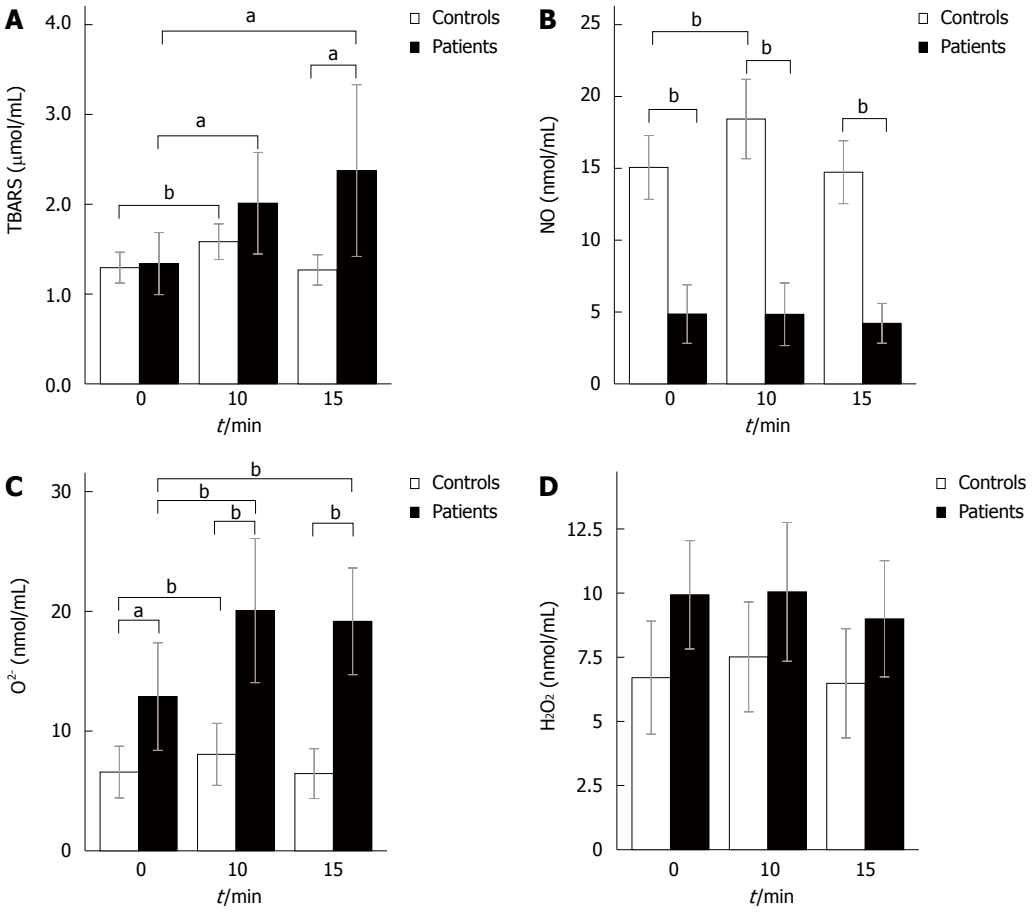

Figure 2 Thiobarbituric acid reactive substances, nitric oxide, O_2^- and H_2O_2 levels after nitroglycerine administration: measured at the beginning of the test, in the 10th and the 15th min of the test (results are expressed as mean ± SE of the mean; $^aP < 0.05$, $^bP < 0.01$). A: Thiobarbituric acid reactive substances levels (TBARS); B: Nitric oxide (NO) levels; C: O_2^- levels; D: H_2O_2 levels.

patients with liver cirrhosis. During the first 10 min, average blood flow velocity decreases simultaneously in both groups due to higher increases in portal cross-sectional area compared to flow rate. Our study disclosed significantly reduced mean blood flow velocity during the first 10 min of the test due to discrepancies between blood flow and CSA. Table 2 shows that blood flow velocity parameters were significantly higher in controls than in patients.

A similar pattern was obtained by using ideal and measured portal blood flow values. The ideal (expected) value was calculated by using equation 1.6 with regard to assumption of the equality of blood viscosity during the study. If the assumption about the equality viscosity is true, then $\alpha = 1$. Nevertheless, a significantly reduced shear rate in patients after GTN administration (49.85 ± 3.07 *vs* 66.50 ± 3.91) led to a nonlinear correlation with shear stress, so α might be different in comparision to the ideal value. In the 10th min of the test, expected (ideal) flow ratio rose up to 2.32 in patients and 2.54 in controls, while at the same time obtained flow ratio (F) was 1.34 *vs* 1.45 and both these differences were not statistically significant between groups. At the same time ideal flow ratio was significantly higher in both groups compared to the experimentally obtained flow ratio. However, ideal flow ratio was significantly higher in controls *vs* patients in the 15th min of test. High compliance does not reflect good elastic properties of vein, as one could presume, it rather reflects a change in geometry[20]. At low pressure the vein's cross section is ellipsoidal and every rise in pressure causes the vein to become more circular, without change in diameter, but with a great increase in cross-sectional area the vein becomes fully rounded and concomitantly the flow velocity decreases due to an inverse relationship with CSA. At higher steady state pressure, every further rise in pressure causes changes in diameter.

This concept is very similar to the observations made in our study. Table 2 shows a significant difference between the diameter in patients *vs* controls after GTN administration. This is, together with mentioned changes in viscoelastic properties (increased elastic modulus) and observed low shear rate in patients, a possible explanation for the proposed "double paradoxes"[21], phenomenon seen in cirrhotic patients, due to obviously increased intrahepatic resistance as a consequence of elevated vasoconstrictor levels. At the same time increased systemic vasodilatation dampened responses to endogenous vasoconstrictors, and the overall effect was markedly increased systemic blood flow. Under physiological conditions vein capacitance does not allow any apparent de-

cline in venous system pressure[22]. However, we assumed that under physiological conditions, increased flow rate (volume overload) due to GTN administration means the portal vein wall is still working in less steep part of the tension-volume curve which further leads to a significant hydrodynamic pressure drop in the control group (15th min). The net effect is lower resistance to pressure changes than we could expect, reflecting the inherently limited distensibility of the portal vein. On the contrary, in some liver diseases increased sinusoidal resistance is responsible for the parallel and leftward shift of the pressure-volume curve. The result of this phenomenon could be a significantly lower hydrodynamic pressure difference in patients *vs* controls and observed significant difference between the groups in 15th min of test with regard to pressure ratio. Indeed, it is obvious that the lower initial velocity in patients led to a significant difference in hydrodynamic pressure during the whole test.

The most interesting observation is lower pressure ratio in controls *vs* patients in the 15th min of the test, which suggested proposed mechanism of geometry and biomechanical changes in portal vein wall. Shear stress is in good linear correlation to the shear rate (equation 1.2) only in Newtonian fluid. The very low shear rate in patients, observed in our study (66.50 ± 24.14 1/s in basal conditions), rules out linear correspondence between these parameters. At low shear rates the apparent viscosity (η) increases markedly. Shear rate measured in the 10th min of the test was significantly lower than the basal value in both groups, while in controls shear rate was significantly higher compared to patients. Below a value of 2001/s the fluid behaviour is non Newtonian[23]. The significance of this observation was stated above in discussion about blood flow discrepancy (double paradoxes). Using the inlet length value, given previously in Table 2, the parabolic velocity profile would not be expected to show complete development, already held flat profile. The reason is a much shorter portal vein (usually 8 cm) compared to inlet length. We used equation 1.8 to approximately assess portal vein pressure difference between the beginning of the test and the 10th min of the test with regard to Bernoulli's rule and Poiseuille's equation. Bernoulli's equation was used, as we mentioned above, due to greater unsheared region in the flat velocity profile and it could at least be useful for explaining pressure change.

Oxidative stress

Increased oxidative stress is a well-known condition in many diseases. Oxidative stress is defined as the tissue damage resulting from an imbalance between an excessive generation of oxidant compounds and insufficient anti-oxidant defence mechanisms[1]. Different cellular enzymes, including xanthine oxidase, cyclooxygenases, lipoxygenase, have been identified as cellular source of ROS.

NO: Nitric oxide, as we expected, did not change significantly in patients in our study. Controls had significantly higher levels of NO at rest, and GTN administration induced its significant increase, observed in the 10th min of the test. NO excessive synthesis might be possible due to mechanisms of flow mediated vasodilatation *via* opening of stretch-activated calcium channels and further intracellular calcium accumulation, which in turn stimulate NO production[24,25]. Increased NO synthesis is also expected in liver cirrhosis environment conditions. However, several mechanisms counteracted the flow-mediated increase in NO synthesis in patients: decreased shear stress induced NO synthesis inhibition; superoxide mediated peroxynitrite formation (superoxide was dramatically higher in patients verus controls). We propose that in portal hypertension, high pressure (P) mediated an exponentially decreased reaction rate constant (K_2) of the ion channel, and altered gating properties of the channel[26]. This mechanism may be explained according to equation 2.0:

$$\frac{k_2}{k_1} = e^{-\frac{P\Delta V}{RT}} \qquad (2.0)$$

K_2 represents the reaction rate constant at pressure P, k_1 is the channel activation constant in basal condition, T is temperature. However, the pressure ratio is similar in both groups, so it is more likely to presume that increased peroxynitrite formation and extensive synthesis of asymmetric dimethyl arginine (ADMA), a potent NOS inhibitor[27], are involved in maintaining the same values of NO in patients. ADMA is downregulated and very much depends on the activity of the enzyme dimethyldiamino-hydrolase (DDHA) which transforms ADMA into citrulline. Increased oxidative stress should be able to reduce the availability of NO, so counteracting excessive NO production in liver cirrhosis. The present data demonstrate that excessive NO synthesis seen in patients with liver cirrhosis might be significantly modified by several described mechanisms.

H_2O_2: Hydrogen peroxide is created in the reaction of superoxide anion and hydrogen cation but this reaction is too slow (K2 < 1.0 $M^{-1}S^{-1}$, $t_{1/2}$ = 1 min) despite the high redox potential (0.89 V). Almost certainly the Fenton and Haber-Weiss reactions are essential for H_2O_2 disassembling. The total redox potential of Haber Weiss and Fenton reaction is 0.78 V, very close to redox potential of synthesis reaction (0.89 V), indicating equilibrium between these opposite reactions (probability that hydrogen peroxide will change it's value in this case is zero, see later) and giving a possible explanation for unexpected lack of changes in hydrogen peroxide values throughout the performed study. Haber-Weiss and Fenton reactions can be deleterious, giving rise to the formation of the highly reactive hydroxyl radical ($OH^{\cdot}$), which induces lipid peroxidation. The concentrations of hydrogen peroxide and superoxide prior to test were in approximately equimolar equilibrium (in controls $O_2^{\cdot-}$: 12.88 nmol/mL and H_2O_2: 9.93 nmol/mL; in patients

O_2^-: 6.57 nmol/mL and H_2O_2: 6.70 nmol/mL). The possibility (P) of some reaction and it's correlation to the redox potentials difference ($\Delta U \approx \varphi_1 - \varphi_2$) is theoretically determined by using equation 2.1:

$$P = e^{-\frac{\Delta U}{RT}} \qquad (2.1)$$

O_2^-: Superoxide basal value was markedly higher in the patients compared to controls, and this observation suggests increased oxidative stress in patients with chronic liver disease. Significantly higher initial nitric oxide levels in controls compared to patients may be due to a "mirror pattern" with superoxide. Taken overall, these findings suggest that superoxide is a good indicator of oxidative stress in patients with chronic liver disease.

Superoxide is produced in accordance to Hund's rule so the probability of it's formation is higher than the probability of reduction with two electrons. The redox potential of superoxide generation is 0.16 V. Superoxide was significantly higher in patients compared to controls during the entire test. There is the possibility, albeit not undisputed, that one major contributing factor is the diameter, as the larger diameter in patients *vs* controls created greater circumferential wall stress according to Laplace's law. Increased oxygen consumption, promoted by increased tension, leads to increased superoxide production. However, there are doubts about whether superoxide levels are in better correlation with shear stress and Voigt's model[28] compared to Laplace's law.

TBARS: Oxidative stress could firstly be evidenced by an increase in TBARS concentration. TBARS continuously increased significantly (compared to basal value) in patients during the test. Basal values did not differ between the groups, but a significant difference was revealed after GTN administration. Indeed, we assumed that increased production of superoxide interferes with increased lipid peroxidation and TBARS concentration.

In conclusion, our study showed that endothelium-independent vasodilatation leads to a significant increase in blood flow and significant decline of mean blood flow velocity and shear rate in participants with chronic liver disease. Increased blood flow was to some extent lower than expected, probably due to increased liver sinusoidal resistance and mentioned double paradoxes. We proved, as a consequence of decreased shear rate far below the critical value, non Newtonian behaviour of portal vein blood flow. Tentative changes in O_2^- and H_2O_2 levels revealed the crucial role of ROS as trigger factors of lipid peroxidation. The preceding findings are in coherence with our assumption of O_2^- and H_2O_2 mediated lipid peroxidation *via* Haber Weiss and Fenton reactions.

Our study could be of great clinical importance, especially regarding the role of oxidative stress in portal vein haemodynamics. This pilot study could be a starting point for further investigation and possible implementation of some antioxidants in the treatment of portal hypertension.

COMMENTS

Background

Oxidative stress is a condition in which the delicate balance that exists between prooxidant (free radicals) production and their subsequent amelioration *via* the antioxidant defense system becomes skewed in favor of free radical expression. The existence of an interference of increased oxidative stress with the vasodilative properties of veins is now a well known fact, resulting in endothelial dysfunction i.e., a loss of nitric oxide (NO) bioactivity in the vessel wall. We hypothesized that the endothelium independent vasodilatation of the portal vein induced by sublingual nitroglycerine administration would lead to increased oxidative stress and significant changes in portal hemodynamics.

Research frontiers

Nitroglycerine is one of the most often used drugs in the treatment of vascular diseases. In some previous studies it was shown that nitroglycerine-induced vasodilation is impaired in some vascular diseases which points to the possible use of that drug in the diagnosis of different vascular diseases.

Innovations and breakthroughs

In previous investigations, application of nitroglycerine in order to test brachial artery reactivity showed that patients suffering from coronary artery disease exhibit loss of endothelium-independent (induced by nitroglycerine) as well as endothelium-dependent vasodilation (induced by short-term artery occlusion) compared to controls. Taking into consideration hyperdynamic portal vein circulation in patients suffering from portal hypertension it seems that some basic vascular mechanisms can correlate with other vascular diseases such as coronary artery disease. In concordance with that, the aim of our study was to determine how sublingual administration of nitroglycerine might be used for the evaluation of portal endothelium-independent vasodilatation through estimating parameters of portal vascular mechanics with special interest in oxidative stress in patients suffering from portal hypertension.

Applications

The study results suggest that nitroglycerine-induced vasodilation could be used as a potential new diagnostic test for evaluation of severity in portal hypertension.

Terminology

Portal hypertension: Increase in blood pressure in the veins of the portal system caused by obstruction in the liver (often associated with alcoholic cirrhosis), causing enlargement of the spleen and collateral veins associated with regional hyperdynamic circulation; Nitroglycerine: Commonly used drug for treatment of vascular diseases, basically coronary artery disease, which acts by inducing endothelium-independent vasodilation; Oxidative stress: A condition in which the delicate balance that exists between prooxidant (free radicals) production and their subsequent amelioration *via* the antioxidant defense system becomes skewed in favor of free radical expression.

Peer review

This is an original article, important for the development of the field of study.

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doi:10.3748/wjg.v18.i4.340

World J Gastroenterol 2012 January 28; 18(4): 340-348
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

α-fetoprotein, vascular endothelial growth factor receptor-1 and early recurrence of hepatoma

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Supported by Japan Society for the Promotion of Science KAKENHI 21390369 [Grant-in-Aid for Science Research (B)]
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Received: July 1, 2011 Revised: August 25, 2011
Accepted: August 31, 2011
Published online: January 28, 2012

Abstract

AIM: To investigate whether α-fetoprotein (AFP) and vascular endothelial growth factor receptor (VEGFR)-1 correlate with early recurrence of hepatoma/hepatocellular carcinoma (HCC).

METHODS: From 2000 to 2005, 114 consecutive patients with HCC underwent primary curative hepatectomy. The mean age was 60.7 (8.7) years and 94 patients were male. The median follow-up period was 71.2 mo (range: 43-100 mo). Immediately prior to commencing laparotomy, 5 mL bone marrow was aspirated from the sternum and collected in citrate-coated test tubes. The initial 2 mL of bone marrow aspirate was discarded in each case. AFP mRNA and VEGFR-1 mRNA in the bone marrow and peripheral blood (BM- and PH-AFP mRNA and BM- and PH-VEGFR-1 mRNA, respectively) were measured by real-time quantitative reverse transcription polymerase chain reaction. As normal controls, VEGFR-1 mRNA in the bone marrow and peripheral blood was also measured in 11 living liver donors. These data were evaluated for any correlation with early recurrence, comparing clinical and pathological outcomes.

RESULTS: The cut-off value of the BM-AFP mRNA and PH-AFP mRNA level in patients with HCC was set at 1.92×10^{-7} and zero, respectively, based on data from the controls. A total of 34 (29.8%) and six (5.4%) patients were positive for BM-AFP mRNA and PH-AFP mRNA, respectively. The BM-VEGFR-1 mRNA levels in all HCC patients were higher than those in the normal controls, and this was the case also for PH-VEGFR-1mRNA. The 25-percentile values for the BM- and PH-VEGFR-1 mRNA in HCC patients were used as the cut-off values for assigning the patients into two groups based on these transcript levels. The High group for BM- VEGFR-1 mRNA contained 81 (71.1%) HCC cases and the Low group was assigned 33 (28.9%) patients. These numbers for PH-VEGFR-1mRNA were 78 (75.0%) and 26 (25.0%), respectively. HCC recurred in 80 patients; in the remnant liver in 48 cases, in the remnant liver and remote tissue in 20, and in the remote tissue alone in 12. BM-AFP mRNA-positive cases showed a significantly higher rate of early recurrence (within 1 year of surgical treatment) compared with BM-AFP mRNA-negative patients (P = 0.0091). Patients were classified into four groups according to the level/status of their BM-VEGFR-1 and BM-AFP mRNA as follows: group A (n = 23), BM-VEGFR-1/BM-AFP mRNA = low/negative; group B (n = 57) high/negative; group C (n = 10) low/positive; group D (n = 24), high/positive. This classification was found to correlate with a recurrence of this

disease within 1 year (P = 0.0228). The disease-free survival curve of group A was significantly better than that of groups B, C or D (P = 0.0437, P = 0.0325, P = 0.0225). No other classification (i.e., PH-VEGF-R1/BM-AFP, BM-VEGF-R1/PH-AFP, and PH-VEGF-R1/PH-AFP mRNA) showed such a correlation.

CONCLUSION: The evaluation of BM-AFP and BM-VEGFR-1 mRNA in patients with HCC may be a valuable predictor of disease recurrence following curative resection.

Key words: α-fetoprotein; Vascular endothelial growth factor receptor-1; mRNA; Early recurrence; Hepatocellular carcinoma

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Kamiyama T, Takahashi M, Nakanishi K, Yokoo H, Kamachi H, Kobayashi N, Ozaki M, Todo S. α-fetoprotein, vascular endothelial growth factor receptor-1 and early recurrence of hepatoma. *World J Gastroenterol* 2012; 18(4): 340-348 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i4/340.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i4.340

INTRODUCTION

Various factors are thought to contribute to hepatocellular carcinoma (HCC) recurrence, which commonly results in death, including multicentric carcinogenesis in the remnant liver due to an underlying hepatitis-B-virus- or hepatitis-C-virus-induced liver cirrhosis[1], hematogenic spread, or micrometastasis of HCC cells prior to surgery or during hepatectomy by manipulation of the liver[2]. Recently, using various molecular biological markers, the detection of malignant cells in the systemic circulation and bone marrow has become possible and the presence of these cells has been found to correlate with the clinical outcome[3-8]. We have also reported from our laboratory that the detection of HCC cells in the bone marrow by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of α-fetoprotein (AFP) mRNA before curative hepatectomy correlates with HCC recurrence and patient survival outcomes. Although early recurrence within 1 year of curative resection for HCC is one of the most important factors affecting the prognosis and clinical outcomes[9,10], the relationship between early recurrence and disseminated cancer cells has not yet been evaluated.

It has been recently hypothesized that metastasis is dependent on both isolated cancer cells and the host response. Kaplan *et al*[11] have reported that bone-marrow-derived hematopoietic progenitor cells that express vascular endothelial growth factor receptor (VEGFR)-1 migrate to tumor-specific pre-metastatic sites and form cellular clusters before the arrival of tumor cells both *in vitro* and *in vivo*. Moreover, it has been reported that the simultaneous presence of isolated tumor cells and VEGFR-1 expression at pre-metastatic sites is clinically significant for disease progression in gastric cancer[12]. With regard to HCC however, there has been no study to date of the association between isolated cancer cells and the expression of VEGFR-1.

In our present study, we examined whether the expression of AFP mRNA and VEGFR-1 in the bone marrow and peripheral blood, detected by sensitive real-time quantitative RT-PCR, could predict early recurrence in consecutive HCC patients who had undergone a curative hepatic resection.

MATERIALS AND METHODS

Ethics

This study was approved by the Institutional Review Board of the Hokkaido University, School of Advanced Medicine. Informed consent was obtained from each patient in accordance with the Ethics Committee Guidelines at our institution.

From July 2000 to June 2005, 114 consecutive patients underwent primary curative hepatectomy at the First Department of Surgery, Hokkaido University Hospital. The mean age was 60.7 (8.7) years and 94 patients were male. The Child-Pugh staging was A in 110 patients and B in four. Patients were discharged from the hospital at an average of 17.5 (7.1) d after surgery. They were followed up at 3-mo intervals by computed tomography (CT), magnetic resonance imaging (MRI), ultrasonography (US) and laboratory tests for AFP, lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and protein induced by vitamin K absence or antagonist-Ⅱ (PIVKA-Ⅱ). The median follow-up period was 71.2 mo (range: 43 mo-100 mo).

As normal controls, VEGFR-1 mRNA in the bone marrow and peripheral blood was also measured in 11 living liver donors. The cut-off value for AFP mRNA/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the bone marrow and peripheral blood was set as described in our previous study[13].

Sample collections

Immediately prior to commencing the laparotomy, 5 mL bone marrow was aspirated from the sternum and collected in citrate-coated test tubes. The initial 2 mL of the bone marrow aspirate was discarded in each case.

RNA isolation and reverse transcription

Bone marrow samples were prepared for the measurement of total RNA using a Blood RNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol with minor modifications. Briefly, 5 mL bone marrow cells were mixed with 25 mL Reagent buffer erythrocyte lysis (EL). They were then cooled on

ice for 15 min, centrifuged, and the cell pellets were collected. The pellets were suspended in 1.35 mL buffer and applied to the reagent columns, and then washed twice with reagent buffer containing ethanol. Total RNA was eluted with RNase-free water. These bone marrow RNA samples were stored at -80 ℃ until use. cDNA was generated from 1 μg total RNA using Moloney murine leukemia virus reverse transcriptase (SuperScript Ⅱ, Life Technologies, Carlsbad, CA, United States), plus 20 pmol/L each dNTP and 10 pmol/L oligo dT primers in a 20-μL final reaction volume at 42 ℃ for 1 h. This was followed by heating at 99 ℃ for 5 min.

Real-time quantitative RT-PCR

A LightCycler PCR and detection system (Roche Diagnostics, Mannheim, Germany) was used for amplification. Online quantification real-time RT-PCR was then performed in glass capillaries according to the manufacturer's protocol. The cDNA was amplified in a 20-μL PCR reaction mixture containing each dNTP (with dUTP instead of dTTP), 1 × PCR buffer, specific primers, and magnesium chloride.

For the detection of AFP, two adjacent oligonucleotide probes were used: the LightCycler Red 640 fluorophore, hAFP-LCR; (5'-CTTGCACACAAAAGCCCACTCCA-3') and a fluorophore labeled at the 3'-end with fluorescein, hAFP-FITC; (5'-TCGATCCCACTTTTCCAAGTT-3') (Nihon Gene Research Laboratories, Sendai, Japan). The sense and antisense primers (kindly supplied by Dr. Hiroaki Nagano at Osaka University) used for the amplification of AFP were as follows: 5'-TGCAGCCAAAGTGAAGAGGGAAGA-3') (hAFP-s) and 5'-CATAGCGAGCAGCCCAAAGAAGAA-3' (hAFP-As). The RT-PCR amplification was carried out for one cycle of 95 ℃ for 10 min, followed by 35 cycles of 95 ℃ for 10 s, 62 ℃ for 15 s, and 72 ℃ for 15 s. The final cycle was followed by a 10-min extension step at 40 ℃.

For the detection of VEGFR-1, two adjacent oligonucleotide probes were used: hVEGFR-1-LCR; 5'-TTCCGTGTCCCCACTGCCAA-3' and hVEGFR-1-FITC; 5'-GGGAAGCTCACTGGCATGGC-3'. The sense and antisense primers for the amplification of VEGFR-1 were as follows: 5'-TCATGAATGTTTCCCTGCAA-3' (h VEGFR-1-S) and 5'-GGAGGTATGGTGCTTCCTGA-3' (h VEGFR-1-As). These primers were designed using sequences described in a previous report[14]. RT-PCR amplification was carried out for one cycle of 95 ℃ for 10 min, followed by 35 cycles of 95 ℃ for 10 s, 60 ℃ for 10 s, and 72 ℃ for 16 s. The final cycle was followed by a 10-min extension step at 40 ℃.

For the detection of GAPDH as an internal control, two adjacent oligonucleotide probes were used: hGAPDH-LCR; 5'-TTCCGTGTCCCCACTGCCAA-3' and hGAPDH-FITC; 5'-GGGAAGCTCACTGGCATGGC-3'. The sense and antisense primers for the amplification of GAPDH were as follows: 5'-GCCTCCTGCACCACCAACTG-3' (hGAPDH-S) and 5'-CGACGCCTGCTTCACCACCTTCT-3' (hGAPDH-As). The RT-PCR amplification was carried out for one cycle of 95 ℃ for 10 min, followed by 35 cycles of 95 ℃ for 10 s, 60 ℃ for 10 s and 72 ℃ for 16 s. The final cycle was followed by a 10-min extension step at 40 ℃.

Quantification analysis

Quantification data were analyzed using the LightCycler analysis software (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer's instructions. In this analysis, the background fluorescence was removed by setting a noise band. The crossing point for the calculation of amplified PCR products was set by the intersection of the best-fit line through the log-linear lesion and the noise band. The standard curve was a plot of the "crossing point" versus the copy number of DNA fragments inserted into the cloning vector.

Statistical analysis

Cumulative survival and disease-free survival (DFS) rates were computed according to the Kaplan-Meier method and compared between groups using the Breslow-Gehan-Wilcoxon test. The Cox proportional hazards model was used for multivariate analysis. Statistical analyses using standard tests (χ^2, *t* test) were performed where appropriate. Significance was defined as $P < 0.05$. Statistical analyses were performed using StatView 5.0 Windows (SAS Institute Inc., Cary, NY, United States).

RESULTS

Analysis of AFP mRNA levels in bone marrow

The mean AFP mRNA/GAPDH ratio in the bone marrow (BM-AFP mRNA) of HCC patients, as determined by real-time quantitative RT-PCR, was 3469.27 × 10^{-7} (range: 0-348 526.19 × 10^{-7}). The cut-off value of the BM-AFP mRNA level was set at 1.92 × 10^{-7} (with reference to a previous report)[13]. The HCC patients were then divided into two groups according to this cut-off value. Accordingly, 80 patients (70.2%) were found to be negative for BM-AFP mRNA and 34 patients (29.8%), assigned to the "High" group, were positive for this transcript.

Expression of AFP mRNA in peripheral blood

No AFP mRNA was detectable in the peripheral blood of the control patients, therefore, the cut-off value for AFP mRNA/GAPDH in the peripheral blood (PH-AFP mRNA) was set at zero. Accordingly, six patients (5.4%) were found to be positive and 105 (94.6%) were negative for AFP mRNA. Due to some sampling loss, peripheral blood samples were unavailable for three patients.

Expression of VEGFR-1 mRNA in bone marrow

The mean VEGFR-1 mRNA/GAPDH in the bone marrow (BM-VEGFR-1 mRNA) of normal controls, again determined by real-time quantitative RT-PCR measurements, was 0.1497 × 10^{-3} (range: 0.0212 × 10^{-3} to 0.3213 × 10^{-3}). The mean BM-VEGFR-1 mRNA level in the HCC patients was 3.8474 × 10^{-3} (range: 0.3481 × 10^{-3} to

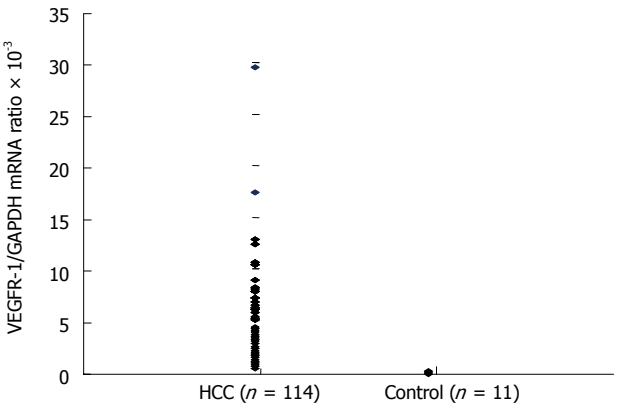

Figure 1 Expression of vascular endothelial growth factor receptor-1 mRNA in bone marrow detected by real-time quantitative reverse transcription polymerase chain reaction. The mean VEGFR-1/GAPDH mRNA ratio in the bone marrow (BM-VEGFR-1 mRNA) of normal controls was 0.1497 × 10^{-3} (range: 0.0212 × 10^{-3}-0.3213 × 10^{-3}). The mean BM-VEGFR-1 mRNA level in HCC patients was measured at 3.8474 × 10^{-3} (range: 0.3481 × 10^{-3}-29.5885 × 10^{-3}). HCC: Hepatocellular carcinoma; VEGFR: Vascular endothelial growth factor receptor; BM: Bone marrow; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

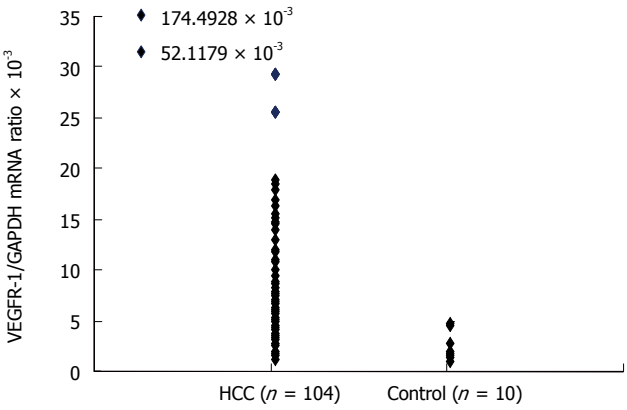

Figure 2 Expression of vascular endothelial growth factor receptor-1 mRNA in peripheral blood detected by real-time quantitative real-time quantitative reverse transcription polymerase chain reaction. The mean VEGFR-1/GAPDH mRNA ratio in the peripheral blood (PH-VEGFR-1 mRNA) of normal controls was 2.4944 × 10^{-3} (range: 1.0730 × 10^{-3}-4.6958 × 10^{-3}). The mean PH-VEGFR-1 mRNA level in HCC patients was 9.1285 × 10^{-3} (range: 1.2774 × 10^{-3}-174.4928 × 10^{-3}). HCC: Hepatocellular carcinoma; VEGFR: Vascular endothelial growth factor receptor; PH: Peripheral blood; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

29.5885 × 10^{-3}). The mean BM-VEGFR-1 mRNA level of all HCC patients was higher than that of the normal controls (Figure 1). The HCC patients were then divided into two groups ("High" and "Low") according to their BM-VEGFR-1 mRNA level; the cut-off value was 1.5664 × 10^{-3}, which was the 25th percentile value of the BM-VEGFR-1 mRNA levels in the HCC cohort. The number of patients in the High group was 81 (71.1%) and 33 (28.9%) were assigned to the Low group.

Expression of VEGFR-1 mRNA in peripheral blood

The mean VEGFR-1 mRNA/GAPDH ratio in the peripheral blood (PH-VEGFR-1 mRNA) of the normal controls was 2.4944 × 10^{-3} (range: 1.0730 × 10^{-3} to 4.6958 × 10^{-3}). The mean PH-VEGFR-1 mRNA level in the HCC patients was 9.1285 × 10^{-3} (range: 1.2774 × 10^{-3} to 174.4928 × 10^{-3}). The PH-VEGFR-1 mRNA level of almost all HCC patients was higher than that of the normal controls (Figure 2). The HCC patients were divided into high and low groups according to their PH-VEGFR-1 mRNA level. The cut-off value was 4.0238 × 10^{-3}, which was in the 25th percentile of the PH-VEGFR-1 mRNA level of HCC patients. The number of patients in the high group was 78 (75.0%) with 26 (25.0%) placed in the Low group. Peripheral blood samples were available for 104 patients only.

Clinical significance of the BM- and PH-VEGFR-1, and BM- and PH-AFP mRNA levels

The status of the BM-AFP mRNA levels was correlated with microscopically detectable portal invasion, whereas that of PH-AFP mRNA was found to correlate with the serum AFP and AFP-L3 levels, the number of tumors, microscopic portal invasion, and microscopic intrahepatic metastasis (Table 1). The number of tumors, serum albumin level, and a noncancerous liver were significantly correlated with the BM-VEGFR-1 mRNA level (Table 2).

Patient outcomes

Mortality: By the end of our study, 42 of the HCC patients under analysis had died; 35 from HCC, three from liver failure and four from another malignant disease. The 1-, 2- and 3-year patient survival rates for this cohort were determined to be 92.1%, 85.9% and 78.7%, respectively.

HCC recurrence: HCC recurred in 80 patients (70.2%); in the remnant liver in 48 cases (60%), in the remnant liver and remote tissue in 20 (25%), and in the remote tissue alone in 12 (15%). The 1-, 2- and 3-year DFS rates were 67.5%, 49.8% and 34.4%, respectively. We found a significant tendency for patients who were positive for BM-AFP mRNA to experience recurrence within 1 year of their surgery compared with patients who were negative for this transcript (Table 3).

HCC classification according to VEGFR-1 and AFP mRNA status

Patients were classified into four groups according to the level/status of their BM-VEGFR-1 and BM-AFP mRNA as follows: group A (n = 23), BM-VEGFR-1/BM-AFP mRNA = low/negative; group B (n = 57) high/negative; group C (n = 10) low/positive; group D (n = 24), high/positive. This classification was correlated with disease recurrence within or more than 1 year after surgery. Significantly, in the groups in which patients were negative for BM-AFP mRNA, only three patients (13.0%) experienced recurrence in group A, whereas 17 (29.0%) in group B experienced recurrence within 1 year of surgery (Table 3). Classification of the HCC cases in the current study cohort by their PH-VEGFR-1 and BM-AFP (P = 0.1024), BM-VEGFR-1 and PH-AFP (P = 0.2100), and

Table 1 Characteristics of hepatocellular carcinoma patients according to their bone marrow-α-fetoprotein and peripheral blood-α-fetoprotein mRNA profile

		BM-AFP mRNA		*P* value	PH-AFP mRNA		*P* value
		Positive (*n* = 34)	Negative (*n* = 80)		Positive (*n* = 6)	Negative (*n* = 105)	
Sex	Male	27	67	0.5774	5	86	0.9294
	Female	7	13		1	19	
Age (yr)	≤ 60	19	34	0.1900	5	47	0.0655
	> 60	15	46		1	58	
HBsAg	+	19	30	0.0697	3	45	0.7312
	-	15	50		3	60	
HCV	+	12	34	0.4731	2	43	0.7116
	-	22	46		4	62	
Albumin	≤ 4.0 mg/dL	13	33	0.7641	3	41	0.5937
	> 4.0 mg/dL	21	47		3	64	
Total bilirubin	≤ 0.7 mg/dL	21	45	0.5853	4	60	0.6461
	≥ 0.8 mg/dL	13	35		2	45	
ICGR15	≤ 15%	22	39	0.1181	3	56	0.8736
	> 15%	12	41		3	49	
Anatomical resection	Yes	25	54	0.5231	4	73	0.8826
	No	9	26		2	32	
AFP	≤ 200 ng/mL	21	57	0.3189	1	76	0.0040
	> 200 ng/nL	13	23		5	29	
AFPL3	≤ 15%	21	58	0.2556	2	76	0.0418
	> 15%	13	22		4	29	
PIVKA-Ⅱ	≤ 40 mAU/mL	8	30	0.1477	1	36	0.3732
	> 40 mAU/mL	26	50		5	69	
Tumor number	Solitary	25	57	0.8804	2	78	0.0259
	Multiple	9	22		4	26	
Tumor size	≤ 2 cm	2	10	0.2922	0	12	0.3806
	> 2 cm	32	70		6	93	
Differentiation	Well	0	7	0.0737	0	7	0.5859
	Moderately	26	49		3	70	
	Poorly	7	24		3	27	
vp	Positive	14	18	0.0423	5	25	0.0014
	Negative	20	62		1	80	
vv	Positive	3	4	0.4366	1	5	0.2098
	Negative	31	76		5	100	
im	Positive	12	18	0.1558	4	25	0.0201
	Negative	22	62		2	80	
Noncancerous liver		11	28	0.6833	1	36	0.3501
Liver cirrhosis							
Non liver cirrhosis		23	49		5	66	

BM: Bone marrow; PH: Peripheral blood; AFP: α-fetoprotein; HBsAg: Hepatitis B surface antigen; HCV: Anti-hepatitis C virus antibody; AR: Patients who underwent anatomical resection; ICGR15: Indocyanine green retention rate at 15 min; vp: Microscopic tumor thrombus in the portal vein; vv: Microscopic tumor thrombus in the hepatic vein; im: Microscopic intrahepatic metastasis.

PH-VEGFR-1 and PH-AFP ($P = 0.2138$) mRNA status showed no such correlation. The DFS curve of group A was significantly better than that of group B, C or D ($P = 0.0437$, $P = 0.0325$, $P = 0.0225$, respectively; Figure 3).

Univariate analysis further revealed that age, hepatitis B surface antigen (HBsAg), albumin, AFP, AFPL3, PIVKA-Ⅱ, the number of tumors, tumor size, portal vein invasion, hepatic vein invasion, intrahepatic metastasis, BM-AFP mRNA and classification by BM-VEGFR-1/BM-AFP mRNA are important risk factors for HCC early recurrence (Table 3). Multivariate analysis revealed that albumin ≤ 4.0 mg/dL and positive portal vein invasion were independent risk factors for recurrence within 1 year of surgery. Although BM-AFP mRNA positivity was not a significant factor by multivariate analysis, it was still found to be an important factor in predicting an early recurrence in HCC cases ($P = 0.0761$, Table 4).

DISCUSSION

In our current study, we found a significant tendency for HCC patients who were positive for BM-AFP mRNA to experience disease recurrence within 1 year of surgery. Patients with low BM-VEGFR-1 mRNA and who were negative for BM-AFP mRNA experienced early recurrence in 3/23 cases, whereas in 57 cases with high BM-VEGFR-1 and BM-AFP mRNA, 17 recurrences were observed. Hence, BM-AFP mRNA positivity is an important predictor of early HCC recurrence after curative hepatectomy due to hematogenic spread. BM-VEGFR-1 mRNA was also found to be associated with early HCC recurrence.

The time between hepatectomy and recurrence of metachronous *de novo* tumors is longer than that of intrahepatic metastases[15], therefore, early recurrence of these

Table 2 Characteristics of hepatocellular carcinoma patients according to their bone marrow-vascular endothelial growth factor receptor-1 and peripheral blood-vascular endothelial growth factor receptor-1 mRNA profile

		BM-VEGFR1		*P* value	PH-VEGFR1		*P* value
		High (n = 81)	Low (n = 33)		High (n = 78)	Low (n = 26)	
Sex	Male	67	27	0.9090	64	22	0.7647
	Female	14	6		14	4	
Age (yr)	≤ 60	40	13	0.3322	37	12	0.9097
	> 60	41	20		41	14	
HBsAg	+	35	14	0.9387	34	12	0.8197
	-	46	19		44	14	
HCV	+	36	10	0.1628	32	9	0.5624
	-	45	23		46	17	
Albumin	≤ 4.0 mg/dL	38	8	0.0252	27	12	0.2926
	> 4.0 mg/dL	43	25		51	14	
Total bilirubin	≤ 0.7 mg/dL	48	18	0.6439	51	11	0.0378
	≥ 0.8 mg/dL	33	15		27	15	
ICGR15	≤ 15%	41	20	0.3322	44	13	0.5695
	> 15%	40	13		34	13	
Anatomical resection	Yes	55	24	0.6124	56	18	0.8026
	No	26	9		22	8	
AFP	≤ 200 ng/mL	54	24	0.5278	57	16	0.2653
	> 200 ng/nL	27	9		21	10	
AFPL3	≤ 15%	54	25	0.3399	59	15	0.0802
	> 15%	27	8		19	11	
PIVKA-Ⅱ	≤ 40 mAU/mL	24	14	0.1888	25	10	0.5491
	> 40 mAU/mL	57	19		53	16	
Tumor number	Solitary	53	29	0.0068	55	18	0.8867
	Multiple	28	3		23	7	
Tumor size	≤ 2 cm	9	3	0.7499	10	2	0.4784
	> 2 cm	72	30		68	24	
Differentiation	Well	4	3	0.1151	6	1	0.1614
	Moderately	51	25		56	11	
	Poorly	26	5		16	14	
vp	Positive	26	6	0.1337	21	7	...
	Negative	55	27		57	19	
vv	Positive	6	1	0.3773	4	1	0.7913
	Negative	75	32		74	25	
im	Positive	23	7	0.4296	24	5	0.2559
	Negative	58	26		54	21	
Noncancerous liver cirrhosis		34	5	0.0061	26	9	0.9962
Non liver cirrhosis		45	27		49	17	

BM: Bone marrow; PH: Peripheral blood; AFP: α-fetoprotein; HBsAg: Hepatitis B surface antigen; VEGFR: Vascular endothelial growth factor receptor; HCV: Anti-hepatitis C virus antibody; AR: Patients who underwent anatomical resection; ICGR15: Indocyanine green retention rate at 15 min; vp: Microscopic tumor thrombus in the portal vein; vv: Microscopic tumor thrombus in the hepatic vein; im: Microscopic intrahepatic metastasis.

lesions (within 1 year) is thought to be dependent on hematogenic spread. By real-time quantitative RT-PCR, we found in our current analyses that, although the AFP/GAPDH mRNA ratios in the liver tissues were generally constant among normal control subjects, they were markedly different among HCC patients. This indicated highly variable AFP synthesis activity among individual HCC cells. It has been shown that high AFP mRNA levels reflect the presence of HCC cells[13]. In our present study, the 1-year survival and DFS rates of HCC patients who were positive for AFP mRNA were 86.5% and 54.5%, respectively. Hence, we analyzed the relationship between early recurrence and the preoperative status of the BM- and PH-AFP, and the BM- and PH-VEGFR-1 mRNA.

Although we found in our present experiments that the BM-AFP mRNA status significantly correlates with early HCC recurrence, the BM-VEGFR-1, PH-VEGFR-1 and PH-AFP mRNA levels did not correlate with this outcome. However, classifying the HCC cases in our cohort using the BM-VEGFR-1/BM-AFP mRNA levels showed a correlation with early recurrence (P = 0.0228). Based on these findings, we speculate that the preoperative presence of cancer cells in the bone marrow is an important and essential driver of early HCC recurrence due to hematogenic spread, although we did not detect any changes in the AFP or VEGFR1 mRNA levels in the bone marrow and peripheral blood after surgical intervention in recurrent cases. The importance of the coexistence of disseminated cancer cells and VEGFR-1-positive hematopoietic bone marrow progenitor cells was further supported by the improved DFS curve of HCC patients that were negative for BM-AFP mRNA, and that showed low BM-VEGFR-1 transcript levels as compared with the other three patient groups. On the other hand, we surmised that the relationship between BM-VEGFR-1 mRNA and hematogenic spread in HCC

Table 3 Clinical factors related to early hepatocellular carcinoma recurrence after curative hepatectomy

		Recurrence over 1 year (77)	Recurrence within 1 year (37)	*P* value
Sex	Male	64	30	0.7980
	Female	13	7	
Age (yr)	≤ 60	30	23	0.0200
	> 60	47	14	
HBsAg	+	24	25	0.0002
	-	53	12	
HCV	+	34	12	0.2322
	-	43	25	
Albumin	≤ 4.0 mg/dL	24	22	0.0039
	> 4.0 mg/dL	53	15	
Total bilirubin	≤ 0.7 mg/dL	47	19	0.3266
	≥ 0.8 mg/dL	30	18	
ICGR15	≤ 15%	43	18	0.4708
	> 15%	34	19	
Anatomical resection	Yes	55	24	0.4769
	No	22	13	
AFP	≤ 200 ng/mL	59	19	0.0066
	> 200 ng/nL	18	18	
AFPL3	≤ 15%	58	21	0.0442
	> 15%	19	16	
PIVKA-II	≤ 40 mAU/mL	32	6	0.0072
	> 40 mAU/mL	45	31	
Tumor number	Solitary	62	20	0.0021
	Multiple	14	17	
Tumor size	≤ 2 cm	36	6	0.0016
	> 2 cm	41	31	
Differentiation	Well	7	0	0.1631
	Moderately	52	13	
	Poorly	18	24	
vp	Positive	11	16	< 0.0001
	Negative	66	21	
vv	Positive	2	5	0.0230
	Negative	75	32	
im	Positive	13	17	0.0010
	Negative	64	20	
BM VEGFR1	Low	24	9	0.4506
	High	53	28	
PH VEGFR1	Low	18	8	
	High	54	24	
BM AFP mRNA	Positive	17	17	0.0091
	Negative	60	20	
PH AFP mRNA	Positive	2	4	0.0569
	Negative	74	31	
BM-AFP mRNA/ BM-VEGFR1	Negative/low	20	3	0.0228
	Negative/high	40	17	
	Positive/low	4	6	
	Positive/high	13	11	

BM: Bone marrow; PH: Peripheral blood; AFP: α-fetoprotein; HBsAg: Hepatitis B surface antigen; VEGFR: Vascular endothelial growth factor receptor; HCV: Anti-hepatitis C virus antibody; ICGR15: Indocyanine green retention rate at 15 min; vp: Microscopic tumor thrombus in the portal vein; vv: Microscopic tumor thrombus in the hepatic vein; im: Microscopic intrahepatic metastasis.

during hematogenic recurrence was not stronger than that in gastric cancer, because it has been reported in a clinically relevant and widely used preclinical study model that blockade of VEGFR-1 activity does not affect the formation of spontaneous metastases[16].

In our present study, the BM-VEGFR-1 mRNA level

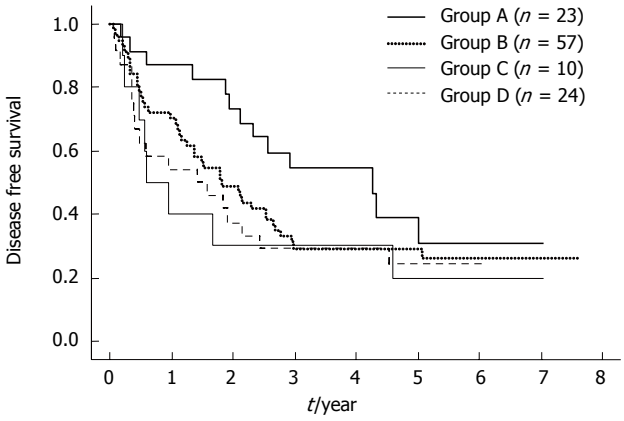

Figure 3 Patients were classified into four groups according to the level/status of their bone marrow-vascular endothelial growth factor receptor-1 and bone marrow-α-fetoprotein mRNA as follows: group A (*n* = 23), bone marrow-vascular endothelial growth factor receptor-1/bone marrow-α-fetoprotein mRNA = low/negative; group B (*n* = 57) high/negative; group C (*n* = 10) low/positive; group D (*n* = 24), high/positive. The disease-free survival (DFS) curve of group A was significantly better than that of groups B, C or D (*P* = 0.0437, *P* = 0.0325, *P* = 0.0225).

of all HCC patients was higher than that in the normal controls, and the PH-VEGFR-1 mRNA levels of almost all of these patients were also higher than in the normal controls. Direct evidence for the role of the chemokine stromal-cell derived factor-1 [SDF-1, also known as chemokine CXC ligand (CXCL)12] in regulating the mobilization of proangiogenic bone marrow cells *in vivo* has been demonstrated by plasma elevation of SDF-1, which stimulates the mobilization of chemokine CXC receptor (CXCR) 4^+ bone marrow cells, including hematopoietic stem cells and endothelial progenitor cells[17,18]. SDF-1 not only promotes revascularization by engaging with CXCR4 expressed on vascular cells but also supports the mobilization of proangiogenic $CXCR4^+$ $VEGFR1^+$ hematopoietic cells[19]. In contrast, Li *et al*[20] have reported a much higher expression level of the CXCL12-CXCR4 axis in HCC specimens than in adjacent, cirrhotic, adenocarcinoma or normal liver tissues. Hence, we speculate that VEGFR-1-positive hematopoietic bone marrow progenitor cells might be regulated and recruited by a mechanism similar to the SDF-1-CXCR4 pathway in most HCC patients. On the basis of our current data and the results of these earlier reports, we further predict that, in almost all patients with HCC, a pre-metastatic niche might have already been initiated by VEGFR-1-positive hematopoietic bone marrow progenitor cells. The levels of BM- and PH-VEGFR-1 mRNA were not found to correlate with early recurrence in each of the HCC patients, although BM-AFP mRNA positivity was significantly associated with early recurrence. These findings thus indicate that the initiation of a pre-metastatic niche be recognized as a first but essential step in the development of metastasis that requires the presence of disseminated cancer cells. This hypothesis is supported by our finding that patients negative for BM-AFP mRNA and with low levels of BM-VEGFR-1 mRNA show the lowest rate of recurrence among all of the groups analyzed.

Table 4 Multivariate analyses of variables that are predictive of early hepatocellular carcinoma recurrence after curative hepatectomy

	P value	Risk ratio	95% CI
Age ≤ 60 yr	0.0899	3.147	0.836-11.838
HBsAg +	0.3601	1.821	0.504-6.571
Albumin ≤ 4.0 mg/dL	0.0038	6.536	1.832-23.256
AFP > 200 ng/nL	0.2571	2.330	0.539-10.067
AFPL3 ≤ 15%	0.4379	1.869	0.385-9.090
PIVKA-II > 40 mAU/mL	0.1494	2.959	0.677-12.987
Tumor number solitary	0.9127	1.088	0.240-4.938
Tumor size > 3 cm	0.1177	3.026	0.756-12.114
vp positive	0.0069	6.639	1.681-26.219
vv positive	0.2221	0.234	0.023-2.408
im positive	0.2307	2.508	0.557-11.289
BM AFP mRNA: positive	0.0761	2.704	0.901-8.113

CI: Confidence interval; BM: Bone marrow; AFP: α-fetoprotein; HBsAg: Hepatitis B surface antigen; vp: Microscopic tumor thrombus in the portal vein; vv: Microscopic tumor thrombus in the hepatic vein; im: Microscopic intrahepatic metastasis.

It has been shown in several previous studies that the detection of micrometastases from solid tumors in bone marrow samples can be an important prognostic indicator with high specificity[3-7,11]. The release of carcinoma cells from the bone marrow into the peripheral blood can be induced by cytokine treatment[21]. Hence, the bone marrow might function as an important reservoir and a source of disseminated cancer cells that can subsequently spread into other organs. Moreover, the bone marrow itself may become altered in response to chemokines produced by the primary tumor and thereby enhance the metastatic capabilities of tumor cells that reside within it[22]. It has been reported that VEGFR-1-positive cells promote tumor adherence and growth[11]. VEGFR signaling is a crucial inducer of angiogenesis, enables primary tumor growth, and probably releases micrometastases from dormancy[23]. In our current study, only the classification by BM-VEGFR-1 and BM-AFP mRNA was correlated with early recurrence. Hence, the coexistence of bone-marrow-derived hematopoietic progenitor cells that express VEGFR-1 in the bone marrow, and not in the peripheral blood, might be advantageous for various cancer cells in the bone marrow in terms of metastasis.

In conclusion, the evaluation of BM-AFP mRNA and BM-VEGFR-1 mRNA in patients with HCC shows great promise as a predictor of recurrence in curatively resected HCC.

ACKNOWLEDGMENTS

The authors wish to thank the staff of the Department of General Surgery, Graduate School of Medicine, Hokkaido University, for their kind cooperation.

COMMENTS

Background

α-fetoprotein (AFP) mRNA, which represents disseminate cancer cells, is related to recurrence of hepatocellular carcinoma (HCC). Bone-marrow-derived hematopoietic progenitor cells that express vascular endothelial growth factor receptor (VEGFR)-1 home to tumor-specific pre-metastatic sites and form cellular clusters before the arrival of tumor cells.

Research frontiers

It has been reported that simultaneous presence of isolated tumor cells and VEGFR-1 expression at pre-metastatic sites is clinically significant for disease progression in gastric cancer. With regard to HCC, there has been no study about the association between the presence of isolated cancer cells and the expression of VEGFR-1. In the present study, we tried to determine whether expression of AFP mRNA and VEGFR-1 in bone marrow and peripheral blood detected by real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) could predict early recurrence in consecutive patients after curative hepatic resection.

Innovations and breakthroughs

There was a significant tendency for patients who were positive for AFP mRNA in bone marrow to experience recurrence within 1 year after surgery compared to those negative for AFP mRNA in bone marrow. The VEGFR-1 mRNA level in bone marrow in all HCC patients was higher than that of normal controls. It was supposed that this initiation of pre-metastatic niche represented by the high level of VEGFR-1mRNA might be recognized as only the first step and as a necessary condition for development of metastasis, and required the subsequent presence of disseminated cancer cells represented by AFP mRNA.

Applications

The evaluation of AFP mRNA and VEGFR-1 mRNA in bone marrow in patients with HCC could be very important for the prediction of recurrence of curatively resected HCC.

Peer review

This study found that the expression of AFP and VEGFR-1 mRNA in bone marrow detected by real-time quantitative RT-PCR predicted early recurrence in consecutive patients after curative hepatic resection. This finding is very important to elucidate the mechanism of metastasis and recurrence by hematogenic spread of HCC cells.

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S- Editor Lv S **L- Editor** Kerr C **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i4.349

World J Gastroenterol 2012 January 28; 18(4): 349-355
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Adenovirus-expressed preS2 antibody inhibits hepatitis B virus infection and hepatic carcinogenesis

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Supported by The National Natural Science Foundation of China, No. 30872998
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Received: April 19, 2011 Revised: June 16, 2011
Accepted: June 23, 2011
Published online: January 28, 2012

Abstract

AIM: To investigate the inhibitory effect of hepatitis B virus (HBV) preS2 antibody (preS2Ab) against HBV infection and HBV-associated hepatic carcinogenesis.

METHODS: An adenoviral vector carrying the full-length light and heavy chains of the HBV preS2Ab gene, Ad315-preS2Ab, was constructed. Enzyme linked immunosorbent assay (ELISA) and Western blotting analyses were used to determine the preS2Ab expression levels *in vitro*. Immunofluorescent techniques were used to examine the binding affinity between the expressed HBV preS2Ab and HBV-positive liver cells. ELISAs were also used to determine hepatitis B surface antigen (HBsAg) levels to assess the inhibitory effect of the preS2Ab against HBV infection in L02 cells. The inhibitory effect of preS2Ab against hepatic carcinogenesis was studied with diethylnitrosamine (DEN)-induced hepatocellular carcinomas (HCCs) in HBV transgenic mice.

RESULTS: The expression of HBV preS2Ab increased with increases in the multiplicity of infection (MOI) of Ad315-preS2Ab in L02 cells, with 350.87 ± 17.37 μg/L of preS2Ab when the MOI was 100 plaque forming units (pfu)/cell. The expressed preS2Abs could recognize liver cells from HBV transgenic mice. ELISA results showed that L02 cells expressing preS2Ab produced less HBsAg after treatment with the serum of HBV patients than parental L02 cells expressing no preS2Ab. HBV transgenic mice treated with Ad315-preS2Ab had fewer and smaller cancerous nodes after induction with DEN than mice treated with a blank Ad315 vector or untreated mice. Additionally, the administration of Ad315-preS2Ab could alleviate hepatic cirrhosis and decrease the serum levels of alanine transaminase and aspartate transaminase.

CONCLUSION: Adenovirus-mediated HBV preS2Ab expression could inhibit HBV infection in L02 cells, and then inhibit DEN-induced hepatocellular carcinogenesis and protect hepatic function in HBV transgenic mice.

Key words: Hepatitis B virus; Adenoviral vector; PreS2 antibody; Hepatocellular carcinoma; Gene therapy

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Zhang Q, Li ZQ, Liu H, Yang JH. Adenovirus-expressed preS2 antibody inhibits hepatitis B virus infection and hepatic carcinogenesis. *World J Gastroenterol* 2012; 18(4): 349-355 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i4/349.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i4.349

INTRODUCTION

Hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) poses a severe threat to human health, and HBV is an important cause of the high HCC incidence in China. The available treatments for recurrent HBV infections in patients include immunization and antiviral therapies. For these therapies, hepatitis B immunoglobulin is administered, which has been proven effective for preventing maternal-infant vertical transmission and HBV recurrence after liver transplantation. However, the immunoglobulin is limited and expensive. Additionally, it has an unsatisfactory neutralization effect and may carry pathogens. Immunoprophylaxis of HBV infection with genetically engineered monoclonal antibodies that are specific to HBV envelope antigens is a promising approach in the clinic. HBV encodes an outer membrane protein, preS2, which binds to polyalbumin and helps the HBV gain entry into liver cells *via* albumin receptors. Blocking preS2 with its antibody may prevent or minimize HBV infection. However, full-length antibodies with large molecular weights have low permeability, and small-molecule antibodies, including Fab, scFv, and dsFv, have relatively low affinity and specificity for antigens.

To overcome these limitations, we have constructed an adenoviral vector carrying the full-length light and heavy chains of the human HBV preS2 antibody (*preS2Ab*) gene and tested its efficacy in the prevention and treatment of HBV-associated HCC. *preS2Ab* gene therapy has the advantages of lower production costs and longer expression durations. The adenoviral vector system can express the humanized HBV preS2Ab with high and stable efficiency, so this system may provide a novel approach for HBV gene therapy and may decrease the incidence of HCC.

MATERIALS AND METHODS

Materials

Nucleic acid synthesis in this study was performed by Shanghai Shenergy Biocolor Bioscience and Technology Company (Shanghai, China). HEK293 and L02 cell lines were purchased from ATCC (Manassas, United States). HBV transgenic Imprinting Control Region (ICR) mice were provided by the Shanghai SLAC Laboratory Animal Center, Chinese Academy of Sciences, (Shanghai, China). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Plasmids were purchased from Microbix Biosystems (Ontario, Canada). The Lipofectamine 2000 reagent is a product of Invitrogen (United States). Restriction endonucleases were from New England Biolabs (Ozyme, France). The enzyme linked immunosorbent assay (ELISA) kit was from R and D Systems (Minneapolis, MN, United States), and diethylnitrosamine (DEN) was from Sigma Chemical Co. (St. Louis, MO, United States).

Construction of adenoviral vector carrying the hepatitis B virus preS2Ab gene

The variable and constant regions of the humanized light and heavy chains of the HBV *preS2Ab* gene were synthesized. A *Bam*HI site was introduced upstream of the heavy chain, and an *Xba*I site was introduced downstream. An *EcoRI* site was introduced upstream of the light chain, and a *SalI* site was introduced downstream. Other restriction sites in the encoding sequence were abolished by same-sense mutations. Next, the light and heavy chains of the *preS2Ab* gene were cloned into the corresponding restriction enzyme sites of pDC315 adenoviral shuttle plasmid. The light and heavy chains were bridged by an internal ribosome entry site (IRES) to yield pDC315-preS2Ab. The pDC315-preS2Ab plasmid was co-transfected with pBHGE3 into HEK293 cells using the Lipofectamine 2000 reagent. Twelve days after transfection, a recombinant adenoviral vector carrying the humanized HBV *preS2Ab* gene, *Ad315-preS2Ab*, was obtained. *Ad315-preS2Ab* was then amplified in HEK293 cells and purified by cesium chloride gradient centrifugation. The recombinant virus titer was determined by TCID50 analysis.

In vitro expression and identification of Ad315-preS2Ab

L02 cells were cultured in 6-well plates for 24 h and then subjected to serum-deprived medium. Ad315-preS2Ab was added for infection according to the multiplicity of infection (MOI) gradient. After 2 h, the cells were cultured with serum-containing medium for 72 h, and the supernatants and cells were collected. ELISA was used to determine the antibody levels in the supernatants; optical density (OD) values (*A* value) were read at 450 nm, and a standard concentration curve was plotted to calculate the antibody levels in the supernatants. The antibody content in the cell lysate solution was determined by Western blotting analysis.

Liver samples from an HBV transgenic ICR mouse and a normal ICR mouse were obtained and made into single-cell suspensions. Cell smears were prepared for immunofluorescent examination using the supernatant of L02 cells infected with Ad315-preS2Ab [MOI = 100 plaque forming units (pfu)/cell] as the primary antibody and FITC-labeled goat anti-human IgG as the secondary antibody. Immunofluorescent labeling was observed under a fluorescence microscope, and photos were taken to assess the binding affinity of the expressed antibody.

Inhibitory effects of Ad315-preS2Ab against hepatitis B virus infection

L02 cells were cultured in 6-well plates for 24 h and then subjected to serum-deprived medium. Ad315-preS2Ab was added for infection at an MOI of 50 pfu/cell, and after 2 h, the cells were cultured with serum-containing medium for 72 h. Sera from HBV patients with HBsAg (+), HBeAg (+) and anti-HBc (+) were collected and

added to L02 cells infected with either Ad315-preS2Ab or the Ad315 blank vector for 7 d. ELISAs were used to determine the HBsAg levels in the supernatants.

Preventive effect of Ad315-preS2Ab against hepatic carcinogenesis

A total of 24 HBV transgenic ICR mice, aged 4 to 6 wk, were evenly divided into 4 groups. Animals in the Ad315-preS2Ab group and Ad315 blank vector group were given the corresponding adenovirus particles *via* tail vein injections; each mouse was injected with 2×10^8 pfu adenoviruses every other day for a total of 5 injections. The total amount for each animal was 1×10^9 pfu. Mice in the non-virus control group and the blank control group were given the same volume of normal saline. DEN was intraperitoneally injected (1 mg/kg, once a week for 4 wk) into animals in the Ad315-preS2Ab, Ad315 vector and non-virus groups one week after the initial injection of adenovirus or normal saline. Mice in the blank control group were not treated with DEN. The animals were sacrificed after 8 mo, and the liver tissues were sectioned at 0.5 cm intervals to observe the diameters of the cancerous nodes. Serial pathological sections were also subjected to hematoxylin/eosin (HE) staining, and all cancerous nodes under a microscope within 5 medium power fields were counted and represented as the mean ± SD.

Protective effects of Ad315-preS2Ab on hepatic function

Sera samples were collected when animals were sacrificed. Hepatic enzyme levels in the sera, including alanine transaminase (ALT) and aspartate transaminase (AST), were measured using an automated biochemical analyzer (SIEMENS ADVIA 2400, Siemens Healthcare Diagnostics, IL, United States).

Statistical analysis

All data are presented as the mean ± SD. Statistical significance was calculated using unpaired Student's *t*-tests. A $P < 0.05$ was considered significant. All analyses were performed using SPSS version 13.0 (SPSS Inc., United States).

RESULTS

Efficient expression of the preS2Ab mediated by Ad315-preS2Ab

TCID50 analysis showed that the titer of Ad315-preS2Ab was 2.1×10^{10} pfu/mL after amplification in HEK293 cells. L02 cells were infected with Ad315-preS2Ab with MOI = 1, 5, 10, 50, and 100 pfu/cell. Seventy-two hours after infection, ELISA and Western blotting results showed that the expression of preS2Ab in cell supernatants or lysates increased with increases in MOI (Figures 1A and B), with a final antibody concentration of 350.87 ± 17.37 ng/mL at an MOI of 100 pfu/cell. The preS2Ab from the supernatant of L02 cells infected with Ad315-preS2Ab (MOI = 100 pfu/cell) recognized liver cells from HBV transgenic ICR mice, as shown by strong immunofluorescent reactions. Liver cells from normal ICR mice demonstrated a lack of immunofluorescent labeling (Figure 1C).

Ad315-preS2Ab-mediated expression of preS2Ab inhibited hepatitis B virus infection

ELISA results showed that, compared with the Ad315 blank vector, Ad315-preS2Ab-mediated preS2Ab expression effectively decreased HBsAg levels in the supernatants of L02 cells treated with the serum of HBV patients. This finding indicated that the preS2Ab could inhibit HBV infection and replication (Figure 2).

Ad315-preS2Ab effectively inhibited diethylnitrosamine -induced hepatic carcinogenesis in HBV transgenic mice

The Ad315 blank vector group and the non-virus group each had one mouse death during the experiment, so the data from 5 mice in each group were analyzed. In the blank control group, there were no cancerous nodes. Slight fibroblastic proliferation and inflammatory cell infiltration were noticed; however, severe hepatic cirrhosis with fibrous septa and pseudolobule formation and cancerous nodes of different numbers and various sizes were found in the non-virus control group after DEN induction. In contrast, only slight-to-moderate hepatic cirrhosis was found in the Ad315-preS2Ab group, and the cancerous nodes were both fewer in number and smaller than those in the non-virus control group (Figure 3).

Ad315-preS2Ab effectively protected hepatic function in hepatitis B virus transgenic mice

To investigate whether the adenovirus Ad315-preS2Ab could protect hepatic function in HBV transgenic mice, the ALT and AST levels in sera were measured to assess hepatic function. Quantitative results for the liver enzyme assays showed that the DEN-treated HBV transgenic mice had higher levels of ALT and AST in sera than the ICR mice that were not treated with DEN. The Ad315 blank vector did not decrease enzyme levels, but 1×10^9 pfu of Ad315-preS2Ab resulted in an obvious decrease of ALT ($P = 0.0141$) and AST ($P = 0.0243$) compared with the DEN-treated control group (Table 1).

DISCUSSION

Globally, there are approximately 300 million HBV carriers. Therefore, it is of great importance to prevent and treat both HBV infection and the subsequent hepatic carcinogenesis. HBV infection is closely associated with the viral proteins. The HBV envelope proteins consist mainly of preS1, preS2 and HBsAg antigens, which can induce production of the corresponding antibodies[1-3]. The HBV preS2 protein is found mainly on tubulose particles and Dane particles, and it is a component of the HBV outer capsid antigen. The preS2 protein possesses stronger antigenicity than the HBsAg; moreover, it also possesses strong antigenic determinants for T and B cells

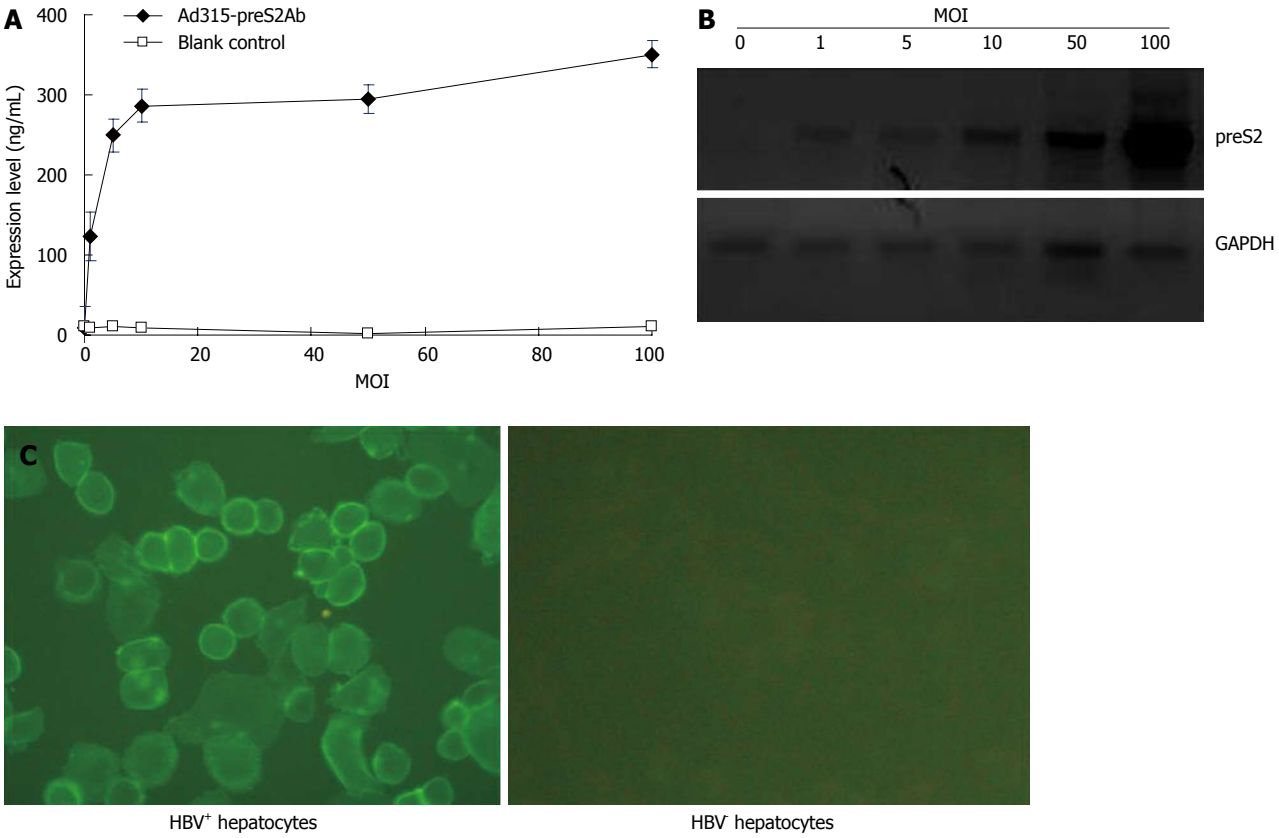

Figure 1 Expression and identification of the Ad315-preS2Ab-mediated preS2Ab. A: ELISA results show that the expression levels of the preS2Ab increase in the supernatants of Ad315-preS2Ab-infected L02 cells with increases in MOI; B: Western blotting shows that the expression of preS2Ab is high at an MOI of 100 pfu/cell. GAPDH is used as the loading control; C: The expressed preS2Ab in the supernatant of Ad315-preS2Ab-infected L02 cells recognizes liver cells from HBV transgenic Imprinting Control Region (ICR) mice as shown by a strong immunofluorescent reaction (left). Liver cells of normal ICR mice do not show labeling (right) (× 200). ELISA: Enzyme linked immunosorbent assay; HBV: Hepatitis B virus; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MOI: Multiplicity of infection.

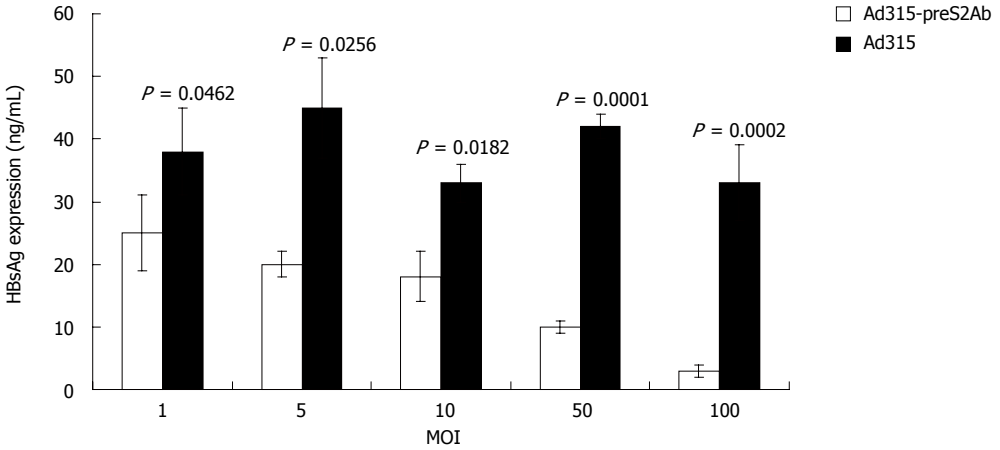

Figure 2 Inhibitory effects of the Ad315-preS2Ab-mediated preS2Ab against hepatitis B virus infection. ELISA results show HBsAg secretion in the supernatants of L02 cells infected with Ad315-preS2Ab. The inhibition of HBV infections increases with increasing Ad315-preS2Ab MOIs, as shown by decreases in HBsAg ($P < 0.05$). Cells infected with the Ad315 blank vector show no inhibitory effects against HBV infection. ELISA: Enzyme linked immunosorbent assay; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; MOI: Multiplicity of infection.

and plays important roles in virus infection, assembly, replication and stimulation of the immune reaction[4,5]. preS2 protein has binding sites for polymerized human serum albumin (pHSA), which possesses determinants for binding with liver cell receptors. Therefore, HBV can enter liver cells by binding to pHSA; this ability is an important reason for the hepatotropism of HBV[6]. It has been found that the strong antigenicity of preS2 can induce immune responses during HBV infection and cause production of the preS2Ab. This antibody can help eliminate HBV and prevent the virus from entering normal liver cells[7]. Therefore, using an antibody against

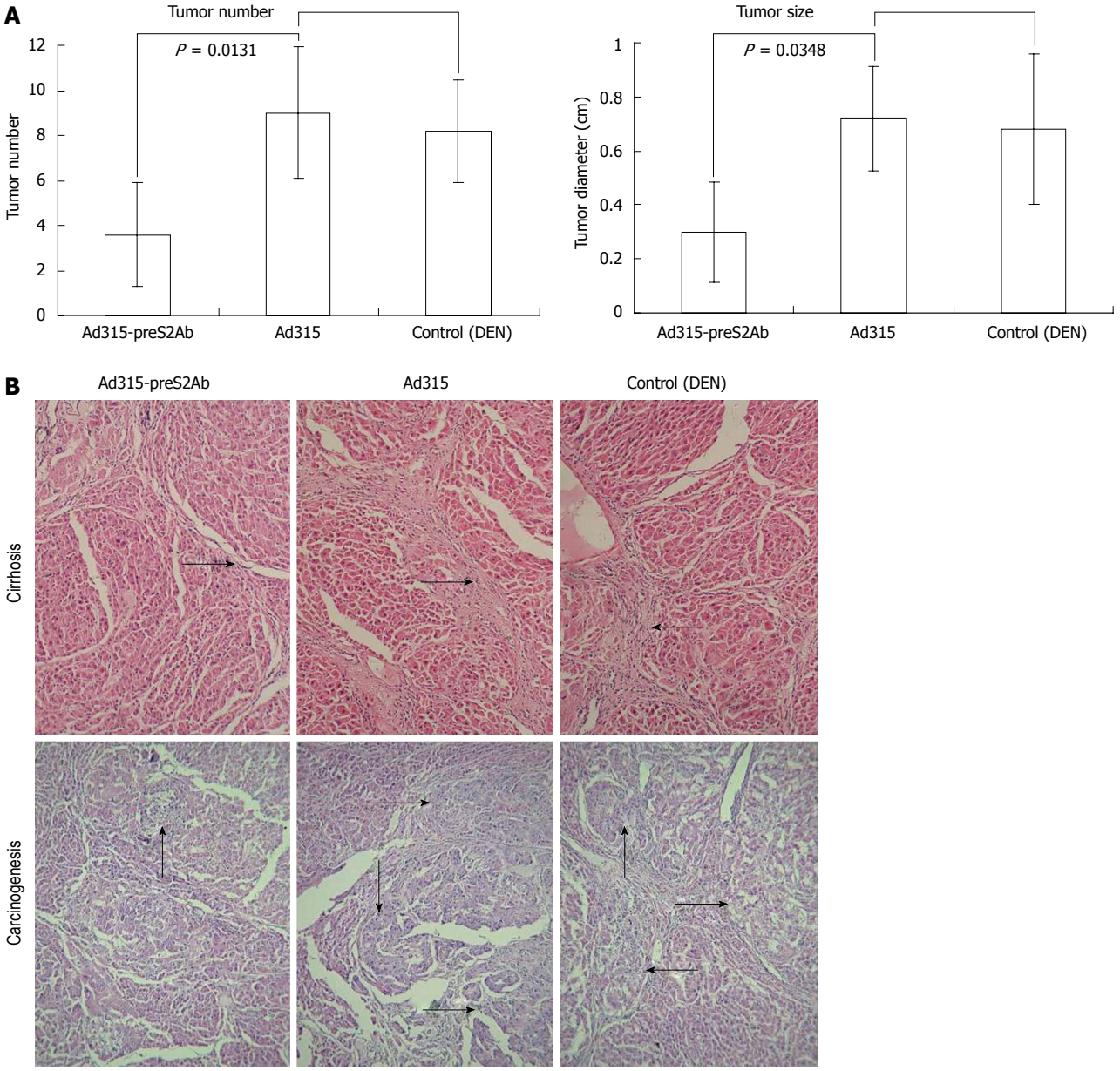

Figure 3 Ad315-preS2Ab effectively inhibited diethylnitrosamine-induced hepatic cirrhosis and carcinogenesis in hepatitis B virus transgenic mice. A: Ad315-preS2Ab-mediated preS2Ab expression demonstrates significant inhibitory effects against diethylnitrosamine-induced hepatic cirrhosis and carcinogenesis in HBV transgenic mice, as shown by significantly fewer (left panel) and smaller cancerous nodes (right panel) ($P < 0.05$); B: Pathological findings show that animals in the Ad315-preS2Ab group have a lesser degree of cirrhosis (upper panel, HE, × 100; arrows: proliferated fibrous septa) as well as fewer and smaller cancerous nodes (lower panel, HE, ×100; arrows: cancerous nodes) compared with the two control groups. HBV: Hepatitis B virus; HE: Hematoxylin and eosin. DEN: Diethylnitrosamine.

Table 1 Serum levels of alanine transaminase and aspartate transaminase in hepatitis B virus transgenic mice

Index	Non-virus control (IU/L)	Blank control (DEN) (IU/L)	Ad315 (IU/L)	Ad315-preS2Ab (IU/L)
Alanine transaminase	47.2 ± 9.4	300.8 ± 76.4	316.4 ± 41.1	167.2 ± 57.4[a]
Aspartate transaminase	118.6 ± 29.5	465.6 ± 156.4	477.4 ± 76.9	239.0 ± 94.7[b]

Data are expressed as the mean ± SD for 5 serum samples in every group. [a]$P = 0.0141$ and [b]$P = 0.0243$ when comparing the treatment samples to the alanine transaminase and aspartate transaminase levels of the diethylnitrosamine -treated blank control group. DEN: Diethylnitrosamine.

the HBV protein to block HBV infection and replication is an important strategy for preventing and treating HBV infection and hepatocellular carcinogenesis.

Hepatitis B immunoglobulin has been shown to be effective in preventing maternal-infant vertical transmission and HBV recurrence after liver transplantation; however, the immunoglobulin has limited sources and is expensive and nonspecific. Additionally, it has an unsatisfactory neutralization effect and may carry pathogens. Artificially-expressed HBsAg antibodies and gene therapy can overcome these shortcomings, indicating a bright future for HBV prevention and treatment. Presently, the best-studied genetically-engineered antibodies

are the small molecular antibodies, which mainly include Fab, ScFv, dsFv and single-domain antibodies. These small antibodies have the advantages of low molecular weight, high permeability, and easy construction and expression[8,9]. However, their low molecular weights also result in a short half-life period *in vivo*, making it difficult for them to reach effective concentrations in the blood. This limitation greatly restricts their clinical applications. Moreover, small molecular antibodies have no Fc segment, which is known to play important roles in the therapeutic effects of antibodies. Currently, there are a dozen monoclonal antibodies which have been used in clinical settings, and even more have been tested in clinical trials[10-12].

To overcome these barriers, we constructed an adenoviral vector carrying the full-length humanized HBV *preS2Ab* gene and examined its expression and inhibitory effects on HBV infection. We also examined the effects of the antibody on the carcinogenesis of HBV-positive liver cells. There are many advantages to gene therapy with adenoviral vectors. The adenoviral particles are stable, and the virus genome rarely undergoes rearrangement, so the inserted genes are kept unchanged after continuous passage of the virus. In addition, the virus genome is easily manageable, and adenovirus can be produced on a large scale. Moreover, adenovirus vectors can infect both dividing and non-dividing cells, and they can be used to transfect pulmonary cells, liver cells, bone cells, blood vessels, muscle cells, and central nervous system cells. Finally, these vectors can be used to achieve high expression of exogenous genes[13-16]. For these reasons, adenoviral vectors have been increasingly used for gene therapy. Additionally, related studies have yielded impressive achievements in China and in other parts of the world. The adenoviral vector constructed by Kim *et al*[17] permanently improved hyperlipidemia in mice, with the induced protein expression lasting for 2.5 years. In a rat hemophilia model, the adenoviral vector constructed by Reddy *et al*[18] continuously expressed factor Ⅷ for more than 9 mo. These findings demonstrate the advantages of adenoviral vectors and indicate a bright future for adenoviral vectors in gene therapy.

In the present study, we successfully constructed an Ad315-preS2Ab vector carrying the full-length *preS2Ab* genes and used this vector to infect L02 liver cells. The production of the *preS2Ab* increased with increases in the MOI of the Ad315-preS2Ab. The expressed preS2Ab recognized liver cells from HBV transgenic ICR mice, as shown by strong immunofluorescent reactions, demonstrating that the Ad315-preS2Ab-mediated preS2Ab possesses a satisfactory binding affinity for the corresponding antigen. We also found that the Ad315-preS2Ab-mediated preS2Ab could efficiently decrease the level of HBsAg in L02 cells infected with the sera of HBV patients, indicating that the expressed *preS2Ab* can inhibit HBV infection and replication. Our *in vivo* study showed that the administration of Ad315-preS2Ab could alleviate hepatic cirrhosis and decrease the number and size of cancerous nodes induced by DEN in HBV transgenic ICR mice, suggesting that Ad315-preS2Ab has an inhibitory effect against hepatocellular carcinogenesis. Our *in vivo* experiment also demonstrated that Ad315-preS2Ab can decrease ALT and AST levels in mouse sera and protect hepatic function in HBV transgenic mice.

In this study, we attempted to establish a complete antibody gene therapy expression system for the prevention and treatment of HBV infection and HBV-associated hepatocellular carcinogenesis. This study will pave the way for gene therapies for HBV infection and HCC.

COMMENTS

Background

Hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) remains a severe threat to human health. Genetically engineered monoclonal antibodies that are specific to HBV envelope antigens are a promising approach for the treatment of recurrent HBV infection. However, full-length antibodies with large molecular weights have low permeability, and small-molecule antibodies have relatively low affinity and specificity for antigens, which affects the efficacy of these antibodies in the clinic.

Research frontiers

An adenoviral vector carrying the HBV preS2 antibody genes can express the humanized HBV preS2 antibody with high and stable efficiency to overcome the limitations of antibody permeability, affinity and specificity.

Innovations and breakthroughs

By encoding the full-length preS2 antibody genes, the Ad315-preS2Ab adenovirus efficiently expressed the HBV preS2 antibody in L02 liver cells, inhibiting HBV infection and replication. Administration of Ad315-preS2Ab could protect hepatic function, alleviate hepatic cirrhosis and suppress hepatocellular carcinogenesis in diethylnitrosamine-treated HBV transgenic mice.

Applications

The preS2 antibody gene therapy may provide a novel approach for inhibiting HBV infection and decreasing the incidence of HCC.

Terminology

HBV encodes an outer membrane protein, preS2, which binds to polyalbumin and helps HBV gain entry into liver cells *via* albumin receptors. This study demonstrates that the adenovirus-expressed preS2 antibody can block preS2, which then prevents or minimizes HBV infection to inhibit HBV-associated hepatocellular carcinogenesis.

Peer review

This is an interesting paper describing the inhibition of HBV infection and subsequent carcinogenesis using a vector carrying the anti-HBV Ab gene.

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S- Editor Zhang SJ **L- Editor** Logan S **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i4.356

World J Gastroenterol 2012 January 28; 18(4): 356-367
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Effects and mechanisms of store-operated calcium channel blockade on hepatic ischemia-reperfusion injury in rats

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Supported by The National Natural Science Foundation of China, No. 30670744 and 81071996; Tsinghua-Yue-Yuen Medical Science Foundation, No. 20240000531 and 20240000547
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Received: May 3, 2011 Revised: July 18, 2011
Accepted: July 25, 2011
Published online: January 28, 2012

Abstract

AIM: To further investigate the important role of store-operated calcium channels (SOCs) in rat hepatocytes and to explore the effects of SOC blockers on hepatic ischemia-reperfusion injury (HIRI).

METHODS: Using freshly isolated hepatocytes from a rat model of HIRI (and controls), we measured cytosolic free Ca^{2+} concentration (by calcium imaging), net Ca^{2+} fluxes (by a non-invasive micro-test technique), the SOC current (I_{SOC}; by whole-cell patch-clamp recording), and taurocholate secretion [by high-performance liquid chromatography and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays].

RESULTS: Ca^{2+} oscillations and net Ca^{2+} fluxes mediated by Ca^{2+} entry *via* SOCs were observed in rat hepatocytes. I_{SOC} was significantly higher in HIRI groups than in controls (57.0 ± 7.5 pA *vs* 31.6 ± 2.7 pA, $P < 0.05$) and was inhibited by La^{3+}. Taurocholate secretion by hepatocytes into culture supernatant was distinctly lower in HIRI hepatocytes than in controls, an effect reversed by SOC blockers.

CONCLUSION: SOCs are pivotal in HIRI. SOC blockers protected against HIRI and assisted the recovery of secretory function in hepatocytes. Thus, they are likely to become a novel class of effective drugs for prevention or therapy of HIRI patients in the future.

Key words: Hepatocyte; Hepatic ischemia-reperfusion injury; Store-operated calcium channel; Non-invasive micro-test technique

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Pan LJ, Zhang ZC, Zhang ZY, Wang WJ, Xu Y, Zhang ZM. Effects and mechanisms of store-operated calcium channel blockade on hepatic ischemia-reperfusion injury in rats. *World J Gastroenterol* 2012; 18(4): 356-367 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i4/356.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i4.356

INTRODUCTION

Hepatic ischemia-reperfusion injury (HIRI) can occur in the liver in response to a wide variety of clinical and operative situations, including hemorrhagic shock, severe hepatic trauma, major hepatic resection/biliary tract operation with temporary clamping of hepatoduodenal ligament, and liver transplantation. HIRI can lead to liver dysfunction (or even loss of function) and thus represents a major therapeutic challenge.

The pathogenesis of HIRI is multifactorial, involv-

ing hepatocellular Ca^{2+} overload[1-5], release of excessive oxygen-derived free radicals[6,7], inflammatory cytokines[8], Kupffer cell activation[9,10], impairment of microvessels[11], apoptosis and nuclear factor kappa B[12]. Ca^{2+} are an important second messenger, and variation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) has been shown to play an important role in regulating a variety of physiological processes in both excitable and non-excitable cells. $[Ca^{2+}]_i$ is a key factor in HIRI because it is integral to the activation of calcium-dependent phospholipases, nucleases and proteases, as well as oxidative phosphorylation. $[Ca^{2+}]_i$ may be increased by the release of Ca^{2+} from the endoplasmic reticulum (ER) (or sarcoplasmic reticulum) or by the stimulation of Ca^{2+} entry from the extracellular space through calcium channels that are voltage-dependent (VDCC), receptor-operated or store-operated[13,14]. Hepatocytes are not known to express VDCC[15-18] but do express store-operated calcium channels (SOCs)[19-21]. The concept of a "store-operated calcium current" was proposed in 1986[22] and has been developed more fully during the past 20 years, including the identification of putative SOC proteins[14]. However, whether SOCs are the dominant Ca^{2+} channel in hepatocytes is uncertain. Studies have found that hepatocellular Ca^{2+} overload plays a crucial role in HIRI, although the exact mechanism of this overload remains unknown, as does the role of SOCs. Thus, no effective drugs currently exist for the prevention or therapy of HIRI.

In the present study, we aim to (1) further verify the importance of SOCs in rat hepatocytes, (2) explore the effects of SOCs in HIRI, and (3) investigate the potential use of SOC blockers for protecting against HIRI.

MATERIALS AND METHODS

Animals and materials

Male Sprague-Dawley rats weighing 180-200 g were obtained from the Beijing Laboratory Animal Research Center (Beijing, China). RPMI 1640 medium, penicillin, streptomycin, Fluo-4-acetoxymethyl (Fluo-4/AM), and poly-D-lysine were purchased from Invitrogen (United States). Collagenase Ⅳ, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), adenosine-triphosphate (ATP), CsOH, Taurocholic acid sodium salt, and SKF-96365 were obtained from Sigma (United States). Ethylene glycol tetraacetic acid (EGTA) was obtained from Solarbio (Beijing, China). Vasopressin, noradrenalin, thapsigargin (TG), and 2-aminoethoxydiphenyl borate (2-APB) were obtained from Merck KcaA (Darmstadt, Germany). Methanol and acetonitrile were of chromatographic grade; all other reagents were of reagent grade.

Fresh isolation of rat hepatocytes

Rat hepatocytes were enzymatically isolated using a modification of the method originally reported by Seglen[23]. Briefly, rats were anesthetized with chloral hydrate (320 mg/kg body weight) and heparinized (1.5 U/g body weight) *via* intraperitoneal injection. A midline laparotomy was performed, and portal vein and the inferior vena cava were cannulated. The liver was initially perfused for 10 min at a constant flow rate of 25-30 mL/min with a modified oxygenated Ca^{2+}-/Mg^{2+}-free Hanks solution containing (in mmol/L) NaCl, 120; KCl, 5; Na_2HPO_4, 0.2; KH_2PO_4, 0.2; $NaHCO_3$, 25; EGTA, 0.5; and glucose, 5 (pH 7.4), followed by perfusion with Type Ⅳ collagenase (0.2 g/L) in RPMI 1640 medium for 10 min. The solution was gassed with 100% O_2 and warmed to 37 ℃. After the perfusions, the three large cephalad lobes of the liver were excised and minced in a Ca^{2+}-/Mg^{2+}-free Hanks solution at 0 ℃, before being filtered through a 74-μm nylon mesh and washed three times by centrifugation at 50 × *g* for 2 min. The isolated hepatocytes (1×10^5/mL; 85%-95% viability assessed by trypan blue exclusion) were incubated in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 ℃ in a 95% air-5% CO_2 incubator for 1 h.

Rat model of hepatic ischemia-reperfusion injury

The rat model of HIRI was established according to our previously reported procedure[10]. Briefly, under anesthesia and heparinization (as above), a midline laparotomy was performed in each rat, and an atraumatic clip was used to interrupt the arterial and portal venous blood supply to the three cephalad lobes. After 20 min of hepatic ischemia, the clip was removed, and the liver was reperfused for a further 40 min. Sham-operated control animals were treated in an identical manner, with the omission of vascular occlusion.

Calcium imaging

Isolated hepatocytes were seeded in 35-mm glass-bottomed dishes (MatTek, Ashland, MA, United States), pretreated with poly-D-lysine (500 μg/mL in borate buffer) for 2 h, and loaded with 6.7 μmol/L fluo-4/AM (Figure 1A) in a recording solution containing (in mmol/L) NaCl, 116; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 0.8; glucose, 5.05; and HEPES, 10 (pH 7.4) for 30 min at 37 ℃, followed by three washes in PBS and a 15-min incubation to allow de-esterification of fluo-4/AM before imaging. A Lambda DG-4 high-speed wavelength switcher (Sutter Instruments, Novato, CA, United States) was used for fluo-4 excitation at 480 nm, and a cooled charge-coupled device (CCD) camera (CoolSnap FX, Roper Scientific, Princeton, NJ, United States) was used for image acquisition. Meta Fluor imaging software (Universal Imaging, Downingtown, PA, United States) was used for hardware control, image acquisition and image analysis. Typically, time-lapse recording of Ca^{2+} signals in hepatocytes was performed for a 2-min control period before and for a 10-min period after the application of the different agonists. The CCD camera was used with a sampling rate of one frame per 2 s, a typical exposure time of 50-350 ms, and a 4 × 4 binning. Quantitative measurements of changes of $[Ca^{2+}]_i$ were obtained by subtracting the average background intensity (measured in cell-free regions) from the average cellular fluo-4 fluorescence intensity values. Changes in $[Ca^{2+}]_i$ for each hepatocyte were then represented by the changes in

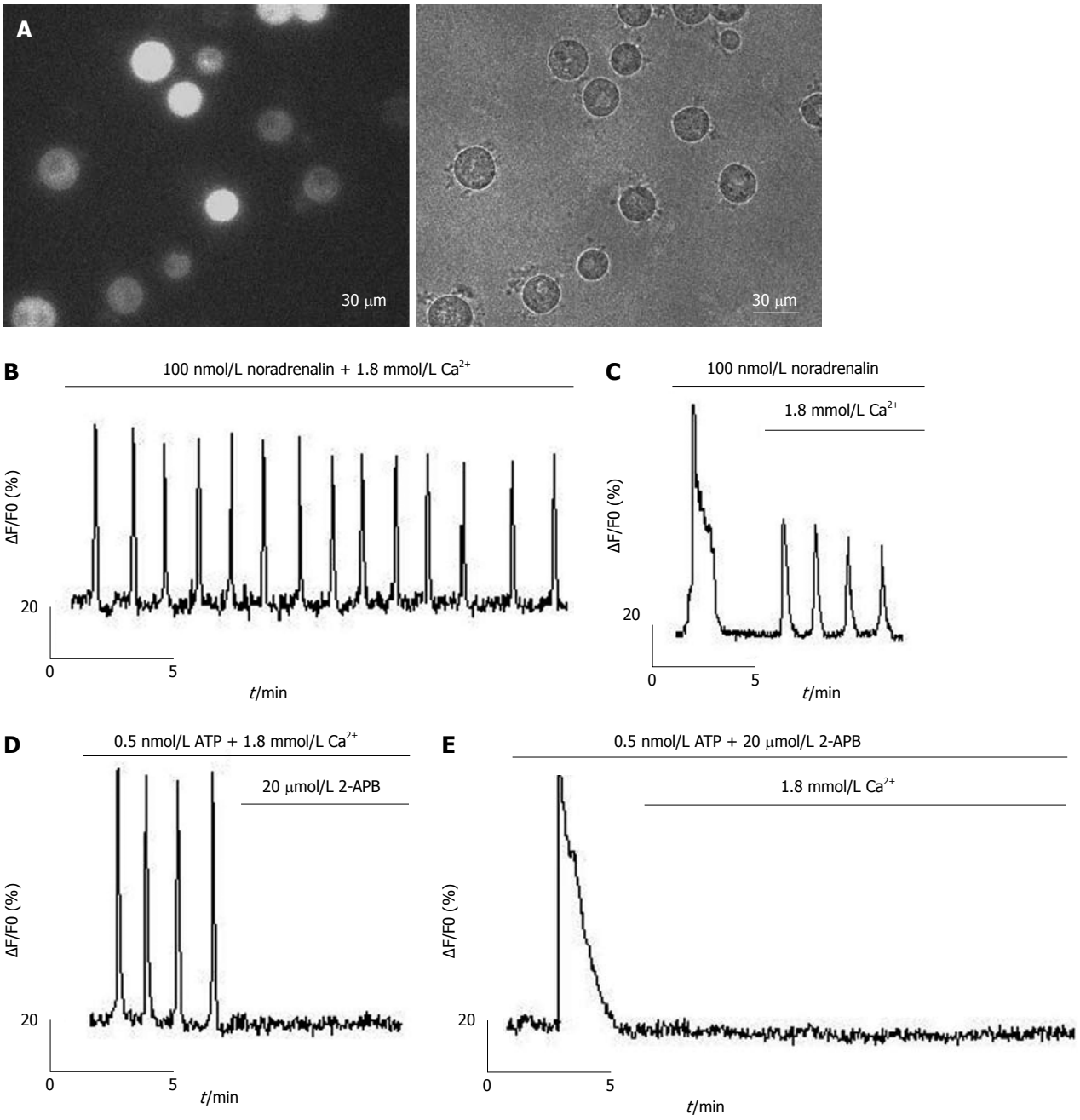

Figure 1 Agonist-induced Ca^{2+} oscillations are inhibited by blocking Ca^{2+} entry in freshly isolated rat hepatocytes. Traces show Ca^{2+} oscillations in freshly isolated rat hepatocytes, with the abscissa axis as time (min), the vertical axis as Fluo-4 fluorescence intensity (ΔF/F0), which demonstrates the changes of [Ca^{2+}]i. The number of hepatocytes measured is indicated by "*n*". A: The fluorescence image (left) and microscopic image (right) of freshly isolated hepatocytes loaded with fluo-4/acetoxymethyl. Ca^{2+} oscillations were initiated by 100 nmol/L noradrenaline in the presence (B, *n* = 27) or absence (C, *n* = 16) of extracellular Ca^{2+}. Ca^{2+} oscillations induced by 0.5 nmol/L adenosine-triphosphate were inhibited by 20 μmol/L 2-APB in the presence (D, *n* = 14) or absence (E, *n* = 11) of extracellular Ca^{2+}. 2-APB: 2-aminoethoxydiphenyl borate.

relative fluo-4 fluorescence ($\Delta F/F0$), where $F0$ was the baseline intensity obtained from the 2-min control period.

Non-invasive micro-test technique measurement

Measurements of net Ca^{2+} influx were performed using the non-invasive micro-test technique (NMT) system (BIO-IM, YoungerUnited States, Amherst, MA, United States) using our previously reported methods[24]. Briefly, isolated hepatocytes plated in a 35-mm dish (Figure 3A) were washed three times with a measuring solution containing (in mmol/L) NaCl, 136; KCl, 2.7; $CaCl_2$, 0.2; KH_2PO_4, 1.5; Na_2HPO_4, 8; and glucose, 5.05 (pH 7.4). The electrode was controlled to move with an excursion of 10 μm at a programmable frequency in the range of 0.3-0.5 Hz, chosen to minimize mixing of the bathing saline. To construct the microelectrodes, borosilicate micropipettes (2-4 μm aperture, XYPG120-2, Xuyue Sci. and Tech. Co., Ltd., Beijing, 100080, China) were silanized with tributylchlorosilane, and the tips were filled with calcium ionophore I-cocktail A (Sigma-Aldrich, St Louis, MO, United States). An Ag/AgCl wire electrode holder (XYEH01-1) was inserted in the back of the electrode to make electri-

cal contact with the electrolyte solution. Only electrodes with Nernstian slopes between 25 and 29 mV/decade were used. Ca^{2+} fluxes were calculated by Fick's law of diffusion: $J_0 = -[D \times (dC/dX)]$, where J_0 represents the net Ca^{2+} flux (in μmol per cm per s), D is the self-diffusion coefficient for Ca^{2+} (in cm^2/s), dC is the difference value of Ca^{2+} concentrations between the two positions, and dX is the 10 μm excursion over which the electrode moved in our experiments. Data and image acquisition, preliminary processing, control of the three-dimensional electrode positioner, and stepper-motor-controlled fine focus of the microscope stage were performed with imFlux® software.

Whole-cell patch-clamp recording

Whole-cell patch-clamp recording was performed at room temperature (22-25 ℃) using a computer-based patch-clamp amplifier (EPC-10, HEKA Electronics, Lambrecht/Pfalz, Germany) and PatchMaster software (HEKA Electronics, Lambrecht/Pfalz, Germany). The isolated hepatocytes were plated in 35-mm dishes and washed with a standard external solution containing (in mmol/L) NaCl, 140; CsCl, 4; $MgCl_2$, 2; $CaCl_2$, 10; glucose, 10; and HEPES, 10 (pH 7.4; adjusted with NaOH). In experiments investigating Ba^{2+} current in rat hepatocytes, the NaCl, $MgCl_2$ and $CaCl_2$ in the external solution were replaced with 30 mmol/L of NaCl and 100 mmol/L of $BaCl_2$. An automatic micropipette puller (Model P-97, Sutter Instruments, Novato, CA, United States) was used to pull the electrodes from the borosilicate glass. The pipette resistance was between 3 and 5 MΩ when filled with the pipette solution containing (in mmol/L) CsCl, 15; Cs glutamate, 135; EGTA, 10; and HEPES, 10 (pH 7.2; adjusted with CsOH). In all whole-cell patch-clamp recording experiments, recording started when the series resistance dropped to below 20 MΩ. After achieving the whole-cell configuration, voltage ramps of 50 ms duration, spanning a range from -100 mV to +100 mV, were immediately delivered from a holding potential of 0 mV every 2 s. Acquired currents were filtered at 2.9 kHz and sampled at 20 kHz. Capacitative currents were determined and compensated automatically by the EPC-10 amplifier. The voltages were corrected for a liquid-junction potential of 17 mV (estimated by JPCalc). The maximum SOC current (I_{SOC}) at -100 mV was applied for statistical analysis.

High-performance liquid chromatography analysis

High-performance liquid chromatography (HPLC) analysis was performed using LC-10A apparatus (Shimizu, Japan) with an Agilent Extend C18 column (416 × 150 mm, 5 μm). To create a fine chromatographic separation of taurocholate, a 60:20 (v/v) mixture of mobile phases A (methanol and acetonitrile 1:1) and B (5 mmol/L KH_2PO_4; pH 3.0) was employed. A constant flow rate of 0.8 mL/min was used for the quantitative determination of the standard solutions and test samples, with a column temperature of 30 ℃, sample size of 10 μL, and detection wavelength of 210 nm. Freshly isolated hepatocytes were plated at 5×10^5/mL in 35-mm dishes at 37 ℃ in a 95% air-5% CO_2 incubator and treated with 100 μmol/L 2-APB, 100 μmol/L La^{3+} or 10 μmol/L SKF-96365 for 12 h. The culture solution was collected and centrifuged at 500 rpm for 3 min. The supernatant was stored frozen at -20 ℃. Supernatant (0.5 mL) was mixed intensively with 0.5 mL of absolute ethyl alcohol, incubated in a 60 ℃ water bath for 3 min, then centrifuged at 3000 rpm for 10 min. Supernatant (0.3 mL) was run through Sep-PAK columns, washed with 10 mL of pure water, and eluted with 3 mL of methanol. This eluent was placed in a water bath (70 ℃), blown dry with nitrogen, and mixed with 3 mL of mobile phase.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The method of Mosmann[25] for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used with modifications for functional studies on cell survival/injury. Freshly isolated rat hepatocytes in 1640 medium were plated at 5×10^4 cells per well in 96-well microtiter plates. After 3 h, various SOC antagonists were added, or 0.025% (v/v) dimethylsulfoxide (DMSO) vehicle was used as control. Cells were cultured after drug exposure for 12 h at 37 ℃ in a 95% air-5% CO_2 incubator, which is sufficient time for evidence of drug-induced cell death, increased cell survival or dampened cell injury to become apparent, as quantified by the generation of the formazan product from the MTT substrate. The number of hepatocytes per microtiter well was proportional to the absorbance of the solubilized formazan. After addition of MTT (5 mg/mL, 20 μL) and incubation for 4 h, the medium was discarded. MTT formazan crystals were then resolubilized with 150 μL DMSO per well and mixed on a microshaker for 10 min. The plate was then read immediately on a scanning multiwell spectrophotometer (Termo Multiscan MK3, Finland) at 490 nm.

Statistical analysis

IGOR Pro 5.01 software (Wavemetrics, Portland, OR, United States) was used to conduct an analysis of whole-cell patch-clamp recording data. All current traces were corrected for leak currents. Mageflux (www.xuyue.net/mageflux) was used to process the data of the NMT. All results are expressed as mean ± SD. Statistical significance of differences between test samples and controls was determined using the Student's *t*-test. *P* values less than 0.05 were considered statistically significant differences.

RESULTS

Ca^{2+} entry mediates Ca^{2+} oscillations in freshly isolated rat hepatocytes

Using calcium imaging, we investigated the relationship between Ca^{2+} entry and Ca^{2+} oscillations in hepatocytes under physiological conditions, with Ca^{2+} oscillations induced by noradrenaline in freshly isolated hepatocytes (Figure 1B). We found that Ca^{2+} oscillations were dependent on Ca^{2+} entry through the plasma membrane (Figure

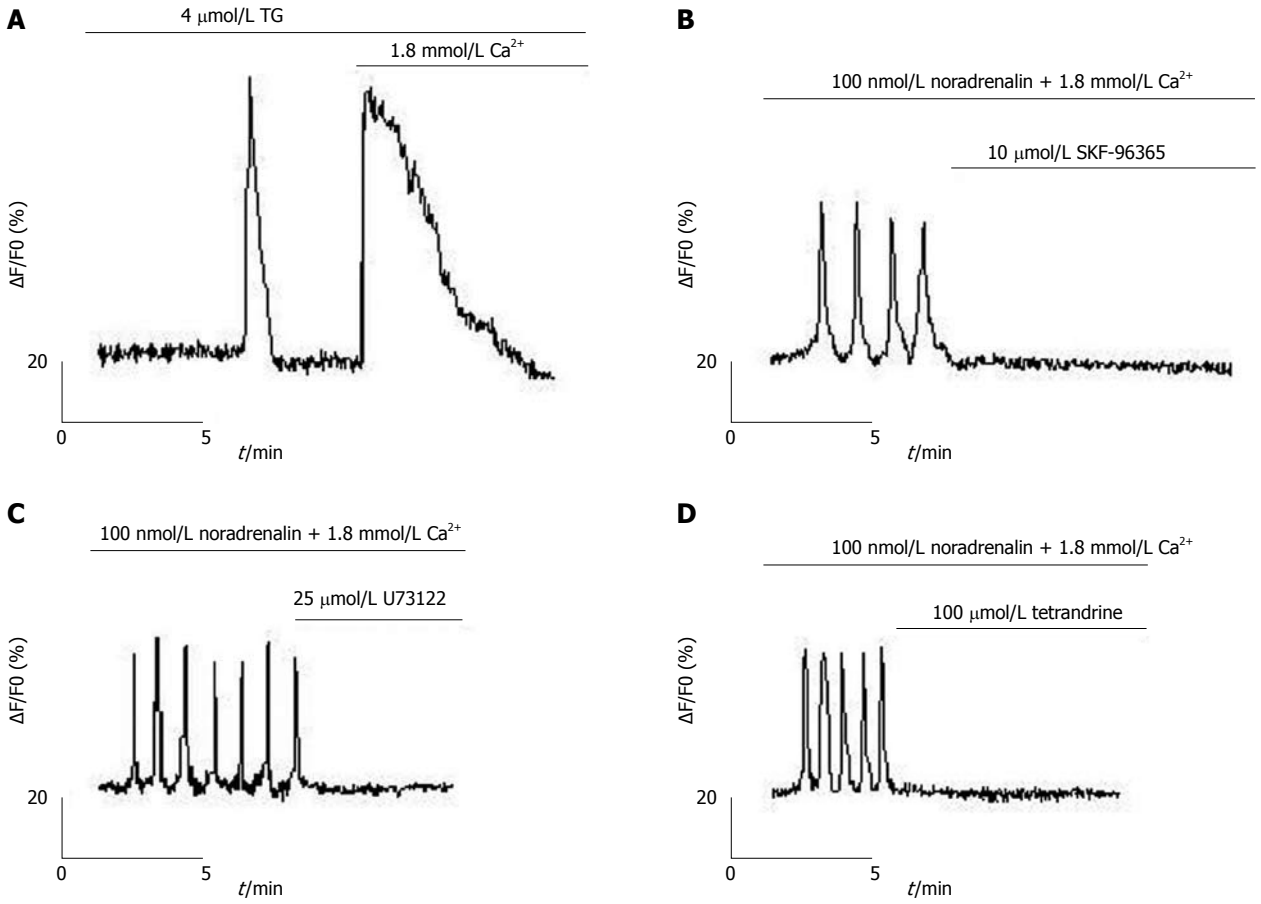

Figure 2 Store-operated calcium channel blockers inhibit Ca^{2+} oscillations of freshly isolated hepatocytes. A: Ca^{2+} stores were depleted with 4 μmol/L thapsigargin (TG), causing two peaks, the first being Ca^{2+} released from endoplasmic reticulum (ER), and the second peak being Ca^{2+} entry through the plasma membrane (n = 16); B: 10 μmol/L SKF-96365 inhibited Ca^{2+} oscillations (n = 12); C: 25 μmol/L U73122 inhibited Ca^{2+} oscillations (n = 10); D: 100 μmol/L tetrandrine inhibited Ca^{2+} oscillations (n = 10).

1C). Ca^{2+} oscillations induced by ATP (activator of phospholipase C) were inhibited by 2-APB[26] (an inhibitor of SOCs, Figure 1D), which inhibited entry of extracellular Ca^{2+} but not release of Ca^{2+} from ER (Figure 1E).

SOCs mediate Ca^{2+} entry in freshly isolated hepatocytes

TG, an inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+} pump (SERCA)[27], was used to passively eliminate hepatocyte ER calcium stores, evoking two peaks (one for the release of Ca^{2+} from the ER and the other for the subsequent Ca^{2+} entry through the plasma membrane; Figure 2A). Ca^{2+} oscillations in freshly isolated rat hepatocytes could be inhibited by 2-APB (Figures 1D and E). Moreover, Ca^{2+} oscillations could also be inhibited by the SOC inhibitor, SKF-96365[28,29] (Figure 2B), the phospholipase C (PLC) inhibitor, U73122[27], and the phospholipase A2 inhibitor, tetrandrine[30,31] (Figures 2C and D).

Ca^{2+} influx in hepatocytes measured by NMT

Using NMT, maximum net Ca^{2+} flux values of 1163 ± 279 nmol cm^{-2} s^{-1} were recorded in freshly isolated hepatocytes (n = 10; Figure 3B). Nifedipine (an inhibitor of VDCCs; 10 μmoL/L) had no effect on net Ca^{2+} fluxes (1108 ± 298 nmol cm^{-2} s^{-1}) (n = 15; Figure 3C). The SOC blockers, SKF-96365, La^{3+} and 2-APB (all at 100 μmol/L), reduced net Ca^{2+} flux to 738 ± 195, 452 ± 136, and 84 ± 37 nmol cm^{-2} s^{-1}, respectively (Figures 3D, E and F). These effects of SOC blockers on net Ca^{2+} fluxes were statistically significant (Figure 3G).

Whole-cell patch-clamp recordings of I_{SOC} in freshly isolated rat hepatocytes

IP_3 (10 μmol/L) and EGTA (10 mmol/L) induced currents in hepatocytes (Figures 4A and C), with a reverse potential of approximately +40 mV. The current-voltage relationship revealed that the currents were inwardly rectifying (Figures 4A, B and C). To further characterize the recorded currents, we substituted Ca^{2+} for Ba^{2+} in the external solution. The Ba^{2+} current (79 ± 7 pA) was significantly greater than the Ca^{2+} current (32 ± 1 pA, t = 5.683, P = 0.002), but much more transient, dropping to a lower level after less than 2 min (Figures 4D and E). The SOC inhibitor, 2-APB (100 mmol/L), inhibited the current induced by 10 mmol/L EGTA (Figure 4F). An inhibitor of non-selective cation channels, A9C (10 μmol/L), did not influence current magnitude (Figure 4G).

SOCs are involved in hepatocellular Ca^{2+} overload in HIRI

Hepatocellular Ca^{2+} overload is thought to play a cru-

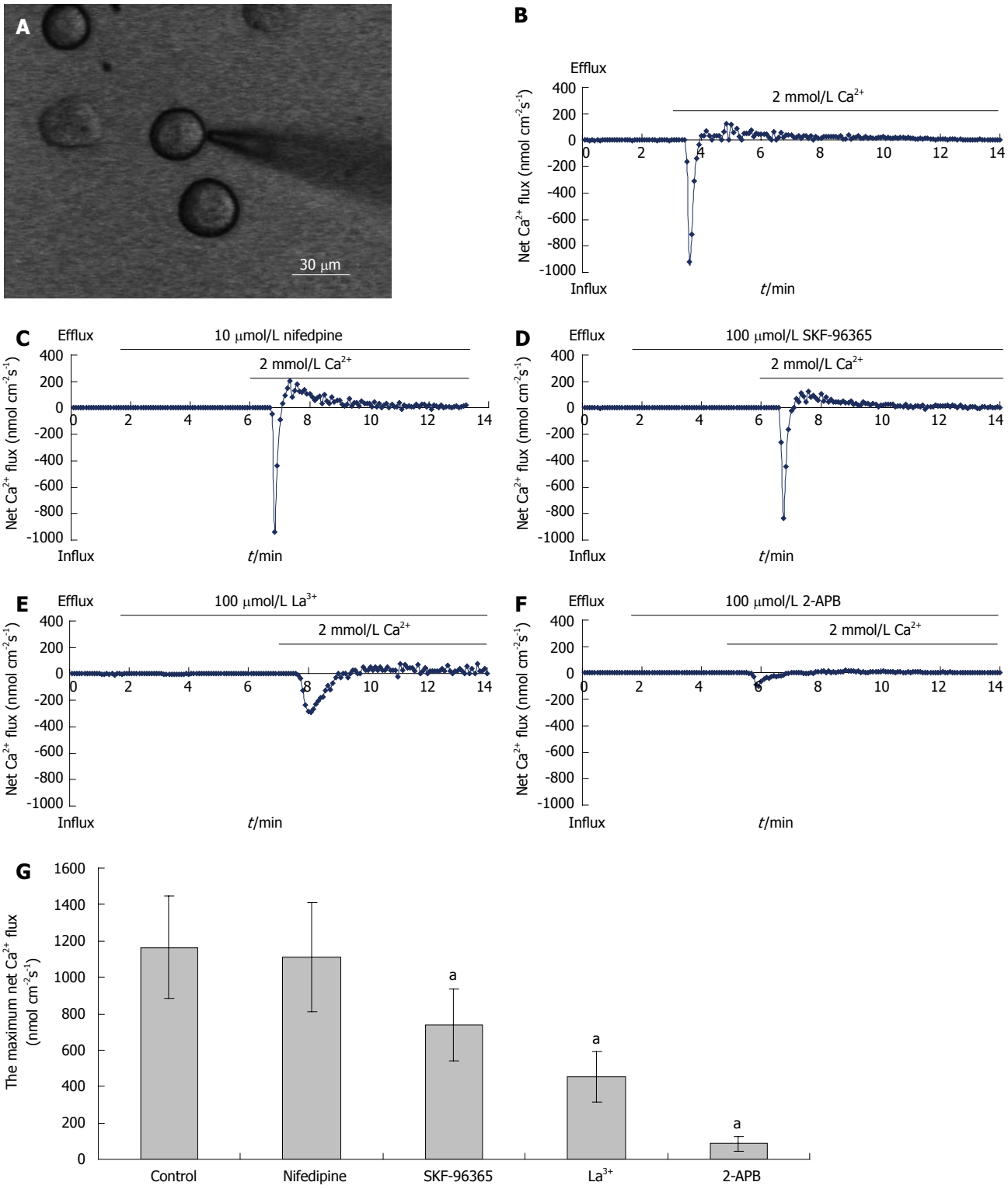

Figure 3 Ca^{2+} flux in freshly isolated hepatocytes. A: A screen-printed picture of a cell measured by NMT; B-F: Net Ca^{2+} fluxes of a freshly isolated rat hepatocyte (B) and repeated in the presence of 10 μmol/L nifedipine (C), 100 μmol/L SKF-96365 (D), 100 μmol/L La^{3+} (E), and 100 μmol/L 2-APB (F); G: Bar graph of the maximum net Ca^{2+} fluxes in five groups. $^{a}P < 0.01$, control group *vs* SKF-96365, La^{3+} or 2-APB group. 2-APB: 2-aminoethoxydiphenyl borate.

cial role in HIRI. To explore the effects of SOCs on the pathogenesis of HIRI, SOC currents induced by 10 mmol/L EGTA in hepatocytes were recorded in the HIRI and control groups (Figures 5A, B and C). The results showed that the SOC currents were significantly increased (from 31.6 ± 2.7 pA in controls to 57.0 ± 7.5 pA in HIRI hepatocytes; $t = 2.682$, $P = 0.036$; Figure 5D). In HIRI hepatocytes, the fully developed inward currents were rapidly reduced by 100 μmol/L La^{3+} (Figures 5E and F).

Evaluation of SOC blocker cytotoxicity

To explore the influence of SOC blockers on cell survival,

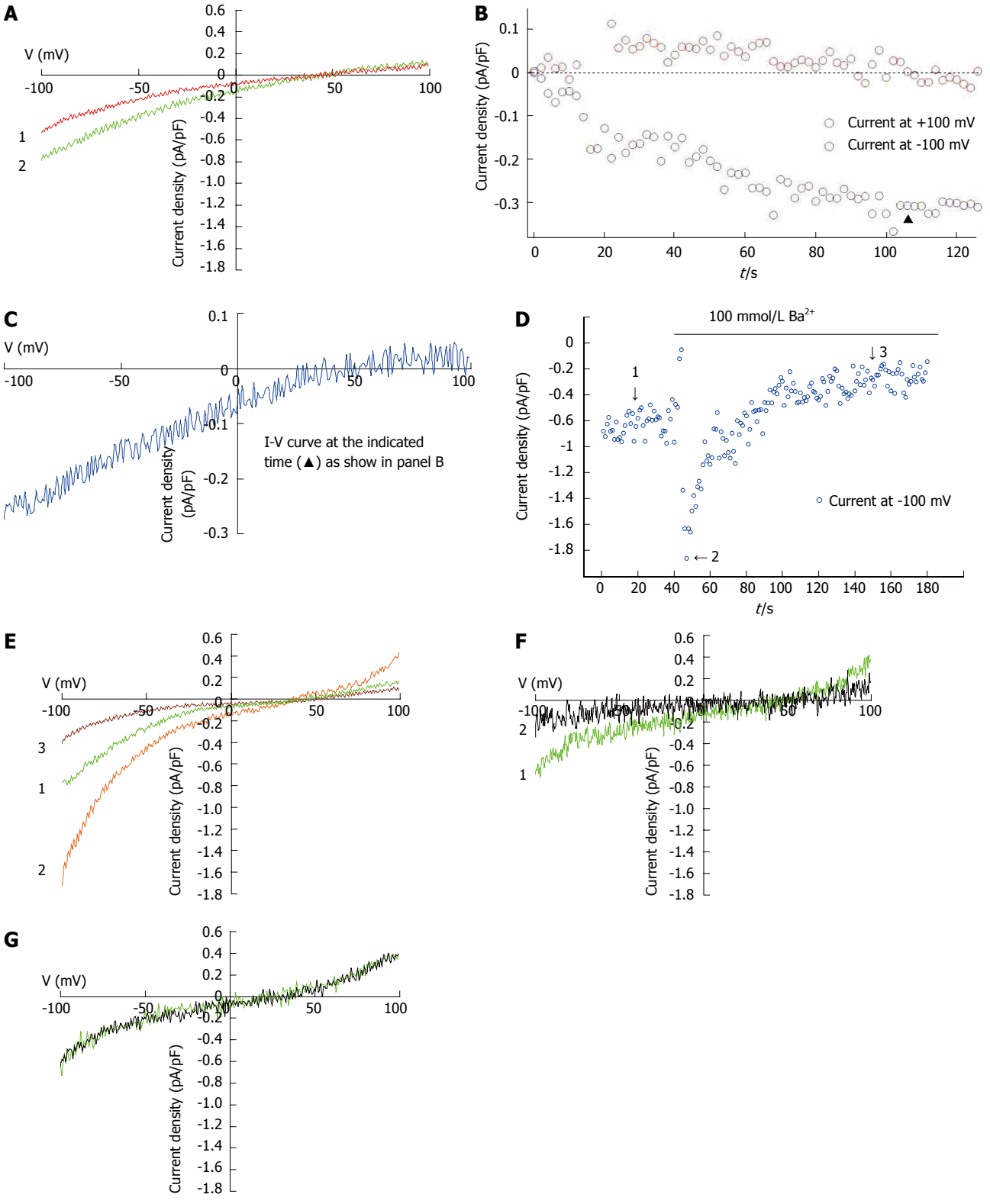

Figure 4 Store-operated calcium currents in freshly isolated hepatocytes. A: I-V curves of I_{SOC} induced by 10 μmol/L IP_3 and 10 mmol/L ethylene glycol tetraacetic acid (EGTA) in hepatocytes, recorded with the voltage ramps from -100 mV to +100 mV, showing the beginning of whole-cell patch-clamp recording (red) and reaching peak current (green) (n = 14); B: Time course of I_{SOC} development, taken at holding potentials of -100 mV and +100 mV for inward and outward currents, respectively; C: I-V curves of I_{SOC} obtained by subtraction of baseline from peak current (the red and green sections of the trace, respectively, as described in A), at the time marked "▲" in B; D: Currents induced by 10 mmol/L EGTA following replacement of Ca^{2+} with Ba^{2+} in the external solution (n = 6); E: I-V curves at the three time points indicated by the arrows in panel D: 1, SOC was fully activated by 10 mmol/L EGTA in the pipette solution, 2, 3, The amplitude of I_{SOC} at -100 mV as a function of time when the external solution was replaced by a solution containing 100 mmol/L Ba^{2+} and no Ca^{2+} or Mg^{2+} for the period indicated in D (n = 6); F: Currents stimulated by 10 mmol/L EGTA. The green trace shows the instantaneous current density-voltage relationship in the range of -100 mV to +100 mV obtained in response to a voltage ramp at the time when inward current was fully developed. The black trace was obtained in response to the presence of 100 μmol/L 2-APB (n = 6); G: Effect of 10 μmol/L A9C on current induced by 10 mmol/L EGTA. The green trace shows the instantaneous current density-voltage relationship in the range of -100 mV to 100 mV obtained in response to a voltage ramp at the time when inward current is fully developed. The black trace was obtained in the presence of 10 μmol/L A9C (n = 5).

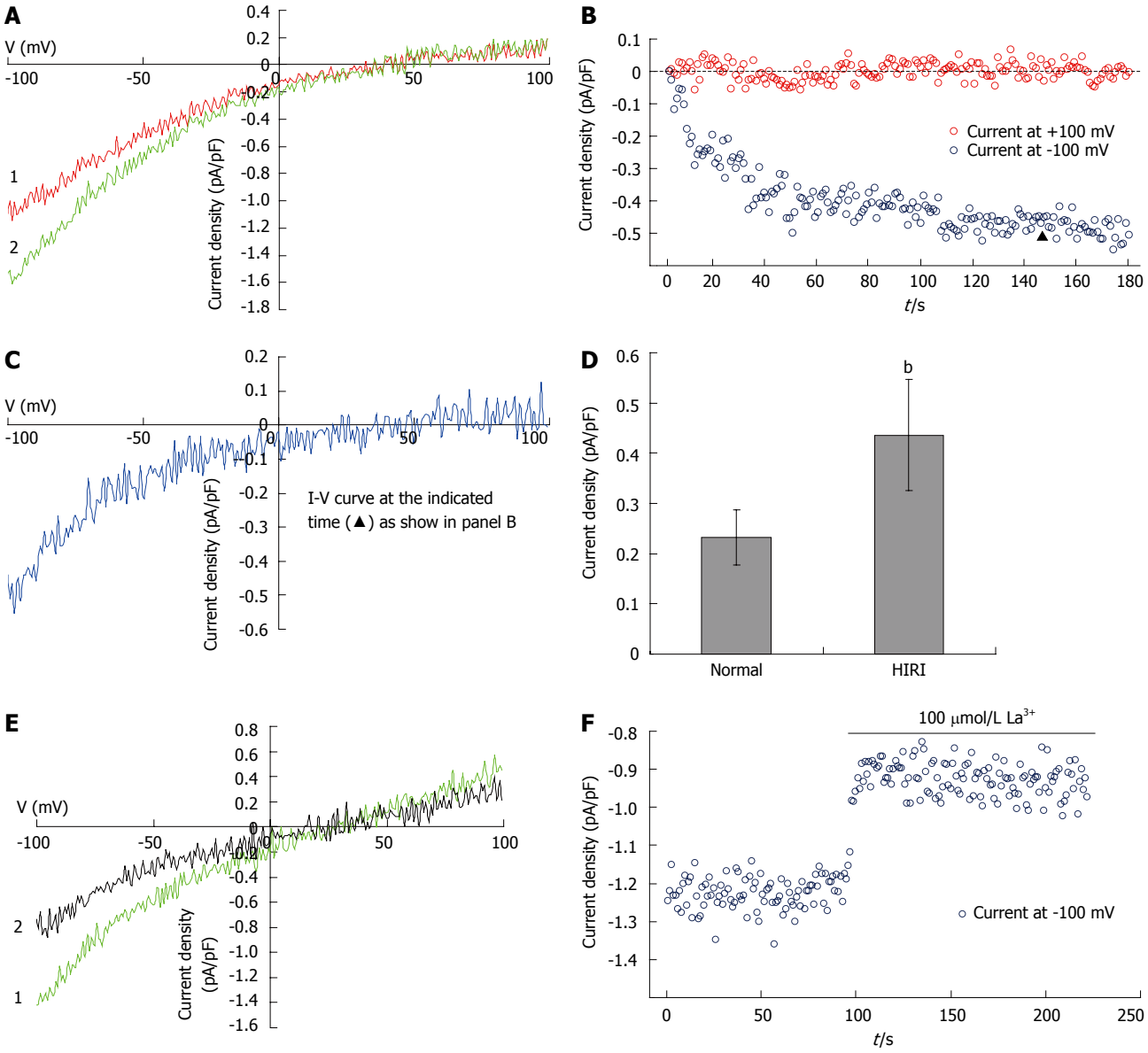

Figure 5 Store-operated calcium channel participates in hepatocellular Ca^{2+} overload in HIRI. A: I-V curves of the I_{SOC} induced by 10 mmol/L ethylene glycol tetraacetic acid (EGTA) in HIRI hepatocytes, recorded with voltage ramps from -100 mV to +100 mV. Red trace shows the beginning of whole-cell patch-clamp recording, the green trace shows the peak current (n = 12); B: The time course for development of I_{SOC} in HIRI hepatocytes (n = 12); C: I-V curves of I_{SOC} obtained by subtraction of baseline from peak current (the red and green sections of the trace, respectively, as described in A) at the time marked "▲" in B; D: Bar graph of the mean current density of maximal I_{SOC} recorded at a holding potential of -100 mV in HIRI hepatocytes (n = 12) and controls (n = 24), $^bP < 0.01$; E: I_{SOC} was recorded in HIRI hepatocytes before (green trace) or after (black trace) the application of 100 μmol/L La^{3+} (n = 7); F: The time course of I_{SOC} development in the presence of 100 μmol/L La^{3+} in HIRI hepatocytes (n = 7). HIRI: Hepatic ischemia-reperfusion injury.

we performed an MTT assay. No significant differences ($P > 0.05$) in cell survival rate (compared with normal hepatocytes) were observed after exposure to the DMSO vehicle or either of the two SOC inhibitors (Figure 6A). In addition, there was a similar survival rate in HIRI hepatocytes compared with hepatocytes pretreated with 0.025% (v/v) DMSO or 100 μmol/L La^{3+}. However, the average optical absorbance value in hepatocytes pretreated with 100 μmol/L 2-APB (0.79 ± 0.05) was higher than in untreated HIRI hepatocytes (0.69 ± 0.04), $P = 0.027$, $t = 4.047$ (Figure 6B). Compared with normal hepatocytes, HIRI hepatocytes incurred cell damage ($P = 0.029$, $t = 2.882$; Figure 6C).

HIRI hepatocytes recover their secretory function in the presence of SOC inhibitors

To explore the influence of SOCs on the secretory function of hepatocytes, taurocholate secretion by hepatocytes was investigated using HPLC analysis. Taurocholate concentration in the culture supernatant of normal hepatocytes was 38.58 ± 7.35 μg/mL (n = 6), notably higher than in hepatocytes treated with 100 μmol/L 2-APB (28.85 ± 8.18 μg/mL; $P < 0.05$, n = 6), 100 μmol/L La^{3+} (27.76 ± 9.86 μg/mL; $P < 0.05$, n = 6) or in HIRI hepatocytes (19.9 ± 3.8 μg/mL; $P < 0.05$, n = 6) (Figure 7). Interestingly, 100 μmol/L2-APB, 100 μmol/L La^{3+} and 10 μmol/L SKF-96365 could reverse the taurocholate level

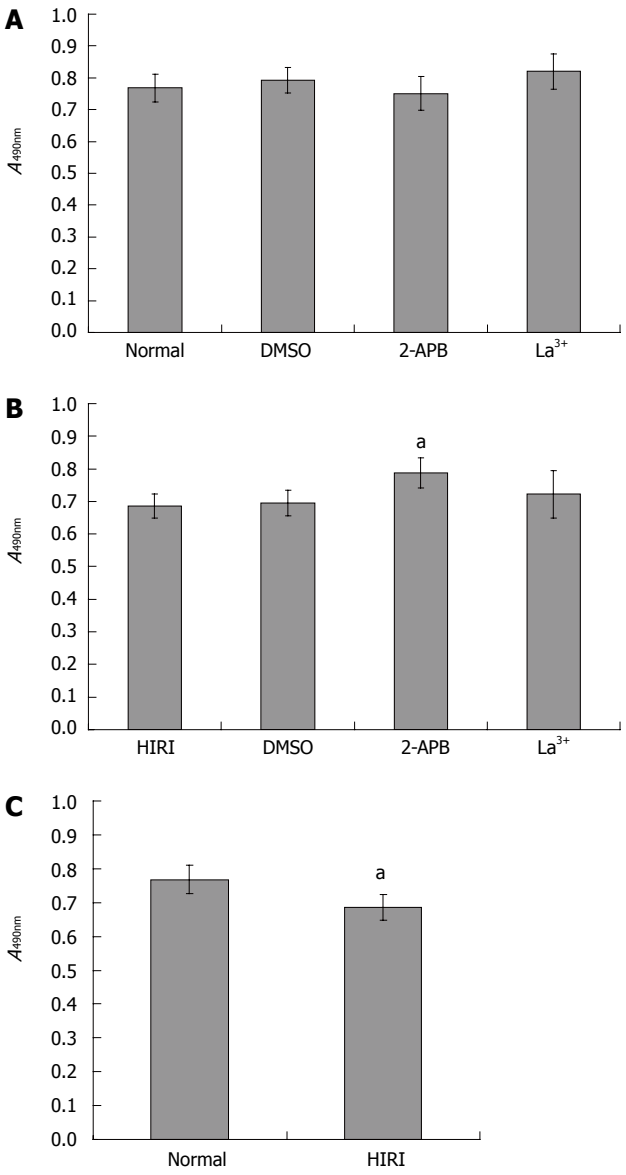

Figure 6 Effects of store-operated calcium channel blockers on cell survival or injuries of normal and hepatic ischemia-reperfusion injuried hepatocytes. A: MTT assay of normal hepatocytes alone or co-cultivated with 0.025% (v/v) DMSO, 100 μmol/L 2-APB, or 100 μmol/L La^{3+} (n = 4), the cell survival rate is not significantly different (P > 0.05); B: MTT assay of HIRI hepatocytes alone or cocultivated with 0.025% (v/v) DMSO, 100 μmol/L 2-APB or 100 μmol/L La^{3+} (n = 4), the average optical absorbance value in hepatocytes pretreated with 100 μmol/L 2-APB (0.79 ± 0.05) was higher than in untreated hepatocytes (0.69 ± 0.04), ^{a}P = 0.027; C: Compared with normal hepatocytes, HIRI hepatocytes incurred cell damage (n = 4), ^{a}P = 0.029. HIRI: Hepatic ischemia-reperfusion injury; 2-APB: 2-aminoethoxydiphenyl borate; DMSO: Dimethylsulfoxide; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

to 27.42 ± 4.74, 26.58 ± 6.67 and 25.52 ± 7.30 μg/mL, respectively, in HIRI hepatocytes, $P < 0.05$, $n = 6$.

DISCUSSION

Ca^{2+} is an important second messenger, and intracellular Ca^{2+} has been shown to play an important role in regulating a variety of physiological processes in both excitable and non-excitable cells. Intracellular Ca^{2+} homoeostasis and signaling are achieved by the complex interplay of Ca^{2+} fluxes among the cytosol, the intracellular stores and the extracellular environment[32]. SOCs are a family of Ca^{2+}-permeable ion channels expressed by most cells. The signal for the activation of SOCs is a decrease in the Ca^{2+} concentration in the ER. Stimulation of a diverse range of plasma membrane receptors converges on and activates phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis by PLC, which results in the generation of diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) and the subsequent activation of (1) Ca^{2+} release from the ER *via* IP_3 receptors and (2) Ca^{2+} influx across the plasma membrane[33]; Stromal interaction molecule 1 (STIM1, identified as the ER Ca^{2+} sensor) and Orai1 (a pore-forming subunit of SOCs) have both been shown to be necessary for SOC function[33,34]. However, the role of SOCs during physiological activation of primary cells has not been extensively investigated, and there is little information on the roles of STIM and Orai proteins in primary cells. Our previous study found that functional interactions among STIM1, Orai1 and TRPC1 (transient receptor potential canonical 1) contribute to activating I_{SOC} in human liver cells[35], but whether SOCs are the dominant Ca^{2+} channels in hepatocytes is still uncertain, and the nature of the Ca^{2+} influx mechanism following hormone activation of hepatocytes is controversial. For this purpose, we aimed to investigate the relationship between Ca^{2+} entry and Ca^{2+} oscillations in hepatocytes under physiological conditions and found that Ca^{2+} oscillations could be induced with noradrenaline, vasopressin or ATP, each of which stimulates PLC and activates both Ca^{2+} influx and intracellular Ca^{2+} release.

NMT is a novel non-invasive technology for obtaining dynamic information on specific ionic/molecular activities on material surfaces. This technique incorporates various temporal and spatial resolution domains from other traditional methods, and its three-dimensional measurement capability enables us to observe the physiological characteristics of biological phenomena that would be difficult or even impossible with other techniques[36]. To date, Ca^{2+}, H^+, K^+, Cl^-, NO^-, Mg^{2+}, Cd^{2+}, Al^{3+}, and O_2 have been detected as sensors for ionic/molecular species. Our previous study found that NMT was a powerful tool for ion channel research, allowing us to demonstrate the existence of TRPC1-dependent Ca^{2+} channels in HL-7702 cells[24]. In the present study, we used NMT to investigate net Ca^{2+} fluxes in freshly isolated hepatocytes and showed that SOC inhibitors (2-APB, SKF-96365 and La^{3+}), but not a VDCC inhibitor (nifedipine), blocked net Ca^{2+} fluxes, suggesting that these Ca^{2+} movements are mediated in rat hepatocytes by SOCs and not by VDCC.

To further explore the importance of SOCs in rat hepatocytes, we used calcium imaging and whole-cell patch-clamp recording techniques to separately measure the cytoplasmic-free Ca^{2+} concentrations and the SOC-mediated currents in rat hepatocytes. An increase in cytoplasmic free Ca^{2+} could be induced by TG-mediated passive depletion of ER calcium stores and subsequent Ca^{2+} entry through the plasma membrane. The Ca^{2+} oscillations could be antagonized by inhibitors of SOCs

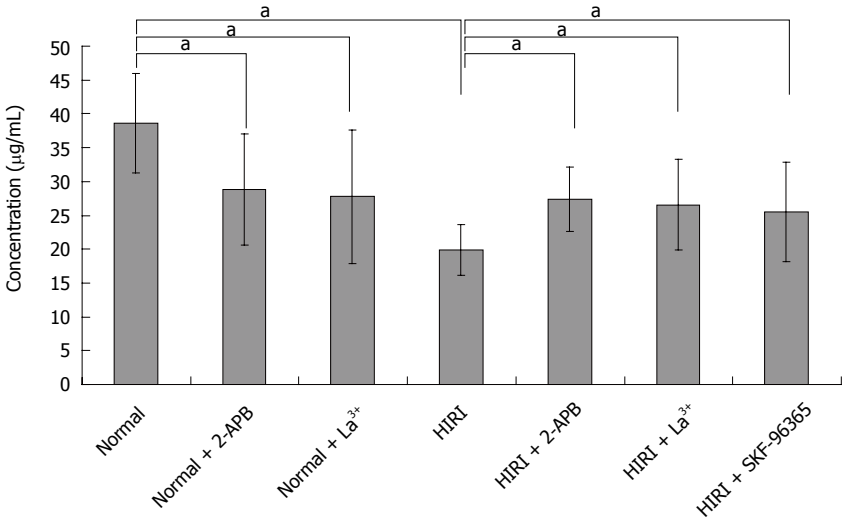

Figure 7 Taurocholate measurements in hepatic ischemia-reperfusion injuried hepatocytes. Bar graph of taurocholate secreted by hepatocytes is distinctly higher in the supernatant of cultured normal hepatocytes (38.58 ± 7.35 μg/mL, n = 6) than for cells after exposure to 100 μmol/L 2-APB (28.85 ± 8.18 μg/mL, $^aP < 0.05$, n = 6), 100 μmol/L La^{3+} (27.76 ± 9.86 μg/mL, $^aP < 0.05$, n = 6) or HIRI hepatocytes (19.92 ± 3.75 μg/mL, $^aP < 0.05$, n = 6). Whereas 100 μmol/L 2-APB, 100 μmol/L La^{3+} and 10 μmol/L SKF-96365 could respectively reverse the taurocholate level to 27.42 ± 4.74, 26.58 ± 6.67 and 25.52 ± 7.30 μg/mL in HIRI hepatocytes, $^aP < 0.05$, n = 6. HIRI: Hepatic ischemia-reperfusion injury; 2-APB: 2-aminoethoxydiphenyl borate.

(2-APB, SKF-96365), PLC (U73122) and phospholipase A_2 (tetrandrine). Tetrandrine, commonly used to inhibit activation of phospholipase A_2 by receptors, is a potent blocker of I_{SOC} in H4IIE cells. We recorded prominent inwardly rectifying currents that could be inhibited by 2-APB. A transient increase in inward current was observed when 100 mmol/L Ba^{2+} was applied to the bath (Figure 4D) which had similar amplitude and time-course properties to those of inward currents activated by IP_3- or TG-mediated depletion of intracellular Ca^{2+} stores. Such changes in the presence of Ba^{2+} have previously been shown for I_{SOC} in H4IIE liver cells[37]. These results are consistent with the properties of SOCs and, thus, strongly implicate the importance of SOCs in rat hepatocytes.

HIRI can occur during hemorrhagic shock or hepatic surgery, including trauma, tumor resection and transplantation. For the purpose of exploring the mechanism of HIRI in hepatocytes and searching for novel and clinically effective therapies, the investigation was based on validating the effects of SOCs on the pathogenesis of HIRI. A rat model of HIRI described previously by Yoshiyuki Yabe[38] that closely reflects the clinical condition was modified and established[10]. In these hepatocytes, SOC currents were significantly increased relative to controls, suggesting that SOCs could be involved in hepatocellular Ca^{2+} overload in HIRI.

The main function of hepatocytes is to synthesize and secrete bile acid; hence, we measured taurocholate secretion as an indicator of the ability of SOC inhibitors to protect and restore hepatocyte function. Taurocholate secretion in normal hepatocytes was significantly reduced in the presence of 100 μmol/L 2-APB and 100 μmol/L La^{3+}, consistent with results from previous experiments[39]. Taurocholate secretion was also reduced in HIRI hepatocytes compared with normal cells. In these cells, non-toxic concentrations (as assessed by MTT assay) of 2-APB (100 μmol/L) or La^{3+} (100 μmol/L) actually reversed this suppression of taurocholate secretion, suggesting that inhibition of SOCs may have beneficial effects on HIRI hepatocyte health and function. Indeed, a concentration of 100 μmol/L 2-APB is in agreement with a previous report[40] that found that 2-APB is (1) effective in preventing HIRI *in vivo* when administered *via* the portal vein before ischemia and (2) able to attenuate HIRI when administered following an ischemic event. Thus, 2-APB could be a potential therapy for HIRI and offers a reduced side-effect profile compared with La^{3+}, a nonspecific blocker of SOCs that also inhibits other types of Ca^{2+} channels on hepatocytes, which is consistent with previous findings by Nathanson *et al*[41]. Concerns about the safety of lanthanides have not yet been completely resolved, and drugs with greater specificity for SOCs are required if a blocker of SOCs is to be considered a viable therapeutic target for HIRI.

The physiological process of bile secretion has been studied extensively. It is believed that natural bile acids exist in the plasma which are taken up actively and concentrated, then secreted, at the biliary pole of the hepatocyte. The mechanisms whereby SOC inhibitors reversed the restraint of taurocholate secretion during HIRI in hepatocytes were determined by regulating the process of hepatic secretion, which is closely related to cytosolic Ca^{2+}. Hepatocytes, as classic epithelial cells, are highly polarized, with transport directed from the sinusoidal or basolateral domain of the cell to the canalicular or apical domain in the physiological process of bile secretion. There is evidence that taurolithocholate and lithocholate increase the cytosolic Ca^{2+} concentration and, meanwhile, inhibit bile secretion[42].

HIRI that occurs in liver surgery, which can be caused by hepatic vascular clamping during partial hepatectomy or liver transplantation, frequently results in cellular dam-

age and organ dysfunction. Although considerable investigation has provided insight into these processes of HIRI in the liver, the exact mechanisms remain only partially elucidated. Intracellular signaling pathways that have been identified as participating in the complex pathophysiological process correlating with cell necrosis and apoptosis involve: the release of multiple bioactive substances, such as cytokines, platelet activating factor (PAF), and free radicals; Ca^{2+}-mediated intracellular Ca^{2+} overload; sinusoidal endothelial cells; Kupffer cells; and cholangiocytes. Among the significant factors is cytosolic Ca^{2+}[43]. Considering that the increase of cytosolic Ca^{2+} appears in the earlier period of intracellular cascade of HIRI[44], the SOC blockers may be effective protective therapies for this clinical problem.

In our experimental results, we found that the sequence of taurocholate secretion seems to be: normal hepatocytes > normal hepatocytes + inhibitors of SOCs > HIRI hepatocytes + inhibitors of SOCs > HIRI hepatocytes. It is known that cellular secretion can not take place without the participation of Ca^{2+}; accordingly, combined with the observation above, we classified the influential factors of cellular secretion roughly into two parts: SOCs and some unknown factors related to Ca^{2+} overload. Owing to blockage of Ca^{2+} influx induced by SOCs, taurocholate secretion was decreased in both normal and HIRI hepatocytes, which is consistent with earlier published papers by other researchers. However, there are unknown factors other than the inhibition of the Ca^{2+} channels that could induce Ca^{2+} overload; thus, it is reasonable that the secretion of HIRI hepatocytes pretreated with 2-APB was lower than normal hepatocytes. We concluded that HIRI hepatocytes recover part of their secretory function in the presence of SOC blockers.

Taken together, our results confirm the important role of SOCs in rat hepatocytes and point toward the effects of SOCs on HIRI. SOC inhibitors could protect against HIRI and are helpful in the recovery of secretory function in hepatocytes. We conclude that SOCs play a vital role in the pathogenesis of HIRI and that SOC blockers could represent a novel class of drugs for the prevention or therapy of HIRI.

COMMENTS

Background

Hepatic ischemia-reperfusion injury (HIRI) can occur in the liver in a wide variety of clinical and operative situations. The pathogenesis of HIRI is multifactorial, such as in hepatocellular Ca^{2+} overload. Variation of $[Ca^{2+}]_i$ has been shown to play a significant role. $[Ca^{2+}]_i$ may be increased by releasing Ca^{2+} from the endoplasmic reticulum (ER) or the sarcoplasmic reticulum (SR), or by stimulating Ca^{2+} entry from the extracellular space through calcium channels. The concept of a "store-operated calcium current" was proposed in 1986, and store-operated calcium channels (SOCs) may play an important role in HIRI.

Research frontiers

SOCs have been verified as Ca^{2+} channels in that the amount of Ca^{2+} in the stores controls the extent of Ca^{2+} influx in non-excitable cells. However, the role of SOCs in rat hepatocytes has not been elucidated. In this study, the authors have further confirmed the important role of SOCs in rat hepatocytes and the pivotal role of SOCs in HIRI. SOC blockers assisted the recovery of secretory function in HIRI hepatocytes.

Innovations and breakthroughs

This is the first study that has used multiple experimental techniques to investigate the important role of SOCs in rat hepatocytes from various perspectives, particularly with regard to pathogenesis of HIRI on the cellular electrophysiological level and on the clinical research level.

Applications

Cytoplasmic Ca^{2+} overload could result in injury of liver cells. By understanding the role of SOCs in HIRI, this research will contribute to clarifying the exact mechanism of hepatocellular Ca^{2+} overload. This study might indicate a novel class of effective drugs targeted at Ca^{2+} channels in hepatocytes for the prevention or therapy of HIRI.

Terminology

SOCs are a family of Ca^{2+}-permeable ion channels expressed by most cells. The signal for the activation of SOCs is a decrease in the $[Ca^{2+}]_i$ in the ER Ca^{2+} store, and this is believed to be an essential and ubiquitous component of Ca^{2+}-signaling pathways. Stimulation of a diverse range of plasma membrane receptors converges on and activates phosphatidylinositol 4,5-bisphosphate hydrolysis by phospholipase C and results in the generation of diacylglycerol and IP_3, which induces the subsequent activation of Ca^{2+} release from the ER *via* IP_3 receptors and Ca^{2+} influx across the plasma membrane. STIM1, identified as the ER Ca^{2+} sensor, and Orai1, as a pore-forming subunit of SOCs, have both been shown to be necessary for SOC function.

Peer review

This is a well conducted study with a clear objective and the data reflects the quality of the work by these investigators. Significance of the data from the study complies with the background objectives. All sections including Materials and Methods, Results, Discussion and References conform well to the style of the journal.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i4.368

World J Gastroenterol 2012 January 28; 18(4): 368-374
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Polymorphisms of ICAM-1 are associated with gastric cancer risk and prognosis

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Supported by Grants from Beijing Municipal Science and Technology Commission NOVA Program, No. 2009BG-02; the National High Technology Research and Development Program of China, No. 2006AA02A402; the Major State Basic Research Program of China, No. 2004CB518702

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Received: August 24, 2011 Revised: October 19, 2011
Accepted: October 26, 2011
Published online: January 28, 2012

Abstract

AIM: To investigate the association between single nucleotide polymorphisms (SNPs) in intercellular adhesion molecule-1 (ICAM-1) and the risk, biological behavior and prognosis of gastric cancer (GC) in Chinese population.

METHODS: The study group consisted of 332 GC patients and 380 healthy controls. Genotyping was performed using polymerase chain reaction and the results were confirmed by sequencing. The association of ICAM-1 K469E polymorphisms and the risk of GC were studied, and the correlation of ICAM-1 K469E polymorphisms with the clinicopathological parameters and prognosis of the patients with complete clinical and follow-up data was analyzed.

RESULTS: Carriers of AA genotype had a significantly increased risk of GC compared with carriers of AG and GG genotypes [odds ratios: 1.36; 95% confidence interval (CI): 1.01-1.84; P = 0.041]. GC patients with AA genotype were more prone to distant metastasis than those carrying AG and GG genotypes (18.9% *vs* 7.0%, respectively; P = 0.002). In addition, patients at stage Ⅳ had significantly more carriers of AA genotype than those of AG and GG genotype (27.4% *vs* 16.9%, respectively; P = 0.046). Follow-up study showed that the overall cumulative survival rate was 23.7% in AA genotype group and 42.9% in AG and GG genotypes group. In univariate analysis, AA genotype was correlated with the overall cumulative survival (P = 0.034). But in multivariate analysis, ICAM-1 polymorphism was not an independent prognostic factor for the overall survival (relative risk, 1.145; 95% CI: 0.851-1.540; P = 0.370).

CONCLUSION: Polymorphisms of ICAM-1 K469E can be a useful biomarker for identifying individuals with higher risk of GC, predicting disease progression, and guiding individualized treatment.

Key words: Intercellular adhesion molecule-1; Gene polymorphism; Gastric cancer; Risk; Prognosis

Peer reviewer: Takaaki Arigami, MD, PhD, Department of Surgical Oncology and Digestive Surgery, Field of Oncology, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 891-0175, Japan; Salvatore Gruttadauria, MD, Assistant Professor, Abdominal Transplant Surgery, ISMETT, Via E. Tricomi, 190127 Palermo, Italy

Tian MM, Sun Y, Li ZW, Wu Y, Zhao AL, Li JY. Polymorphisms of ICAM-1 are associated with gastric cancer risk and prognosis. *World J Gastroenterol* 2012; 18(4): 368-374 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i4/368.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i4.368

INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer in the world and has the second highest mortality rate[1]. GC is a biologically and genetically heterogenous tumor and develops as a result of the interplay among genetic and environmental factors. Genetic polymorphism plays a role in determining how hosts respond to various environmental factors[2-4].

As a risk factor for GC, *Helicobacter pylori* (*H. pylori*) infection is closely correlated with the occurrence of GC. A number of studies have confirmed that many polymorphisms of inflammation-related genes are significantly associated with the risk of GC[5-8]. Intercellular adhesion molecule-1 (ICAM-1) is a cell adhesion molecule belonging to the immunoglobulin superfamily and is the ligand of β2 integrin (LFA-1 and Mac-1)[9]. *H. pylori* infection can induce increased expression of ICAM-1, possibly due to the stimulation of local mucosal inflammation and inflammatory cytokines. ICAM-1 is expressed in various cell types, including leukocytes, epithelial and endothelial cells, fibroblasts and keratinocytes[9,10]. ICAM-1 plays an important role in cell-to-cell, cell-to-extracellular matrix interaction and cell signaling. Two single-nucleotide polymorphisms (SNPs) in the ICAM1 gene have been recognized, the first at position +241 (+241G/A or G241R) located in exon 4 (GGG→AGG; Gly→Arg), and the second one at position +469 (+469 A/G or K469E) located in exon 6 and coding the Ig-like domain 5 (AAG→GAG; Lys→Glu)[11]. Some studies have shown that ICAM-1 plays an important role in the development and progression of human cancers. Overexpression of ICAM-1 in melanoma and renal cell carcinoma has been reported[12,13]. There is also a slightly increased expression of ICAM-1 in other tumors, such as gastric, breast, and colorectal cancers[14-16]. The soluble form of ICAM-1, sICAM-1, is partially detectable in the serum of healthy subjects, but its level is elevated in malignancies. It has been observed that sICAM-1 serum level was positively correlated with tumor size, lymph node and distant metastasis of cancers, such as breast cancer, pancreatic cancer, gastric cancer, lung cancer and hepatocellular carcinoma[17-22]. The SNP in ICAM1 gene was also found associated with the risk of cancers, including breast cancer, colorectal cancer and melanoma[23-28].

There has been no report on the relationship between ICAM1 gene polymorphisms and GC risk. The polymorphism of G241R is rare in Chinese populations[28,29]. In this study, we aimed to examine the frequency of the ICAM-1 K469E polymorphism in the Chinese population and its correlation with the risk of GC using a large case-control cohort from our hospital. We also analyzed the relationship between K469E polymorphism and the clinicopathological parameters and prognosis of GC patients with complete clinical and follow-up data.

MATERIALS AND METHODS

Patients and specimens

In this hospital-based case-control study, histologically normal tissue specimens of 332 patients (124 females and 208 males, median age 59 years, range 29-91 years) who underwent radical gastrectomy for GC without any adjuvant therapy prior to surgery in the Beijing Cancer Hospital from May 1999 to June 2004 were collected prospectively. Various clinicopathological data were evaluated or collected from patient files by a pathologist. Cancer staging followed the 7th edition of the TNM classification of the International Union Against Cancer (UICC)[30]. Of the 332 patients, 301 patients had complete follow-up records. The survival time was counted in months from the time of surgery to the date of death.

A total of 380 subjects (186 females and 194 males, median age 57 years, range 25-84 years) who received endoscopy in the Beijing Cancer Hospital from 2002 to 2005 with a pathological diagnosis of superficial gastritis served as controls. All patients and controls were of Chinese Han descent and lived in and around Beijing.

A total of 102 serum samples were provided by the Beijing Cancer Hospital tissue bank. *H. pylori* infection was detected by immunohistochemistry.

Experimental methods

Histologically normal tissues from patients and biopsied tissues from controls, fixed in formalin and embedded in paraffin, were used for extraction of genomic DNA. Tissues were dewaxed using xylene and ethanol and then dried. After addition of 1 mL lysis buffer and 10 μL proteinase K solution (20 mg/mL), the tissues were incubated at 37 ℃ overnight. The solution was extracted with equal volumes of saturated phenol, phenol : chloroform (1:1), and chloroform : isoamyl alcohol (24:1). After addition to the supernatant of 1/10 volume of NaOAc (3 mol/L) and 2.5 times the volume of ice-cold ethanol, the solution was precipitated at -20 ℃ overnight. The precipitate was washed with 70% ethanol to remove the salt. After the precipitate was dissolved in 1 × TE solution (pH 7.6), the DNA was stored at -20 ℃.

According to GenBank full-length sequence of human ICAM1 gene, we applied Primer 5.0 software to design 469K/E locus-specific primers. The primers used were 5'-GCTCAAGTGTCTAAAGGATGGC-3' (forward) and 5'-CTCACAGAGCACATTCACGG-3' (reverse). The annealing temperature was 60 ℃ and the length of polymerase chain reaction (PCR) products was 148 bp. The PCR products were sequenced using an ABI377 automatic sequencer (Applied Biosystems, Foster City, CA, United States).

sICAM-1 was measured using an enzyme linked immunosorbent assay (ELISA) kit. ICAM-1 expression in GC tissues was detected by immunohistochemistry using an ICAM-1 mouse monoclonal antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, United States). As a membrane protein, the positive staining for ICAM-1 was located in the cytoplasm and at the cell membrane. If > 10% cells were positively stained in each slice, the tissue was considered ICAM-1 positive[14]. *H. pylori* infection was detected by immunohistochemistry using an *H. pylori* rabbit monoclonal antibody (1:200; Zeta Corporation,

Table 1 General status of gastric cancer patients and controls

Variables	Patients (n = 332) (%)	Controls (n = 380) (%)	P value
Age (yr)			
mean ± SE	57.37 ± 0.68	56.92 ± 0.66	0.638
Gender			
Male	208 (62.7)	194 (51.1)	0.002
Female	124 (37.3)	185 (48.9)	
Helicobacter pylori infection			
Yes	138 (41.6)	156 (41.1)	0.89
No	194 (58.4)	224 (58.9)	
Tobacco smoking			
Yes	86 (25.9)	82 (21.6)	0.175
No	246 (74.1)	298 (78.4)	
Alcohol consumption			
Yes	63 (19.0)	73 (19.2)	0.937
No	269 (81.0)	307 (80.8)	

Table 2 Genotype and allele distribution of ICAM-1 +469 A/G polymorphism

	Patients (n = 332) (%)	Controls (n = 380) (%)	P value[a]
Genotype			
AA	190 (57.2)	187 (49.2)	0.032[b]
AG	116 (34.9)	169 (42.4)	
GG	26 (7.8)	24 (8.4)	
Allele			
A	496 (74.7)	543 (71.4)	0.070
G	168 (25.3)	217 (28.6)	

a: Pearson χ^2 test; b: AA *vs* AG + GG. ICAM-1: Intercellular adhesion molecule-1.

Sierra Madre, CA, United States).

Statistical analysis

All analyses were performed using SPSS statistical analysis software, version 10.0 (SPSS, Chicago, IL, United States). Differences in age, sex, *H. pylori* infection, tobacco smoking, alcohol consumption, and the distribution of the genetic polymorphism between cases and controls were calculated using Pearson's χ^2 test. It was also determined if the genotype distribution was consistent with Hardy-Weinberg equilibrium. $P < 0.05$ (both sides) was considered statistically significant.

The correlation between the polymorphism and clinicopathologic parameters of GC was also examined by Pearson's χ^2 test, and $P < 0.05$ (both sides) was considered statistically significant. The Kaplan-Meier method and log-rank test were used to evaluate the correlation between the polymorphism and patient survival. Odds ratios (OR) and confidence intervals (CI) (95%) were calculated by Cox regression model to examine the impact of the polymorphism on prognosis. P value < 0.05 was considered statistically significant.

RESULTS

Characteristics of GC patients and controls

Baseline characteristics of the 332 GC patients and 380 control subjects are summarized in Table 1. There were more males among the patients with GC (62.7%) compared with the control group (51.1%, $P = 0.002$). However, no significant difference was observed between the cases and controls in age, *H. pylori* infection, tobacco smoking, and alcohol consumption (Figure 1).

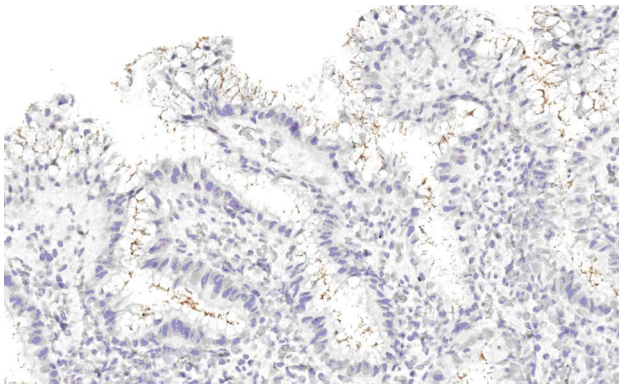

Figure 1 *Helicobacter pylori* infection was detected by immunohistochemistry.

ICAM-1 +469 A/G polymorphism and its relationship with GC risk

As is shown in Table 2, the frequency of the AA genotype was 57.2% and 49.2% in patients and controls, whereas the frequency of the AG genotype was 34.9% and 42.4% in patients and controls, respectively. On the other hand, the frequency of GG genotype was 7.8% and 8.4% in patients *vs* controls, respectively. The most common genotype was AA followed by AG. Significantly more AA carriers were found among the patients with GC compared with the controls ($P = 0.032$, Figure 2). It is noticeable that both patients and controls were in Hardy-Weinberg equilibrium.

Considering the low frequency of the GG genotype, we combined the GG and AG genotypes for use as the reference. After adjustment for age, sex, *H. pylori* infection, smoking, and alcohol consumption, subjects with AA genotype had a 1.36-fold increase in GC risk compared with those with AG + GG genotypes (OR = 1.36, 95% CI = 1.01-1.84, $P = 0.041$).

Association between ICAM-1 +469 A/G polymorphism and clinicopathological parameters of GC

The potential associations of the ICAM-1 +469 A/G polymorphism with tumor characteristics are presented in Table 3. Among the 332 patients with GC, the frequencies of the three genotypes were 57.2% (AA, 190/332), 34.9% (AG, 116/332), and 7.8% (GG, 26/332), respectively. No correlation was found between +469 A/G genotypes and tumor size, differentiation, presence of lymph node metastases and vascular invasion, or age and gender at diagnosis. However, AA genotype carriers had distant metastasis more frequently than AG and GG carriers (18.9% *vs* 7.0%, $P = 0.002$). In addition, there were more stage IV patients carrying the AA genotype compared with patients with the AG and GG genotypes

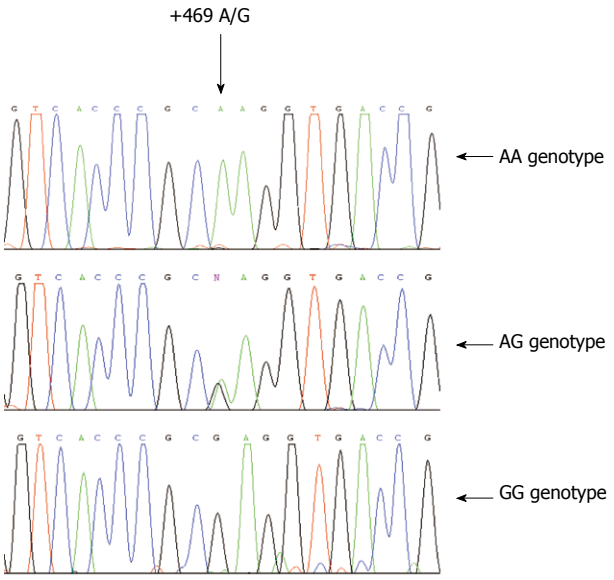

Figure 2 Sequence data of intercellular adhesion molecule-1 K469E genotype. These three graphs represent AA, AG and GG genotypes.

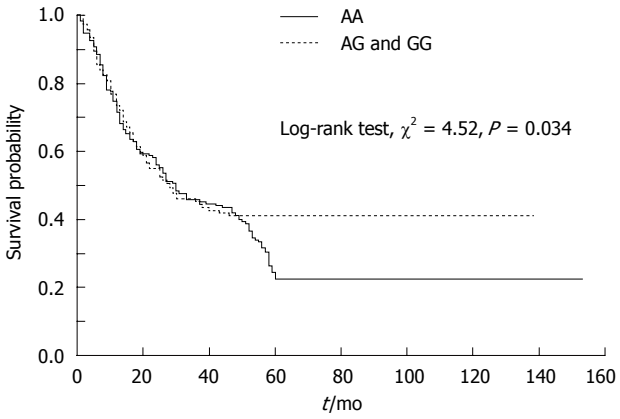

Figure 3 Kaplan-Meier survival analyses of gastric cancer patients.

(27.4% *vs* 16.9%, $P = 0.046$).

ICAM-1 +469 A/G polymorphism and GC prognosis

To evaluate whether the ICAM-1 +469 A/G polymorphism in GC correlates with a worse prognosis, Kaplan-Meier survival curves were constructed using overall cumulative survival to compare the patients with AA genotype to those with AG and GG genotypes. Our data revealed that AA genotype was correlated with overall cumulative survival ($P = 0.034$, log-rank test; Figure 3). The overall cumulative survival rate was 23.7% in AA genotype group and 42.9% in AG and GG genotypes group. In addition, we performed a multivariate analysis including ICAM-1 polymorphism, age, sex, tumor size, differentiation, depth of invasion, lymph node metastasis, distant metastasis, and TNM stage for GC, and found that ICAM-1 polymorphism is not an independent prognostic factor for the overall survival (relative risk, 1.145; 95% CI, 0.851-1.540; $P = 0.370$).

Table 3 Association between ICAM-1 +469 A/G polymorphism and clinicopathological parameters of patients with gastric cancer

Variables	Total (n = 332)	ICAM-1 +469 A/G genotype		P value
		AA (n = 190) (%)	AG + GG (n = 142) (%)	
Age (yr)				0.733
mean ± SE		57.17 ± 0.94	57.64 ± 0.99	
Gender				0.355
Male	208	115 (60.5)	93 (65.5)	
Female	124	75 (39.5)	49 (34.5)	
Tumor size				0.154
≤ 5cm	174	106 (55.8)	68 (47.9)	
> 5cm	158	84 (44.2)	74 (52.1)	
Differentiation				0.760
Well	120	70 (36.8)	50 (35.2)	
Poor	212	120 (63.2)	92 (34.8)	
Lymph node metastasis				0.928
Negative	102	58 (30.5)	44 (31.0)	
Positive	230	132 (69.5)	98 (69.0)	
Vascular invasion				0.408
Negative	176	97 (51.1)	79 (55.6)	
Positive	156	93 (48.9)	63 (44.4)	
Distant metastasis				0.002
Negative	286	154 (81.1)	132 (93.0)	
Positive	46	36 (18.9)	10 (7.0)	
TNM stages				0.046[a]
Ⅰ	62	35 (18.4)	27 (19.0)	
Ⅱ	60	35 (18.4)	25 (17.6)	
Ⅲ	134	68 (35.8)	66 (46.5)	
Ⅳ	76	52 (27.4)	24 (16.9)	

a: Ⅳ *vs* Ⅰ + Ⅱ + Ⅲ. ICAM-1: Intercellular adhesion molecule-1.

ICAM-1 +469 A/G polymorphism and sICAM-1 and ICAM-1 expression in GC

Among the 332 patients with GC, 102 had available serum samples. The serum sICAM-1 levels in these patients were measured using an ELISA kit. ICAM-1 immunohistochemical staining of paraffin sections from these patients showed that ICAM-1 was mainly expressed in the invasive front cells (Figure 4). Pearson's χ^2 test showed that the ICAM-1 +469 A/G polymorphism was not significantly associated with sICAM-1 or ICAM-1 expression in GC (Table 4).

DISCUSSION

In the present case-control study, we found that ICAM-1 K469E polymorphisms were associated with significantly increased risk of GC. Consistent with our results, Theodoropoulos *et al*[31] reported that this SNP in ICAM1 gene was associated with increased risk of colorectal carcinoma. Gastrointestinal carcinomas might largely be secondary to inflammation and immune response. Gastric carcinogenesis is closely related to chronic inflammatory lesion in gastric mucosa mainly caused by *H. polyri* infection. ICAM-1 plays a pivotal role in leucocyte migration across endothelial cells and facilitates leukocyte recruit-

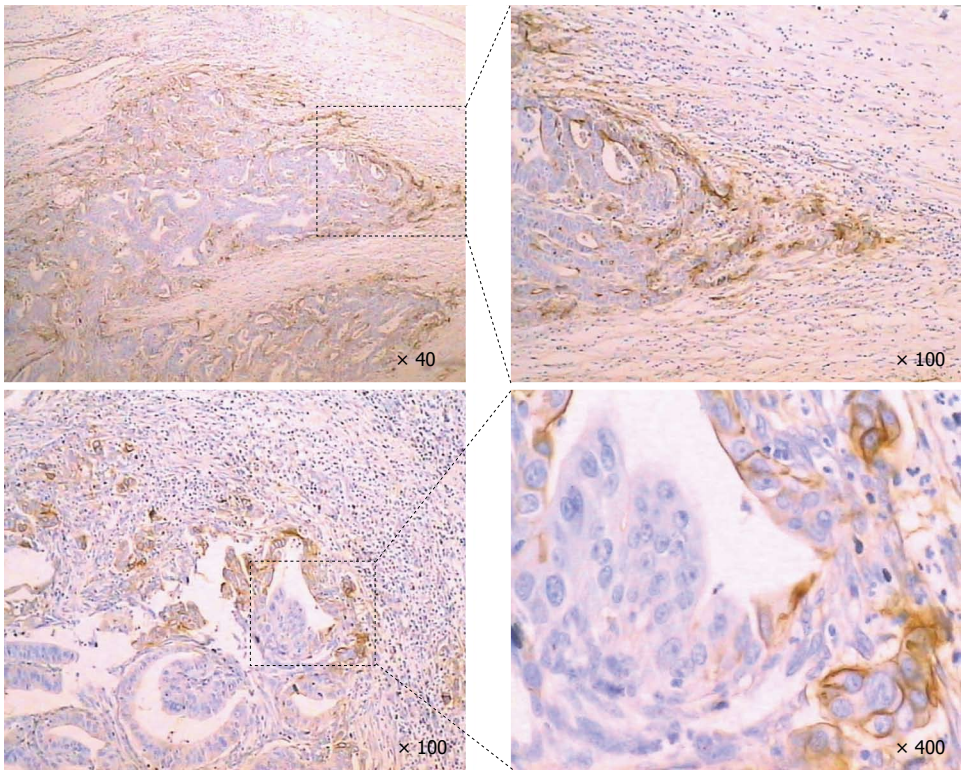

Figure 4 Intercellular adhesion molecule-1 expression in gastric cancer. Intercellular adhesion molecule-1 (ICAM-1) was mainly expressed in the invasive front cells.

Table 4 ICAM-1 +469 A/G polymorphism and sICAM-1 and ICAM-1 expression in gastric cancer

Characteristics	Total	Genotype		*P* value
	(*n* = 102)	AA	AG + GG	
Serum sICAM-1				0.990
mean ± SE		176.00 ± 7.72	175.85 ± 9.40	
ICAM-1 expression				0.197
Positive	29	13 (44.8)	16 (55.2)	
Negative	73	43 (58.9)	30 (41.1)	

ICAM-1: Intercellular adhesion molecule-1; sICAM-1: The soluble form of ICAM-1.

ment into inflammatory sites. K469E amino acid changes in the ICAM1 gene may influence its functional role, as it is located at the Mac-1 binding domain and the immunoglobin-like domain 5. The ICAM-1 K469E polymorphism may result in the functional alteration of the gene product that facilitates the inflammation and immune response. The mixture of cytokine produced in inflammation and immune response contributes to the establishment of oncogenic microenvironment that plays a key role in initiation of gastric carcinogenesis by stimulating angiogenesis, damaging DNA and epithelial cell proliferation and subsequent malignant transformation[32,33]. For example, the pro-inflammatory and acid inhibiting properties of TNFα seem to enhance *H. pylori* oncogenic or other effects on gastric mucosa. In another study, we found that ICAM-1 K469E polymorphism was strongly associated with the progression of gastric lesions from gastritis to dysplasia (data not shown).

Since ICAM-1 K469E polymorphism could lead to the functional alteration of the gene product, it may influence its role in pathogenesis as well as in the progression and subsequent prognosis. This study revealed that K469E polymorphism was strongly associated with distant metastasis and advanced staging (stage Ⅳ) of GC. In addition, this polymorphism was correlated with poor survival of the patients, although there was no correlation between polymorphism and expression and soluble level of ICAM-1. Our results showed that ICAM-1 was mainly expressed in the invasive front cells. These findings are in agreement with previous reports on the involvement of ICAM-1 in tumor progression[34-37]. The possible mechanism of this phenomenon is that tumor cells with high ICAM-1 expression could bind to infiltrating lymphocytes through an ICAM-1/LFA-1 interaction. As a result, cancer cells could become detached from the tumor mass and migrate into the blood circulation. Additionally, cells would not be damaged when transiting through the blood vessels and could easily adhere to the capillaries or lymphatic sinus, leading to metastasis.

Our data analysis relied on extracted genomic DNA from tissue samples preserved in paraffin blocks. Rae *et al*[38] reported that the fixation process used for making paraffin blocks did not affect genotype detection. They found that the results of genotype detection with genomic DNA from paraffin blocks were consistent with the results of genotype detection using genomic DNA from peripheral

blood and suggested that accurate and reliable genotyping results with paraffin samples could be achieved.

To the best of our knowledge, this is the first evidence to indicate that individuals carrying the AA genotype of the ICAM-1 K469E polymorphism might be more susceptible to GC. In addition, patients with the AA genotype were more likely to develop distant metastasis and have a higher risk of death compared with other patients. These results suggested that the ICAM-1 K469E polymorphism could be used as a new molecular marker to screen for individuals with higher risk of GC, predict disease progression, and guide individualized treatment.

COMMENTS

Background

Gastric cancer (GC) is a biologically and genetically heterogenous tumor and develops as a result of the interplay among genetic and environmental factors. Genetic polymorphism plays a role in determining how hosts respond to various environmental factors. A number of studies have confirmed that polymorphisms of many inflammation-related genes are significantly associated with the risk of GC.

Research frontiers

According to the authors of this paper, there has been no report on the relationship between intercellular adhesion molecule-1 (ICAM-1) gene polymorphisms and the risk, biological behavior and prognosis of GC.

Innovations and breakthroughs

In this study, the authors investigated the association between single nucleotide polymorphisms in ICAM-1 and the risk, biological behavior and prognosis of GC in Chinese population. This is the first evidence to indicate that individuals carrying the AA genotype of the ICAM-1 K469E polymorphism might be more susceptible to GC. In addition, patients with AA genotype were more likely to develop distant metastasis and have a higher risk of death compared with other patients.

Applications

The ICAM-1 K469E polymorphism could be used as a new molecular marker to screen for individuals with higher risk of GC, predict disease progression, and guide individualized treatment.

Terminology

ICAM-1 is a cell adhesion molecule belonging to the immunoglobulin superfamily and is the ligand of β2 integrin (LFA-1 and Mac-1). ICAM-1 plays an important role in cell-to-cell, cell-to-extracellular matrix interaction and cell signaling.

Peer review

The authors investigated K469E polymorphism of ICAM-1 in patients with gastric cancer. The number of cases enrolled in this study is enough and the results are very important.

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S- Editor Cheng JX **L- Editor** Ma JY **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i4.375

World J Gastroenterol 2012 January 28; 18(4): 375-382
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Population-based study of DNA image cytometry as screening method for esophageal cancer

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Supported by Grants from the Ministry of Health of China, No. 200902002-8; Grants from Cancer Institute/Hospital Chinese Academy of Medical Sciences and Peking Union Medical College, No. 2009YF50
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Received: June 7, 2011 Revised: August 22, 2011
Accepted: October 28, 2011
Published online: January 28, 2012

Abstract

AIM: To explore the DNA image cytometry (DNA-ICM) technique as a primary screening method for esophageal squamous precancerous lesions.

METHODS: This study was designed as a population-based screening study. A total of 582 local residents aged 40 years-69 years were recruited from Linzhou in Henan and Feicheng in Shandong. However, only 452 subjects had results of liquid-based cytology, DNA-ICM and pathology. The sensitivity and specificity of DNA-ICM were calculated and compared with liquid-based cytology in moderate dysplasia or worse.

RESULTS: Sensitivities of DNA-ICM ranging from at least 1 to 4 aneuploid cells were 90.91%, 86.36%, 79.55% and 77.27%, respectively, which were better than that of liquid-based cytology (75%). Specificities of DNA-ICM were 70.83%, 84.07%, 92.65% and 96.81%, but the specificity of liquid-based cytology was 91.91%. The sensitivity and specificity of a combination of liquid-based cytology and DNA-ICM were 84.09% and 85.78%, respectively.

CONCLUSION: It is possible to use DNA-ICM technique as a primary screening method for esophageal squamous precancerous lesions.

Key words: DNA image cytometry; Aneuploidy; Screening; Esophageal cancer; Precancerous lesions

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Zhao L, Wei WQ, Zhao DL, Hao CQ, Lin DM, Pan QJ, Li XQ, Lei FH, Wang JW, Wang GQ, Shang Q, Qiao YL. Population-based study of DNA image cytometry as screening method for esophageal cancer. *World J Gastroenterol* 2012; 18(4): 375-382 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i4/375.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i4.375

INTRODUCTION

Esophageal cancer, the world's eighth most common cancer, is curable if detected early. In China, the survival rates are over 85% when found at an early stage of esophageal squamous cell carcinoma (ESCC), but less than 10% when diagnosed at the advanced stage[1]. Patients in early stages are asymptomatic, and therefore, screening is important for detecting patients before they progress to advanced stage disease.

Presently, several screening methods have been explored in high-risk areas of China including conventional cytology[2-5], liquid-based cytology[6], occult blood detection[7] and endoscopic biopsy examination with Lugol's iodine staining[8]. Conventional cytology had been used in high-risk areas of China in the 1970s, but it has been replaced by liquid-based cytology. Occult blood detection has been explored in several areas in China, but because of low sensitivity and specificity, this method has not been used widely yet. Endoscopic examination with Lugol's iodine staining is mostly used in high-risk areas in China now. Although endoscopic examination with Lugol iodine staining has high sensitivity and specificity of more than 90%[8], because of the high cost, this screening method is difficult to be accepted by residents in rural high-risk areas. Meanwhile, endoscopic and pathological doctors require a lengthy qualification process. Liquid-based cytology may improve the quality of the cytological procedure and provide satisfactory diagnostic slides compared with conventional cytology, but it has limits for improving the sensitivity and specificity of screening[6,9]. Furthermore, there is a skill shortage in rural high-risk areas due to the lack of experienced cytologists.

DNA image cytometry (DNA-ICM) has been proposed as a simple, easy, sensitive and high specificity method for screening of ESCC. Changes in DNA ploidy occurring in human tumors have been shown to be a global reflection of the chromosomal and subchromosomal genetic changes which are important in tumor development and progression[10]. DNA ploidy is analyzed by DNA-ICM as a screening marker to reveal the development of ESCC. Until now, few studies have investigated the relationship between DNA ploidy and ESCC diagnosis application[11-13]. In these previous studies, researchers obtained specimens from surgical tissue, paraffin-embedded tissue and endoscopic biopsy. They have analyzed the prognostic value of DNA ploidy in the progression of ESCC, and predicted the prognosis of ESCC. Furthermore, these studies have not been performed by a population-based method, but were small sample clinical studies. DNA-ICM has been used as a screening method in cervical cancer[14], and was shown to have a higher sensitivity than that of cytology. However, no population-based screening study has focused on DNA-ICM for assessment of esophageal squamous carcinoma and precancerous lesions.

In order to evaluate sensitivity and specificity of ESCC screening by DNA-ICM, we conducted a population-based study in two high-risk areas of China.

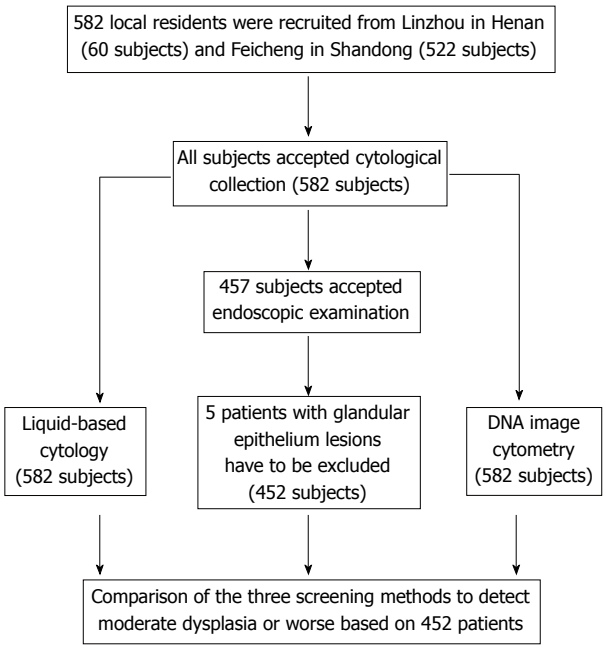

Figure 1 Study flow chart, procedure, and subjects included in the study.

MATERIALS AND METHODS

Study design and subjects recruitment

This was a population-based screening study. In two areas at high risk of ESCC, namely Linzhou in Henan province and Feicheng in Shandong province, local trained village doctors visited every family in June 2010, gathered local residents to the village hospital, and then explained this study to them. Finally, 70% of the local residents consented to participate in our study. Inclusion criteria were: (1) local resident; (2) age 40 to 69 years; (3) no contraindications for endoscopic examinations (e.g., history of reaction to iodine or lidocaine, serious cardiovascular disease, poor health status); and (4) voluntarily consenting to participate in screening and signing the informed consent document. Exclusion criteria were: participants who had a history of liver cirrhosis, esophageal varices, hematemesis, a bleeding disorder, uncontrolled congestive heart failure, unstable angina or a reaction to topical anesthetics or iodine.

The details of subject recruitment and exclusion, those with cytology and endoscopy examinations, and the study procedure are described in Figure 1.

This study was approved by the Institutional Review

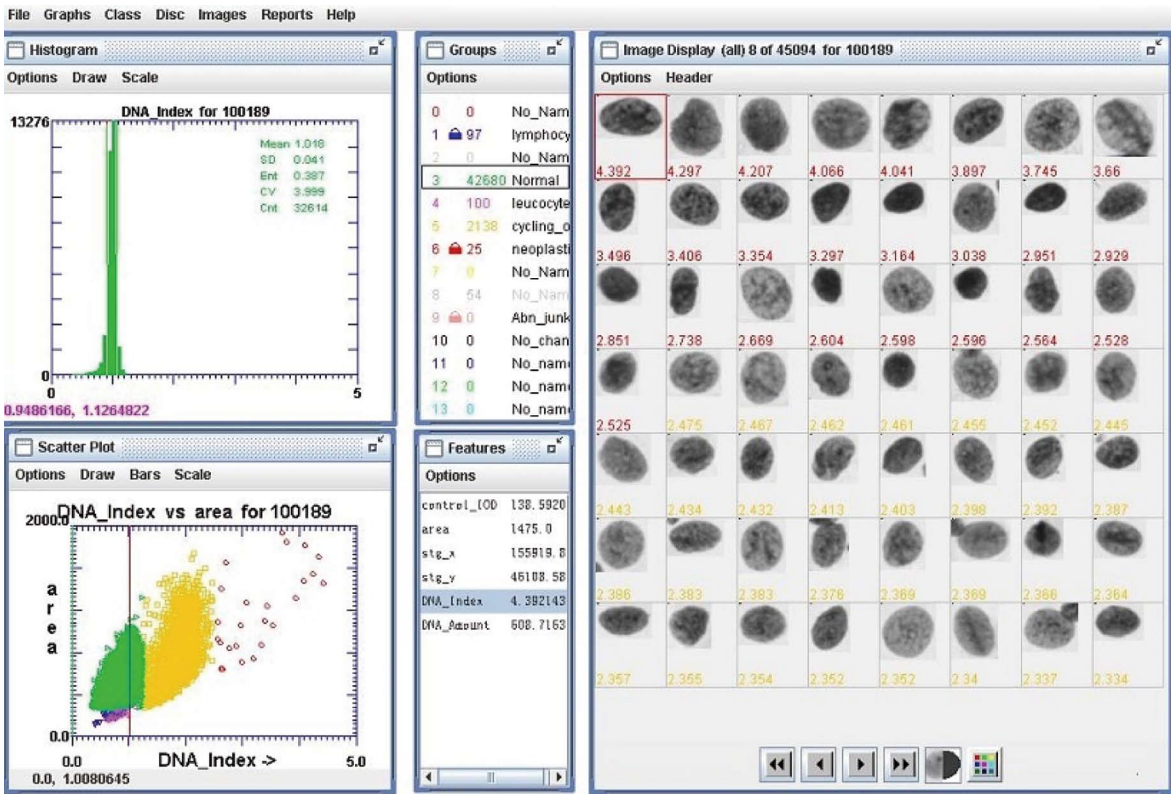

Figure 2 Image picture from a Feulgen-stained Thinprep slide visualized by DNA imaging software shows the DNA index of every nucleus of different cells in one slide.

Boards of the Cancer Institute/Hospital of the Chinese Academy of Medical Sciences (CICAMS) and Peking Union Medical College.

Cytological sample collection

Preparation for balloon examination: All cytological examinations were performed in the villages. After completing informed consent and a short questionnaire, patients fasted overnight prior to examination. Before balloon examination, an inflatable balloon was soaked in 75% alcohol for at least 12 h.

Collection of cytological samples[6]: Balloon examination was performed by an experienced local doctor. After cleansing the oral cavity, the balloon was inserted into the back of the throat and swallowed by the patient. Once in the stomach, the balloon and covering mesh were expanded and then gradually pulled up the esophagus. When the balloon reached the upper esophageal sphincter, it was deflated and withdrawn completely. After removal, the balloon was placed in a 50 mL centrifuge tube including Thinprep digestive fluid, the catheter was cut off, and the centrifuge tube was sealed and transferred to a CICAMS laboratory.

Reparation of cytological slides: Each sample was vortexed for 10 min to remove adherent cells from the balloon, which was then taken out of the tube. The remaining cell suspension was centrifuged at 2500 r/min for 5 min. Excess supernatant was discarded and the cell deposits remained in the tube. All cell deposits were then transferred to Eppendorf tubes, which included 20 mL of Thinprep preserving cyto-solution. Every sample in the eppendorf tube was prepared for two cytological slides. Every subject had two slides, which were divided randomly for a Pap smear or Feulgen staining. We adopted a crossover design to eliminate the impact of numbers of cells on the diagnosis.

The slides were reviewed by two cytopathologists independently, and discrepancies were adjudicated by joint review. The slides were read blindly without the pathological results. Diagnostic criteria were adapted from criteria of the original Bethesda System[15].

DNA image analysis on liquid-based specimens

The specimens were stained by the Feulgen-Thionin method as detailed in one previous study[16]. The hardware of the system included a MOTIC BA600 microscope, DELL370 workstation, automated microscope control-box, Moticam 1501 camera and assistant accessories. The software for the DNA-ICM system came from Canada[17].

This system automatically loaded each slide, scanned the areas of Thinprep deposition, stored an image of every object detected, calculated a set of 132 features for each cell nucleus, ordered the DNA content of the nucleus, and used multilevel decision-making to classify each object as either nucleus or 'junk'. Finally, two cytopathologists checked blindly the image of every nucleus repeatedly, then excluded an unsatisfactory nucleus. Figure 2 shows the interface of DNA-ICM which is used to

Table 1 Number of diploid and aneuploid cells by DNA ploidy analysis based on pathological diagnoses *n* (%)

DNA ploidy	Pathological diagnoses				
	Normal	mD	MD	SD	ESCC
Diploid	244 (72.6)	45 (62.5)	4 (13.8)	0 (0)	0 (0)
Aneuploid	92 (27.4)	27 (37.5)	25 (86.2)	10 (100)	5 (100)
No. of aneuploid cells ≤ 5	90 (26.8)	25 (34.7)	7 (24.1)	1 (10)	0 (0)
No. of aneuploid cells ≤ 10	2 (0.6)	2 (2.8)	2 (6.9)	2 (20)	0 (0)
No. of aneuploid cells ≤ 20	0 (0)	0 (0)	8 (27.6)	5 (50)	2 (40)
No. of aneuploid cells > 20	0 (0)	0 (0)	8 (27.6)	2 (20)	3 (60)
Total	336	72	29	10	5

mD: Mild dysplasia; MD: Moderate dysplasia; SD: Severe dysplasia; ESCC: Esophageal squamous cell carcinoma.

assess the image of the cell nucleus with its corresponding features.

At least 400 normal epithelial cells were taken as the internal reference diploid population in each specimen. The coefficient of variation (CV) of the DNA quantity of these reference cells never exceeded 3%. This value was lower than the CV of 5% recommended by the European Society for Analytical Cellular Pathology (ESAPC)[18,19]. The DNA content of every nucleus was measured by integrated optical density (IOD).

The resulting DNA ploidy value is expressed as a "c" value for the chromosome. A DNA ploidy value of 2c indicates a normal diploid cell, 4c is a tetraploid cell, 5c is a cutoff used for aneuploidy by most authors[18].

The quality control process was implemented and depended on the reports of ESAPC[18,19].

DNA index = DNA IOD value of detected cell/Average DNA (G0/G1) IOD among reference cells nuclei

Endoscopic examination

Endoscopic screening was completed by local doctors after training by and under the supervision of experienced doctors from the CICAMS. The technical processes of endoscopic screening were as follows:

Pre-endoscopy: Firstly, subjects signed the informed consent, then were anesthetized with 5 mL 1% lidocaine *via* the mouth.

Endoscopy: Subjects were placed in the left lateral position. The entire esophagus and stomach were visually examined including careful examination of the cardiac mucosa spinal roots.

Iodine staining: During the endoscopic procedure, Lugol's iodine (1.2%) solution was used to stain the normal glycogen-containing tissue, which left the suspicious lesions unstained. Unstained foci were targeted and multiple biopsies were taken. Cleaning and disinfection of endoscopes was carried out using 2% alkaline glutaraldehyde solution.

Pathological diagnoses: Biopsy specimens were fixed in 10% buffered formalin, embedded in paraffin, cut in 5 μm sections, and stained with hematoxylin and eosin. The biopsy slides were read blindly by two experienced pathologists (WJW, LFH) without knowledge of the visual endoscopic results. Subjects with mild dysplasia needed to be followed up, but with moderate dysplasia or worse would be offered argon plasma coagulation and/or endoscopic mucosal resection, or surgery, depending on the grade of the lesion. Therefore, diagnosis of moderate dysplasia or worse has clinical implications.

Statistical analysis

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of DNA-ICM were calculated by SAS 9.2 software. The results of DNA-ICM ranging from 1 to 15 aneuploid cells were based on different esophageal precancerous lesions (mild dysplasia, moderate dysplasia, severe dysplasia and squamous cell carcinoma).

RESULTS

Subject demographics

The study consisted of 452 subjects, including 171 men and 281 women. The mean age of the participants was 55 years (range, 40-69 years). Overall, 60 subjects (13.3%) came from Linzhou in Henan province and 392 subjects (86.7%) from Feicheng in Shandong province. The analytic database was limited to 452 subjects who had results of liquid-based cytology, histopathology and DNA-ICM.

Results of histopathology, liquid-based cytology and DNA-ICM

There were 336 of 452 (74.3%) subjects with "negative" histology, 10 (2.2%) had severe dysplasia, 29 (6.4%) had moderate dysplasia, 72 (15.9%) had mild dysplasia and 5 (1.1%) were diagnosed with ESCC. For diagnoses with liquid-based cytology, 386 subjects were normal (85.4%) and 66 (14.6%) had cytological abnormalities, of which 39 had atypical squamous cells of undetermined significance (ASCUS), 19 had low-grade squamous intraepithelial lesions (LSIL), 3 had high grade squamous intraepithelial lesion (HSIL) and 5 cases had ESCC. For DNA-ICM, 293 of 452 (64.8%) subjects had no aneuploid cells, and 159 (35.2%) subjects had aneuploid cells.

Table 1 shows diploid cells and aneuploidy classified by pathological diagnoses. The results showed that 27.4%

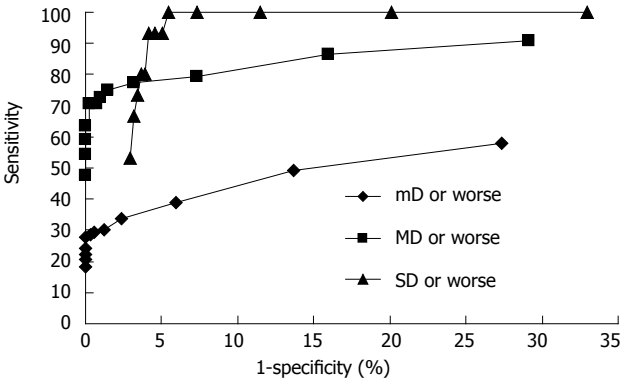

Figure 3 Receiver operating characteristics curves of DNA image cytometry based on histopathological results including mD or worse, MD and worse or SD or worse. For each curve, the 15 points from right to left are from at least one aneuploid cell to at least 15 aneuploid cells. mD: Mild dysplasia; MD: Moderate dysplasia; SD: Severe dysplasia.

Table 2 Comparison of sensitivity, specificity, positive predictive value, negative predictive value between liquid-based cytology and DNA ploidy method based on moderate dysplasia or worse as threshold

	Threshold for positivity	Total	Se (%)	Sp (%)	PPV (%)	NPV (%)
Cytology	ASCUS+	452	75.00	91.91	50.00	97.15
	LSIL+	452	52.27	99.02	85.19	95.06
	HSIL+	452	18.18	100.00	100.00	91.89
DNA ploidy	At least 1 5cER cell	452	90.91	70.83	25.16	98.63
	At least 2 5cER cell	452	86.36	84.07	36.89	98.28
	At least 3 5cER cell	452	79.55	92.65	53.85	97.67
	At least 4 5cER cell	452	77.27	96.81	72.34	97.53
	At least 5 5cER cell	452	75.00	98.53	84.62	97.34

ASCUS: Atypical squamous cells of undetermined significance; LSIL: Low-grade squamous intraepithelial lesions; HSIL: High grade squamous intraepithelial lesion; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

subjects with normal esophageal epithelia presented with aneuploid cells, but the proportions of aneuploid cells for subjects with mild dysplasia, moderate dysplasia, severe dysplasia, and ESCC were 37.5%, 86.2%, 100% and 100%, respectively. In particular, among subjects with severe dysplasia or ESCC, the proportion of diploid cells was zero. Furthermore, the larger the proportion of aneuploid cells, the worse the lesions.

The sensitivity and specificity of DNA-ICM compared with histopathology and were showed in ROC curve

Figure 3 shows receiver operating characteristics curves of the DNA-ICM method based on mild dysplasia or worse, moderate dysplasia or worse and severe dysplasia or worse. It also shows the sensitivity and specificity of DNA ploidy by category, which is the minimum number of cells required to define a subject as aneuploid. It is difficult to determine the exact threshold value, so optimal limits of the threshold for DNA-ICM method were determined. According to Figure 3, the optimal values for sensitivity and specificity fell around a minimum number of aneuploid cells ranging from at least 1 to 5.

Table 3 Sensitivity and specificity of a combination of cytology and DNA image cytometry based on moderate dysplasia or worse as threshold

Cytology or DNA ploidy	Se (%)	Sp (%)
⩾ ASCUS or ⩾ 1 aneuploid cells	93.18	67.65
⩾ ASCUS or ⩾ 2 aneuploid cells	88.64	79.41
⩾ ASCUS or ⩾ 3 aneuploid cells	84.09	85.78
⩾ ASCUS or ⩾ 4 aneuploid cells	81.82	88.97
⩾ ASCUS or ⩾ 5 aneuploid cells	81.82	90.69

ASCUS: Atypical squamous cells of undetermined significance; Se: Sensitivity; Sp: Specificity.

Comparison of liquid-based cytology and DNA-ICM

Table 2 shows the sensitivity, specificity, PPV and NPV of liquid-based cytology and DNA-ICM in our laboratory using these samples as determined by two blinded reviewers. ASCUS, LSIL and HSIL thresholds for cytology, and 1-5 aneuploid cells present at a DNA index of 2.5 for ploidy. For the threshold of moderate dysplasia or worse, sensitivities of the DNA-ICM method ranging from at least 1 aneuploid cell to 4 aneuploid cells were 90.91%, 86.36%, 79.55% and 77.27%, respectively. The specificities were 70.83%, 84.07%, 92.65% and 96.81%, respectively, but the sensitivity and specificity of liquid-based cytology for ASCUS or worse was 75% and 91.91%, respectively.

Combined liquid-based cytology with DNA ploidy

Table 3 shows the sensitivity and specificity of a combination of liquid-based cytology and DNA-ICM. Liquid-based cytology took ASCUS as the threshold value. The threshold value of DNA-ICM ranged from at least 1 aneuploid cell to at least 5 aneuploid cells. The combination improved sensitivity and the ability to screen for positive cases. The group "⩾ ASCUS or ⩾ 3 aneuploid cells" delivered optimal sensitivity and specificity, 84.09% and 85.78%, respectively.

DISCUSSION

This is the first population-based study to explore DNA image analysis technology as a screening method in ESCC. Our study indicated that the higher the grade of esophageal precancerous lesions the higher the proportion of aneuploid cells. This result is similar to the conclusion that early malignant changes in the esophagus are associated with alterations in DNA content in the clinical study by Blant *et al*[13] in Switzerland. Therefore, DNA image analysis may have potential clinical application as a screening method for ESCC.

Our study also showed that the sensitivities of the

DNA-ICM method ranging from at least 1 aneuploid cell to 4 aneuploid cells were 90.91%, 86.36%, 79.55% and 77.27%, respectively, in identifying moderate dysplasia or worse disease. These sensitivities were much higher than that of liquid-based cytological testing (75%) using moderate dysplasia or worse as the threshold.

One study on cervical cancer showed that the sensitivity of DNA cytometry testing for cervical cancer was 91.7%, whereas the sensitivity of cytological testing was 44.5%, and the specificity was 54.1% for DNA cytometry testing and 70.6% for cytological testing[14]. Singh *et al*[20] proposed the idea of combination screening using the DNA-ICM method, and in cervical cancer, human papillomavirus screening with cytology would be an optimal method to detect progressive lesions with the greatest possible sensitivity and specificity. In our study, the aim of combining liquid-based cytology and DNA-ICM was to explore whether the combination could improve the sensitivity in screening for ESCC and precancerous lesions. The results indicated that the group "$\geqslant$ ASCUS or $\geqslant$ 3 aneuploid cells" had the optimal sensitivity (84.09%) and specificity (85.78%). This improved the sensitivity of liquid-based cytological testing (75%) based on moderate dysplasia or worse. However, the specificities of combined cytology and DNA-ICM were lower than for the individual methodologies. If both the results of cytology and DNA-ICM are negative, the specificities of the combination were negative. This led to the low specificities. All in all, a combination of the two methods was only an indication for screening, and was not suitable for screening ESCC and precancerous lesions.

It is important to note one parameter of DNA-ICM in this study: the DNA index. In this study, we took the 5c value as the threshold value of the aneuploid cell. The ESAPC suggest that 5c is the cut-off value for aneuploidy[18]. A few studies suggested the threshold value for aneuploidy in other cancers was 5c, based on clinical samples[21-23]. DNA-ICM, used in cervical cancer screening, also set 5c as the cut-off value since results had showed that optimal values for sensitivity and specificity fell around a DNA ploidy of 5c[17]. However, this value has not been verified in esophageal cancer screening. It may be a limitation of our study, and future studies should confirm the cut-off value of DNA-ICM in esophageal cancer.

Our study is also the first study using cytological samples for DNA-ICM with Feulgen staining by inflatable balloon. Previous studies[24,25] on esophageal cancer and DNA-ICM collected biopsy specimens and then analyzed the DNA content of the nucleus. The sample collection procedure in our study was simple, and easy to perform as a screening method. Most ESCC cases occur in rural areas in China where residents have little knowledge of the capacity of medical diagnosis, and there is little heath education and inadequate treatment. These areas also lack experienced cytologists, endoscopists, and pathologists, as medical training is arduous and long. However, for DNA-ICM, a technician could work independently after 3 wk training, and 90% of slides could be diagnosed in 1 min, so this method would be easier to perform. Cytological diagnosis depends largely on the numbers of cytologists, but DNA-ICM is more objective as it is read by computer[26]. Our aim was to explore DNA-ICM as a primary screening method, which is certain to miss some positive cases compared with the "golden standard" of endoscopic examination. Nevertheless, we should not ignore the advantages of DNA-ICM: it is simple, objective, and likely to be accepted by participants. According to the data analysis, of 116 subjects with mild dysplasia or worse, 49 results of DNA-ICM were negative (false negative rate 42.2%). However, all of the 49 cases (100%) had mild or moderate dysplasia. This means that no advanced stage esophageal lesions had been missed. We also have relevant data about the efficiency of cell collection by inflatable balloon. is the so-called adequacy codes are classified into three grades: adequate for evaluation; satisfactory for evaluation but limited; and unsatisfactory. The number of subjects with adequate for evaluation cytological samples was 288, which is 63.7% of all subjects; with satisfactory for evaluation but limited samples was 163 (36.1%), and the sample of one subject was unsatisfactory (0.2%). Thus, we could say that the efficiency of cell collection by inflatable balloon was good for diagnosis by DNA-ICM.

DNA-ICM could also be used to predict the progress of lesions, as ESCC patients with diploid cells lived longer than ones with polyploidy[12]. One study in 2001 showed that the progression rate from oral dysplasia to invasive or carcinoma, based on the detection of DNA aneuploidy was only 10% after 1 year but increased significantly, to 90%, after 5 years[27]. DNA-ICM has been used as a diagnostic tool and aneuploidy tended to correlate with progression to invasive SCC[13]. Esophageal squamous cell carcinogenesis is thought to be a multistep process, influenced by multiple factors. Changes in DNA ploidy occurring in human tumors are shown to be a global reflection of the chromosomal and subchromosomal genetic changes which play a key role in tumor development and progression[10]. It had been indicated that the aneuploid cell is important in the early stage of carcinogenesis[28,29]. Therefore, we believe that aneuploid cells as a biomarker can be detected by DNA-ICM, which not only may be used for screening precancerous lesions of ESCC, but also for clinical diagnoses, prognostic evaluation and, in particular, patient follow-up and management after screening.

In summary, this is the first population-based study to focus on the relationship between the alteration in DNA content and ESCC and its precancerous lesions. We found that higher grade esophageal precancerous lesions had a higher proportion of aneuploid cells. Confirmed by pathological diagnoses from endoscopic biopsy, DNA-ICM had higher sensitivity, based on moderate dysplasia or worse as threshold, in ESCC screening than liquid-based cytology. Thus, it is possible to use DNA-ICM as the primary screening method in ESCC in high-risk areas

of China. Further larger sample studies are under consideration.

ACKNOWLEDGMENTS

We thank the staff of Feicheng People's Hospital, Feicheng, Shandong Province and Linzhou Cancer Hospital, Linzhou, Henan Province, China, for their cooperation with esophageal specimen and data collection.

COMMENTS

Background

Several screening methods have limitation in screening esophageal squamous cell carcinoma (ESCC) and precancerous lesions. Thus, it is necessary to explore a simple, objective, sensitive method as a primary screening method.

Research frontiers

In several previous studies, researchers obtained specimens from surgical tissue and endoscopic biopsy. They analyzed the prognostic value of DNA ploidy in the progression of ESCC and predicted the prognosis of ESCC. These studies were not population-based studies, but small sample clinical studies. DNA image cytometry (DNA-ICM) has been used as a screening method in cervical cancer, which showed that the sensitivity of DNA-ICM was higher than that of cytology.

Innovations and breakthroughs

This is the first population-based study to explore DNA image analysis technology as a screening method in ESCC. Our study is also the first study using cytological sampling for DNA-ICM with Feulgen staining by inflatable balloon. According to our results, the specificities of DNA-ICM were higher than those of liquid-based cytology based for moderate dysplasia or worse.

Applications

It is possible to use DNA-ICM as a primary method in ESCC and precancerous lesions according to our results. Further larger sample studies are under consideration to verify our results.

Terminology

The diploid cell is normal according to diagnosis by DNA imaging, which means it has a normal DNA content. The aneuploid cell has abnormal DNA content.

Peer review

The authors address an important issue considering the shortage of experienced cytologists, pathologists and endoscopic doctors in rural high-risk areas.

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S- Editor Tian L **L- Editor** Cant MR **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i4.383

World J Gastroenterol 2012 January 28; 18(4): 383-387
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

A polymorphism within *ErbB4* is associated with risk for hepatocellular carcinoma in Chinese population

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Supported by The Applied Basic Research Programs of Science and Technology Commission Foundation of Suzhou, No. sys201047

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Received: April 26, 2011 Revised: July 28, 2011
Accepted: August 4, 2011
Published online: January 28, 2012

Abstract

AIM: To investigate the association between hepatocellular carcinoma (HCC) susceptibility and a 12-bp insertion/deletion polymorphism (rs6147150) in the 3'UTR of *ErbB4*.

METHODS: Using a case-control design, the rs6147150 genotypes in 270 patients with HCC and 270 healthy controls were determined by direct polymerase chain reaction and polyacrylamide gel electrophoresis. Logistic regression was used to analyze the association between the polymorphism and cancer risk.

RESULTS: Computational modeling suggested that rs6147150 was located in the seed region of hsa-let-7c, a potential target sequence in *ErbB4* 3'UTR. Logistic regression analysis showed that, compared with individuals homozygous for wild-type, heterozygotes [adjusted odds ratio (OR) = 1.48, 95% confidence interval (CI) = 1.03-2.17, P = 0.034] and individuals homozygous for 12-bp del/del (OR = 2.50, 95% CI = 1.37-4.56, P = 0.001) were at significantly higher risk of HCC. Carriers of the "del" allele of rs6147150 had a 1.59-fold increased risk for HCC (95% CI = 1.22-2.07, P = 0.003).

CONCLUSION: rs6147150 may be associated with HCC risk, in part through let-7c-mediated regulation, and may be involved in the pathogenesis of HCC in Chinese populations.

Key words: Hepatocellular carcinoma; *ErbB4*; rs6147150; Insertion/deletion polymorphism

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Yu Q, Zhou CX, Chen NS, Zheng SD, Shen LM, Zhang JK. A polymorphism within *ErbB4* is associated with risk for hepatocellular carcinoma in Chinese population. *World J Gastroenterol* 2012; 18(4): 383-387 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i4/383.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i4.383

INTRODUCTION

Hepatocellular carcinoma (HCC) is an epithelial cancer originating from hepatocytes or their progenitor cells and is the fifth most common malignancy worldwide[1]. Carcinogenesis of HCC is a complex, multistep process, associated with various risk factors, including chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, cirrhosis and exposure to carcinogens[2]. Chronic HBV infection is by far the most important risk factor for HCC in China and sub-Saharan Africa, the regions of highest incidence of HCC. Epidemiological studies

have provided evidence that genetic factors are important in determining an individual's susceptibility to HCC. Advances in knowledge of the molecular pathogenesis of HCC have resulted in significant improvements in the therapeutic management of the disease[3]. For example, genetic variants within nuclear factor-κ B (NF-κ B) signaling pathways may be involved in HBV-associated hepatocarcinogenesis[4]. Significant progress in understanding the genetic predisposition to HCC has been provided by genome-wide association studies (GWAS) and intensive international collaboration[5]. However, the genetic basis of susceptibility to HCC is still poorly understood and early detection is limited by the lack of reliable clinical and molecular markers.

miRNAs are a class of endogenous, small (21-23 nucleotide), noncoding but functional RNAs. Mature miRNAs can be generated by sequential processing of primary miRNA transcripts by Drosha and Dicer enzymes, and may act as posttranscriptional regulators of gene expression by complementary base pairing to messenger RNAs[6]. Misregulation of miRNA expression has been linked to many types of cancer[7]. miRNAs recognize their targets mainly through limited base-pairing interactions between the 5' end of the miRNA (i.e., nucleotides 2-8, the seed region) and complementary sequences in the 3' untranslated regions (3'UTRs) of the target mRNAs[6]. The binding of miRNA to mRNA is critical for regulating mRNA level and protein expression. Genetic changes in the 3'UTR targeted by miRNAs have been found to alter the strength of miRNA binding, affecting the regulation of target genes and an individual's risk of cancer[8-10].

The epidermal growth factor (EGF) family of receptor tyrosine kinases consists of four members, ErbB1, ErbB2, ErbB3 and ErbB4[11]. The four ErbB receptors are selectively activated by a number of EGF-like growth factors leading to cellular responses, such as proliferation, differentiation, migration, and/or survival. Aberrant expression or activity of EGFR and ErbB2 have been strongly linked to the etiology of several human epithelial cancers including head and neck squamous cell carcinoma, non-small-cell lung cancer, colorectal cancer, and breast cancer[12]. Activating mutations in ErbB4 have been observed in metastatic melanoma, providing strong evidence for an oncogenic role of ErbB4 in cancer[13]. Furthermore, ErbB4 expression is lower in HCC than in adjacent noncancerous tissues[14]. However, no studies to date have examined the effects of *ErbB4* polymorphisms on susceptibility to HCC. Using *in-silico* analysis, we identified a 12-bp (AAAATAGGATTG) insertion/deletion polymorphism (rs6147150) in the 3'UTR of *ErbB4*, located within the seed region of the hsa-let-7c potential target sequence. Using a case-control design, we assessed whether this rs6147150 polymorphism influences susceptibility to HCC in a Chinese population.

MATERIALS AND METHODS

In-silico analysis of microRNA-binding

The mature human microRNA sequences were obtained from the microRNA database (miRBase) (http://microrna.sanger.ac.uk). A region consisting of rs6147150 plus 15 bp 5' and 3' was used to analyze hybridization of putative microRNAs. The minimum free energy required for hybridization of putative microRNA and polymorphisms was predicted by miRanda software with default parameters[15]. The difference in energies between wild type and variant was computed as ΔΔG and used to assess the impact of the polymorphism on miRNA binding.

Study populations

Peripheral blood samples were obtained from 270 HCC patients newly diagnosed, hospitalized, and treated in our department from 2004 to 2009, and from 270 cancer-free subjects matched by sex and age and selected from a community-wide nutritional survey conducted in the same regions and at the same time as the cancer patients were recruited. All subjects were unrelated ethnic Han Chinese, and none of the 270 HCC patients had received any medical treatment. HCC patients were excluded if they had: (1) primary or secondary biliary cirrhosis or Budd-Chiari syndrome; (2) autoimmune hepatitis or toxic hepatitis; (3) recurrence of HCC; (4) tumors other than HCC; or (5) liver disease due to parasitosis, diabetes, fatty liver, metabolism disorders or severe cardiovascular diseases. HCC diagnosis was confirmed by a pathological examination combined with positive results on magnetic resonance imaging and/or computerized tomography. Tumor stages were determined according to a modified American Joint Committee on Cancer (AJCC) and International Union Against Cancer (UICC) standard. Subjects who smoked more than two cigarettes per day for more than 1 year were classified as smokers; others were defined as non-smokers. Subjects who consumed at least one drink of alcohol per week were considered alcohol drinkers; others were considered non-drinkers. All participants were negative for antibodies to hepatitis C virus, hepatitis D virus and human immunodeficiency virus. The design of the study was approved by the Ethical Committee of Suzhou Municipal Hospital, and all participants provided written informed consent.

DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood samples using a Chelex method[16]. DNA fragments containing the polymorphism were amplified using the primers 5'-ATTCCAGAGGCCAATTGTA-3' (forward) and 5'-TTTCCTCACCTGTTTACCAC-3' (reverse). Polymerase chain reaction (PCR) reactions were performed in a total volume of 20 μL, containing 2.0 μL 10 × PCR buffer, 1.5 mmol/L $MgCl_2$, 0.25 mmol/L of each dNTP, 0.5 mmol/L of each primer, 100 ng of genomic DNA, and 1.0 U of Taq DNA polymerase. The amplification protocol consisted of an initial denaturation at 94 ℃ for 5 min, followed by 35 cycles of denaturation for 30 s at 94 ℃, annealing for 30 s at 58 ℃, and extension for 30 s at 72 ℃, followed by a final extension at 72 ℃ for 5 min. The PCR products were analyzed by 7% non-denaturing polyacrylamide gel electrophoresis (PAGE) and visual-

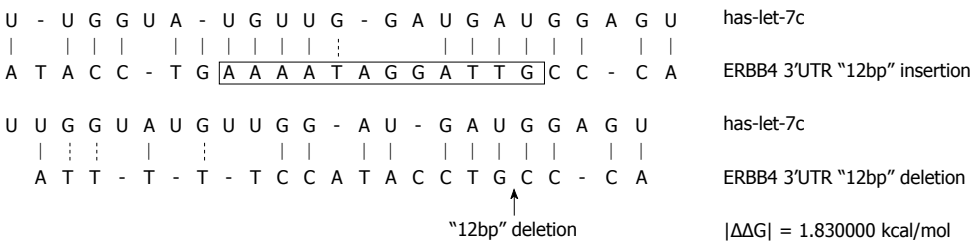

Figure 1 ***In-silico*** **analysis of microRNA-binding.** rs6147150 was located at the fifth nucleotide from the 5' end of has-let-7c. The ΔΔG value between the 12-bp insertion (up) and the 12-bp deletion (down) alleles was calculated to be 1.830000 kcal/mol.

Table 1 Demographic characteristics of the hepatocellular carcinoma case and control groups

Characteristics	Case		Control		*P* value
	n = 270	Frequency (%)	*n* = 270	Frequency (%)	
Age, (yr) (mean ± SD)	50.3 ± 9.2		50.7 ± 9.4		0.91[a]
Gender					
Male	187	69.3	190	70.4	0.78[b]
Female	83	30.7	80	29.6	
Smoking status					
Smoker	79	29.3	85	31.5	0.57[b]
Non-smoker	191	70.7	185	68.5	
Drinking status					
Drinker	120	44.4	116	43.0	0.73[b]
Non-drinker	150	55.6	154	57.0	
Tumor stages					
Ⅰa + Ⅰb	193	71.5			
Ⅱa + Ⅱb	58	21.5			
Ⅲa + Ⅲb	19	7.0			
HBsAg, *n* (%)					
Positive	201	74.4	26	9.6	< 0.0001[b]
Negative	69	25.6	244	90.4	

[a]Two-sided two-sample *t*-tests. [b]χ^2 tests. HBsAg: Hepatitis B surface antigen.

ized by silver staining[17]. The 12-bp deletion allele of rs6147150 yielded a band of 181 bp and the insertion allele yielded a band of 193 bp. To validate the genotyping method, we analyzed 20 randomly selected DNA samples by both direct sequencing and the PCR method; the concurrence rate of these two methods was 100%, indicating that the PCR method is reliable. Genotyping was performed without knowledge of case or control status. Moreover, 10% of the samples were randomly selected for testing by two different persons, with a reproducibility of 100%.

Statistical analysis

Hardy-Weinberg equilibrium was assessed using a goodness-of-fit χ^2 test for biallelic markers. The adjusted odds ratios (ORs) with their 95% confidence intervals (CIs) of the association between polymorphism and HCC risk were estimated by multiple logistic regression models after controlling for sex, age, smoking status, drinking status, tumor stage and HBV infection. In all cases, homozygosity for the most common allele (i.e., ins/ins) was used as the reference category. A binary logistic regression model was used for stratification by HBV infection status. All statistical analyses were performed using Statistic Analysis System software (version 8.0, SAS Institute), with statistical significance defined as $P < 0.05$ and statistical power calculated using PS software (http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize).

Table 2 Genotype and allele frequencies of rs6147150 among cases and controls, and risk of hepatocellular carcinoma *n* (%)

Genotype/allele	Cases		Controls		OR (95% CI)[1]	*P* value
12N ins/ins	109	40.4	143	53.0	1.00 (Reference)	-
12N ins/del	117	43.3	104	38.5	1.48 (1.03-2.17)	0.034
12N del/del	44	16.3	23	8.5	2.50 (1.37-4.56)	0.001
P_{trend}						0.0006
12N ins	335	62.0	390	72.2	1.00 (Reference)	-
12N del	205	38.0	150	27.8	1.59 (1.22-2.07)	0.0003

[1]adjusted for sex, age, smoking status, drinking status, HBV infection and tumor stage. HBV: Hepatitis B virus; OR: Odds ratio; CI: Confidence interval.

RESULTS

In-silico analysis of microRNA-binding

Computational modeling of the rs6147150 polymorphism suggested that it was located within the hsa-let-7c potential target sequence of the *ErbB4* 3'UTR (Figure 1). The ΔΔG value was calculated as 1.83 kcal/mol.

Association of HCC with the rs6147150 polymorphism

The demographic characteristics of the 270 HCC patients and 270 controls are summarized in Table 1. There were no statistically significant differences in sex distribution, age, or smoking or drinking status between the two groups. As expected, HBV infection was a significant risk factor for HCC. About 74.4% of the cases and 9.6% of the controls were HBsAg-positive ($P < 0.0001$). Genotype distributions showed no deviation from Hardy-Weinberg equilibrium in either cases ($P = 0.149$) or controls ($P = 0.416$). We found that rs6147150 was significantly associated with HCC susceptibility at both the allele and genotype levels (Table 2). Comparing with individuals homozygous for 12N ins/ins, those heterozygous for 12N ins/del (adjusted OR = 1.48, 95% CI = 1.03-2.17, $P = 0.034$) and homozygous for 12N del/del (OR = 2.50, 95% CI = 1.37-4.56, $P = 0.001$) were at significantly in-

Table 3 Stratification analysis based on hepatitis B virus infection status in cases and controls

Genotype	HBV positive					HBV negative				
	Case	%	Control	%	OR[1] (95% CI)	Case	%	Control	%	OR[1] (95% CI)
12N ins/ins	82	40.8	14	53.8	1.00 (Reference)	28	40.6	127	52.0	1.00 (Reference)
12N ins/del	88	43.8	10	38.5	1.51(0.57-3.91)	30	43.5	90	36.9	1.52 (0.79-2.94)
12N del/del	31	15.4	2	7.7	2.64 (0.51-17.59)	11	15.9	27	11.1	1.87 (0.74-4.54)
P_{trend}					P = 0.156					P = 0.083

[1]Adjusted for sex, age, smoking status, drinking status and tumor stage. HBV: Hepatitis B virus; OR: Odds ratio; CI: Confidence interval.

creased risk of HCC after controlling for other covariates (Table 2). We also found that the frequency of the 12-bp deletion or insertion allele differed significantly between the HCC and control groups. The presence of the 12-bp deletion allele was associated with a significantly increased risk of developing HCC (OR = 1.59, 95% CI 1.22-2.07, P = 0.003). HBV stratification analysis showed no significant difference in allele frequency between HBV-positive and HBV-negative groups (Table 3). Using PS software, we estimated a power of 0.94 with an α set at 0.05 to obtain an OR of 2.0.

DISCUSSION

In addition to environmental factors, such as viral infection, an increasing number of novel genetic components identified by GWAS have been found to predispose individuals to HCC. Thus, assessments of functional variants are necessary to determine risks of developing HCC. To our knowledge, this study is the first to evaluate the association between genetic variants in *ErbB4* and HCC susceptibility. Our results indicate that rs6147150 is associated with HCC susceptibility in a Chinese population, possibly through let-7c mediated regulation.

Altered ErbB signaling has been frequently observed during malignant transformation, with ErbB overactivity often implicated in the pathogenesis of several epithelial malignancies[18,19]. In contrast, growth factor receptors with tyrosine kinase activity are known to contribute greatly to the regulation of cell behavior, such as cell growth, proliferation and mortality. ErbB4 is frequently expressed in tumors[20-22]; although, in contrast to EGFR and ErbB2, its role as a tumor-driving oncogene is unclear[23]. Although there is little evidence of an association between ErbB4 and HCC, miRNAs have been shown to modulate ErbB receptor expression and downstream signaling activity, stimulating intense interest in the development of miRNAs as therapeutic molecules and clinical biomarkers in cancer[24]. Because HCC is an epithelial cancer originating from hepatocytes or their progenitors, genetic polymorphisms in *ErbB4* may be associated with susceptibility to HCC.

Since bioinformatics analysis suggests that rs6147150 lies within a predicted binding site (seed region) for let-7c, we hypothesized that let-7c would bind tightly to ErbB4 mRNA transcripts containing the 12-bp deletion allele, negatively regulating ErbB4 expression. Conversely, binding with mRNA transcripts containing the 12-bp insertion allele would be disrupted, resulting in increased ErbB4 expression. An ErbB4-specific ligand, heparin-binding EGF-like growth factor (HB-EGF), has been shown to be involved in the development and/or progression of human HCC in an autocrine and/or a paracrine manner, especially during the early stages of HCC[25,26]. Therefore, aberrant expression of ErbB4 would influence the specific binding of HB-EGF and increase the risk for HCC. miRNAs of the let-7 family are highly conserved in bilateral animals and control stem cell division and differentiation[27]. They also function as tumor suppressors and inhibit cell proliferation and tumorigenesis. Low levels of expression of let-7 have been observed in a variety of cancers, including HCC[28].

Taken together, our results suggest that common genetic polymorphisms in *ErbB4* may influence HCC risk, at least in part by let-7c-mediated regulation which may be involved in the pathogenesis of HCC. Our results may provide a greater understanding of the mechanisms of hepatocarcinogenesis and may help in the identification of diagnostic markers for HCC. However, it is important to determine whether the association between this polymorphism and HCC risk also applies to other populations. Finally, additional functional studies are required to fully understand the involvement of *ErbB4* polymorphisms in predisposition to HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver, with an increasing incidence worldwide. Epidemiological studies have indicated that genetic factors are important in determining an individual's susceptibility to HCC. However, the genetic basis of susceptibility to HCC is still poorly understood and early detection of HCC is infrequent because of the lack of reliable markers. Studies focusing on functional variants in these findings are therefore indispensable.

Research frontiers

Recent genome-wide association studies, which are routinely used to identify common polymorphisms that underlie disease susceptibility in a large population, have enhanced our understanding of the genetic predisposition to HCC. These findings have led to the identification of several genetic variants that may modulate the risk of HCC.

Innovations and breakthroughs

Relative to individuals homozygous for the rs6147150 12-bp ins/ins allele, homozygotes for 12-bp del/del and heterozygotes are at significantly higher risk of HCC. Carriers of the "del" allele are associated with a 1.59-fold increased risk of HCC relative to non-carriers. These findings suggest that common genetic polymorphisms in *ErbB4* may influence HCC risk, at least in part *via* let-

7c-mediated regulation, which may be involved in the pathogenesis of HCC in Chinese populations.

Applications

This preliminary report may help clarify the molecular pathogenesis of hepatocarcinogenesis. Polymorphisms within *ErbB4* may be used as potential markers for HCC predisposition in Chinese populations.

Peer review

This study shows that rs6147150 alleles are significantly associated with the risk of HCC, at least in part through let-7c-mediated regulation, which may be involved in the pathogenesis of HCC. The study design is reasonable and sample size is acceptable.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i4.388

World J Gastroenterol 2012 January 28; 18(4): 388-392
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

No association between *IRF3* polymorphism and susceptibility to hepatitis B virus infection in Chinese patients

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Supported by Grants from the National Natural Science Foundation of China, No. 81072342; the National Pre-973 Program Projects, No. 2009CB526411
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Received: April 25, 2011 Revised: July 28, 2011
Accepted: October 27, 2011
Published online: January 28, 2012

Abstract

AIM: To investigate the association between three tag single nucleotide polymorphisms (tagSNPs) in interferon regulatory factors (*IRF3*) and the genetic susceptibility to chronic hepatitis B virus (HBV) infection.

METHODS: We performed a case-control study of 985 Chinese cases of chronic HBV infection and 294 self-limiting HBV-infected individuals as controls. Three tagSNPs in *IRF3* (rs10415576, rs2304204, rs2304206) were genotyped with the Multiplex SNaPshot technique. The genotype and allele frequencies were calculated and analyzed.

RESULTS: The three SNPs showed no significant genotype/allele associations with chronic HBV infection. Overall allele *P* values were: rs10415576, *P* = 0.0908, odds ratio (OR) [95% confidence interval (CI)] = 1.1798 (0.9740-1.4291); rs2304204, *P* = 0.5959, OR (95% CI) = 1.0597 (0.8552-1.3133); rs2304206, *P* = 0.8372, OR (95% CI) = 1.0250 (0.8097-1.2976). Overall genotype *P* values were: rs10415576, *P* = 0.2106; rs2304204, *P* = 0.8458; rs2304206, *P* = 0.8315. There were no statistically significant differences between patients with chronic HBV infection and controls. Haplotypes generated by these three SNPs were also not significantly different between the two groups.

CONCLUSION: The three tagSNPs of *IRF3* are not associated with HBV infection in the Han Chinese population.

Key words: Chronic hepatitis B virus infection; Interferon regulatory factors tag single nucleotide polymorphisms; Genetic susceptibility; Haplotype

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Yan F, Gao YF, Lv F, Zhang TC, Li X, Yin HF. No association between *IRF3* polymorphism and susceptibility to hepatitis B virus infection in Chinese patients. *World J Gastroenterol* 2012; 18(4): 388-392 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i4/388.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i4.388

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is considered a multifactorial and polygenic disorder with viral, environmental, genetic, and immune components. Host immunological and genetic factors may influence the course of the disease[1,2]. Genetics of the host such as single nucleotide polymorphisms (SNPs) in a variety of genes have been implicated in the diversity of the clinical course of HBV[3-8].

Interferon regulatory factors (IRFs) are transcriptional mediators of virus- and interferon (IFN)-induced signaling pathways and have been shown to be involved in antiviral defense, immune response, and cell growth regulation[9]. Among the nine identified members of the IRF family (IRF1-IRF9), three IRFs (IRF3, IRF5 and IRF7) were found to function as direct transducers of virus-mediated signaling and play a crucial role in the expression of type I *IFN* genes[10]. Interferon regulatory factor 3 (*IRF3*) is a member of the IRF family which plays a major role in gene expression of IFN regulatory factors, and is closely related to the level of interferon gene expression in condition of virus infection[11-13]. Patients with chronic HBV infection failed to activate *IRF3* following virus contraction and thereby are unable to secrete enough IFN-β to eradicate the HBV virus, which may partly contribute to persistent infection of HBV[14].

IRF3 plays an important role in the response of the innate immune system to viral infection[15]. Polymorphisms in *IRF3* can affect induction of *IFN-β1* expression[16], and *IRF3* is differentially activated during type 1 IFN responses in human macrophages[17]. The aim of this study was to clarify whether *IRF-3* polymorphisms can serve as candidates for predicting clinical outcomes of the disease caused by HBV infection.

MATERIALS AND METHODS

Participants

Samples were obtained from 1279 unrelated Chinese Han patients with HBV infection. Chronic HBV-infected patients (n = 985; 687 males and 298 females) were recruited from the Infectious Disease Department as cases, and 294 self-limiting HBV-infected individuals were recruited as controls (132 females and 162 males). The average age was 44.2 years for the HBV chronic carriers and 44.9 years for the controls. All the patients with chronic HBV infection fulfilled the diagnostic criteria of the Proposal of Prevention and Treatment of Viral Hepatitis, 2005, issued by the Chinese Society of Infectious Diseases and Parasitology and the Society of Hepatology of the Chinese Medical Association[18]. Clinical criteria for self-limiting HBV infection were positive for HBsAb and HBcAb, negative for HBsAg, and no history of HBV vaccination. Controls were age- and gender-matched to the cases.

Genomic DNA extraction

Blood samples were obtained from all participants, and DNA was extracted from peripheral blood leukocytes using a DNA extraction kit (Yuan Ping-Hao Biotechnology Co. Ltd., Tianjin, China). DNA samples (100 ng/μL) were stored at -80 ℃.

Tag SNP selection

We selected SNPs on the basis of the following principal criteria: tag SNPs (tagSNP) were identified using genotype data from the panel (Han Chinese in Beijing) of the phase II HapMap Project. The criteria for tagSNPs were $r^2 > 0.8$, minor allele frequency MAF > 0.1, functional relevance and importance, and SNPs significantly associated with diseases in previous studies. A total of three tagSNPs in *IRF3* gene (rs10415576, rs2304204 and rs2304206) were selected, which captured 100% of common SNPs (minor allele frequency > 0.1) in the HapMap Chinese database at $r^2 > 0.8$.

Genotyping

The three SNPs in *IRF3* were genotyped using the Multiplex SNaPshot technique. The primers and probes were designed by primer5.0 software and were rs10415576 (5' to 3'): forward primer: CGAGTGCAACCAGGACGTGAT, reverse primer: ATGCTCCCAAGGATGCTCAGTG, and extension primer: GCTGGTTGGCATTCAGTCC; both rs2304204 and rs2304206, forward primer: TCCCATCGGCTTTTGGGTCT, reverse primer: CTTTCCCGCTCCTCGCTCTC, and extension primer: TTTTTGTTGGTTTTATTTCAAGAAGTCGATC. The polymerase chain reaction (PCR) amplification conditions were: a 15-μL final volume containing 10 × 1.5 μL buffer, 0.3 μL dNTPs (10 mmol/L), 0.9 μL $MgCl_2$ (25 mmol/L), 0.1 μL Taq DNA polymerase (TAKARA Biotechnology Co. Ltd., Dalian, China), 0.5 μL each primer (10 pmol/L), and 1 μL DNA template (20 mg/L). Conditions for the multiplex PCR reaction using Touch-down PCR response procedures included initial denaturation at 95 ℃ for 15 min, denaturation at 94 ℃ for 40 s, annealing at 63 ℃ for 1 min, and recursive-descent 0.5 ℃, followed by extension at 72 ℃ for 1.5 min, for a total of 15 cycles. This was followed by 25 cycles of denaturation at 94 ℃ for 40 s, annealing at 56 ℃ for 40 s, and extension at 72 ℃ for 1.5 min, with a final extension at 72 ℃ for 8 min. Amplified samples were stored at 4 ℃. After amplification, 1.5 μL PCR product was examined on an agarose gel to test for successful amplification.

SNaPshot reaction: take the purified PCR product, each concentration of 0.2 μmol/L SNaPshot primer mixture, SNaPshot fluorescent mixtures (containing Taq DNA polymerase and different fluorescently labeled ddNTP, TAKARA Biotechnology Co. Ltd., Dalian,China) consisting of an PCR reaction system. SNaPshot response procedures: initial denaturation at 96 ℃ for 10 s; denaturation at 96 ℃ for 10 s, annealing at 53 ℃ for 5 s, extension at 60 ℃ for 30s, for a total of 25 cycles, and finally extension at 60 ℃ for 30s. Amplified samples were stored at 4 ℃. SNaPshot PCR products using SAP purification, in 10 μL SNaPshot PCR product with 1 U SAP or

Table 1 Genotype and allele distributions of interferon regulatory factors tag single nucleotide polymorphisms in patients with chronic hepatitis B virus infection and with self-limiting hepatitis B virus infection *n* (%)

IRF3	Chronic HBV infection	Acute HBV infection	*P* value	OR (95% CI)
rs10415576	*n* = 985	*n* = 294		
AA	347 (35.23)	119 (40.48)		1.0
GG	135 (13.71)	33 (11.22)	0.125	1.4029 (0.9091-2.1650)
AG	503 (51.07)	142 (48.30)	0.172	1.2148 (0.9187-1.6063)
A	1197 (60.76)	380 (64.63)	0.091	1.1798 (0.9740-1.4291)
G	773 (39.24)	208 (35.37)		
rs2304204	*n* = 985	*n* = 294		
AA	542 (55.03)	166 (56.46)		
GG	54 (5.48)	14 (4.76)	0.594	1.1813 (0.6400-2.1807)
AG	389 (39.49)	114 (38.78)	0.75	1.0451 (0.7965-1.3713)
A	1473 (74.77)	446 (75.85)	0.596	1.0579 (0.8552-1.3133)
G	497 (25.23)	142 (24.15)		
rs2304206	*n* = 985	*n* = 294		
CC	639 (64.87)	191 (64.97)		1.0
TT	30 (3.05)	7 (2.38)	0.562	1.2810 (0.5539-2.9627)
CT	316 (32.08)	96 (32.65)	0.909	0.9839 (0.7437-1.3016)
C	1594 (80.91)	478 (81.29)	0.837	1.0250 (0.8097-1.2976)
T	376 (19.09)	110 (18.71)		

IRF3: Interferon regulatory factors 3. HBV: Hepatitis B virus; OR: Odds ratio; CI: Confidence interval.

1 U CIP, were mixed and insulated at 37 ℃ for 1 h, and 75 ℃ for 15 min to inactivate the enzyme. The samples can be stored at 4 ℃ for 24 h or -20 ℃ permanently.

DNA sequencing: the SNaPshot product was diluted 20-fold. In a total volume of 10 μL we mixed 8.6 μL HiDiFormamide (high-purity formamide), 0.9 μL GeneScan-120 LIZ Size Standard, and 0.5 μL SNaPshot purification product. Samples were incubated at 95 ℃ for 5 min, chilled quickly for 4 min, and then loaded on an ABI 3730XL Genetic Analyzer (Applied Biosystems, CA, United States) for capillary electrophoresis, running GeneMapper4.0 software analysis of experimental results.

Statistical analysis

Allele and genotype frequencies were obtained by direct counting, and the Chi-square test was used to compare allele and genotype distributions. The quality of the genotype data was assessed by Hardy-Weinberg equilibrium in the case and control samples using Fisher's exact test ($P > 0.05$). Odds ratios (OR) and 95% confidence intervals [95% confidence interval (CI)] were calculated according to Woolf's method

RESULTS

We investigated the distribution of the three SNPs in 985 Chinese HBV-infected patients (cases) and 294 self-limiting HBV-infected patients (controls). All genotypes of the *IRF-3* polymorphisms were in Hardy-Weinberg equilibrium in both the cases and controls.

The genotype frequencies and allele distributions of *IRF3* polymorphisms in each subgroup of HBV-infected patients are summarized in Table 1. The genotype frequencies for AA, GG, and AG of *IRF3* rs10415576 were 35.23%, 13.71%, and 51.07% in case samples, and 40.48%, 11.22%, and 48.30% in control samples, respectively, without significant differences between cases and controls ($P = 0.2105$). The genotype frequencies for AA, GG, and AG of *IRF3* rs2304204 were 55.03%, 5.48%, and 39.49% in case samples, and 56.46%, 4.76%, and 38.78% in control samples, respectively, without significant differences between cases and controls ($P = 0.8372$). The genotype frequencies for CC, TT, and CT of *IRF3* rs10415576 were 64.87%, 3.05%, and 32.08% in case samples, and 64.97%, 2.38%, and 32.65% in control samples, and no significant differences were noted ($P = 0.8315$).

Table 2 Distribution of haplotypes of interferon regulatory factor tag single nucleotide polymorphisms rs10415576, rs2304204 and rs2304206 in patients with chronic hepatitis B virus infection and self-limiting hepatitis B virus infection

Haplotypes	Frequency (cases)	Frequency (controls)	χ^2	*P* value	OR (95% CI)
AAC	0.6066	0.6446	2.8246	0.0929	1.1787 (0.9729-1.4279)
GAC	0.1411	0.1139	2.8473	0.0916	0.7833 (0.5895-1.0408)
GGC	0.0609	0.0527	0.5420	0.4616	0.8586 (0.5721-1.2888)
GGT	0.1904	0.1871	0.0293	0.8642	0.9796 (0.7738-1.2402)

OR: Odds ratio; CI: Confidence interval.

In addition, no statistically significant differences were found when the allele frequencies of SNPs rs10415576, rs2304204 and rs2304206 were compared between patients with chronic HBV infection and controls. Overall allele *P* values were: rs10415576, $P = 0.0908$, OR (95% CI) = 1.1798 (0.9740-1.4291); rs2304204, $P = 0.5959$, OR (95% CI) = 1.0597(0.8552-1.3133); rs2304206, $P = 0.8372$, OR (95% CI) = 1.0250(0.8097-1.2976).

Haplotype analysis

We also estimated the *IRF3* haplotype frequencies and evaluated the association among these variants and HBV infection. We observed four haplotype combinations, but no significant association was found in the distribution of the haplotype frequencies between cases and controls ($P > 0.05$). Haplotype frequencies lower than 0.03 were ignored in the analysis (Table 2).

DISCUSSION

The human *IRF3* gene is located on chromosome 19ql3.3-ql3.4, and encodes a 50-KDa protein[19]. The ubiquitously expressed *IRF3* is activated in infected cells upon recognition of double-stranded RNA, which is considered the common signature of virus-infected cells[19,20]. Lau *et al*[21] indicated that hepatitis C virus (HCV) can transiently trigger *IRF3* activation during virus spread and that in chronic HCV, *IRF3* activation within infected hepatocytes occurs but is limited.

Several studies have described the importance of *IRF3* polymorphisms. The effect of the A to T substitution on the splicing efficiency of intron 5 of mouse *IRF3*

was confirmed using an *in vitro* splicing assay. The A to T polymorphism in intron 5 of *IRF3* affects induction of IFN-β1 expression[16]. Zhang *et al*[22] showed that SNPs in codon 427 of human *IRF3* may be related to the susceptibility to esophageal cancer. The risk of esophageal cancer in participants with the C allele is 2.38 folds higher that in those with the G allele. However, Sánchez *et al*[23] suggest that the *IRF3* polymorphisms (rs2304204, rs7251 and rs2304207) do not appear to play a major role in the susceptibility or severity of systemic lupus erythematosus in a Spanish population. We selected three SNPs in *IRF3* (rs10415576, rs2304204, and rs2304206) using genotype data from the panel (Han Chinese in Beijing) of the phase II HapMap Project. The three tagSNPs captured 100% of common SNPs (minor allele frequency > 0.1) in the HapMap Chinese database at $r^2 > 0.8$. We analyzed the associations of the three SNP alleles with HBV-infected patients compared with spontaneously cleared HBV controls. The results indicated that there were no significant differences between cases and controls in the genotype or haplotype frequencies of any of the three SNPs we analyzed, suggesting that tagSNPs of *IRF3* do not predict the outcome of HBV infection.

In conclusion, although *IRF3* is a functional gene with a relevant role in HBV pathogenesis, our study demonstrates that the tagSNPs of *IRF3* do not correlate with genetic susceptibility to chronic HBV infection in Chinese patients. Further genetic studies are needed to examine the polymorphisms in other *IRF3* SNPs and the possible association with disease progress in chronic HBV infection.

COMMENTS

Background

Persistent hepatitis B virus (HBV) infection is considered a multifactorial and polygenic disorder with viral, environmental, and genetic components, as well as contributions from HBV genomic variability, host age, gender, concurrent infection with the hepatitis C virus, hepatitis D virus, and human immune deficiency virus. Interferon regulatory factors 3 (*IRF3*) plays an important role in the response of the innate immune system to viral infection. *IRF3* polymorphisms affect induction of interferon (IFN)-β1 expression.

Research frontiers

This study is the first to investigate the association between three tag single nucleotide polymorphisms (tagSNPs) (rs10415576, rs2304204 and rs2304206) of *IRF3* and the genetic susceptibility to chronic HBV infection in Chinese patients using the Multiplex SNaPshot technique.

Innovations and breakthroughs

The three tagSNPs of *IRF3* were not related to genetic susceptibility to HBV infection.

Applications

Based on the results of this study, further genetic studies are needed to examine the roles of other *IRF3* SNPs and their association with disease progress in chronic HBV infection.

Terminology

IRF3 is located on chromosome 19ql3.3-ql3.4, and encodes a 50-KDa protein.

Peer review

The manuscript analyzed the association between polymorphisms of *IRF3* and clinical outcomes of HBV infection. Three SNPs of IRF3 were genotyped using Multiplex SNaPshot technique and compared between chronic HBV infection and self-limiting HBV infection patients. The overall data are negative and showed that SNPs of IRF3 did not affect the outcome of HBV infection. The data may have a significant clinical implication.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2012 January 28; 18(4): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2012 January 28; 18(4): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

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American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

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Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

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The Liver Meeting
San Francisco, CA 94101, United States

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American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
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Online Submissions: http://www.wjgnet.com/1007-9327office
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www.wjgnet.com

World J Gastroenterol 2012 January 28; 18(4): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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Name of journal

World Journal of Gastroenterology

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

Editorial office
World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
Ocean International Center,
No. 62 Dongsihuan Zhonglu,
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Indexed and abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifer, and Directory of Open Access Journals. ISI, Thomson Reuters, 2010 Impact Factor: 2.240 (35/71 Gastroenterology and Hepatology).

Published by
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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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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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World Journal of
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Volume 18 Number 5
February 7, 2012

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Volume 18 Number 5 Feb 07 2012

ISSN 1007-9327 (print)
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WJG

World Journal of Gastroenterology

Contents

Weekly Volume 18 Number 5 February 7, 2012

World Journal of Gastroenterology
Volume 18 Number 5 February 7, 2012

Contents

NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

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PUBLISHER
Baishideng Publishing Group Co., Limited
Room 1701, 17/F, Henan Building,
No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-31158812
Telephone: +852-58042046
E-mail: bpg@baishideng.com
http://www.wjgnet.com

PRINT SUBSCRIPTION
RMB 300 Yuan for each issue, RMB 14400 Yuan for one year.

PUBLICATION DATE
February 7, 2012

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

ONLINE SUBMISSION
http://www.wjgnet.com/1007-9327office/

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i5.393

World J Gastroenterol 2012 February 7; 18(5): 393-400
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Estrogen, male dominance and esophageal adenocarcinoma: Is there a link?

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Received: May 28, 2011 Revised: August 11, 2011
Accepted: August 15, 2011
Published online: February 7, 2012

Abstract

Esophageal adenocarcinoma is a cancer with poor prognosis, and its incidence has risen sharply over recent decades. Obesity is a major risk factor for developing this cancer and there is a clear male gender bias in the incidence that cannot be fully explained by known risk factors. It is possible that a difference in the expression of estrogen, or its signaling axes, may contribute to this gender bias. We undertook a comprehensive literature search and analyzed the available data regarding estrogen and estrogen receptor expression, and the possible sex-specific links with esophageal adenocarcinoma development. Potentially relevant associations between visceral *vs* subcutaneous fat deposition and estrogen expression, and the effect of crosstalk between estrogen and leptin signaling were identified. We also found limited studies suggesting a role for estrogen receptor β expression in esophageal adenocarcinoma development. The current literature supports speculation on an etiological role for estrogen in the male gender bias in esophageal adenocarcinoma, but further studies are required.

Key words: Estrogen; Estrogen receptors; Male dominance; Esophageal adenocarcinoma

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Yang H, Sukocheva OA, Hussey DJ, Watson DI. Estrogen, male dominance and esophageal adenocarcinoma: Is there a link? *World J Gastroenterol* 2012; 18(5): 393-400 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i5/393.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i5.393

INTRODUCTION

Esophageal carcinoma is the eighth most common cancer worldwide, and over the last three decades its incidence has risen significantly in all Western countries[1-4]. This change is entirely due to an increase in the adenocarcinoma subtype, and this has predominantly occurred in males[2,5-9]. Recent Western experiences report that the male:female ratio for patients undergoing esophagectomy for adenocarcinoma now exceeds 8:1. However, the identified risk factors for esophageal adenocarcinoma, including gastroesophageal reflux disease, Barrett's esophagus, obesity, alcohol, and tobacco consumption cannot adequately explain this profound gender difference. The dramatic gender difference for esophageal adenocarcinoma suggests there should be a gender-related mechanism underpinning this phenomenon. Estrogens, the primary female sex hormones, are mechanistically linked to aspects of cancer risk and cancer development. Therefore it seems reasonable to consider that estrogens might

contribute towards the gender difference for esophageal adenocarcinoma.

A link between estrogen-activated signaling and carcinogenesis in many organs, including mammary glands[10], ovaries and colon[11] has been clearly defined, although it is unclear whether a similar connection exists for the esophagus, and esophageal adenocarcinoma in particular. Additionally, estrogen is actively involved in the regulation of metabolism in adipose tissues[12], and it can be synthesized locally by activated aromatase in adipocytes in both men and women[13-15]. Involvement of estrogen signaling in regulation of adipose tissue metabolism indicates a possible connection between the effects of estrogen and male obesity - one of the main risk factors for esophageal adenocarcinoma. Given the established regulatory role for estrogen in carcinogenesis and metabolic homeostasis for other cancers, and the strong gender differences for the incidence of esophageal adenocarcinoma, it is plausible to suggest that the estrogen signaling network is involved in the progression of this cancer, and an understanding of estrogen and estrogen receptor (ER) roles in the regulation of carcinogenesis, and how this might be relevant in the esophagus, could provide a basis for developing either preventive measures or new treatments.

In this paper we review potential links between estrogen signaling and esophageal adenocarcinoma, to determine whether this might contribute to the dominance of esophageal adenocarcinoma in males. Literature pertinent to gender specific differences in estrogen synthesis, estrogen-regulated carcinogenesis, specific differences between ERs and signaling in cancer cells, and available information about estrogen signaling in esophageal adenocarcinoma is reviewed.

ESTROGEN IN WOMEN AND MEN: AGE RELATED CHANGES

In premenopausal women the ovaries are the principal source of estrogen[16]. Serum estradiol concentration is much higher in premenopausal women, compared to men, but decreases substantially after the menopause, and ultimately becomes lower than in elderly men[17]. Mass spectrometry has shown that average levels of serum estradiol in elderly men are approximately 73 pmol/L, whereas levels in postmenopausal women are markedly lower (about 15 pmol/L)[17].

When the ovaries cease to produce estrogens in postmenopausal women the main characteristics of estradiol function change, and it is produced in extragonadal sites, and acts locally at these sites as a paracrine or intracrine factor[16-20]. These sites are similar in men and postmenopausal women and include the mesenchymal cells of adipose tissue[13], osteoblasts and chondrocytes of bone[21], the vascular endothelium and aortic smooth muscle cells[22], and numerous sites in the brain[16,17,23].

Importantly, in men and postmenopausal women, circulating estrogens are not the main drivers of estrogen action, but locally produced estrogens originating in extragonadal sites are responsible for the majority of paracrine and intracrine effects of these hormones[20]. The total amount of estrogen synthesized by these extragonadal sites may be small, but the local tissue concentrations achieved are probably high and exert biological influence locally. This might impact on tumor biology. For example, it has been determined that the concentration of estradiol present in breast tumors in postmenopausal women is at least 20-fold higher than in the plasma. Aromatase inhibitor therapy is associated with a major decrease in intratumoral concentrations of estradiol and estrone and loss of intratumoral aromatase activity, which is followed by downregulation of cancer cell growth[10,24]. Local estrogen biosynthesis has also been demonstrated in men, where aromatase expression in adipose tissue is greatly increased by this process[25,26]. However, with respect to esophageal adenocarcinoma, no studies have evaluated whether the amount of estrogen synthesized in abdominal adipose tissue is sufficient to exert any paracrine effect in the esophagus.

ESTROGEN RECEPTORS: STRUCTURE AND FUNCTIONS

The effects of estrogens are mediated by their ligation to ERα and ERβ. ERα and ERβ both belong to the nuclear steroid/thyroid hormone receptor family and they are encoded by two distinct genes [encoding estrogen receptor 1 (*ESR1*) and *ESR2*] which are located on two different chromosomes 6q25.1 and 14q22-24[27,28]. ERα and ERβ have distinct cellular distributions and regulate separate sets of genes. ERα is predominantly expressed in female sex organs such as the breast, uterus and ovaries especially during the reproductive years. ERβ is widely expressed in many other tissues in both genders, but to a lesser degree in males compared to females[15,29]. Although the role of ERs in male physiology has long been neglected, there is growing evidence for estrogen involvement in multiple areas of male physiology[15-17].

The mechanism for ER signaling has been widely investigated. ERα and ERβ share common functional domains, with a conserved central DNA-binding domain which is often involved in receptor dimerization[30,31]. ERs possess two activation function domains; activation function-1 and activation function-2, with the former interacting with non-ER transcription factors, and the latter containing the ligand binding domain[31,32]. One of the most important differences between ERα and ERβ is that activation function-1 in ERβ lacks functional activity[30]. Also, it has been suggested that the main function of ERβ is to bind ERα and suppress its activation, so that ERα and ERβ as a dimer might exert inverse biological effects. Another difference between ERα and ERβ signaling is their interaction with the activator protein-1[32-35]. The activator protein-1 complex of Jun/Fos hetero- or homo-dimers is a key regulator of cell proliferation, with one of its target genes identified as cyclin

D1[33]. Depending on whether ERα or ERβ is activated, the activator protein-1 complex acts in a reciprocal fashion to stimulate or inhibit cell proliferation[35].

After binding estrogen, the receptor ligand-binding domain undergoes a conformational and surface-charge change that results in receptor dimerization. Ligand-binding is accompanied by the dissociation of intracellular ER from chaperone proteins, subsequently releasing the hormone/ER complex for attachment to estrogen response elements in the promoter region of target genes. The dimer then binds DNA to regulate gene expression at specific regions of the DNA named hormone response elements[31,35]. As a consequence, transcription of 17β-estradiol-responsive genes increases, and proliferation or differentiation of steroid-sensitive tissue is augmented. Although most steroid hormone receptors primarily localize to the nuclei, additional ERs have been identified in the cytoplasm and on the plasma membrane. Activation of cytoplasm signaling cascades has been detected after estrogen binding to its plasma membrane receptors[36].

Several isoforms of ERβ able to mediate estrogen signaling have also been found. The isoforms can exert diverse functions, and significantly complicate understanding of cellular responses to estrogens. ERβ isoforms can inhibit ERα transcriptional activity at the estrogen response elements and potentially reverse estrogen signaling[34]. A splice variant of ERβ, termed ERβcx, has been characterized[37]. ERβcx is expressed in the breast[38], the prostate and testis[37], the esophagus[39], and in gastric tissue[40]. Interestingly, ERβcx does not bind estrogen[41]. Instead it inhibits ERα from binding DNA, whilst it does not influence ERβ. The role and mechanism of ERβcx downstream signaling in esophageal tissue is largely unclear and needs to be further investigated.

ROLE OF ESTROGEN AND ESTROGEN RECEPTORS IN VARIOUS MALIGNANCIES: HARMFUL OR HELPFUL?

The biological significance of ERs in breast tumorigenesis has been studied extensively. In breast tumors, ER signaling promotes malignancy due to oncogenic mutations, sustained exposure of ERα with endogenous or exogenous estrogen, and abnormal coupling of estrogen-activated cytoplasmic machinery to growth and anti-apoptosis, all well established causative triggers of cancer in postmenopausal women[41]. Several large prospective studies have confirmed the role of estrogen in stimulation of breast tumor growth, and have demonstrated that the risk of breast cancer is increased in women taking estradiol after the menopause[42-44].

In females with breast cancer, ERα is instrumental in promoting cell proliferation and cancer progression, whereas ERβ exerts anti-proliferative effects by induction of cell cycle and growth arrest[34]. For instance, the downregulation of the cyclin D1 gene by ERβ prevents cellular progression from the G1 to S-phase of the cell cycle[45]. Loss of ERβ expression is considered to be a common feature in estrogen-dependent breast tumor progression[34,35] supporting the hypothesis that ERβ acts as a protector against the mitogenic activity of estrogen in breast pre-malignant tissues.

Estrogen is also critical for the progression of ovarian cancer[46-48]. A strong association between long-term estrogen replacement therapy and increased risk of ovarian cancer has been detected in several studies[45-47]. Similar to breast cancer, the imbalance between ERα and ERβ, along with decreasing expression of ERβ in the ovaries can also lead to uncontrolled cellular proliferation, subsequent malignancy and metastasis[49,50]. Thus, ERβ appears to be pro-apoptotic, facilitating the destruction of malignant cells, whereas ERα has anti-apoptotic activity, indicating its growth stimulatory role[34,45,49]. Confirming the role of ERβ as a tumor-suppressor, deletion of chromosome 14q, where ERβ co-localizes with some other tumor suppressors, is often detected in breast, colon, ovarian and prostate malignant tissue[51-54].

In contrast to the cancer-promoting role of estrogen in breast and ovarian cancers, it has been shown that estrogen works as a cancer suppressor for several gastrointestinal malignancies[41,42,44-56]. The Women's Health Initiative study, which included a cohort of 16 608 women randomized to hormone replacement therapy (HRT) *vs* no HRT, showed that the risk of colorectal cancer was almost halved in women using HRT[55]. A similar study in the United Kingdom of patients with esophageal and gastric cancer concluded that HRT was associated with a 50% reduction in the risk of gastric and colon adenocarcinoma, but had no significant benefit for esophageal adenocarcinoma[56]. However, due to the relatively small number of females with esophageal adenocarcinoma in this study (n = 299), the power of the study was limited and the question remains, thus, unresolved[41,42].

The male predominance of approximately 2:1 in gastric cancer incidence across the world cannot be explained on the basis of gender differences for the prevalence of known risk factors[57]. It has been hypothesized that estrogens play a protective role against gastric cancer. This statement has gained further support from a clinical study of a male cohort of patients with prostate cancer. In this study the risk of developing gastric cancer was lower amongst those who had been treated with estrogen than in those without such treatment (standardized incidence ratio, 0.87; 95% confidence interval, 0.78-0.98)[58]. Further supporting this argument are studies which have shown decreased ERβ expression in other gastrointestinal cancers, such as colon cancer, compared to benign tumors and normal tissues[59]. Tamoxifen exposure has also been shown to be a risk factor for gastric cancer[60,61], adding support to the idea that estrogen signaling has a protective role against gastrointestinal cancer.

FAT DISTRIBUTION, LEPTIN AND ESTROGEN: IS THERE A LINK?

There is a growing appreciation that estrogens are not only directly involved in the reproductive process and in regulation of carcinogenesis, but also have general metabolic roles in both sexes[15-17]. Estrogen signaling has a complex relationship with obesity that differs for premenopausal and postmenopausal women[12]. Importantly, obesity is a risk factor for esophageal adenocarcinoma in both women[62] and men[63]. In a recent study of 23 women with esophageal adenocarcinoma[63], 21 (91.3%) were in the top half of the distribution of the studied cohort with regard to waist-to-hip ratio, waist circumference, and body mass index. Multiple studies of male cohorts have demonstrated a strong association between increased abdominal diameter and esophageal adenocarcinoma, after controlling for body mass index and gastroesophageal reflux[63-68]. It is possible that associations between obesity and esophageal cancer are similar for both sexes, even though the regulation of adiposity in men and women differs significantly. For instance, distribution of body fat in men is characterized by the accumulation of visceral fat, but in women by subcutaneous fat.

Subcutaneous and visceral fat tissues express variable levels of both types of ER[69-71]. However, only ERα has a significant influence on energy homeostasis. The role of ERα in estradiol regulation of body weight and obesity is supported by the following observations: (1) both male and female mice that have been genetically altered to reduce the ability to produce estrogen by knocking out aromatase (an enzyme that catalyzes the conversion of androgen to estrogen) became obese when fed the same amounts as normal mice[72]; and (2) increased white adipose tissue and body fat were seen in both sexually mature male and female ERα-knockout mice[73,74]. Further supporting a role for estrogen signaling through ERα in the regulation of body weight are the findings that abnormal adiposity has been associated with the XbaI polymorphism of the human ERα gene[75,76].

The role of ERβ in estradiol regulation of body weight and obesity is less clear and somewhat controversial suggesting that ERβ functions more as a modulator of estrogen actions[71].

Estrogen has also been shown to contribute to the regulation of body adiposity and fat distribution through ERs in the brain[77], and by interacting with leptin signaling pathways[78]. 17β-estradiol increases leptin mRNA levels in adipose tissue[79]. Consistently, estrogen deficiency impairs central leptin sensitivity[77,78]. In women, leptin fluctuations during the menstrual cycle correlate directly with secretion of estrogen[79,80]. Estrogen has also been found to influence leptin receptor expression and hypothalamic sensitivity to leptin driving subcutaneous body fat accrual over visceral fat during the estrous cycle in rats[81]. Hence, visceral fat varies inversely with estrogen levels. Visceral fat accumulates in females when circulating estrogen levels become sufficiently low, as in postmenopausal women[76,78,82]. The accumulation of visceral fat is associated with an increased risk of various gastrointestinal malignancies, including esophageal adenocarcinoma[83]. Thus, estrogen regulation of leptin levels in women may play a protective role, directing accumulation of subcutaneous in preference to visceral fat.

The situation for men, however, is less clear, although a high level of leptin is considered to be a risk factor for males to develop esophageal adenocarcinoma[63,83]. Speculatively, the production of, and sensitivity to, leptin in men may be increased in visceral fat, and locally in tissues located in close proximity to adipose tissue where estrogen synthesis may be increased. However, mechanisms of ER and leptin signaling in males remain obscure, mostly because the majority of laboratory findings and clinical investigations of leptin and estrogen signaling have used tissues from females. To address this issue, studies are needed that specifically address the role of estrogen signaling in male adipose tissue.

IMPLICATIONS OF ESTROGEN RECEPTOR EXPRESSION IN ESOPHAGEAL ADENOCARCINOMA

In 1998, Lagergren *et al*[83] hypothesized that high estrogen and/or progesterone levels, low testosterone, or a combination of both, might contribute to the lower incidence of esophageal carcinoma in women. Epidemiological data for esophageal adenocarcinoma demonstrates a profound gender difference, with the male: female ratio exceeding 8:1, strongly supporting this hypothesis[1-4]. There are no detailed studies that compare the expression of ERs in esophageal tissues between males and females, but a limited number of studies have provided some preliminary data comparing ER expression in esophageal adenocarcinoma and its precursor lesion, Barrett's esophagus. These studies are summarized in Table 1.

In contrast to the anti-tumor role of ERβ in other cancers, some studies have identified a positive association between ERβ expression and esophageal adenocarcinoma development. Akgun *et al*[84] determined ERβ expression in the esophageal mucosa from patients with Barrett's metaplasia negative for dysplasia, Barrett's metaplasia with low grade dysplasia and Barrett's metaplasia with high grade dysplasia. The results of this study showed significant expression of ERβ (more than 50% of cells positive) in all patients with esophageal adenocarcinoma, and there was a trend towards increased expression of ERβ as the esophageal lesions progressed[85]. These results raise the possibility of ERβ as a target of therapy for esophageal adenocarcinoma. Similarly, another investigation showed a moderate increase in ER expression in tissue samples from men and women with Barrett's esophagus and esophageal adenocarcinoma. However, the subtype of ER was not determined in this study[39].

As ERβ has several isoforms, and these isoforms

Table 1 Estrogen receptor expression in patients with esophageal adenocarcinoma

	No. of patients with EAC	ERα	ERβ	Conclusion
Akgun *et al*[84]	31	Not expressed	Increased expression as esophageal lesions progressed	ERβ is suggested as a EAC therapy target
Tiffin *et al*[85]	20 (8)	Type of ER was not specified; ER were detected in EAC patients		ER may be important for further investigation
Liu *et al*[39]	33	Not expressed	Expressed in EAC, but not in Barrett's esophagus	Anti-estrogen treatment could be a promising therapeutic target for EAC
Kalayaransan *et al*[86]	45 (15)	Not expressed	Detected in all 45 patients; Expressed higher in EAC, compared to normal esophageal mucosa	ERβ suggested as marker and/or prognostic factor

EAC: Esophageal adenocarcinoma; ER: Estrogen receptor.

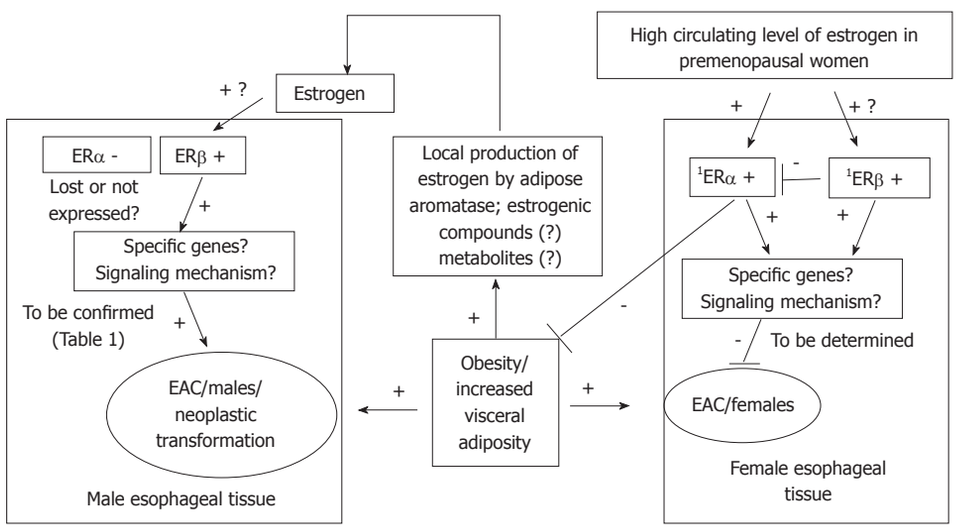

Figure 1 Role of estrogen-activated signaling in the development of esophageal adenocarcinoma: hypothetical pathway. [1]Expression level of estrogen receptor (ER) needs to be determined in esophageal tissue from females and compared to expression in males. EAC: Esophageal adenocarcinoma.

have different functions, Liu *et al*[39] identified which isoforms of ERβ were expressed in esophageal adenocarcinoma but not in Barrett's esophagus. All isoforms of ERβ showed much higher expression in esophageal adenocarcinoma, than in its precursor lesion, Barrett' s esophagus. Thus, a possible role for ERβ isoforms in the maintenance and evolution of esophageal adenocarcinoma was suggested. Although the study did not find a correlation between immunoreactivity and cancer proliferative activity, it showed that ERβ1 tended to have higher expression in invasive tumors which had penetrated the full thickness of the esophageal wall, compared to tumors limited to the esophageal wall ($P = 0.05$), and ERβ1 immunostaining tended to be most prominent in invasive esophageal adenocarcinoma. Conclusively, the study detected the presence of ERβ isoforms in esophageal adenocarcinoma and suggested the potential use of anti-estrogen treatment as a therapeutic target for esophageal adenocarcinoma. Manipulation of ERβ signaling may be considered as a potential prevention strategy to delay or block progression from dysplasia to esophageal adenocarcinoma. Figure 1 summarizes a potential mechanism for interaction between estrogen, ERs and esophageal adenocarcinoma.

Another study by Kalayaransan *et al*[86] determined the expression of ERα and ERβ in esophageal adenocarcinoma across various classifications of tumor stage, and compared expression with adjacent normal esophageal mucosa. No significant expression levels of ERα were found in esophageal adenocarcinoma, suggesting ERα is unlikely to mediate the growth of esophageal adenocarcinoma. However, immunostaining with ERβ antibodies yielded significantly higher results in esophageal adenocarcinoma, compared to normal esophageal mucosa[87]. In each group with the same degree of tumor differentiation, tumor samples had significantly higher staining scores compared to normal esophageal mucosa. Tumors with good or moderate differentiation had lower staining scores than those which were poorly differentiated, indicating that the potential effect of estrogen on esophageal adenocarcinoma could be mediated by ERβ[84,86,87]. Overall, most studies that have evaluated esophageal adenocarcinoma are consistent in suggesting a detrimental effect and prognostic value for ERβ.

Unfortunately, these clinical findings have not yet been supported by *in vitro* experiments using esopha-

geal adenocarcinoma cells. The few *in vitro* studies that have addressed the role of estrogen in the regulation of esophageal cell growth were conducted using squamous cancer cells[87,88]. It has been shown that the growth of an ER-positive esophageal squamous carcinoma cell line (ES-25C) is significantly inhibited by 17β-estradiol, whereas this effect is not observed in an ER-negative squamous carcinoma cell line (ES-8C)[87]. A similar finding was seen in another study, in which the proliferation of the ER-positive KSE-1 esophageal squamous carcinoma cell line was inhibited by 17β-estradiol[88]. In addition, *in vivo* growth of this cell line in both female and male mice was suppressed by the administration of 17β-estradiol, raising the possibility of manipulating the growth of esophageal carcinoma by manipulating the estrogen-ER system[88]. However, esophageal squamous cell carcinoma and esophageal adenocarcinoma are two biologically distinct diseases, so estrogen responsiveness in squamous cell carcinoma lines does not automatically mean that esophageal adenocarcinoma cell lines will also respond. Similar experiments need to be performed on esophageal adenocarcinoma cell lines in order to explore this possibility further.

FUTURE PERSPECTIVES

Current literature provides only limited evidence for a link between estrogen and the development of esophageal adenocarcinoma. Hence, a series of questions can be proposed, and further studies will be needed to determine whether there is any link. It is unclear whether there is a gender difference for the expression of ERβ, or correlation between tumor stage and the expression of ERβ. Most previous studies have not compared estrogen effects in both genders, and have only addressed men and women separately. Detailed comparisons have not been done for various esophageal pathologies *vs* normal esophageal mucosa within both gender groups. Another limitation of previous studies is the small number of patients studied, and for this reason reported data is yet to be verified. A systematic study which includes a sufficiently large number of men and women is needed to determine whether, within each gender group, ERβ expression is associated with the development and progression of esophageal adenocarcinoma. A confirmed link might provide support for ERβ to be used as a target for therapy, or as a prognostic marker.

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S- Editor Tian L **L- Editor** Cant MR **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i5.401

World J Gastroenterol 2012 February 7; 18(5): 401-411
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

Anastasios Koulaouzidis, MD, MRCP, *Series Editor*

Update of endoscopy in liver disease: More than just treating varices

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Received: April 11, 2011 Revised: June 2, 2011
Accepted: June 9, 2011
Published online: February 7, 2012

Key words: Cirrhosis; Portal hypertension; Varices; Endoscopic ultrasound; Esophageal capsule; Endoscopic retrograde cholangiopancreatography; Endomicroscopy; Spyglass

Peer reviewers: Erwin Biecker, MD, PhD, Department of Gastroenterology and Hepatology, Helios Klinikum Siegburg, Siegburg 53343, Germany; Yu-Yuan Li, Professor, Department of Gastroenterology, First Municipal People's Hospital of Guangzhou, 1 Panfu Road, Guangzhou 510180, Guangdong Province, China

Krystallis C, Masterton GS, Hayes PC, Plevris JN. Update of endoscopy in liver disease: More than just treating varices. *World J Gastroenterol* 2012; 18(5): 401-411 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i5/401.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i5.401

Abstract

The management of complications in liver disease is often complex and challenging. Endoscopy has undergone a period of rapid expansion with numerous novel and specialized endoscopic modalities that are of increasing value in the investigation and management of the patient with liver disease. In this review, relevant literature search and expert opinions have been used to provide a brief overview and update of the current endoscopic management of patients with liver disease and portal hypertension. The main areas covered are safety of endoscopy in patients with liver disease, the use of standard endoscopy for the treatment of varices and the role of new endoscopic modalities such as endoscopic ultrasound, esophageal capsule, argon plasma coagulation, spyglass and endomicroscopy in the investigation and treatment of liver-related gastrointestinal and biliary pathology. It is clear that the role of the endoscopy in liver disease is well beyond that of just treating varices. As the technology in endoscopy expands, so does the role of the endoscopist in liver disease.

INTRODUCTION

Liver disease and cirrhosis are common causes of mortality worldwide[1]. The role of endoscopy in liver disease is both diagnostic and interventional: endoscopy should be offered to patients with relevant symptoms (unsuspected liver disease may be diagnosed in this manner) and for variceal screening and treatment. Patients with liver disease can be challenging to sedate, and the complexity of endoscopy in liver disease continues to increase with rising numbers of patients with a liver transplant, and the advent of new endoscopic modalities such as capsule endoscopy and endoscopic ultrasound (EUS).

SEDATION AND ANALGESIA IN LIVER DISEASE

Pharmacodynamics are altered in advanced liver disease as a result of changes in hepatic conjugation and oxidation, shunting, decreased protein binding and an increased vol-

ume of distribution[2]. The common agents used for sedation in endoscopy are discussed, however, specific doses cannot be recommended because these are dependent on patient factors. We would recommend that an endoscopist or anesthetist who has experience with this patient group undertakes the sedation of liver patients.

Benzodiazepines

Midazolam is the benzodiazepine of choice in most endoscopy units. It is protein bound and metabolized in the liver by cytochrome P3A4. In cirrhosis, clearance of midazolam is impaired and elimination half-life is doubled. As a result, midazolam should be used with caution in patients with cirrhosis[3].

Opiates

Pethidine and fentanyl are the most commonly used analgesics for endoscopic procedures. The liver is the major site of biotransformation for most opiates. The oxidation of pethidine is reduced in patients with cirrhosis and its clearance is diminished. Therefore, there is increased bioavailability, and pethidine should be avoided in patients with liver disease[4]. The half-life of fentanyl is shorter and does not seem to be influenced by cirrhosis. Its use is preferred to pethidine[5].

Anesthetic agents

The pharmacokinetics of propofol, an anesthetic agent that is widely used in endoscopy, appears to be unaffected by cirrhosis; again, perhaps secondary to its short half-life. One study has suggested that the use of propofol rather than midazolam in patients with compensated cirrhosis facilitates a faster recovery time with less exacerbation of subclinical encephalopathy[6].

Endotracheal intubation

Gastrointestinal (GI) bleeding in patients with liver disease may be life threatening. If bleeding varices are suspected or patients are hemodynamically unstable, there is often a low threshold for endotracheal intubation to protect the airway. There is little literature on prophylactic intubation for airway protection in such patients, and two retrospective studies[7,8] have concluded that it does not prevent cardiopulmonary complications, or pneumonia. We are of the opinion that airway protection at emergency endoscopy is extremely important in patients with suspected variceal bleeding, who present with hematemesis, and particularly in hemodynamically unstable patients and those with hepatic encephalopathy or alcohol withdrawal symptoms. In such patients, endoscopy is best undertaken in a critical care environment with immediate access to anesthetic support and endotracheal intubation[9].

ENDOSCOPY IN THE CIRRHOTIC PATIENTS WITH COAGULATION ABNORMALITIES

Coagulopathy and thrombocytopenia are common in patients with chronic liver disease. The mechanisms behind coagulation abnormalities are often complex, and it is now thought that prolongation of prothrombin time may not directly relate to the risk of bleeding, and rather, it is the balance of pro- and antithrombotic factors that is important. In practical terms, there is currently no reliable way of quantifying this. Routine correction of coagulopathy at endoscopy is not recommended, although patients with chronic liver disease should receive vitamin K to correct any dietary deficiency that may result in coagulopathy.

It is recognized that diagnostic endoscopy is a low-risk procedure and safe in patients with altered coagulation. However, high-risk endoscopic therapeutic procedures have a significantly increased risk of hemorrhage and, as such, coagulopathy should be treated[10]. It is therefore common practice in cirrhotic patients to correct significant thrombocytopenia ($< 50 \times 10^6$/mL) with platelet transfusions and to correct coagulopathy with fresh frozen plasma (FFP) if prothrombin time is > 20 s to an international normalized ratio < 1.5, before high-risk procedures. Platelet and FFP transfusions are particularly helpful during an acute bleeding episode if the prothrombin time is prolonged or platelets are low, similar to the previously mentioned values[11].

Novel treatments include recombinant factor Ⅶa. This has been used as a hemostatic agent in acute variceal bleeding, but failed to show efficacy in a large randomized study[12]. Another trial has demonstrated that addition of desmopressin does not improve and may worsen the efficacy of terlipressin in controlling acute variceal bleeding in cirrhotic patients[13].

Certain endoscopic investigations have been shown to be safe in coagulopathic patients with cirrhosis, despite being relatively invasive; EUS-fine needle aspiration of the liver has been shown to be a safe alternative to percutaneous liver biopsy, particularly in patients with advanced liver disease, coagulopathy and high risk of bleeding[14]. At endoscopic retrograde cholangiopancreatography (ERCP), endoscopic papillary balloon dilation is safer than endoscopic biliary sphincterotomy for the treatment of choledocholithiasis in patients with advanced cirrhosis and coagulopathy, because it has a reduced risk of bleeding[15].

COMMON ENDOSCOPIC DIAGNOSES AND MANAGEMENT IN PATIENTS WITH LIVER DISEASE

Peptic ulcer disease

The correlation between peptic ulcer disease and cirrhosis is well described. Both duodenal and gastric ulcers are more common in cirrhosis: the reported prevalence is 24.1%[16]. It is recognized that the prevalence of gastric ulceration increases with the severity of liver disease and is related to changes in the hepatic venous pressure gradient[16,17].

A high prevalence of *Helicobacter pylori* (*H. pylori*), up

Table 1 Summary of primary prophylaxis of esophageal varices

Grade	Appearance	High-risk stigmata	Treatment
Grade 1: Small varices	Barely noticeable varices; disappear easily with insufflation	No red signs	No treatment
Grade 2: Small/medium varices	Small or medium varices; do not easily disappear with insufflation	± Red signs	NSBB or VBL
Grade 3: Medium/large varices	Medium or large varies; do not disappear with insufflation	± Red signs	NSBB or VBL

NSBB: Non-selective b blockers; VBL: Variceal band ligation.

to 89%, in patients with cirrhosis has been reported[18]. 13C urea breath testing and gastric body histology remain highly accurate in detecting *H. pylori* in cirrhosis, whereas rapid urease tests and serology are less reliable than in non-cirrhotic patients[19]. A meta-analysis of seven studies with almost 1000 patients has strongly suggested that, as with non-cirrhotic patients, *H. pylori* infection increases the risk for peptic ulcer disease in cirrhosis[20]. *H. pylori* eradication therapy is effective in chronic liver disease[21]. However, two recent studies have suggested that *H. pylori* eradication in cirrhotic patients with duodenal ulcers is not as effective at reducing ulcer recurrence as it is in the general population. These patients require maintenance acid suppression therapy[22,23].

Portal hypertension

The development of portal hypertension and formation of portosystemic shunts is a major event in the natural history of liver disease. Measurement of the portal pressure gradient is invasive and not widely available for clinical use; instead the hepatic venous pressure gradient (HVPG) is commonly used in clinical practice and it is of prognostic value: HPVG $\geqslant$ 10 mmHg strongly predicts the development of esophageal varices[24]. Similarly, the most significant risk factor associated with failure to control bleeding or early rebleeding of esophageal varices is HVPG > 20 mmHg. This is also associated with increased mortality[25].

Gastroesophageal varices are present in > 50% of patients with portal hypertension and are more likely as liver disease progresses[26]. Ectopic varices are located in sites other than the gastroesophageal region and are more common than previously thought: duodenal or colonic varices are seen at angiography or colonoscopy in up to 40% of patients with intrahepatic portal hypertension[27].

Esophageal varices

It is recommended that all patients undergo endoscopy to assess the presence and the size of varices at the time of the diagnosis of cirrhosis. Thereafter, guidelines for the interval of endoscopic screening vary. Currently, the American Association for the Study of the Liver (AASLD) recommends that, if no varices are present at index endoscopy, this should be repeated at 2-3 years in compensated cirrhosis and annually in decompensated cirrhosis[11]. The British Society of Gastroenterology recommends annual screening if grade 1 varices are present at initial screening (Table 1, grading and treatment of esophageal varices), and an interval of 3 years if there is no evidence of varices at index endoscopy[28].

Esophageal variceal bleeding occurs at a rate of 5%-15% per year in untreated patients. The main risk factors for bleeding are variceal size (grade 2 or 3), decompensated cirrhosis, and the presence of high-risk stigmata at endoscopy[29]. Variceal bleeding is a significant clinical event with a mortality rate of approximately 20% at 6 wk, and a recurrence rate of up to 60% at 2 years if secondary prophylaxis is not commenced[30].

The management of esophageal varices may be divided into pre-primary, primary and secondary prophylaxis and control of active bleeding. At present, there is no evidence to support treatment to prevent the development of varices in patients with liver disease (pre-primary prophylaxis)[31,24].

For primary prophylaxis of esophageal varices, there is no evidence that variceal band ligation (VBL) is superior to β-blockade. Due to issues with access to endoscopy and patient preference, non-selective β-blockade, typically with propranolol, is often first line when treatment is indicated[32]. Carvedilol is a potent non-selective β-blocker, with weak vasodilating properties. A reduction in the HVPG in the range of 10%-43% has been reported with a 12.5 mg/d dose. Carvedilol has therefore been adopted as the β-blocker of choice for primary prophylaxis of variceal bleeding in some centers[33-35]. Primary prophylaxis with VBL is recommended if there are contraindications to β-blockers, or concerns about patient compliance[11].

Secondary prophylaxis is indicated for patients who have had an episode of variceal hemorrhage. β-blocker monotherapy is not used as secondary prophylaxis, and patients should either be entered into a variceal banding program or receive a combination of a β-blocker and nitrate[36]. AASLD recommends a combination of β blockade and VBL[11]. However, there is no strong evidence to suggest that this strategy is associated with improved mortality[37] and our local practice is to use VBL alone. VBL should be repeated every 2 wk until obliteration of varices is achieved. Following this, a surveillance endoscopy at 1-3 mo to confirm eradication is required, and this should be repeated every 6-12 mo[11]. VBL is a safe technique: although asymptomatic banding ulcers are common after VBL, the rate of bleeding from these and requiring hospitalization does not exceed 5%[38].

The combination of terlipressin and VBL is the preferred treatment for acute variceal bleeding in many centers, and using terlipressin before endoscopy is not unreasonable if there is a delay to the endoscopy. Endo-

scopic hemostasis is usually achieved in the majority of cases[11]. Transjugular intrahepatic portal systemic shunt (TIPSS) may be considered if VBL has been unsuccessful or there is an early re-bleed (defined at Baveno V as a repeat bleed within 5 d of the index bleed). A reduction in HVPG below 12 mmHg or a 20% reduction from the baseline value, even without reaching < 12 mmHg, protects against rebleeding[39].

The use of sclerosing agents (variceal sclerotherapy) is no longer recommended as first-line treatment, because of increased mortality rates[40], nor for secondary prophylaxis because VBL treatment has been shown to be safer and more effective[41].

Recently, endoscopic placement of a specifically designed self-expanding covered metal stent has proved effective in the treatment of esophageal varices in patients in whom initial endoscopic methods have failed to achieve hemostasis[42]. This method appears to be a safe and effective means of controlling ongoing bleeding. The stent is usually removed 1 wk after the acute bleed. Currently, this technique is limited by its relative complexity of stent insertion in acute bleeding, but stenting with covered biodegradable stents when available, which do not require removal, may play an important role in the management of acute esophageal variceal bleeding[43].

Gastric and ectopic varices

Gastric varices are less prevalent than esophageal varices and less prone to bleeding: around 25% over a 2-year period[44]. There is no evidence to support the primary prophylaxis of gastric varices. The tissue adhesive cyanoacrylate ("glue") is used widely in the management of acutely bleeding gastric varices. Cyanoacrylate is a liquid with consistency similar to water, which when added to a physiological fluid like blood, polymerizes to form a solid substance[45]. Two randomized controlled studies have compared cyanoacrylate with VBL for management of bleeding gastric varices[46,47]. In one study, cyanoacrylate was more effective than VBL in achieving homeostasis, and in the second, no difference was reported, although both reported less recurrence of bleeding. Current evidence suggests that cyanoacrylate achieves control of bleeding in 87%-93% of cases, and that bleeding-related mortality is between 6.5% and 10%[46,47].

The use of bovine or more recently, human thrombin has been described as an alternative treatment for active gastric variceal bleeding. Thrombin [activated factor Ⅱ (Ⅱa)] is a serine protease that converts soluble fibrinogen into insoluble strands of fibrin clot. It has additional effects including promotion of platelet aggregation. Initial hemostasis rates have been reported at 94%-100%, and rebleeding rates of between 23% and 25%[48,49]. A single center experience of 13 patients treated with thrombin for bleeding gastric varices has reported efficient hemostasis and an overall mortality of 38% in a median follow-up of 22 mo[50].

Technical difficulties that include the risk of equipment damage and reports of severe thromboembolic complications may limit the use of cyanoacrylate, and thrombin (Figure 1) may become more widespread in the future[51,52].

The role of TIPSS as primary treatment in actively bleeding gastric varices has also been explored. Although TIPSS has a comparable mortality and rebleeding rate to cyanoacrylate, it is associated with significantly higher morbidity and is not used as first-line treatment[53,54], but remains an effective treatment when endoscopy fails to control bleeding.

Nonvariceal manifestations of portal hypertension

Portal hypertensive gastropathy (PHG), with its typical "snake skin" appearance, is present in approximately 80% of patients with cirrhosis[55†]. PHG accounts for 8% of nonvariceal bleeds in patients with liver disease, although this condition more commonly presents with anemia[56]. Patients with cirrhosis and severe PHG-related bleeding may respond to β-blockade. Endoscopic measures such as argon plasma coagulation (APC) therapy can reduce bleeding, thus controlling anemia. TIPSS should be reserved for those patients with pharmacological treatment failure[57].

The prevalence of portal hypertensive enteropathy (PHE), determined by capsule endoscopy, is as high as 63% in patients with end-stage liver disease who also have esophageal or gastric varices[58]. Portal hypertensive duodenopathy is present in around half of patients with cirrhosis, and it is more common in patients with severe PHG[59].

Gastric antral vascular ectasia

Gastric antral vascular ectasia (GAVE) is related to portal hypertension in about 30% of patients, and accounts for 4% of nonvariceal upper GI bleeds[60]. Unlike PHG, GAVE does not respond well to reduction in portal pressure[61]. The Nd:YAG laser has been widely used in the treatment of GAVE and is the most commonly reported endoscopic modality in cirrhotic and non-cirrhotic patients, with a reported overall success rate of almost 90%, although the authors did not distinguish the etiology of GAVE when reporting the outcomes[62].

APC has also proved to be effective for the treatment of GAVE-related bleeding, and it is extensively used in our unit, with a success rate of > 85%; success is defined as control of bleeding, stabilization of hemoglobin at > 100 g/dL, or hemoglobin increase > 10% from pretreatment level, and reduction of transfusion requirements by > 50% in transfusion-dependent patients. An average of four sessions of APC is usually required (Figures 2 and 3). In two published studies, with a total of 37 patients with cirrhosis, success rates in controlling bleeding were very high, and the reported rebleeding rates were between 12% and 20% after 2 years follow-up[62,63].

Although the success rates of the two aforementioned modalities are comparable, APC treatment is probably the therapy of choice due to the technical ease, safety and low cost[61]. Other endoscopic techniques have been positively reported in small series, such as VBL[64],

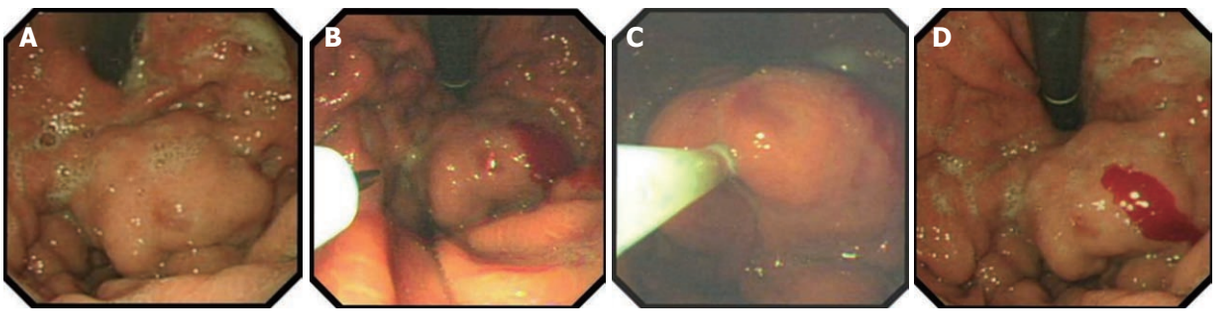

Figure 1 Endoscopic images of fundic gastric varices before (A), during (B, C) and after (D) thrombin injection.

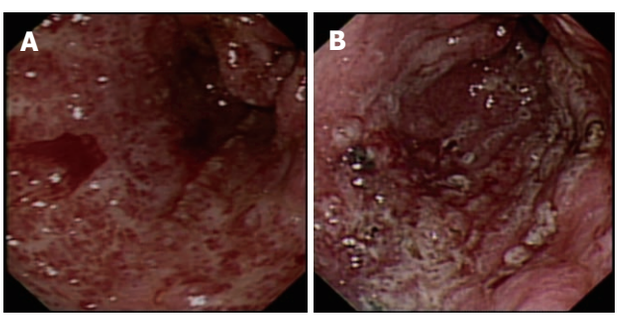

Figure 2 Endoscopic image. A: Gastric antral vascular ectasia (GAVE) (diffuse type) with active bleeding prior to argon plasma coagulation (APC) treatment; B: GAVE (diffuse type) immediately after APC treatment.

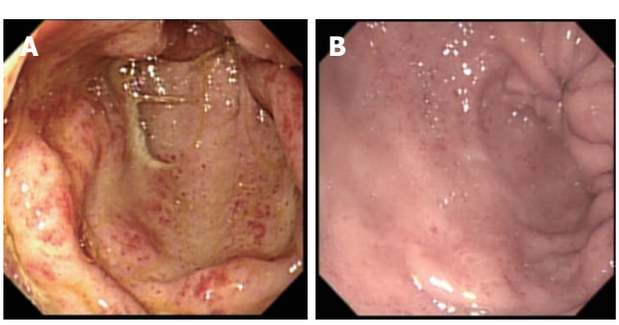

Figure 3 Endoscopic image. A: Gastric antral vascular ectasia-induced symptomatic anemia; B: Endoscopic image of the same patient 2 years later, after several argon plasma coagulation sessions. The number of angioectatic lesions in the gastric outlet had dramatically decreased.

endoscopic mucosal ablation[65] and cryotherapy[66].

ADVANCED ENDOSCOPIC PROCEDURES AND THEIR VALUE IN LIVER DISEASE

EUS

Recently, EUS has been used to assist in the management of portal hypertension. Doppler EUS is of significant value in differentiating ectopic varices from other submucosal lesions[67] (Figure 4), and several EUS-assisted techniques have been used to identify the precise site for the intravariceal injection of sclerosant agents. Linear color EUS-guided sclerotherapy has proved to be effective in the eradication of esophageal varices, in two small studies with recurrence reported at 0% and 8.3%, respectively[68,69]. EUS catheter probes and high-frequency (20 MHz) miniprobes have both been used successfully before and after esophageal variceal sclerosant injection in two different studies to assess eradication and variceal recurrence. After a mean follow-up of 24 mo, variceal recurrence was reported at 16.6% and 26.3%, respectively[70,71].

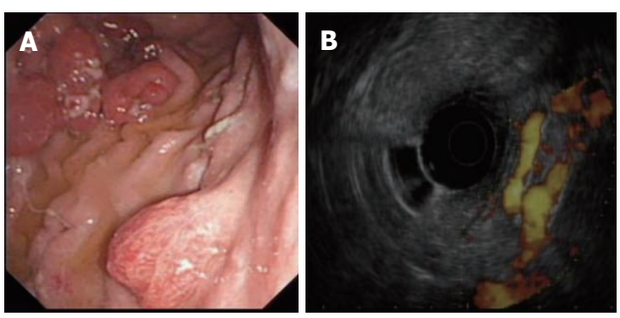

Figure 4 Endoscopic image of gastric irregular submucosal lesion. A: Gastric irregular submucosal lesion in a patient with portal hypertension; B: The same lesion examined under color Doppler endoscopic ultrasound. The submucosal lesion was hypervascular and represented a gastric varix.

Although the overall patient numbers are small, linear EUS seems to be the superior modality in assisting treatment of esophageal varices, because it permits the targeting of the feeding vessels.

EUS-assisted injection of cyanoacrylate for the treatment of gastric varices has been described in 54 patients with a mean follow-up of 24 mo. Varices recurred in 35% of patients[72]. Furthermore, a series of 15 patients with gastric or ectopic varices treated with thrombin injection in conjunction with a variety of EUS techniques (Figure 5) has recently been reported in our unit, and this proved to be effective in controlling active bleeding and achieving variceal eradication[73].

Capsule endoscopy

Esophageal capsule endoscopy (OCE) is an alternative to conventional upper GI endoscopy for the diagnosis of varices in complex patients with portal hypertension. In a recent meta-analysis of seven studies involving 446 patients, OCE had a sensitivity of 85.8% and specificity 80.5% in detecting esophageal varices[74]. However, a multicenter trial evaluating the efficacy of OCE in esophageal varices screening was less encouraging, because the standard of < 10% difference between capsule

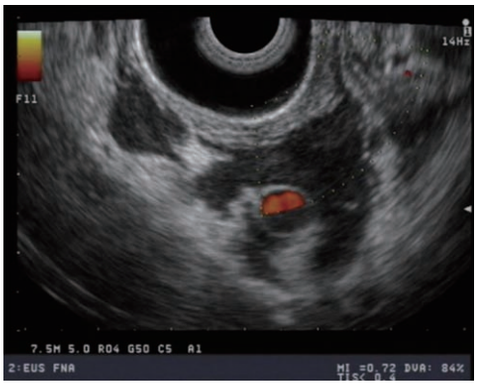

Figure 5 Color doppler endoscopic ultrasound image of duodenal varices after thrombin injection. The absence of blood flow and the speckled appearances were suggestive of thrombus formation.

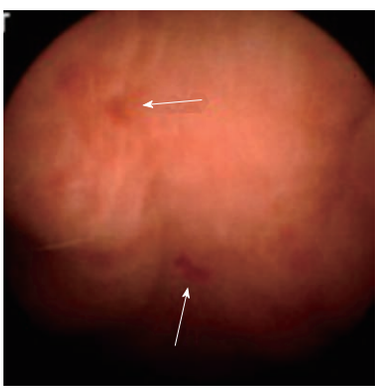

Figure 6 Small bowel capsule image of portal hypertensive enteropathy and stigmata of recent bleeding. Engorged small bowel villi and micro-hemorrhagic spots were visible.

and conventional endoscopy was not met[75]. This is not surprising, because the two techniques differ in that during conventional endoscopy, the esophagus is inevitably insufflated with air and varices can appear more flattened than during OCE examination. Further studies to take this into account are necessary. Nevertheless, OCE remains a useful tool for screening of varices in certain patient groups; patients who poorly tolerate endoscopy or who have significant comorbidity, thus increasing the risks of repeated endoscopy, and patients with high risk of variant Creutzfeldt-Jakob disease. Although this technique is limited by availability and high costs, OCE can be cost-effective for variceal screening of patients with coagulation abnormalities (e.g., hemophilia) with coexisting liver disease, because it does not require prophylactic clotting factor administration, unlike conventional endoscopy. Serial capsule examinations in the same patient may provide significant diagnostic information regarding progression of varices.

Small bowel capsule endoscopy (SBCE) has been used to characterize PHE (Figure 6), and is of value in the diagnosis of this condition in patients with advanced liver disease who continue to bleed despite treatment of esophageal/gastric varices or portal gastropathy (Figure 7).

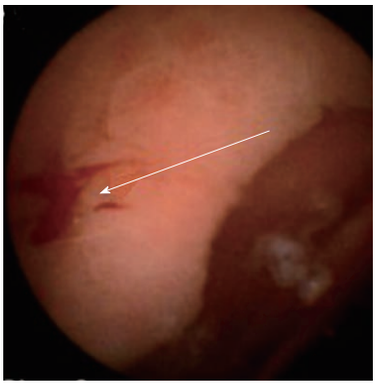

Figure 7 Small bowel capsule image of portal hypertensive enteropathy with snake-skin-like appearance of the mucosa and red spots as stigmata of recent bleeding.

The role of SBCE in portal hypertension has yet to be defined, but it is likely that it will remain a valuable tool in certain groups of patients with liver disease.

Endoscopic retrograde cholangiography

Primary sclerosing cholangitis (PSC) is characterized by fibrosis of both intrahepatic and/or extrahepatic biliary ducts. These patients are at risk of developing infectious cholangitis and up to 20% develop cholangiocarcinoma. A strategy of initial magnetic resonance cholangiopancreatography (MRCP) followed, if necessary, by ERCP is currently the most cost-effective approach to the work-up of patients with suspected sclerosing cholangitis[76†]. ERCP plus diagnostic brushing have a sensitivity of 60%-100%, and specificity of 85%-89% in differentiating between a benign dominant stricture and cholangiocarcinoma[77,78]. Recently, two advanced cytological techniques (digital image analysis and fluorescence *in situ* hybridization) have been used for the detection of malignancy in PSC-related strictures and have proved to be more sensitive and equally specific to conventional cytology[79†].

In addition, ERCP permits therapeutic interventions with balloon dilation or stent placement as appropriate.

Novel techniques and the biliary tree

Novel endoscopic modalities have been compared with conventional ERCP and brush cytology. Transpapillary cholangioscopy with tissue sampling has proved to be more sensitive (92% *vs* 66%) and specific (93% *vs* 51%) than ERCP to detect cholangiocarcinoma in PSC[80]. In a small study, narrow band imaging has demonstrated superior visualization of biliary lesions compared with conventional white light imaging[81]. In another study, transpapillary intraductal ultrasound was superior to ERCP for the detection of cholangiocarcinoma in PSC in terms of sensitivity (87.5% *vs* 62.5%) and specificity (90.6% *vs* 53.1%)[82].

Spyglass™ is a new single-operator system used for the diagnosis of a variety of pancreatobiliary disorders, such as the definition of indeterminate strictures and filling defects prior to stone extraction[83]. Although the

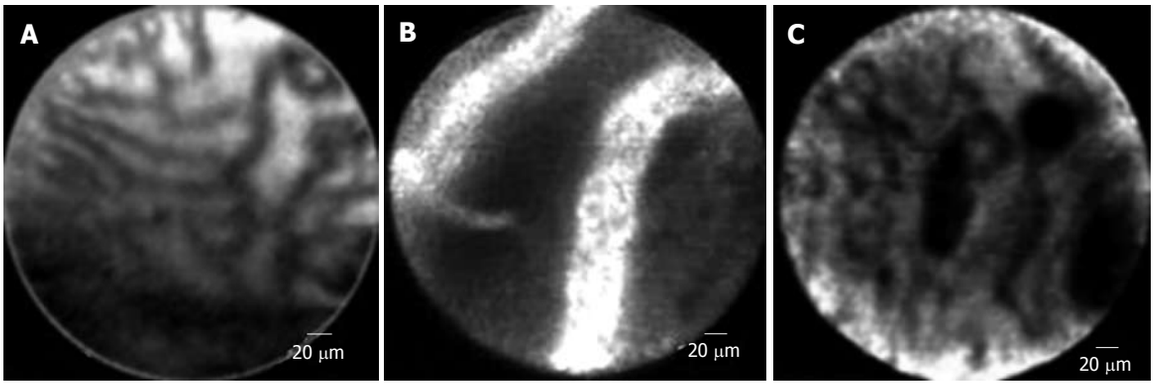

Figure 8 Endomicroscopy image. A: Image from Cellvizio® bile duct endomicroscopy. The regular reticular pattern of thin dark structures with low signal (dark) characterized the normal bile duct (Image courtesy of www. cellvizio.net); B: Abnormal bile duct appearances in Cellvizio® endomicroscopy; isolated blood vessels with very strong signal (with strands) suggestive of tumor neovascularization of cholangiocarcinoma (Image courtesy of www.cellvizio.net); C: Reticular pattern of dark bands and dark clumps or glands suggestive of cholangiocarcinoma (Image courtesy of www.cellvizio.net).

initial experience is promising, the modality has been only been tested in a limited number of PSC-related strictures, and unlike ERCP, this is a purely diagnostic technique.

Confocal laser endomicroscopy ("miniprobe") is a new field of endoluminal imaging that offers extremely high magnification and resolution. This technique allows visualization of pancreatic and biliary ducts. In a pilot study of 14 patients with biliary strictures, miniprobe-based microscopy after fluorescein administration proved to be more accurate than brushings and biopsy in distinguishing benign from malignant strictures (Figure 8)[84].

A novel alternative is direct cholangioscopy using ultra-slim endoscopes (4.9-5.9 mm). These endoscopes, initially developed for transnasal endoscopy, can be safely inserted into the bile duct following sphincterotomy, and not only permit high-resolution images, but also biopsy and other interventional procedures in the bile ducts, such as hydraulic lithotripsy and division of strictures in benign biliary disease. This technique is currently under development and after a full range of endoscopic accessories are available for endobiliary interventions, it could be an effective and safe approach for patients with difficult to manage biliary disease[85].

Endoscopic modalities in the lower GI tract

In comparison to the upper GI tract, colonic manifestations of portal hypertension much less often present with acute bleeding, and are more often found incidentally or during investigation of anemia. As such, data are sparse and less consistent. The reported prevalence of portal hypertensive colonopathy is 24%[86].

The most significant feature of portal hypertension in the colon is arguably the presence of rectal varices. These can be present in up to 44% of patients with cirrhosis at colonoscopy, although the reported prevalence varies widely. They are more frequent in patients with advanced liver disease[87].

Although bleeding from rectal varices is uncommon, it can be life threatening. Due to their rarity, no firm guidelines have been established for the management of bleeding colonic varices, and there is a limited evidence base. The most commonly used treatment modalities are sclerotherapy and band ligation. In a small retrospective comparative study of 15 patients, endoscopic injection sclerotherapy proved to be superior to endoscopic band ligation and achieved lower recurrence rates[88]. In our unit, thrombin has been successfully used to manage rectal variceal bleeding. Patients may require TIPSS if bleeding cannot be controlled endoscopically.

ENDOSCOPY AND THE LIVER TRANSPLANT PATIENT

Luminal diseases in liver transplant patients

Peptic ulcer disease is the most common cause of GI bleeding in post-orthotopic liver transplant (OLT) recipients, accounting for 27% of all bleeding[89]. Varices rarely recur post-transplant, and if present, require investigation to exclude portal vein thrombosis or disease recurrence[90].

Liver transplant patients are at increased risk of opportunistic infections, particularly candidiasis and cytomegalovirus[91]. These often present with GI symptoms and require endoscopic evaluation with biopsies or brushings to confirm the diagnosis.

The association of PSC with ulcerative colitis is well recognized and is an additional risk factor for the development of colorectal cancer in immunosuppressed transplant patients. It is currently recommended that these patients have an annual surveillance colonoscopy commencing 10 years after the onset of bowel symptoms[92]. Colectomy is safe in patients who have undergone OLT, and in some high-risk cases, such as when high-grade dysplasia has already been identified, a prophylactic colectomy may be performed at the time of transplantation[93].

Transplant-related biliary disease

Biliary complications (biliary strictures and leaks) following liver transplantation are a challenging and common

issue that affects 10%-30% of OLT patients. Biliary strictures are classified as anastomotic or non-anastomotic[94]. The initial approach in suspected post-transplant biliary strictures is usually MRCP, restricting the use of ERCP to patients who require intervention, or where MRCP results are equivocal[95]. Further imaging techniques include SpyGlass™, which has been successfully used for the investigation of post-transplant biliary strictures[96], and contrast-enhanced ultrasound. This is a non-invasive technique for the detection of strictures relating to hepatic artery stenosis in liver transplant patients. It provides details on the presence, location, degree, and type of stricture[97]. Treatment comprises a combination of balloon dilation and stent placement, repeated if necessary until stricture resolution.

Biliary leaks can occur in up to 22% of patients. Evidence suggests that sphincterotomy with stent placement is the best treatment option for biliary leaks following OLT[94,98]. Surgical revision and biliary reconstruction with the formation of hepaticojejunostomy is indicated when endoscopic or percutaneous treatment fails[94,98].

In patients who have a roux-en-Y anastomosis, the technique of double balloon ERCP has been devised with promising results. The technique uses a double balloon colonoscope to approach the ampulla, although there are some limitations regarding endoscopic accessories. With further development of this technique, the endoscopic treatment of biliary complications may become easier and will play a larger role in the management of such patients[99].

CONCLUSION

Endoscopy has undergone rapid expansion with numerous novel endoscopic modalities and techniques directly applicable to the diagnosis and management of complications of liver disease. Although conventional upper GI endoscopy is still the modality of choice for esophageal variceal surveillance and treatment, further options are now available with the use of capsule endoscopy and EUS. Therapeutic options for the management of upper GI bleeding in portal hypertension have also been developed. Band ligation remains the treatment of choice for esophageal variceal bleeding, whereas for gastric and ectopic varices, the use of sclerosants, particularly "glue" and thrombin are increasingly being used. APC is the preferred modality for GAVE and PHG. Biliary strictures and the risk of cholangiocarcinoma are major issues in patients with PSC. ERCP is both diagnostic and therapeutic in this setting and can differentiate benign from malignant lesions in the majority of cases. Novel endoscopic techniques such as transpapillary cholangioscopy, Spyglass Direct Visualization System, confocal laser endomicroscopy ("miniprobe") and ultra-thin cholangioscopy are increasingly being used to assist diagnosis in selected patients. Finally, in the post-liver transplant patient, upper and lower endoscopies are used to detect gastrointestinal opportunistic infections, as well as to screen for colorectal cancer in high-risk patients. Biliary complications are common after transplantation and ERCP is the modality of choice for treating such patients.

ACKNOWLEDGMENTS

We would like to thank the Hellenic Foundation of Gastroenterology and Nutrition for providing a research scholarship to Dr. C Krystallis.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i5.412

World J Gastroenterol 2012 February 7; 18(5): 412-424
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Role of *ATG16L*, *NOD2* and *IL23R* in Crohn's disease pathogenesis

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Supported by The Broad Foundation grant, No. IBD-0207R
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Received: April 1, 2011 Revised: August 8, 2011
Accepted: August 27, 2011
Published online: February 7, 2012

Abstract

Inflammatory bowel disease is a group of diseases that includes Crohn's disease (CD) and ulcerative colitis. CD is characterized as a chronic inflammatory disease of the gastrointestinal tract, ranging from the mouth to the anus. Although there are gross pathological and histological similarities between CD and Johne's disease of cattle, the cause of CD remains controversial. It is vital to understand fully the cause of this disease because it affects approximately 500 000 people in North America and Europe. It ranges from 27 to 48 cases per 100 000 people. There are many theories on the cause of CD ranging from possible association with environmental factors including microorganisms to imbalance in the intestinal normal flora of the patients. Regardless of the environmental trigger, there is strong evidence that a genetic disposition is a major key in acquiring CD. Many studies have proven the link between mutations in the *ATG16L*, *NOD2/CARD15*, *IBD5*, *CTLA4*, *TNFSF15* and *IL23R* genes, and CD. The purpose of this review is to examine all genetic aspects and theories of CD, including up to date multiple population studies performed worldwide.

Key words: Crohn's disease; *ATG16L*; *NOD2/CARD15*; *IBD5*; *CTLA4*; *TNFSF15*; *IL23R*

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Naser SA, Arce M, Khaja A, Fernandez M, Naser N, Elwasila S, Thanigachalam S. Role of *ATG16L*, *NOD2* and *IL23R* in Crohn's disease pathogenesis. *World J Gastroenterol* 2012; 18(5): 412-424 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i5/412.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i5.412

INTRODUCTION

The first description of Crohn's disease (CD) was made in 1769 by an Italian physician, Giovanni Battista Morgagni, when he diagnosed a man with chronic diarrhea. In 1898, consecutive cases were reported by John Berg, and then in 1904 by Antoni Lesniowski. Throughout the 1920s and 1930s, young adults were thought to have this same condition as they suffered from symptoms, such as abdominal cramps, diarrhea, fever and significant weight loss. In 1923, surgeons at Mt. Sinai Hospital in New York had also identified patients with comparable symptoms. In addition, in 1930, Dr. Burrill B Crohn saw a connection between this unknown debilitating condition and two of his patients. Consequently, Dr. Crohn and his

colleagues presented a paper in 1932, "Regional ileitis: a pathologic and chronic entity", describing the features of this disease to the American Medical Association. CD was named after Burrill Crohn and it became an official medical entity in 1932.

CD is a chronic inflammatory disease that can affect any portion of the digestive tract including the mouth, esophagus, and small and large intestines, but is most common in the ileum. It is also characterized as an autoimmune disease in which the body attacks itself and causes inflammation. In its mild form, it causes erosions called aphthous ulcers in the inner surface of the bowel. In severe cases, deeper and larger ulcers develop that can lead to bowel obstruction and holes in the bowel wall. If a hole in the bowel wall arises, infection to neighboring organs can occur. CD branches off to many forms depending on the location of erosion. If these symptoms take place within the large intestine, it is called Crohn's or granulomatous colitis. If it occurs in the small intestine, it is known as Crohn's enteritis, and more specifically if it occurs in the ileum, it is called Crohn's ileitis. During severe cases in which the small and large intestine are both involved, it is known as Crohn's enterocolitis or ileocolitis. CD is separated into three phenotypes: non-stricturing and non-penetrating, stricturing and penetrating CD[1,2].

CD is most prevalent in North America and Europe, and least prevalent among African Americans and Asians[3]. It affects approximately 500 000 people in North America and Europe. It ranges from 27 to 48 cases per 100 000 people. There is no difference in prevalence among males or females. Individuals with siblings affected by CD have a higher risk of acquiring the disease. There is evidence of a cause from environmental factors, thus, there are a higher number of cases in western industrialized countries. Symptoms of CD typically begin in the teens and twenties and then go into remission on and off with appropriate therapies. There is a peak incidence between 50 years and 70 years of age, which often leads to major complications due to age and the necessity of surgery.

Although the etiology is still unknown, there are many theories about what causes CD and ulcerative colitis (UC). Many believe it is caused by environmental factors, such as certain foods, bacteria, viruses, or cigarette smoke, which all can trigger an immune system response. Scientists have linked inflammatory bowel diseases (IBDs) as an autoimmune problem. In a healthy person, the immune system defends the body against harmful microbes that have entered it. Upon triggering the immune system, an inflammatory response occurs in which immune cells aggregate at the site of infection and overcome the threat. There are microbes native to our bodies that are useful rather than harmful to which the immune system does not trigger a response. In patients with CD, the immune system will attack these native luminal bacteria, disrupting the normal flora, thus characterizing this condition as an autoimmune disease.

Recent research has indicated specific genetic variations as a direct cause of CD and UC. The genetic aspects of CD have been linked by observing familial clustering of IBD cases[4]. Genetic variations in the *ATG16L*, *NOD2/CARD15* and *IL23R* genes have strongly been linked to the onset of CD[5]. Not only are individual gene mutations listed as a cause of CD, but a combination of them has also been shown in CD patients by conducting many population studies. There have also been many studies that have predicted surgical outcomes in both adults and children with specific genetic variations.

Diagnosis of CD can be tricky and requires a number of tests to be certain. Colonoscopy is the most effective way to diagnose CD but not in all cases because it only allows the physician to visualize the colon, ileum, and lower portion of the small intestine[6]. If the ulcers are located within the upper portion of the small intestine, this test will not be effective. In this case, a barium follow-through X-ray is useful because barium sulfate gives fluoroscopic images of the bowel and the physician can see areas of inflammation and narrowing[6]. Another effective method of diagnosis is the use of white blood cell scans. During this procedure, white blood cells are tagged with a radioisotope and then injected back into the patient. At specific intervals of time, the scan can locate accumulations of white blood cells in the intestine at the site of CD. This method is also useful to monitor the disease and show effectiveness in other therapies. Furthermore, a simple blood test can also diagnose CD because it can determine whether the patient is anemic or has a vitamin B12 deficiency because vitamin B12 is absorbed in the ileum and a deficiency can be due to ileitis.

CD causes a wide variety of symptoms and can be confused with UC, which is a similar disease, both under the group of IBDs. UC is only found in the colon and affects the mucosal membrane, whereas CD can occur anywhere throughout the gastrointestinal (GI) tract and affects the thickness of the GI wall. Individuals with CD can experience flare-ups followed by remissions. It can vary from one flare-up in a lifetime to multiple flare-ups that need surgical treatment. Symptoms include persistent diarrhea, abdominal pain in the affected area, fever, and weight loss[6]. There are also signs and symptoms that may occur unrelated to the GI tract, such as reddening and inflammation of the eye, joint pain, skin lesions, and sores inside the mouth.

Currently, there is no cure for CD. Treatment is focused on relieving the symptoms and putting it into remission. Since CD is characterized as an autoimmune disease, medications to suppress the immune system include 5-aminosalicylic acid and steroids, such as prednisone[6]. Antibiotics such as clarithromycin, ampicillin and metronidazole, can also be used. More than 50% of patients with CD will have to undergo surgical treatment to correct a fistula, drain an abscess, open a narrow or obstructed bowel, or remove a segment of infected intestine.

AUTOPHAGY-RELATED 16-LIKE 1 PROTEIN COMPLEX

Autophagy is a catabolic process of intracellular degradation in which cytoplasmic components are sequestered within vesicles and delivered to the lysosomes. Cells use this pathway during nutrient starvation because they can break down non-vital components and use them as nutrients. Autophagy plays a role during infection by helping rid the cell of foreign antigens by breakdown of the pathogen. This pathway can also be implemented as a repair mechanism to degrade damaged organelles and proteins. Autophagosomes, formed by the fusion of lysosomes and vesicles, are also implicated in the processing of intracellular bacteria. If the gene responsible for autophagy is mutated, it can cause a shift in normal flora as previously mentioned, and lead to many GI problems, which has been pointed out as a possible cause of CD. If the cell cannot regain nutrients or fight off foreign antigens within the GI tract, these cells will undergo programmed cell death and cause tissue damage. This damage can be seen as lesions and ulcers within the intestines, creating dead infected patches of tissue along the GI tract. The only option of treatment is surgery to remove the sections of the intestines with the diseased tissue so that the necrosis will not spread among neighboring cells.

An autophagosome, a double-membraned vesicle formed by autophagy, envelops part of the cytoplasm and delivers it to the lysosomes where it is degraded and recycled. There have been approximately 30 autophagy-related (Atg) genes identified, with two proteins having ubiquitin-like characteristics, Atg12 and Atg8[7]. These proteins covalently modify their target protein with molecules such as ubiquitin-like proteins to tag them for degradation. Both proteins also contain a conserved ubiquitin-fold region[7]. Autophagosomes use two conjugation systems, the Atg12 and LC3-Ⅱ systems[8]. These systems were first discovered during yeast genetic studies revealing a set of 17 ATG genes involved in the autophagy pathway[9]. In the Atg12 conjugation system, an Atg12-Atg5-Atg16L complex forms, and dissociates from the membrane just before or after completion of the autophagosome[8]. The ATG16L1 protein is expressed in the colon, small intestine, intestinal epithelial cells, leukocytes, and spleen[8]. Recent independent studies have shown that an ATG16L mutation, located on chromosome 2, is associated with the onset of ileal CD, and is therefore a key molecule in elucidating the genetic aspects of this disease[10].

Multiple studies have been performed with each resulting in the same conclusion that ATG16L is implicated in CD. During a genome-wide survey of 19 779 non-synonymous single nucleotide polymorphisms (SNPs), Thr300Ala within the N terminus of ATG16L was found to be highly associated with CD by using a haplotype and regression analysis[4]. This study used a total of 735 CD patients, 368 controls and 72 SNPs. A second report from the North American CD genome-wide study submitted by Rioux and colleagues also shows an association with ATG16L using a case-control analysis in 988 CD patients and 1007 controls[11]. Another group of German and British collaborators demonstrated that rs2241880, another non-synonymous variant of the *ATG16L* gene on chromosome 2q37.1, is implicated in the autophagy pathway[8].

In a study performed on an Italian cohort, the same polymorphism, rs2241880, was observed in 667 CD and 668 UC patients[12]. Both the frequency of the G allele and number of carriers of the G allele were increased in CD patients when compared to the controls[12]. These differences were only significant in the adult subgroup, which could be due to the small sample size of the pediatric subgroup. In comparison, there were no significant allele or genotype frequencies found between UC patients and controls for the groups as a whole[12]. During analysis of genotype/phenotype correlation of the rs2241880 SNP, there were no associations with disease location, behavior, and age at diagnosis based on the Montreal Classification of CD[12]. There were also no associations found in sex, smoking, and perianal fistulae[12]. For the rs2241880 variant, recent studies, specifically by Prescott[13], demonstrate an association with the ileal form of CD with or without colonic involvement, but not with isolated colonic disease[12].

In addition, a study from Oxford compared 645 CD patients with 1190 controls and showed an association of ATG16L1 with CD[14]. To understand fully the function of this gene, a study utilized oligo-based silencing RNA directed against ATG16L1 isoforms, where autophagy was induced by *Salmonella typhimurium* in ATG16L1 knockdown HEK293 cells. There was a significant difference between the knockdown cells com-pared to the control cells during the autophagy pathway[11]. It is clear to say that variants of this gene have been proven without a doubt to be directly associated with CD because autophagy plays a critical role in disease pathogenesis. Further research needs to focus on understanding how ATG16L1 variants contribute to disease susceptibility in IBD patients, and their possible therapeutic implications.

TUMOR NECROSIS FACTOR SUPER FAMILY 15

Tumor necrosis factor super family 15 (TNFSF15) is a Th-1 polarizing cytokine involved in systemic inflammation. TNF functions to regulate immune cells, induce apoptosis, induce inflammation, and inhibit tumorigenesis. TNFs are produced by macrophages, lymphoid cells, mast cells and endothelial cells. During immunological studies, it has been found that CD patients have an increased expression of TNFSF by multiple cells in the intestinal tissues when compared to controls[7]. The *TNFSF15* gene is a candidate for increased susceptibility in IBD. It binds to a specific T-cell receptor to enhance

cytokine-induced interferon expression in mucosal $CD4^+$ T cells[7].

In 2005, the first genome study involving IBD tested nearly 80 000 SNPs in Japanese CD patients. This study identified haplotypes within the *TNFSF15* gene, which included seven SNPs within a 280-kb region on chromosome 9q32[15]. By resequencing TNFSF15 from the same CD cases but with a new control group, TNFSF15 was found to be strongly associated with CD with an odds ratio (OR) of 2.17 (95% CI: 1.78-2.66), $P = 1.71 \times 10^{-14}$[15]. The Japanese wanted to see if the same patterns were seen in other population groups so they replicated their associate in two panels from Oxford, United Kingdom. Although the risk haplotype was identified in both cohorts, there was a weaker effect size ($P = 0.02$ in both family-based and case-control association panels). In addition, another study involving a Jewish cohort also showed an association of TNFSF15 with CD, and also suggested that in response to FC-gamma receptor stimulation, *TNFSF15* gene variation aggravates induction of TNFSF15[16]. However, in a separate study using a Belgian CD cohort, no significant association was observed between CD and TNFSF15[4]. This may have been due to different marker genotypes or differences in susceptibility genes between Asian and European cohorts[4]. Not enough studies have been performed regarding TNFSF15 and its possible implications in CD. Future studies have to focus on different populations to provide efficient insight.

NOD2/CARD15 GENE

Nucleotide-binding oligomerization domain containing 2 (NOD2), located on chromosome 16q12, is a protein that plays an essential role in the immune system by controlling commensal bacterial flora in the intestine[17]. NOD2 belongs to a nucleotide-binding domain, leucine-rich repeat family of cytoplasmic proteins that may detect a variety of bacteria by acting as an intracellular sensor for bacterial peptidoglycan[18]. It has the ability to respond to N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) *via* the leucine-rich repeat domain. The MDP is conserved in both Gram-negative and positive bacteria[10,19]. This leucine-rich repeat domain plays a role in protein-protein interactions and the middle portion of the protein is responsible for self-oligomerization. The N-terminal portion of NOD2 contains two caspase recruitment domains (CARDs) which play a role in apoptosis. The *CARD15* gene, which encodes for the CARD domain within NOD2, has been specifically identified as a genetic factor for CD. Three SNPs were found to be independently associated with CD: rs2066844, rs2066845, and an insertion mutation 3020insC[20]. Each variant may result in distinct phenotypic expression of CD[4]. To activate NOD2, Rip2 kinase is required because it is necessary for downstream signaling of NOD2 and signaling cascades such as nuclear factor (NF)-κB and mitogen-activated protein kinase cascades[21].

The intestinal mucosa is constantly exposed to a large number of commensal microorganisms; the majority of which inhabit the large intestine. In a healthy individual, there is a basal immune response elicited from the interaction between the intestinal immune system and commensal bacteria. This immune response is constantly present to protect the host from pathogenic and non-pathogenic bacteria. When there are changes within this balance, the intestines are susceptible to chronic intestinal inflammatory conditions, such as CD[21]. Multiple genetic studies have linked NOD2 with susceptibility to CD. However, NOD2-deficient mice do not develop colitis, suggesting that dysregulation of the NOD2 pathway is not sufficient to provoke CD[22]. This is not a surprise because the pathogenesis of CD is caused by several factors including environmental, dysfunctional immune system, and a shift in normal bacterial flora[21].

NOD2 is expressed in Paneth cells, which are found in the intestines. The exact functions of Paneth cells are still unknown, but they are likely to contribute to the host defense by secreting antibacterial compounds due to the presence of lysozyme. A NOD2 mutation can alter the function of Paneth cells, which alters their antimicrobial activity and leads to the development of ileal lesions, which correspond to the location of these cells. Of the three NOD2 mutations associated with CD, both rs2066844 and rs2066845 are the result of a two-amino-acid substitution in which rs2066844 is encoded by exon 4 and rs2066845 is encoded by exon 8[23]. The variant 1007fs is created by a frameshift mutation in exon 11[23]. Each of the three mutations occurs within or near the leucine-rich repeat and decrease the cells ability to activate NF-κB in response to peptidoglycan[18]. Interestingly, these mutations are observed in Caucasian patients but not in Japanese, Chinese and Korean patients with IBD, and they are very rare in African Americans with IBD[24,25]. An individual heterozygous for at least one NOD2 mutation is at a 2-4-fold increased risk of developing CD, whereas a homozygous individual for at least one NOD2 mutation is at a 20-40-fold increased risk when compared to healthy individuals[14].

Studies of each individual mutation in NOD2 have shown that 1007fs causes a decrease in defensin expression[26]. Defensins are cells of the immune system that assist in killing phagocytized bacteria. They function by binding to the microbial cell membrane and forming a pore in the membrane that allows outward flow of nutrients and essential ions. CD patients who are homozygous and/or heterozygous for NOD2 mutations typically have lower defensin levels in their ileostomy fluid[17]. As a result of a mutation in NOD2, the function of this protein is diminished, therefore allowing subsequent entry of bacteria into epithelial cells because they are no longer able to recognize them. This in turn alters the bacterial population in the intestines and thus, defensins are not able to function correctly because they are working in an impaired bactericidal capacity[22].

In a recent study by Van Limbergen *et al*[17], it was

proven that NOD2 is required for the regulation of commensal microbiota in the intestine. The regulation of NOD2 depends on the downstream kinase Rip2 because Rip2-deficient mice fail to establish and regulate commensal bacteria in their terminal ileum. NOD2-deficient mice do not develop spontaneous intestinal inflammation. In conclusion, the NOD2–Rip2 pathway is critical for the regulation of homeostasis between the body's normal bacterial flora and innate immunity[17]. As a controlled balance, it has been found that the expression of NOD2 and Rip2 is dependent upon and regulated by the presence of commensal bacteria[17]. This creates a negative feedback by which the commensal bacteria positively regulate NOD2, and in turn negatively regulate the normal flora. NOD2 mutations directly affect the ileum in CD patients, thus, it is possible that they are responsible for the composition of the bacterial flora in the terminal ileum[17]. This may facilitate both disease pathology and progression.

Although the exact mechanism by which NOD2 contributes to the control of commensals in the intestines is still not known, there are many possible theories proposed by this study. The first theory entertains the possibility that NOD2 regulates the commensal flora through the bactericidal activity of ileal crypt secretions[17]. The second theory is that NOD2 regulates the adaptive immune system by inducing lymphoid tissue genesis[17]. The third theory describes how NOD2 expression in myeloid lineage cells contributes indirectly to maintain the normal microbiota flora[17]. The fourth theory states that there may be other cells in the intestines, in addition to Paneth cells, that may play a role in the regulation of commensal bacteria in the intestines[17]. Further research is needed to elucidate this mechanism.

It has been reported that both adults and pediatric cohorts share a strong association between the NOD2 variants and ileal disease location[23]. Some studies have suggested that adult patients show a correlation in fibrostenotic behavior and NOD2 mutation status, whereas other studies have failed to replicate the same phenotypic effect[12]. It is necessary to study different CD cohorts from different countries to obtain an accurate effect of each mutation. Exploring the genotype-phenotype interactions in children is beneficial because they show a higher gene dosage and have less environmental influences.

A study by Lacher and colleagues has explored the association of the NOD2 mutation in German pediatric CD patients and the risk of surgery. The risk of surgery in children is important to predict because surgery is sometimes the only method of treatment. Out of 171 young CD patients, 78 (45.6%) carried at least one NOD2 mutation, with 11 being compound heterozygous, and 14 being homozygous for two NOD2 mutations[14]. The presence of rs2066844 was found in 29 (17%) children, rs2066845 was found in 18 (10.5%), and 1007fs was found in 42 (24.6%)[14]. Overall, one out of three German children with CD had at least one NOD2 mutation. In comparison, 36% of adult CD patients were heterozygous for at least one NOD2 mutation: 17.2% for rs2066844, 9.6% for rs2066845, and 11.7% for 1007fs[14]. In conclusion, the genetic alterations were observed more predominantly in German pediatric CD patients than in adults. The study also looked into each mutation and its association with localization and symptoms of the disease. A 4.73-fold increased risk of isolated ileal localization was observed in patients that were identified as 1007fs carriers when compared to children with none of the NOD2 mutations[14]. Although the 1007fs carriers were predisposed to ileal disease, there was no involvement of the ileocolonic or upper GI tract[14]. The next characterization the study explored was the association of NOD2 mutations and stricturing disease or perianal fistulae in their German pediatric CD cohort. Only 17% of patients showed a stricturing phenotype and 18.1% had a perianal fistula[14]. Among the children that showed a stricturing phenotype, 79.3% required surgery and those with the 1007fs mutation had surgical complications[14]. The outcome showed a 9.8-fold increase in surgical complications when the child carried at least one allele for the 1007fs mutation[14]. Not only is the 1007fs mutation strongly associated with isolated ileal disease, but children are at a high risk for surgical intervention. Therefore, this mutation can act as a prognostic tool in Caucasian children with CD[14].

Another study by Jurgens and colleagues has investigated the presence of fistulas and their association with NOD2 homozygosity and how they predict intestinal stenosis in CD patients. It was observed that patients with fistulas had simultaneous intestinal stenosis[15]. In another study using the same research, it was found that NOD2/CARD15 variants, especially with 1007fs homozygosity, could predict the occurrence of intestinal stenosis[27]. By using a strict screening process based on phenotypes including stenoses (stricturing CD) and fistulae (penetrating CD), and genotype, the study isolated a total of 145 patients. One hundred and twenty-five of the patients had penetrating CD with simultaneous stenosis within a 6-mo interval[28]. It was also found that all 14 CD patients homozygous for NOD2 variants suffered from stenosis[28]. To no one's surprise, 11 out of the 14 patients with stenosis and fistulas carried the NOD2 1007fs mutation[28]. These data confirm the strong risk factor of the 1007fs NOD2 mutation in the prevalence of intestinal stenosis, which may be related to the decreased intestinal barrier function found in CD patients with NOD2/CARD15 variant mutations[28]. This study has isolated the 1007fs variant as the cause in disrupting the intestinal barrier and causing the many problems often observed in CD patients.

To investigate whether stenosis occurs in a specific anatomical region, 223 patients with 248 stenoses located at different intestinal regions were examined[28]. The most common anatomical region was the terminal ileum with 68.8% of stenoses, followed by 11.7% found in the rectosigmoidal segment, and 9.3% in the jejunum or proximal ileum[28]. In conclusion, homozygosity in the NOD2/

CARD15 mutation is a strong risk factor for intestinal stenosis[28]. When the study looked into the possible association between stenosis and interleukin-23 receptor (IL-23R mutation variants, there was a weak connection with no influence on stenosis[28]. This study suggests classifying CD into four disease phenotypes instead of the current three in the Montreal Classification System. The four new classifications would be: non-stricturing[12], non-fistulizing CD[12]; stricturing, non-fistulizing CD[18]; non-stricturing, fistulizing CD[29]; and stricturing, fistulizing CD[28]. It is important to link any possible association between fistulas and stenoses because there is a strong risk factor for recurrence of CD after surgery[28].

Many studies have investigated North American, European and Asian countries and their CD patients with specific genotypes. Interestingly, the NOD2/CARD15 mutations are very rare and even absent in Asians (Japanese, Chinese and Korean), Arabs, Africans and African Americans[28]. A study by Baptista and colleagues has concentrated on a South American population for the first time. There were a total of 187 CD patients used for the study with a median age of 33 years and a median age of onset of 23 years[24]. Their patients were ethnically classified into the following groups: 58.8% were in the Brazilian subgroup, 36.9% shared a common European ancestry and were in the European-Brazilian subgroup, three were Amerindian-Brazilian, and two were Afro-Brazilian[24]. The alleles related to CD within the CARD15, rs2066844 and 3020insC variants were significant for CD susceptibility[24]. Both the rs2066845 and 1007fs variants failed to show any significant association[24]. Among their patients, 30% had at least one NOD2/CARD15 variant allele[24]. The frequency of rs2066844 (9.63%) in the Brazilian CD cohort was consistent with the reports for European populations[24]. In conclusion, this study confirmed that CARD15 variants lead to greater susceptibility to CD in the Brazilian population[24].

INTERLEUKIN-23 RECEPTOR

IL-23R is a protein consisting of an IL-12β1 and an IL-23R chain[27]. The molecular location of the *IL23R* gene is on chromosome 1 and is formed by the binding of IL-12p40 and a p19 protein[30]. It is highly expressed on the cell membrane of memory T cells and other immune cells, such as natural killer cells, monocytes, and dendritic cells, which identify foreign substances to defend the body against infection. It is highly involved in the mediation of proinflammatory activities by the production of IL-17 *via* the activation of Th17 lymphocytes[20]. IL-23R interacts with IL-23, which is a cytokine that regulates the activity of immune cells and plays an important role in the inflammatory response against infection by bacteria and viruses. It also has been suggested that the functional IL23R pathway polymorphisms play a role in modulating neonatal development of intestinal tolerance and bacterial colonization[4].

Th-17 lymphocytes are a distinct subset of T-helper cells, which mainly produce IL-17 and to a lesser extent IL-6 and TNF-α[26]. IL-17 *in vitro* and *in vivo* acts as a potent inflammatory cytokine and is involved in the destruction of cartilage and bone, as seen in rheumatoid arthritis[12]. It has been reported that IL-23 could be a key regulator in the differentiation of Th-17 lymphocytes from memory T cells. It also has been suggested that IL-23 plays a role in providing a survival advantage to already differentiated Th-17 cells[21]. The expression of the heterodimeric receptor complex, IL-23R and IL-12Rβ1, regulates activities of IL-23[12]. Therefore, the IL-23-IL17 cytokine axis is a key pathogenic mechanism that mediates the development and progress of inflammation by Th-17 cells. The role for the IL23-IL17 axis in CD patients was supported in human patients and animal models of colitis[31]. Both cytokines are increased in knockout mouse models of IBD. More specifically, IL-17 levels are increased in both intestinal mucosa and in the serum of CD patients[12]. In a study using IL-17R knockout mouse models, an association was found with colitis and disease severity[27]. Therefore, the use of anti-IL-12p40 antibody to treat CD patients is a therapeutic option because it is known to reduce production of IL-23 and IL-17 in the lamina propria cells[12]. The mechanism of IL-23R is clearly important to understand because it is directly associated with CD.

During a genome-wide association study, 2877 DNA samples from IBD patients (two-thirds CD and one-third UC), identified rs11209026 as a possible protective variant, in the *IL23* gene on chromosome 1p31[4]. There are many other variants within IL23R that are associated with IBD, but rs11209026 has the strongest association with conferring protection against CD[4]. Although the effect of IL23R variants is greatest in CD, it may have an overall effect on susceptibility to chronic intestinal inflammation.

Additional studies have confirmed the susceptibility of the *IL23R* gene to CD in North American and European populations. They include cohorts ranging from Scottish pediatric IBD, Belgian CD and an independent cohort of 883 families[4]. Due to the role of IL-23 in activation of inflammatory responses, targeting this pathway may be a good therapeutic approach. Some promising research is underway using anti-p40, which blocks IL-23 and IL-12 activities. The variant allele, rs11209026, could be exploited to define clinical outcomes, such as a pharmacological approach to mimic the rs11209026 polymorphism[4].

A study by Schmechel and colleagues has shown a link in susceptibility to CD with Th17 cell function, IL-22 serum levels, and IL23R genotype. IL-22 is a strong activator of proinflammatory gene expression and upregulates SOC3 mRNA in intestinal epithelial cells[29]. Recent evidence has shown that Th17 cells expressing IL23R play a key role in the mechanism by which IL23R modulates IBD susceptibility[32]. Previous studies have shown that Th17 plays a role in autoimmune diseases,

such as rheumatoid arthritis and CD. However, Th17 is responsible for the important function of antimicrobial immunity at epithelial barriers where it produces cytokines such as IL-22. Because it produces IL-22 in epithelial barriers such as in the intestines, theoretically by testing for increased IL-22 serum levels, a physician can determine CD and disease activity.

It has been confirmed that IL-22 serum level is increased in CD and correlates with disease activity[33]. IL-22 serum levels are also independent of CD phenotype and CARD15 genotype, but are modulated by IL23R polymorphisms[33]. The study investigated IL-17 serum because Th17 cells also produce IL-17. There was no correlation between IL-22 and IL-17 serum levels[33]. Currently, serum levels of TNF-α and IL-6 are used as inflammation markers in determining CD. There was no difference in TNF-α and IL-6 levels whether CD was active or in remission, whereas IL-22 levels were significantly higher in active CD compared to CD in remission[33]. Therefore, measuring IL-22 levels are clinically relevant in determining the disease activity in CD patients[33].

There are also strong associations between IL-22 serum levels and *IL23R* gene variants. Previous studies have shown both protective and inducing variants of IL23R in susceptibility to CD. Although rs1004819 is the CD increasing variant of IL23R, rs11209026 has shown to be a protective IL23R variant against CD. As predicted, when the IL-22 mean serum levels were tested against each variant, the serum levels were highest among SNPs that increased CD risk as opposed to the identified protective SNPs of IL23R, which had low serum levels[33]. In contrast, the three main variants within NOD2/CARD15 did not show any differences in IL-22 serum levels[33]. Although, the exact function of IL-22 in human IBD is still unknown, recent studies have observed increased β-defensin-2 expression and intestinal epithelial cell migration and proliferation upon stimulation with IL-22[29]. These data ultimately suggests a clinically useful marker to assess disease severity and Th17 cell activity in CD patients[33].

As mentioned above, a rare glutamine allele, rs11209026, in IL23R conferred protection against CD during a recent genome-wide association study. Recent studies have proven that this variant protects against CD in both Jewish and non-Jewish populations. In addition, in a study involving IBD (both UC and CD) patients of Spanish Caucasian origin, the rs11209026 variant was seen to be most significantly associated with IBD protection (OR: 0.4; 95% CI: 0.3-0.7)[34]. A study by Dubinsky and colleagues has investigated this rare allele further and how it protects pediatric CD patients. They used the transmission disequilibrium test (TDT) analysis and genotyping of whole blood samples from children with IBD and their two parents. This rare rs11209026 SNP was present in 2.67% non-Jewish CD patients and 2.94% of non-Jewish UC patients[35]. The TDT demonstrated that the allele was under-transmitted in all CD offspring and confirmed the negative association between rs11209026 and CD[35].

Another way to confirm the association of alleles with CD and UC is to use a population that is genetically isolated, such as the Finnish population. This will provide an advantage in molecular genetic studies in complex disorders[36]. A study by Lappalainen has used such a strategy to confirm the association of IL23R, TNFRSF1A, and the HLA-DRB1*0103 allele variants. The strongest association of IL23R with the marker rs2201841 (IL-23R risk variant) showed a frequency of 37.2% in CD. In most studies, the non-synonymous SNP, rs11209026 (the protective IL23R allele), has had the highest association, but is only marginally associated with Finnish CD[36]. No association has been observed between the IL23R markers and UC patients[36].

Previous studies have shown that the HLA-DRB1*0103 allele is associated with both UC and CD. In the Finnish population, this allele only has a frequency of 0.6%, which is not statistically significant, whereas it is significant for UC and IBD[36]. When looking into the genotype-phenotype association, patients carrying the rare HLA-DRB1*0103 allele have colonic involvement in CD[36]. During the TNFRSF1A analysis, the investigators genotyped a rare A36G variant and an IVS6+10A (rs1800693) variant. CD patients with both variants often show ileocolonic disease in comparison with patients without these two variants[36]. The weak association is probably due to the small sample size. Interestingly, when the protective haplotype of IL-23R (described in the above paragraph) was sequenced and compared to North American Caucasian CD patients, there was a one-nucleotide difference between Finnish (CCTGATCG) and North American (CGTGATCG) CD patients[36]. In conclusion, the HLA-DRB1*0103 allele has been confirmed in CD patients, which shows an inherited susceptibility of colonic inflammation. The *TNFRSF1A* gene variants are markers of ileocolonic involvement in CD[36]. Although this study shows a weaker association of the *IL23R* gene, it confirms the genetic involvement within a Finnish population.

In another population study among French-Canadian and English-Canadian children, the association between genetic variants of the *IL23R* gene and early-onset of CD has been investigated. To study the associations accurately, they have carried out both a case-control and a family-based study. They have targeted the 10 SNPs in IL23R achieved by the genome-wide study and the three CARD15 SNPs. In total, 259 CD patients and 139 controls were recruited with a mean age at diagnosis of 13.3 years (range: 2.6-20 years)[12]. The IL23R protective allele, rs11209026, was only present in 2% of CD case chromosomes and 6% of the control chromosomes[12]. All CARD15 variant allele frequencies were higher among CD patients when compared to the control group[12]. In the *IL23R* gene, they observed a significant association among four SNPs that did not posses any CARD15 variants[12]. Therefore, variants in the *IL23R* gene were associated with early-onset CD among Canadian children.

This study confirms previously reported findings in CD patients among North Americans.

In continuation of a study that was described in the ATG16L section of this paper, Latiano and colleagues have shown that the replication of IL23R is associated in adult and pediatric onset of IBD in Italy. Approximately 730 CD patients were genotyped for the rs7517847 and rs11209026 variants. For the rs7517847 polymorphism, significant reductions were found in minor allele (G) frequency in CD patients when compared with controls and in the number of carriers[12]. No differences were found in UC patients. When the rs11209026 polymorphism was examined, a significant increase in the frequency of the risk genotype was observed in CD patients. In either SNP, there were no correlations between phenotypes of CD and risk alleles or genotypes of the *IL23R* gene.

To specify which IL23R variant is the main disease associated variant, a study using German CD patients was conducted. Among all 10 IL23R SNPs, they chose to focus their study on the rs1004819 variant because it had the strongest association to CD when compared to controls. This variant showed high prevalence of ileal involvement when carriers had the TT genotype versus the CC wild-type genotype[28]. This identification is different from recent data published by Roberts *et al*[37], which have identified the rs7517847 variant as having the strongest association with CD, along with other overlapping North American study populations. They have only analyzed ileal cases of CD, therefore, it is assumed that IL23R variants are predisposed to an ileal disease phenotype[37]. In a recent British study, they could not identify any association between disease phenotype and IL23R variants[9]. Interestingly, they found that the rs1004819 variant was 1000-fold weaker than that reported in the German study.

Not only are the IL23R variants highly associated with CD, they are also associated with other chronic inflammatory diseases, such as the rs10489629 variant with chronic periodontitis. In psoriasis, the variant rs11209026 has been described as a predisposing haplotype[17]. In both CD and psoriasis, treatment with an anti-p40 IL-12/23 antibody has shown promising results. Antibodies to the p40 subunit block both IL-12 and IL-23, although in knockout mice studies, it has been proven that IL-23 drives chronic intestinal inflammation[28]. Therefore, IL23R can serve as a therapeutic target in many different chronic inflammatory diseases, although the variants may differ among the different diseases. It is hypothesized that this is due to alternative mRNA splicing, which results in corresponding IL23R isoforms with different tissue distribution[28].

IBD5 GENE

The *IBD5* gene is about 250 kb and is located at position 5q31. During a genome-wide linkage analysis, mapping studies have identified a risk haplotype within the IBD5 locus. Many studies have linked this gene and its two variants, SLC22A4 (OCTN1) and SLC22A5 (OCTN2), to CD. IBD is believed to originate from an uncontrolled mucosal immunity of the GI tract[17]. Although these variants are associated with CD, they act independently because there is no statistical evidence for interaction between *IBD5* and the *IL23R*, *ATG16L1* or *CARD15* genes[12].

CTLA4 VARIANTS

The *CTLA4* gene is a member of the immunoglobulin superfamily and is expressed on the surface of helper T cells. It is located within the 2q33 region, translates into a protein that plays a role in the immune system, and may have a genetic association with IBD. It is a T-cell suppressor, which is essential in the function of the $CD25^+$ $CD4^+$ regulatory cells[7]. These regulator cells control the process of intestinal inflammation. Many SNPs have been studied within this gene and it has been found that the rs3087243 variant shows the most association with IBD followed by the rs11571302, rs7565213 and rs11571297 variants[35]. Although studies have shown that the three variants in the *CTLA4* gene, g.49A > G (rs231775), g.-318C > T (rs5742909), and rs3087243, have no association with CD, other work has suggested that these variants may control the phenotype of CD[14].

A study performed by Hradsky has shown no crude association between CD and SNPs within the CTLA4 gene[12]. The study explored the possibility of interactions in CTLA4 SNPs with variants in IL23R and NOD2. The R-project package SNPassoc was used and significant interactions between the three CTLA4 variants with NOD2 p.Leu1007fsX1008 and IL23R rs11209026 were observed[12]. This may be due to complex gene-gene interactions. To characterize further the different variants and whether they determine phenotype in CD patients, the study used a case-only design. They observed a difference of minor allele frequency at the rs3087243 gene between pediatric-onset and adult-onset of CD[12]. It seems that a genetic factor has a greater impact in early-onset patients when compared to the adult-onset patients[38]. Within this study, the age of diagnosis and localization of the disease was strongly associated with the rs3087243, rs11571302 and rs11571297 variants[12].

NOD2, IL23R, OCTN1/2 AND ATG16L1 POLYMORPHISMS

Gene-gene interactions

Interactions of the major IBD alleles show a high susceptibility in CD patients. Gene-gene interaction can either enhance or weaken the effects of an individual gene, therefore making it more important than independent studies looking into the effects of single susceptibility genes. Csongei and colleagues have performed a gene-gene interaction analysis in the Hungarian CD population[5]. They concentrated on the two IL23R gene risk variants (rs2201841 and rs1004819), the ATG16L1 gene variant (Thr300Ala), and the three NOD2/CARD15

variants (rs2066844, L1007fs and rs2066845). Logistic regression analysis showed that the IL23R variants, both rs1004819 and rs2201841 and the NOD2/CARD15 variants (rs2066844 and L1007fs) conferred significant risk for CD. When the patients were homozygous for IL23R (rs1004819 and rs2201841) variants or ATG16L1, there was a highly increased risk for CD. When they analyzed possible statistical interactions between pairs of ATG16L1, IL23R and CARD15 variants, no evidence of interactions was found. Therefore, all examined loci contribute independently to CD risk. Although significant statistical interactions were not detected, these susceptibility factors may have a cumulative effect in the Hungarian population.

Further gene-gene interaction studies have been performed in another population study, as well as assessment of CD genetic risk factors. The study included five SNP variants for IL23R, the functional variants SLC22A4 and SLC22A5, and the ATG16L1 mis-sense risk polymorphism Thr300Ala. All the SNPs, except for rs1495965 of IL23R, showed significant association when carriers were either homozygous or heterozygous for the alleles[2]. Among them all, rs10889677 of IL23R, showed the strongest association with CD risk[2]. Unsurprisingly, ATG16L1 also showed a strong association with CD[2]. None of the SNPs showed an association with UC. When they evaluated correlations between genotype and phenotype for intestinal complications, carriers of the rs7517848 allele of IL23R, particularly those who were homozygous for the allele, were found to be at risk for ileal disease. No other SNPs showed differences in genotype or carrier frequencies when compared to CD patients and their history of complications.

Homozygote variants from the IBD5 and ATG16L1 genes had a greater risk than heterozygotes, suggesting a gene dosage effect[2]. When SNPs were considered two at a time, the best interactions were shown between IL23R_rs10889677 and IBD5_rs17622208, which were not statistically significant[2]. However, when using a logistic regression approach between these two markers, the IBD5_rs17622208 risk was only significant in the presence of the IL23R_rs10889677 risk allele[2]. When SNPs were considered three at a time, the model suggested statistically significant interactions between IL23R_rs10889677, IBD5_rs11739135, and ATG16L1_rs2241880[2]. When SNPs were considered four at a time, the model suggested interaction between IL23R_rs2201841, IL23R_rs7517847, and IBD5_rs11739135[2]. A study in Oxford, UK has hown that certain IL23R polymorphisms have an association with CD only when the person is positive for IBD5[2]. In conclusion, the logistic regression model did not show any significant evidence of gene-gene interaction, due to the small size of the study, even though there was a consistently small association between IBD5 and IL23R.

Childhood- vs adult-onset CD

The pathogenesis of pediatric and adult IBD differ. The age of childhood and adult onset varies between cultures to culture because different cultures base it on different aspects such as physical, mental, or puberty. North American studies choose an age cut-off of 18 years, and < 18 years is considered a child, even though an individual at this age is physically mature. This is why Canadian studies have chosen to describe a child as < 16 years old. The belief is that the lower the age cut-off, the better the results are when comparing pediatric and adult onset of disease. Recent studies have shown that a subgroup of patients with early-onset IBD may have specific phenotypes that differ from adult-onset IBD[39]. Many believe that pediatric-onset IBD is influenced by genetics compared to adult onset because there is less time for exposure to environmental modifiers to influence the onset of disease. Adult-onset is probably due to a mixture of genetics and abundant environmental exposure[25]. For example, smoking is a major variable in adult IBD patients, but has little influence on pediatric IBD cohorts. Currently, there are conflicting studies on whether NOD2/CARD15 polymorphisms are associated with the age of onset of IBD because some show an association towards a younger age, while others show no effect[40].

A study by Gazouli *et al*[25] has investigated the main polymorphisms and their association with childhood-onset of CD in a Greek cohort. While investigating the genotype and allele frequencies of the NOD2/CARD15 polymorphisms, rs2066844, rs2066845 and 3020insC, a statistically significant association between rs2066844 and adult-onset CD was observed. In both pediatric- and adult-onset CD, individuals with at least one NOD2/CARD15 polymorphism showed a genotype-phenotype correlation with ileal involvement[25]. The study also confirmed the recently described association between IL23R variants in both child- and adult-onset CD[35]. There has been conflicting evidence in studies regarding the ATG16L1 SNPs. One study has shown no association with early-onset or adult-onset CD[25]. Recent research has indicated that the ATG16L1 rs2144880 variant is associated with adult-pediatric-onset CD, whereas other studies have demonstrated an association with diagnosis at an earlier age[25]. In conclusion, the 3020insC variant in the NOD2/CARD15 gene is associated with CD and occurs considerably more often in childhood- than in adult-onset patients with CD[25].

There is growing evidence that pediatric-onset IBD shows distinct differences when compared to its adult counterpart. Familial aggregation studies have shown an age-adjusted risk of developing IBD in first-degree relatives of affected individuals compared to the general population[23]. The risk increases to > 30% for children when both parents are affected with IBD, suggesting that family history is the strongest risk factor[5]. This is especially true among CD patients. Familial cases of CD occur at a younger age with greater severity than random sporadic cases[36]. One main difference observed between pediatric- and adult-onset is that early onset shows a distinct and

more aggressive phenotype, such as the need for surgery, than similar IBD in individuals > 20 years old[41].

Although NOD2/CARD15 mutations are neither sufficient nor necessary for the development of IBD, they are associated with a younger age of onset, presence of ileal involvement, and the development of strictures[23]. There is also a gene dosage effect for CD location and complications. For example, stricture complications occur more frequently in CD children with the 1007fs mutation in the *NOD2/CARD15* gene compared to children without this variant due to early surgery[42]. Therefore, children with this mutation have a sixfold increased risk for developing a stricture complication[23].

There has been conflicting evidence among studies that have attempted to show a link between the IBD5 locus and early-onset CD. A study by Rioux has found that the IBD5 locus is associated with early-onset CD where children were defined with an age of onset of < 16 years old[23]. However, studies from pediatric-onset CD cohorts have demonstrated that the risk of IBD5 is lower compared to that in adult-onset CD, while others have demonstrated enhanced risk of developing CD when an individual has both SLC22A4-A5 and NOD2/CARD15 mutations[43]. This connection may be due to a common pathophysiological mechanism[23].

Although there is no sex difference among patients with adult-onset IBD, there is a clear and distinct difference among children with CD. Many studies from pediatric CD cohorts in the United States, Canada and the United Kingdom have shown an increase in male incidence. The higher male to female ratio continues to be unexplained. These differences are not observed among UC pediatric patients[23]. It seems that sex is an age-dependent variable that has more influence on children than adults with IBD.

There are also phenotypic differences, such as disease location, among children and adult CD. For example, increased rates of upper GI tract disease and pure colonic disease in pediatric-onset CD have been identified. This difference may be due to the amount of examination during the onset of disease. Children undergo extensive GI endoscopy, whereas adults do not[23]. These findings may be artificial or represent a true disease distinction among children and adults. Another distinction is the occurrence of colon-predominant disease during childhood-onset IBD under the age of 10 years old. With children < 5 years old, all have colon-only disease[44]. During a study of approximately 1400 North American early-onset patients, data showed a colon-predominant phenotype in children < 8 years old. In another study in Europe, the acquisition of ileal CD became increasingly common as an individual approached 16 years old[23]. These data confirm an association of colon-predominant phenotype in early diagnosed children that changes as they grow older.

Population studies with NOD2, IL23R and ATG16LI polymorphisms

NOD2, IL23R and ATG16L1 polymorphisms were studied in a Lithuanian cohort with IBD. The study included 57 unrelated patients with CD, 123 with UC and 186 healthy individuals as controls. The three NOD2 variants, the IL23R variant rs11209026, and the ATG16L1 variant Thr300Ala, were genotyped among the population sample. No individuals were carriers of all three NOD2 risk alleles, whereas two CD patients were compound heterozygotes[10]. Carriers of at least one NOD2 variant were highest among CD patients[10]. There were no significant differences observed between UC patients and the controls. The NOD2 variant, Leu1007insC, was significantly associated with increased susceptibility in the Lithuanian CD population[10]. In comparison to the other two NOD2 variants, the frequencies were very low and not significant in controls and among the IBD patients[10]. In contrast to other European studies, a positive association between rs2066844, rs2066845 and CD was not found[10]. When they further tried to analyze IL23R and ATG16L1, they were unable to replicate previous findings of increased susceptibility to IBD within the Lithuanian population[10]. They were was a trend for a possible association with the ATG16L1 risk allele[10]. It is of particular interest to study this population because Baltic countries have low IBD incidence rates, especially for CD. To confirm distinct IBD subtypes, a study using a larger North-Eastern European IBD sample needs to be investigated.

Another population study was performed among New Zealand Caucasians with IBD. Their cohort included 466 UC patients, 496 CD patients and 591 controls. All individuals were genotyped for the IL23R rs11209026 SNP, the ATG16L1 rs2241880 SNP, and the CARD15 variants. Significant interaction was detected between variants in ATG16L1, IL23R and CARD15 and CD susceptibility, whereas no significant association was observed between IL23R or ATG16L1 genotypes and IBD sub-phenotypes[37]. The strongest association occurred between the ATG16L1 rs2241880 variant and CD, with no association detected with UC[37]. ATG16L1 is suggested to have a CD-specific susceptibility locus. Unlike ATG16L1, the IL23R rs11209026 variant was strongly associated with both CD and UC. In their patient cohort, there was no evidence that IL23R or ATG16L1 genotypes influenced disease behavior, age of onset, location, or the need for surgical bowel resection[37]. On the other hand, CARD15 was consistently a susceptibility factor and predictor of CD phenotype. Similar to many other studies, all three CARD15 SNPs are significantly overrepresented in patients with IBD family history, early onset of disease, ileal disease involvement, and development of complications[33].

Interactions between NOD2 and IL23R variants with toll-like receptor-9 polymorphisms

Toll-like receptors (TLRs) are single, membrane-spanning proteins that play a key role in the innate immune system. These receptors recognize microbes that have structurally conserved molecules that breach the physical

Table 1 Key gene polymorphisms and their significance in Crohn's disease

Gene	Polymorphism	Relationship significance	Ref.
ATG16L	rs2241880	Associated with ileal form of CD with or without colonic involvement	[4,12]
	Thr300Ala	Highly associated with CD	
NOD2/CARD15	rs2066844, rs2066845, 3020insC, 1007fs	Independently associated with CD	[20]
IBD5	SLC22A4 (OCTN1), SLC22A5 (OCTN2)	Independently associated with CD	[12]
CTLA4	rs3087243, rs11571302, rs11571297, rs7565213	Associated with IBD; no crude association to CD	[35]
TNFSF15	80 000 SNPs tested, including 7 SNPs within a 280 kb region on chromosome 9q32	Strongly associated with CD for Japanese and Jewish cohorts, but not for Europeans	[4,15,16]
IL23R	rs11209026	Strongly associated with conferring protection against CD	[4,33]
	rs1004819	Highly associated with CD	

SNPs: Single-nucleotide polymorphism; CD: Crohn's disease.

barriers. Once bound, TLRs activate the immune system. The responsiveness of the GI tract to luminal bacteria is dependent on the interaction of transmembrane TLRs and the intracellular NOD2 receptor[18]. Specifically, TLR9 plays a role in the maintenance of intestinal inflammation in IBD. TLR9 is also responsible for stimulating NOD2, which in turn enhances innate immune responses. Patients with two NOD2 mutations lose the synergistic affect between NOD2 and TLR9 stimulation. Therefore, interactions of both receptors have implications for intestinal homeostasis and inflammation[18]. The TLR9 gene is located on chromosome 3p21.3, which is close to other CD susceptible loci[9]. There are four SNPs in TLR9, but two of them are sufficient to distinguish between the haplotypes, which are rs5743836 and rs352140.

There might be a synergistic effect of NOD2 and TLR9 stimulation, therefore, Torok and colleagues have tested for gene interactions between TLR9 and CD-associated variants of NOD2[18]. Significant associations between the two were observed that were specific to CD[18]. The controls and UC showed no difference in distribution of TLR9 polymorphisms and NOD2 variants[18]. Other CD variants in IL23R, ATG16L1 and IBD5 were analyzed for epistatic interactions. Aside from NOD2, the most significant association was found in the IL23R variant rs1004819, with ATG16L1 showing weaker associations with CD. There was no significant association between TLR9 polymorphisms with CD or UC phenotypes[18]. Along with previous studies, they also showed that NOD2 mutations were associated with younger age of diagnosis of CD, ileal disease, and need for surgery[18]. When there were two NOD2 mutations, there was a higher frequency of penetrating disease[18].

In conclusion, a new association between CD and a TLR9 polymorphism has been found. There is evidence that when CD patients carry CD-associated NOD2 variants, they have an increased incidence of TLR9 polymorphisms. This is not surprising because there is a synergistic effect of NOD2 and TLR9 stimulation and it is important for the maintenance of intestinal homeostasis and inflammation. TLR9 also demonstrates significant epistatic interactions with IL23R variants, but unlike NOD2, there is no association with the frequency in TLR9 polymorphisms present. This study shows the first evidence for interaction between polymorphisms in TLR9 and variants between NOD2 and IL23R[18].

CONCLUSION

CD is an autoimmune disease characteristic of chronic intestinal inflammation and lesions. It can affect people of all ages and ethnicities worldwide. Recent genome-wide studies have shown significant genetic associations of several variants and susceptibility to CD. This is true for North American, South American and European populations. Certain variants have been linked only to Asian and African cohorts. It is vital to understand the pathology of CD and the underlying genetic interactions to increase efficiency of diagnosis and develop drugs that target specific immune system pathways. There have been several genetic variants highly associated with CD, which include ATG16L1, TNFSF15, NOD2/CARD15, IL23R and IBD5 (Table 1).

The process of autophagy is an important aspect of our immune system. It is a way to destroy foreign pathogens that enter the body. The Thr300Ala variant within the N terminus of ATG16L, which is part of a complex that forms autophagosomes, has significant associations with CD. Different ethnicities show different genotype markers. This is seen in TNFSF15, which is involved in systemic inflammation and regulation of various immune cells. Seven haplotypes have been identified within a Japanese cohort, but not in other ethnic cohorts. More research needs to be conducted to characterize fully this possible genetic link to CD. The most significant association with CD is observed among NOD2/CARD15 and IL23R variants. NOD/CARD15 plays an essential role in maintaining the intestinal normal flora. There are many theories about the cause of CD, and one of them includes a shift in the intestinal bacterial flora. Therefore, it is no surprise that NOD2 variants are highly associated with CD. There are three independent variants associated with CD: rs2066844, rs2066845 and 1007fs. The other highly associated genetic link is observed within the IL23R gene. One variant that is highly associated with CD is rs1004819, whereas rs11209026 confers protection against CD. IL23R plays an essential role in

mediating proinflammatory activities. Another highly associated genetic link with CD is observed in two variants within IBD5. The two variants, SLC22A4 (OCTN1) and SLC22A5 (OCTN2), show significant association with CD, but no interactions between these variants and other CD-associated genetic variants have been observed. The last highly associated genetic link with CD is observed within the *CTLA4* gene. This gene plays a role in the immune system. The four polymorphisms associated with IBD are rs3087243, rs11571302, rs7565213 and rs11571297.

Many CD variants are associated with a specific CD phenotype, age of diagnosis, severity and location of CD, and surgical outcome. Gene-gene interactions have also been characterized among the different variants and other immune system receptors. We need to understand fully the pathogenesis of CD to target pathways for effective treatment. Further research is needed to explore all possible gene-gene interactions due to a gene dose affect associated with CD. It seems that new genetic associations are constantly being uncovered within IBD and then associated with either CD or UC. Overall, CD shows a greater genetic link than UC. Recent discoveries have led to therapies and treatments that show much promise. Researchers need to continue the search for genetic links and diseases because this may be the only way to understand the pathology of CD and develop effective treatments.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i5.425

World J Gastroenterol 2012 February 7; 18(5): 425-434
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Effect of *Helicobacter pylori cdrA* on interleukin-8 secretions and nuclear factor kappa B activation

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Supported by The Project Research Fund from Kochi University, to Takeuchi H; and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, No. 21590631 and 21590629, in part
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Received: April 15, 2011 Revised: May 19, 2011
Accepted: May 26, 2011
Published online: February 7, 2012

Abstract

AIM: To investigate genetic diversity of *Helicobacter pylori* (*H. pylori*) cell division-related gene A (*cdrA*) and its effect on the host response.

METHODS: Inactivation of *H. pylori cdrA*, which is involved in cell division and morphological elongation, has a role in chronic persistent infections. Genetic property of *H. pylori cdrA* was evaluated using polymerase chain reaction and sequencing in 128 (77 American and 51 Japanese) clinical isolates obtained from 48 and 51 patients, respectively. Enzyme-linked immunosorbent assay was performed to measure interleukin-8 (IL-8) secretion with gastric biopsy specimens obtained from American patients colonized with *cdrA*-positive or -negative strains and AGS cells co-cultured with wild-type HPK5 (*cdrA*-positive) or its derivative HPKT510 (*cdrA*-disruptant). Furthermore, the cytotoxin-associated gene A (*cagA*) status (translocation and phosphorylation) and kinetics of transcription factors [nuclear factor-kappa B (NF-κB) and inhibition kappa B] were investigated in AGS cells co-cultured with HPK5, HPKT510 and its derivative HPK5CA (*cagA*-disruptant) by western blotting analysis with immunoprecipitation.

RESULTS: Genetic diversity of the *H. pylori cdrA* gene demonstrated that the *cdrA* status segregated into two categories including four allele types, *cdrA*-positive (allele types; Ⅰ and Ⅱ) and *cdrA*-negative (allele types; Ⅲ and Ⅳ) categories, respectively. Almost all Japanese isolates were *cdrA*-positive (Ⅰ: 7.8% and Ⅱ: 90.2%), whereas 16.9% of American isolates were *cdrA*-positive (Ⅱ) and 83.1% were *cdrA*-negative (Ⅲ: 37.7% and Ⅳ: 45.5%), indicating extended diversity of *cdrA* in individual American isolates. Comparison of each isolate from different regions (antrum and corpus) in the stomach of 29 Americans revealed that *cdrA* status was identical in both isolates from different regions in 17 cases. However, 12 cases had a different *cdrA* allele and 6 of them exhibited a different *cdrA* category between two regions in the stomach. Furthermore, in 5 of the 6 cases possessing a different *cdrA* category, *cdrA*-negative isolate existed in the corpus, suggesting that *cdrA*-negative strain is more adaptable to colonization in the corpus. IL-8 secretions from AGS revealed that IL-8 levels induced by a *cdrA*-disrupted HPKT510 was significantly lower ($P < 0.01$) compared to wild-type HPK5: corresponding to 50%-60% of those of

wild-type HPK5. These data coincided with *in vivo* data that an average value of IL-8 in biopsy specimens from *cdrA*-positive and *cdrA*-negative groups was 215.6 and 135.9 pg/mL, respectively. Western blotting analysis documented that HPKT510 had no effect on CagA translocation and phosphorylation, however, nuclear accumulation of NF-κB was lower by HPKT510 compared to HPK5.

CONCLUSION: Colonization by a *cdrA*-negative or *cdrA*-dysfunctional strain resulted in decreased IL-8 production and repression of NF-κB, and hence, attenuate the host immunity leading to persistent infection.

Key words: *Helicobacter pylori* cell division-related gene A; Genetic diversity; Host immune response; Interleukin-8 secretion; Nuclear factor kappa B; Persistent infection

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Takeuchi H, Zhang YN, Israel DA, Peek Jr RM, Kamioka M, Yanai H, Morimoto N, Sugiura T. Effect of *Helicobacter pylori cdrA* on interleukin-8 secretions and nuclear factor kappa B activation. *World J Gastroenterol* 2012; 18(5): 425-434 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i5/425.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i5.425

INTRODUCTION

Helicobacter pylori (*H. pylori*), a Gram-negative spiral bacterium, colonizes the human stomach and causes chronic inflammation, which may progress to peptic ulceration, atrophic gastritis and gastric cancer[1]. Although the *H. pylori* population structure appears to be clonal over short periods of time, isolates obtained from different individuals exhibit substantial genetic diversity, consistent with extensive recombination and a panmictic population structure[2-7]. Putative mechanisms for the generation of diversity within *H. pylori* include frequent horizontal genetic exchange among strains and a high level of spontaneous mutation occurring over a long evolutionary time period within a highly restricted niche[6,8].

Infection by cytotoxin-associated gene A (*cagA*)-positive *H. pylori* is a known risk factor for the development of gastroduodenal disease due to major changes in cellular morphology and the release of molecules, including cytokines, from the gastric epithelium. *H. pylori* infection up-regulates secretion of various inflammatory cytokines, including interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor α, and contributes to the pronounced inflammatory response[9]. IL-8 production induced by *H. pylori in vitro* and *in vivo* is recognized as a host response to microbes[10,11], but the role of CagA on IL-8 production is less clear[12]. IL-8 is a potent pro-inflammatory cytokine which regulates neutrophil infiltration of gastric mucosa in *H. pylori* gastritis and is released through the signaling pathway concerning nuclear factor-kappa B (NF-κB), which includes extracellular signal-regulated kinase (ERK) activity[12,13] and mitogen-activated protein kinase[14]. Among the strongest transcriptional regulators of *H. pylori*-induced cytokine expression is the NF-κB family of transcription factors[15]. Activation of NF-κB can affect the expression of several hundred genes, and activation of its signal transduction pathway occurs in response to a wide range of stimuli and results in nuclear accumulation of NF-κB transcription factors, causing changes in the expression of target genes involved in innate and adaptive immunity, and inflammation.

We described previously that the *H. pylori* cell division-related gene A (*cdrA*) not only has a repressive role on cell division but is also involved in cell elongation and cell death *via* cell wall synthesis at the division site[16,17]. The *cdrA*-disrupted mutant HPKT510 was able to survive for the long-term in liquid medium, even under serum-free and aerobic conditions, and was more resistant to bactericidal of beta-lactam antibiotics than the wild-type HPK5[16]. Inactivation of *cdrA* during infection in the stomach may have contributed to ensuring persistent infection by altering its ability to adapt to the microenvironment. In fact, the *cdrA* gene was found to be absent in 3 out of 4 colonies recovered from a mouse infected with *H. pylori* strain B128[18]. Furthermore, additional isolates of the sequenced *H. pylori* strain J99 from a patient after a 6-year interval were subjected to microarray analysis, which indicated that the *cdrA* gene was missing in additional isolates[19]. Thus, we hypothesize that a loss of *cdrA* in *H. pylori* during infection might be an evolutionary event to alter its biological characteristics, which affects the host immune response to the microbes and promotes persistent infection.

In this study, the level of IL-8 secretion induced by a *cdrA*-negative strain was approximately 50% lower than those induced by a *cdrA*-positive strain *in vitro* and *in vivo*. Genetic diversity of *cdrA* was extended in American isolates and *cdrA*-negative isolates might be more adaptable to colonize in the corpus. Western blotting analysis documented that the CagA status at the point of its translocation and phosphorylation was not different between HPK5 and HPKT510 strains. However, expression of NF-κB was lower in HPKT510 than that in HPK5, indicating that *H. pylori* with inactivation of *cdrA* might escape from rigorous immune clearance and facilitate chronic persistent infection caused by decreased levels of IL-8 and nuclear accumulation of NF-κB.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Wild-type *H. pylori* HPK5 and its derivatives such as HPKT510, a *cdrA*-disrupted mutant carrying *xylE-Km* cassette[17], and HPK5CA, a *cagA*-disrupted mutant carrying *xylE-Km* cassette[20], were cultivated in Brucella broth (Becton Dickinson, United States) supplemented with 10% horse serum at 37 ℃ in an atmosphere containing 10% CO_2 as previously described[21]. Bacteria grown in 10 mL of Brucella-serum medium in 100-mL conical flasks following sub-culture were subjected to co-culture with AGS cells. Bacterial growth was measured by determining absorbance at 600 nm (A_{600}) with a spectrophotometer (GENEQUANT pro, Amersham Pharmacia Biotech), and colony forming units were determined for bacterial viability. Additional clinical isolates, 77 American and 51 Japanese isolates endoscopically obtained from 48 and 51 patients, respectively, were cultured on Brucella-serum agar at 37 ℃ in an atmosphere containing 10% CO_2. Isolates from different regions in the stomach, such as the antrum and corpus, were also included in 29 of 77 American isolates. The bacterial genomic DNA extracted by a DNA kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions were subjected to polymerase chain reaction (PCR) with specific primer pairs to examine *cdrA* gene diversity.

AGS cell culture conditions

AGS cells (human gastric adenocarcinoma epithelial cell line, CRL1739c) were grown in RPMI 1640 + 7 medium including streptomycin (20 μg/mL) and kanamycin (60 μg/mL) (Nikken bio medical Laboratory, Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Biowest, France) at 37 ℃ in an atmosphere containing 5% CO_2. AGS cells were seeded at a density of 0.5 × 10^6 cells in six-well plates (SUMITOMO BAKELIFE, Japan) and grown to about 80% confluence prior to co-culture experiments.

Co-culture of AGS cells with H. pylori strains

Subconfluent AGS cells (1 × 10^6) cultured in RPMI 1640 + 7 medium supplemented with 10% serum were washed twice by phosphate-buffered saline (PBS) and subsequently cultured with or without each *H. pylori* strain (HPK5, HPKT510 and HPK5CA) at a multiplicity of infection (MOI) of 150 or 300 in 1.5 mL of RPMI 1640 medium (Gibco BRL, Eggenstein, Germany) alone for 48 h at 37 ℃ in an atmosphere containing 10% CO_2. The supernatant was collected at various times (3-48 h), centrifuged at 7000 r/min for 5 min to pellet bacteria and AGS cells, and subjected to sandwich enzyme-linked immunosorbent assay (ELISA) to measure IL-8 productions. AGS cells after co-culture were collected at the appropriate time and used in the following analyses.

Preparation of whole-cell and nuclear extracts

AGS cells were washed five times in ice-cold PBS, incubated with lysis buffer containing 50 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L NaF, 100 μmol/L sodium orthovanadate, 10 μmol/L phenylmethylsulfonyl fluoride (PMSF) and a commercially available protease inhibitor mixture tablet (Complete, Roche Molecular biochemical, Indianapolis, IN), harvested by scraping and transferred to a microcentrifuge tube. Debris was removed by centrifugation at 10 000 *g* for 10 min (TOMY MRX-150) to collect the total proteins for whole-cell extracts. Nuclear proteins were prepared by Cellytic Nu-CLEAR Extraction Kit (SIGMA, Japan) according to a previous report[22]. Briefly, AGS cells were incubated with lysis buffer (10 mmol/L Hepes, pH 7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 1 mmol/L DTT and 0.25 mmol/L PMSF) on ice for 15 min. The pellet precipitated containing nuclei following centrifugation for 30 s at 10 000 *g* was resuspended in 100 μL extraction buffer (20 mmol/L Hepes, pH 7.9, 1.5 mmol/L $MgCl_2$, 0.42 mol/L NaCl, 0.2 mmol/L EDTA, 25% glycerol, 1 mmol/L DTT and 0.25 mmol/L PMSF) for 30 min at 4 ℃ with agitation. After centrifuging for 5 min at 20 000 *g*, the supernatant (nuclear fraction) was collected for nuclear extracts.

Immunoprecipitation

One milligram of the lysate proteins was incubated with polyclonal rabbit anti-*H. pylori* CagA antibody (Austral Biologicals, CA, United States) for 1 h at 4 ℃, followed by an overnight incubation with a 20 μL aliquot of Protein G Plus-agarose beads (Santa Cruz, United States) at 4 ℃, as previously described[20]. Briefly, the beads were washed with lysis buffer and boiled for 10 min in 2 × electrophoresis sample buffer (50 mmol/L Tris (pH 6.8), 10% sodium dodecyl sulfate, 12% 2-mercaptoethanol, 20% (wt/vol) glycine and 1% (v/v) bromophenol blue) to elute the immunoprecipitated proteins.

Western blotting analysis

For detection of CagA and its phosphorylation, equivalent amounts of the immunoprecipitated proteins (100 μg) were resolved by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrotransferred onto nitrocellulose membranes (Millipore Corporation, United States) by a semidry blotting apparatus KS-8460 (System Instruments Co., Ltd. Tokyo). The blots were blocked overnight with 4% (wt/vol) dried skim milk in Tris-buffered saline with Tween 20 at room temperature and then incubated with monoclonal antibody PY99 (diluted 1/1000; Santa Cruz, United States) for 1 h and anti-mouse IgG peroxidase-linked species-specific whole secondary antibody (diluted 1/1000; GE Healthcare Biosciences, Co. Ltd., United Kingdom) for 1 h to detect phosphorylated tyrosine protein. Immunodetection was performed by enhanced chemiluminescence Plus Western Blotting Detection Reagents (GE Healthcare Biosciences). Next, blots were stripped, reprobed with a specific polyclonal rabbit anti-*H. pylori* CagA antibody (diluted 1/1000; Austral Biologicals. CA, United States) and incubated with a horseradish perox-

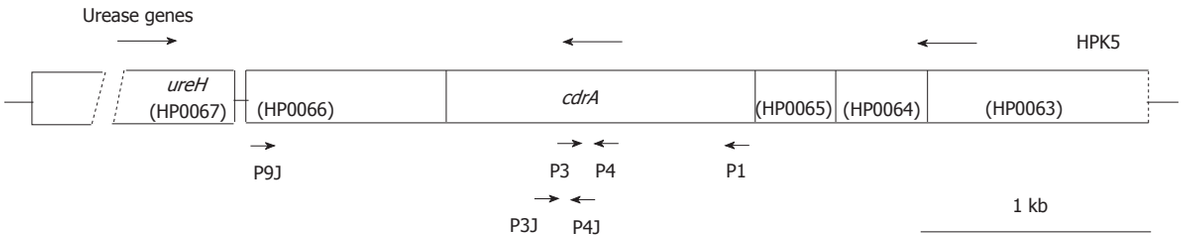

Figure 1 Location of primers used for cell division-related gene A in the region downstream of the urease gene cluster of *Helicobacter pylori* HPK5. Arrows above and below the map depict the direction of transcription and primers, respectively. The open reading frames (HP0063 to HP0067) are shown based on strain 26695.

idase-conjugated goat anti-rabbit secondary antibody (diluted 1/10 000; Jackson ImmunoResearch, PA, United States)[20]. Each 10 μg of nuclear and total proteins was subjected to 12% SDS-PAGE for detection of NF-κB and inhibition kappa B (IκB), respectively. Procedures of western blot analysis were followed as described above. Rabbit anti-human-NF-κB (p65) (diluted 1/200; Cell Signaling Technology, United States) and rabbit anti-human-IκB antibodies (diluted 1/200; Santa Cruz Biotechnology Inc, United States), and an anti-rabbit IgG antibody-HRP (diluted 1/2000; Santa Cruz Biotechnology Inc, United States) were used as primary and secondary antibodies, respectively. For a standard control, a rabbit anti-human-ERK2 antibody (diluted 1/200; Santa Cruz Biotechnology Inc, United States) was used to detect unphosphorylated ERK2 to confirm equal protein load[23]. The analyses were performed for least three independent experiments.

Measurement of IL-8 secretion from AGS cells

The amount of IL-8 secreted into culture medium after co-culture with *H. pylori* strains was determined by ELISA using the CytoSets system (BioSource International) according to the manufacturer's instructions. Fifty μL aliquots of the supernatant was briefly centrifuged at 7000 r/min for 10 min to remove bacteria and AGS cells, after which supernatants were added to an equal volume of regent on the 96-well ELISA plate. The absorbance of samples was measured at 550 nm using a 96-well microplate reader (Multiskan JX ver1.1, Thermo Labsystems, Finland), and the data was expressed as pg/mL. All samples were measured in triplicate in at least three independent experiments.

Measurement of mucosal IL-8 secretion

Frozen gastric antral biopsy specimens from 25 clinical patients infected with *H. pylori* were homogenized in 1 mL of PBS, and supernatants obtained by centrifugation were used for determination of IL-8 proteins by ELISA, as described previously[24]. Cytokine concentrations in homogenates were normalized in terms of total protein concentration of the biopsy specimen and were expressed as pg/mL.

PCR for cdrA gene diversity

To compare the *in vivo* IL-8 level in the biopsy specimens between the patients infected with *cdrA*-positive or -negative strains, PCR was utilized to examine the *cdrA* status in clinical isolates. The genomic DNA of clinical isolates, including 77 American and 51 Japanese strains, were subjected to PCR with specific primers for the *cdrA* gene of the HPK5 strain such as P1 (forward), P3 (reverse) and P4 (forward), previously described[17]. Additional new primers, P9J (reverse), P3J (reverse) and P4J (forward), based on the sequence of the *cdrA* gene of J99 strains, were also utilized in this study. The sequence of primer P1 was identical in strains, HPK5 and J99. The conditions used for PCR to amplify the 194-bp or 336-bp products in the central region of *cdrA*, which is highly conserved among strains using P3-P4 or P3J-P4J primer pairs, respectively, were as follows: pre-heat for 2 min at 96 ℃, followed by 40 cycles of 96 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 30 s. To amplify the N-terminal region of *cdrA* using P1-P3 or P1-P3J primer pairs, PCR was performed with the following conditions: pre-heat for 2 min at 96 ℃, followed by 40 cycles of 96 ℃ for 30 s, 52 ℃ for 30 s, and 72 ℃ for 2.5 min. PCR was carried out at least two times with all sets of primer pairs, P1-P3, P1-P3J, P3-P4 and P3J-P4J, for determining the standing of the *cdrA* gene. In particular, for the strains representing no PCR product using various primer pairs mentioned above, P9J-P4 or P9J-P4J primer pairs were used to confirm the existence of the region from central to C-terminal on *cdrA* corresponding to flanking the downstream region of urease gene cluster. The PCR conditions were as follows: pre-heat for 2 min at 96 ℃, followed by 40 cycles of 96 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 1 min. The location of these primers used for *cdrA* amplification (Figure 1) and primer sequences are shown (Table 1).

Statistical analysis

Fischer's exact test and the χ^2 test with Yates' continuity correction were applied using SPSS version 10.0 for Windows to compare differences on the level of IL-8 productions induced by *cdrA*-positive and -negative *H. pylori* strains. P value of < 0.05 were considered significant.

RESULTS

IL-8 secretion from AGS cells

To determine the effect of host response to *H. pylori*

Table 1 The sequence of primers used and the target region of cell division-related gene A amplified with combination of primers

Primers	Sequence (5′-3′)	Reference	Combination of primers	Target region of cell division-related gene A
P1 (forward)	TGAAGCACACGAAAGGA	17	P1-P3	N-terminal to central
P3 (reverse)	CATGCTCTAAAATCGTCG	17	P1-P3J	N-terminal to central
P3J (reverse)	ATCACCACGATCAGTCTG	This study	P3-P4	Central
P4 (forward)	TTTTGAGATCGGTGAAGC	17	P3J-P4J	Central
P4J (forward)	AACCACACGCTCATTTGC	This study	P4-P9J	Central to C-terminal
P9J (reverse)	GCTGAAAGGCCTGAATTCAG	This study	P4J-P9J	Central to C-terminal

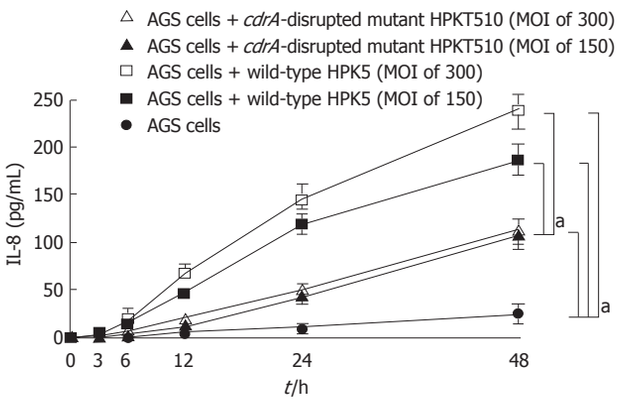

Figure 2 Interleukin-8 production from AGS cells induced by either wild-type or cell division-related gene A-disrupted mutant strains. [a]$P < 0.01$.

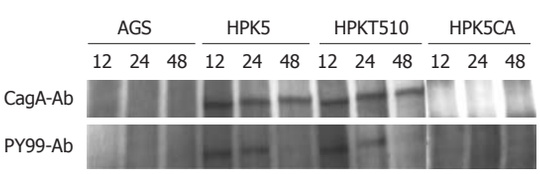

Figure 3 Cytotoxin-associated gene A status in AGS cells co-cultured with or without *Helicobacter pylori* strains. The immunoprecipitated proteins with anti-*Helicobacter pylori* (*H. pylori*) cytotoxin-associated gene A (CagA) antibody (CagA-Ab) were subjected to Western blotting with CagA-Ab (upper) and PY-99 antibody (PY99-Ab) (bottom), respectively. The assay was carried out with strains at MOI of 150. HPK5: Wild-type; HPKT510: Cell division-related gene A-disrupted mutant; HPK5CA: *cagA*-disrupted mutant.

during persistent infection in the human stomach, *in vitro* levels of IL-8 production from AGS cells co-cultured with HPK5 or HPKT510 strains were examined relative to time (Figure 2). Both strains induced the secretion of IL-8 from AGS cells and the levels increased time-dependently, but HPKT510, the *cdrA*-disrupted mutant, showed significantly lower levels, corresponding to 50%-60% of levels induced by wild-type HPK5 at MOI of 150 ($P < 0.01$). Furthermore, the levels of IL-8 were increased dose (MOI)-dependently by HPK5, but not HPKT510, which showed identical levels between MOI of 150 and 300.

Western blotting for CagA

Ultrastructural analyses revealed that the HPKT510 strain had a slightly wider periplasmic space between the inner and the outer membrane than that of HPK5[16]. To determine whether such morphological difference affects translocation of CagA into AGS *via* type Ⅳ secretory component system (T4SS) and its phosphorylation, which are considered to be risk factor for development of gastroduodenal disease, western blotting following immunoprecipitation with anti-*H. pylori* CagA antibody was carried out. CagA was detected in AGS cells co-cultured with HPK5 or HPKT510 strains, but no CagA was observed in AGS alone or when co-cultured with a *cagA*-disrupted mutant, HPK5CA as a negative control (Figure 3). The intensities of each CagA band are similar between both strains. Phosphorylation of CagA was detected up to 24 h after co-culture with HPK5 or HPKT510 strains and had similar intensity, indicating that there were no differences in the status of CagA between both strains.

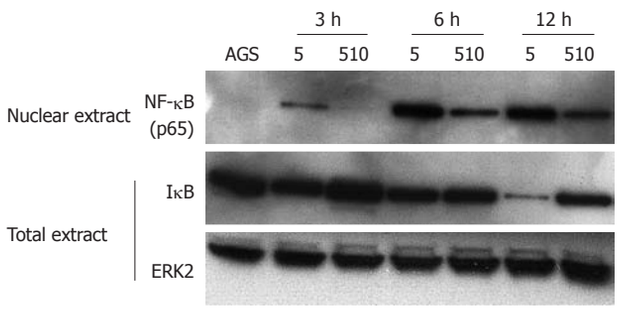

Figure 4 Detection of nuclear factor kappa B (p65) (upper), inhibition kappa B (middle) and extracellular signal-regulated kinase 2 (bottom) in nuclear and total extracts, respectively at 3, 6 and 12 h after being co-cultured with *Helicobacter pylori*. Unphosphorylated extracellular signal–regulated kinase 2 (ERK2) was detected as the control in this study[23]. Molecular weights of nuclear factor kappa B (NF-κB), inhibition kappa B (IκB) and ERK2 are 65, 35-37 and 42 kDa, respectively. The assay was carried out with strains at a MOI of 150. AGS: AGS cells co-cultured without *H. pylori*; HPK5: AGS cells co-cultured with wild-type HPK5; HPKT510: AGS cells co-cultured with *cdrA*-disrupted mutant HPKT510.

Western blotting for NF-κB (p65) and IκB

To determine how the expression level of NF-κB transcription factor affects the expression of many genes involved in inflammation and immunities, western blotting was carried out utilizing AGS cells co-cultured with HPK5 or HPKT510 strains for 3, 6 and 12 h. Overall, NF-κB levels in the nuclear extracts from AGS cells with HPKT510 were definitely lower compared to those with HPK5, whereas IκB levels in total extracts were found to be higher in HPKT510 (Figure 4), indicating that activation of NF-κB was relatively repressed in AGS cells stimulated by *H. pylori* with disruption of *cdrA*.

Table 2 ***Helicobacter pylori*** **cell division-related gene A status (positive or negative) and genotype (allele type) in clinical isolates *n* (%)**

H. pylori cdrA			
Status	**Genotype**	**United States (48 patients)[1]**	**Japan (51 patients)**
Positive	Type I	0 (0)	4 (7.8)
	Type Ⅱ	13 (16.9)	46 (90.2)
Negative	Type Ⅲ	29 (37.7)	1 (2.0)
	Type Ⅳ	35 (45.4)	0 (0)
Total		77[2]	51

H. pylori cdrA: *Helicobacter pylori* cell division-related gene A. [1]In 29 out of 48 patients, each isolate was obtained from both antrum and corpus in same stomach; [2]The total 77 isolates include 44 and 33 isolates obtained from antrum and corpus, respectively.

Table 3 Comparison of cell division-related gene A status and genotype (allete type) between antrum and corpus isolates in 29 American patients

	***cdrA* status[1]**		***cdrA* genetype (allele type)**		
cdrA positive (6 cases)	Same[2]: 1	Antrum (+)[3] Corpus (+)	Identical[4]: 1	Antrum Type Ⅱ	Corpus Type Ⅱ
	Difference: 5	Antrum (+) Corpus (-)	Difference: 5	**4 Type** Ⅱ	**Type** Ⅲ
				1 Type Ⅱ	**Type** Ⅳ
cdrA negative (23 cases)	Same: 22	Antrum (-) Corpus (-)	Identical: 16	5 Type Ⅲ	Type Ⅲ
				11 Type Ⅳ	Type Ⅳ
	Difference: 1	Antrum (-) Corpus (+)	Difference: 7	4 Type Ⅲ	Type Ⅳ
				2 Type Ⅳ	Type Ⅲ
				1 Type Ⅳ	**Type** Ⅱ
Total (%)	Same: 23 (79)		Same: 17 (59)		
	Difference: 6 (21)		Difference: 12 (41)		

[1]The status of cell division-related gene A (*cdrA*) determined with isolate from antrum was divided to two category groups (*cdrA* positive and negative); [2]Same or difference: *cdrA* status is same or difference between isolate (antrum and corpus); [3]+: *cdrA* positive isolate; -: *cdrA* negative isolate; [4]Identical or difference: the genotypes of *cdrA* is identical or difference between isolate (antrum and corpus). Bold denotes the 6 cases representing differences at *cdrA* status and genotype between isolates (antrum and corpus).

Genetic diversity of H. pylori cdrA

To examine the *cdrA* status [category such as *cdrA*-positive and -negative, including 4 allele types (Ⅰ to Ⅳ)] in 128 clinical isolates, PCR was performed with appropriate specific primer pairs. In a previous report, PCR with P1-P3 yielded the 731 bp (short fragment) and approximately 2 kb (long fragment) in HPK5 and other three strains examined respectively, demonstrating that the shorter product of HPK5 was due to partial deletion in the N-terminal region of *cdrA*. However, *cdrA* was functionally involved in cell division, morphology, long-term survival and susceptibilities to beta-lactam and its transcript could be detected[16,17]. On the other hand, the 194-bp amplified in the central region of *cdrA* was detected in all strains, indicating that size diversity exists within the N-terminal region of *cdrA* among individual strains[17]. In this study, 128 clinical isolates (77 American and 51 Japanese) were subjected to PCR with N-terminal primer pairs (P1-P3 and P1-P3J) which demonstrated that the short fragment (allele type Ⅰ) was obtained in only 4 Japanese isolates (7.8%) including HPK5 and the long fragment (allele type Ⅱ) was detected in 13 American (16.9%) and 46 Japanese (90.2%) isolates. In addition, no product was observed in the 64 American (83.1%) and 1 Japanese (2.0%) isolates by PCR with central region primer pairs (P3-P4 and P3J-P4J). Of the 64 American isolates showing no amplification on the central region, 35 isolates have no product amplified on the C-terminal region with P9J-P4J and P9J-P4 pairs. The results revealed that the downstream region of the urease gene cluster, including *cdrA,* was absent in the 35 isolates (allele type Ⅳ). However, PCR product on the C-terminal region with P9J-P4J or P9J-P4 pairs was detected in remaining 29 isolates, demonstrating that they have partial *cdrA* sequence loss between the N-terminal and central regions (allele type Ⅲ). Taken together, the status of the *cdrA* gene among individual isolates examined was divided into two categories; *cdrA*-positive including allele types Ⅰ (HPK5) and Ⅱ (J99 and 26695), and *cdrA*-negative including allele types Ⅲ and Ⅳ (Table 2).

Comparison of cdrA status between antrum and corpus

The *cdrA* allele type of each isolate from the antrum and corpus in the stomach of 29 American patients demonstrated that *cdrA* allele between the regions was identical in 17 patients (59%); however, 9 of the remaining 12 patients possessing a different allele had a isolate with more loss of the *cdrA* sequence in the corpus compared to the antrum (Table 3). Regarding the status of *cdrA*, 6 out of 29 patients had different *cdrA* status between the regions, and of which, 5 patients harbored *cdrA*-positive and -negative isolates in the antrum and corpus, respectively (Table 3).

Measurement of mucosal IL-8 secretion

The *cdrA*-disrupted mutant HPKT510 showed significantly lower levels of IL-8 secretion *in vitro* compared to wild-type HPK5. To investigate whether *cdrA* is associated with IL-8 production *in vivo*, mucosal IL-8 secretion of biopsy specimens was measured using samples obtained from 20 American patients infected with *cdrA*-negative and 5 American patients with *cdrA*-positive *H. pylori*. Measurement of IL-8 in these specimens demonstrated that the *cdrA*-negative group exhibited lower IL-8 levels than those of *cdrA*-positive group. The average value of IL-8 in the *cdrA*-negative group was 111 pg/mL, corresponding to the average of 60% in the *cdrA*-positive group (156 pg/mL). This tendency was consisted with *in vitro* data, revealing that *cdrA*-negative *H. pylori* does not induce IL-8 secretion as strongly in the human stomach as *cdrA*-positive strains.

DISCUSSION

H. pylori colonizes more than half of the world's population. While it is clear that this organism induces a strong innate and adaptive immune response leading to active inflammation in the gastric mucosa, the ability of *H. pylori* to establish persistent infection so efficiently has not been fully elucidated. *H. pylori* possesses a number

of virulence factors, and some, such as urease, flagella and lipopolysaccharide (LPS), contribute to its persistence[25-27], whereas others, such as CagA and vacuolating cytotoxin, appear to confer increased virulence[28,29]. Furthermore, the strain-specific genes found outside of the *cag* pathogenicity island, especially genes in the plasticity regions[30] and genes with variable structures/genotypes, have considerable interest in their contribution to pathogenesis and the host immune response. Nearly half of the strain-specific genes of *H. pylori* are located in the plasticity regions in strains 26695[31] and J99[30,32]. There is evidence that genetic recombination among *H. pylori* may occur during the course of infection in the stomach. The diversity or phase variation in *H. pylori* genes such as *babA*[33] and *fucT1*[26] likely contributes to the evasion of the host immune response and thereby has a role in the establishment of persistent infection in the stomach. Based on the genomic information of the sequenced *H. pylori* strains 26695[31] and J99[32], more than a third of *H. pylori* genes have unknown function, suggesting that there may be strain-specific genes such as *ctkA*[34] involved in the mechanisms of persistence as well as the host immune response.

One of unique genes found in the HPK5 strain, *cdrA* located in the downstream region of urease gene cluster, has not only a inhibitory role for cell division, but is also involved in elongation and death of cells *via* cell wall synthesis at the site of division[16,17]. Furthermore, the *cdrA*-disrupted mutant HPKT510 had a longer survival time compared to the wild-type HPK5 in both liquid and solid media, as well as in serum-free medium and aerobic conditions[16]. Loss of the *cdrA* gene during infection is frequently found in a mouse infected with *H. pylori* strain B128[18] and in the human stomach infected with J99[19]. The present study found that *cdrA*-negative strains resulted in lower levels of IL-8 production *in vitro* and *in vivo* compared to *cdrA*-positive strains. *In vitro* data showed that increased IL-8 production was dose-dependently observed by *cdrA*-positive HPK5, but not *cdrA*-disrupted HPKT510. In addition, nuclear accumulation of NF-κB in AGS co-cultured with HPKT510 was lower compared to HPK5, suggesting that activation of the NF-κB signaling pathway was relatively repressed by stimulation of the *cdrA*-negative strain, which coincided with decreased IL-8 production. These indicated that *cdrA*-defective *H. pylori* may evade immune clearance due to limiting bactericidal effects of pro-inflammatory molecules leading to promotion for persistent infection, likely *via* repression of the NF-κB signaling pathway. Persistent infection was not observed in IL-10-deficient mice with strong inflammation, implying that attenuate host immunity is necessary for *H. pylori* colonization[35]. Accordingly, it is acceptable that *H. pylori* infection occurred in the stomach with immature immunity during infantile generation. The proper NF-κB transcriptional response is primarily regulated by post-translational modification of NF-κB signaling components. On the other hand, alternative splicing of NF-κB signaling components is another way to control the NF-κB signaling pathway[36,37]. Alternative splicing of some NF-κB signaling components can be induced by prolonged exposure to a NF-κB-activating signal, such as LPS, suggesting a mechanism for negative feedback to dampen excessive NF-κB signaling. In particular, alternative splicing events in Toll/interleukin-1 NF-κB signaling pathways *via* Toll like receptors can inhibit the NF-κB response[38]. This raises the possibility that changes in bacterial constituents concerned with disruption of *cdrA*, such as cell envelope components including LPS, may exert on the complicated NF-κB signaling pathway.

As HPKT510 had a slightly wider periplasmic space[16], the effector protein, CagA, was investigated to determine whether this morphological change altered the function of T4SS. The CagA production, its translocation and phosphorylation were indistinguishable between HPK5 and HPKT510 strains, indicating that such morphological differences related with inactivation of *cdrA* had no effect on CagA-associated pathogenicity, including CagA-related signaling pathway and T4SS. Cytoplasmic nucleotide-binding oligomerization domain (Nod) molecules, Nod1 and Nod2, have been shown to be specific ligands of diaminopimelic acid-containing muropeptides and muramyl dipeptide, respectively[39,40], and were important components of the innate immune response. A peptidoglycan-derived muropeptide possessing a Nod1 motif was translocated through T4SS and affected cell signaling *via* activation of NF-κB in the host cell, leading to the stimulation of an intracellular pro-inflammatory response[41]. In terms of conserved domains, part of HPK5 *cdrA* belongs to the SM1-NRK4 family, beta glucan and cell wall synthesis enzyme family, and another belongs to the SpoIIE-FtsK-ATPase family. The properties of penicillin-binding proteins such as PBP1, PBP2, PBP3 and PBP4 varied between HPK5 and HPKT510 strains[16], suggesting that an alteration of such peptidoglycans influences the level of IL-8 production *via* the Nod1-NF-κB signaling pathway. In fact, the measurement of *nod1* transcript level in AGS by real-time RT-PCR documented that HPKT510 induced lower level of *nod1* transcript compared to HPK5 (data not shown), which supports the Nod1-NF-κB pathway involved in the difference of IL-8 production.

The *cdrA* status in almost all Japanese isolates were *cdrA*-positive (98%), but not in isolates obtained from Americans, demonstrating that American isolates had a greater diversity and higher prevalence of *cdrA*-negative strains (83.1%). Evaluation of *cdrA* status in isolates obtained from different regions of the stomach revealed that in 5 of the 6 cases possessing different status between the antrum and corpus of the stomach, the *cdrA*-negative isolate was from the corpus, suggesting that a *cdrA*-negative strain might be more adaptable to colonize in the corpus over a longer time period than the *cdrA*-positive strain. A *cdrA*-disrupted mutant could survive

longer under the stresses, such as presence of beta-lactam antibiotics, serum-free and aerobic conditions, than the wild-type strain[16]. It is possible that changes in biological behavior accompanied by inactivation of *cdrA* are necessary to stay balanced for the establishment of long-term infection in the human stomach. When the HPK5 *cdrA* sequence was compared to reference strain 26695[31], the *cdrA* sequence was a fusion of the ~130 codon hp0064 gene to the middle ~one third of the hp0066 gene, with deletion of the interstitial hp0065 and first part of hp0066. A similar fusion is evident with respect to the reference strain J99 (jhp0059 and jhp0061) or HPAG1 (hpag1_0064 and hpag1_0067)[42]. These two reference strains might represent the majority class in the Japanese population, which gave 2 kb instead of 730 bp PCR products. In contrast, two other sequenced strains, Shi470[43] and G27[44], seem to contain only the ~130 codon ORF, not the longer one, and thus might possibly represent one of the major classes in the United States population. The HPK5 type *cdrA* stems from gene fusion and synthesizes a partially defective protein that impacts cell wall synthesis or structure and a greater release of peptidoglycan fragments, which can be proinflammatory through the Nod1-NF-κB pathway. More studies are required in the future to dissect the mechanistic role of *cdrA* and the alteration of comprehensive genomix/proteomix affected by inactivation of *cdrA*. Analysis for putative deletion alleles might actually provide us with insights to elucidate whether the phenomenon resulted from such sources as large insertions or genome rearrangements.

In histological observation, as far the confined tissue sections of small biopsy specimens were examined, no significant finding was observed in the gastric mucosa colonized with either *cdrA*-positive or *cdrA*-negative *H. pylori* strains. We may need more examinations in detail with extended sections and specimens to confirm the differences in histological findings, including inflammation with neutrophil infiltration.

We concluded that the presence of *H. pylori cdrA* was associated with effective production of pro-inflammatory cytokine, IL-8, both *in vitro* and *in vivo*. Therefore, *cdrA*-inactivated *H. pylori* strains may result in attenuated host immunity and evade immune clearance due to repression of the NF-κB signaling pathway in the host cell, leading to persistent infection. Finally, our studies suggest that *cdrA*-negative *H. pylori* strains are more likely to colonize in the corpus over long time periods compared with *cdrA*-positive strains.

COMMENTS

Background

Helicobacter pylori (H. pylori) infection up-regulates secretion of various inflammatory cytokines including interleukin-8 (IL-8), whose production *in vitro* and *in vivo* is recognized as a host response to microbes. The levels of IL-8 production differ among individual strains, suggesting the existence of unique genes involved in host response and genetic diversity during infection in the stomach. Furthermore, the effect of fundamental constituents belonging to cell division of *H. pylori* on host response is unclear.

Research frontiers

H. pylori infection and its persistence in the stomach is the most important event to actually lead a variety of diseases. However, the functions and practices of the cell division of strains colonized in an individual stomach remain unclear. Furthermore, how the bacterial behaviors concerned with the host response is still unknown. In this study, the authors demonstrate that *H. pylori* cell division-related gene A (*cdrA*) required for cell division and morphological shape could be a potential role for mediating IL-8 secretion.

Innovations and breakthroughs

Reports have highlighted the importance of *H. pylori*-host interaction to understand host immunity and pathogenesis. Certain molecules, such as cytotoxin-associated gene A (*CagA*) and outer membrane proteins, were shown to be involved in these phenomena; however, the relationship between cell division- and morphological shape-related molecules and host response associated with persistent infection in the stomach is unknown. This is the first study to report that *cdrA* influenced IL-8 secretion (*vivo* and *vitro*) and loss of *cdrA* may attenuate the host immunity due to repression of NF-κB, leading to persistence.

Applications

In this study, how *H. pylori cdrA* functions and influences bacterium-host interaction was investigated, which provides insights into understanding bacterial fundamental components and their effect on the stomach, including host response, and a future strategy for therapeutic intervention in the treatment of patients with *H. pylori* infection.

Terminology

H. pylori cdrA, one of the unique genes discovered in strain HPK5, has not only a repressive role on cell division but is also involved in cell elongation and cell death *via* cell wall synthesis (mainly penicillin binding proteins) at the division site. The *cdrA*-disrupted mutant is able to survive for long time periods and is more resistant to the bactericidal of beta-lactam antibiotics than the wild-type. The *cdrA* gene tends to be lost during infection to facilitate adaption in the stomach, resulting in a persistent infection.

Peer review

This interesting manuscript showed that the presence of *H. pylori cdrA* was associated with the effective production of IL-8 compared to the inactivation of *cdrA in vitro* and *in vivo*. Additionally, they observed that *cdrA*-inactivated *H. pylori* strains may result in attenuated host immunity and evade immune clearance due to repression of the NF-κB pathway, leading to persistent infection.

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S- Editor Tian L **L- Editor** Rutherford A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i5.435

World J Gastroenterol 2012 February 7; 18(5): 435-444
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

A *Macaca mulatta* model of fulminant hepatic failure

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Supported by National Basic Research Program of China, No. 2009CB522401; and grand from Natural Science Foundation of China, No. 30870983 and 30971118
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Received: May 19, 2011 Revised: June 23, 2011
Accepted: June 30, 2011
Published online: February 7, 2012

Abstract

AIM: To establish an appropriate primate model of fulminant hepatic failure (FHF).

METHODS: We have, for the first time, established a large animal model of FHF in *Macaca mulatta* by intraperitoneal infusion of amatoxin and endotoxin. Clinical features, biochemical indexes, histopathology and iconography were examined to dynamically investigate the progress and outcome of the animal model.

RESULTS: Our results showed that the enzymes and serum bilirubin were markedly increased and the enzyme-bilirubin segregation emerged 36 h after toxin administration. Coagulation activity was significantly decreased. Gradually deteriorated parenchymal abnormality was detected by magnetic resonance imaging (MRI) and ultrasonography at 48 h. The liver biopsy showed marked hepatocyte steatosis and massive parenchymal necrosis at 36 h and 49 h, respectively. The autopsy showed typical yellow atrophy of the liver. Hepatic encephalopathy of the models was also confirmed by hepatic coma, MRI and pathological changes of cerebral edema. The lethal effects of the extrahepatic organ dysfunction were ruled out by their biochemical indices, imaging and histopathology.

CONCLUSION: We have established an appropriate large primate model of FHF, which is closely similar to clinic cases, and can be used for investigation of the mechanism of FHF and for evaluation of potential medical therapies.

Key words: Fulminant hepatic failure; *Macaca mulatta*; Biochemistry; Imaging; Pathology

Peer reviewer: Dr. Yasuji Arase, Department of Gastroenterology, Toranomon Hospital, 2-2-2 Toranomon Minato-ku, Tokyo 105-8470, Japan

Zhou P, Xia J, Guo G, Huang ZX, Lu Q, Li L, Li HX, Shi YJ, Bu H. A *Macaca mulatta* model of fulminant hepatic failure. *World J Gastroenterol* 2012; 18(5): 435-444 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i5/435.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i5.435

INTRODUCTION

Fulminant hepatic failure (FHF) is an uncommon and challenging clinical disease characterized by sudden and severe hepatic injury and dysfunction, with many different symptoms and complications, and high mortality. The term was first used in 1970 to describe a potentially reversible disorder that was the result of severe liver injury in the absence of pre-existing liver disease, with an onset of encephalopathy within 8 wk of symptom appearance[1]. The incidence and etiology of FHF vary according to different geographic regions[1,2], and viral hepatitis and drug or toxin ingestion are the most common causes. As the pathophysiological changes are complicated and still need to be investigated, a clinically relevant large-animal model would be an indispensable tool. Orthotopic liver transplantation has been proved to be an effective treatment[3-6], but the shortage of donor organs has restricted its wide application[7-9]. The development of new therapies, including hepatocyte transplantation, stem cell transplantation, tissue engineered liver and bioartificial liver support systems, is still under investigation[3-6]. Before these treatments can be used clinically, their effects should be determined in large animal models. For a satisfactory animal model of FHF, seven criteria were suggested by Terblanche *et al*[10] and Fourneau *et al*[11]: (1) reversibility; (2) reproducibility; (3) death from liver failure; (4) presence of a therapeutic window; (5) a large animal model; (6) minimal hazard to personnel; and (7) a conscious animal model. Previous reports have described different animal models of FHF induced by various methods[7,12,13], but all the established models did not entirely satisfy all these criteria. Moreover, selection of a species with similar metabolic and physiological properties to humans is of importance. In this article, we describe, for the first time, the establishment of a *Macaca mulatta* model of FHF and the dynamic biochemical, pathological and imaging changes are also described.

MATERIALS AND METHODS

Chemicals

Lipopolysaccharide (LPS) from *Escherichia coli* was purchased from Sigma-Aldrich. Inc., St. Louis, MO. α-amanitin was from Alexis Biochemicals Co., Lausen, Switzerland. Gd-BOPTA (multihance) and SonoVue, contrast media for magnetic resonance imaging (MRI) and ultrasonography, respectively, were from Bracco Sine Pharmaceutical Co., Shanghai, China.

Animals

Two healthy female *Macaca mulattas*, 3 and 4 years old and with body weights of 5.5 kg and 4.6 kg, respectively, were purchased from the Safe and Secure Experimental Animal Breeding Base in Chengdu, China. They were housed in a large animal care facility with a constant temperature of 20 ℃ ± 1 ℃ and a 6 am to 6 pm light cycle. Two weeks were allowed for the animals to acclimatize to the animal facility before the study. They were fed with standard dry monkey food, washed apples and water *ad libitum*. Animal procedures and care were conducted in accordance with the institutional guidelines and in compliance with national and international laws and policies.

Study design

After premedication with ketamine (15 mg/kg im), skin preparation was performed on the posterior legs for blood collection from the saphenous vein and on the abdomen for intraperitoneal infusion and hepatic needle biopsy. LPS (1 μg/kg) and α-amanitin (0.1 mg/kg) were mixed in 50 mL physiological saline and slowly infused into the peritoneal cavity. Before their administration, blood biochemical parameters of the liver, kidney, heart and pancreas were measured, and total body MRI and ultrasonography were performed to acquire the physiological data as baseline values (expressed as "0" in the figures). After administration, blood biochemical parameters were measured every 12 h. An imaging examination was performed every 24 h.

Core needle biopsy of the liver guided by ultrasonography scan was performed 12 h after administration and repeated every 24 h. Part of the needle biopsy specimens was subjected to frozen section and oil red O staining.

The state of consciousness and behavior of the animals was observed and the hepatic encephalopathy was defined according to the West-Haven criteria in humans[14].

The two *Macaca mulattas* underwent necropsies after death, which included a full macroscopy and a histological evaluation of the liver, kidneys, lungs, heart, brain, spleen, pancreas and lymph nodes. Tissues were fixed in 10% neutral buffered formalin, sectioned at 5 μm and stained with hematoxylin and eosin (H and E).

RESULTS

Clinical features and animal survival

The animals showed a gradual increase in listlessness and loss of appetite after toxin administration. Anorexia and vomiting occurred 24 h after administration, followed by grasping disability, mental indifference, drowsiness and coma. Hepatic coma appeared at 42 h and 56 h, and decease occurred at 49 h and 70 h, respectively.

Biochemical results

We measured the liver enzymes, bilirubin and coagulation indices to determine the extent of liver injury. The data below were the average values from the two *Macaca mulatta* models when both were alive, or from one monkey when one died. After administration, the values of alanine aminotransferase and aspartate aminotransferase began to increase significantly and reached a peak at 36 h with over 300-fold and 200-fold increases from the baseline, respectively (Figure 1A), then the values sharply decreased. The total bilirubin, direct bilirubin and indirect bilirubin all increased gradually, and the final measured values exceeded a 30-fold rise from the corresponding

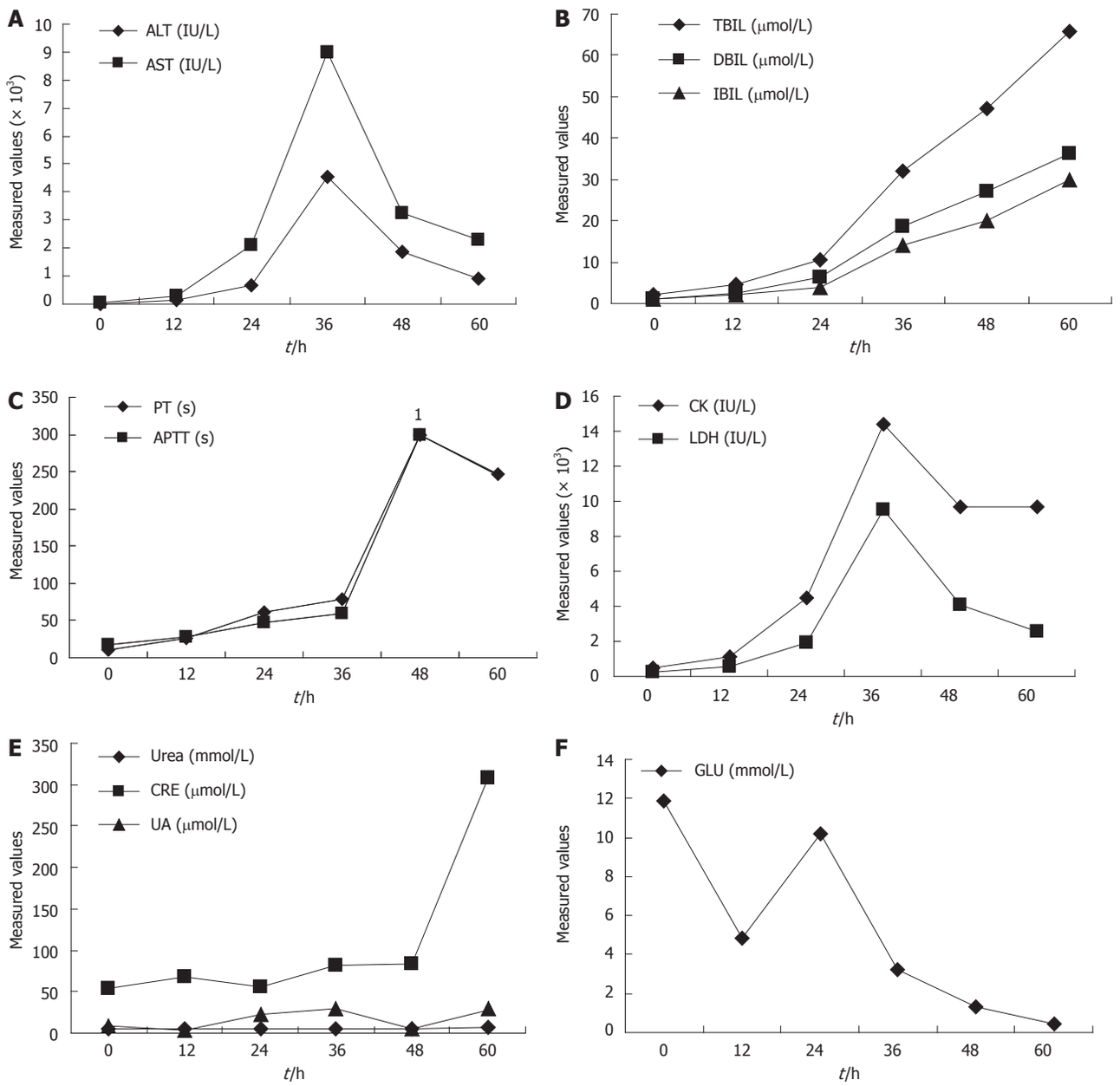

Figure 1 Blood biochemical parameters of the *Macaca mulatta* model of fulminant hepatic failure. The abscissa and ordinate represent measured time and value, respectively. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; DBIL: Direct bilirubin; IBIL: Indirect bilirubin; PT: Prothrombin time; APTT: Activated partial thromboplastin time; CK: Creatine kinase; LDH: Lactate dehydrogenase; CRE: Creatinine; UA: Uric acid; GLU: Glucose; FHF: Fulminant hepatic failure. [1]Represents values > 300 s, which exceeded the range of the equipment, so we did not obtain an exact measurement.

baseline values (Figure 1B). The consistent increase in bilirubin and the notable decrease in enzymes after 36 h suggest the emergence of enzyme-bilirubin segregation. The prothrombin time (PT) and activated partial thromboplastin time (APTT) were also prolonged after administration. At 48 h, both the levels exceeded 300 s so that we could not measure the exact number using the machine. Even the last two measured levels of PT and APTT showed little diminution, and at 250 s were still 14- to 22-fold of the baseline (Figure 1C). The ratios of prothrombin activity were 1.18% and 5.53%.

To determine whether the toxins damaged other organs, the biochemical parameters of the heart, kidneys and pancreas were measured. Creatine kinase (CK) and lactate dehydrogenase (LDH), markers of myocardial injury, also displayed peaks at 36 h with values 27-fold and 38-fold that of baseline, respectively (Figure 1D). Of the three kidney-associated parameters, i.e., urea, creatinine (CRE) and uric acid, only the level of CRE obviously increased after 48 h (Figure 1E). The blood glucose concentration decreased (Figure 1F), and the serum amylase of the pancreas did not show any significant changes.

Imaging

MRI and ultrasonography were performed every 24 h, and the final scans were performed when the animal

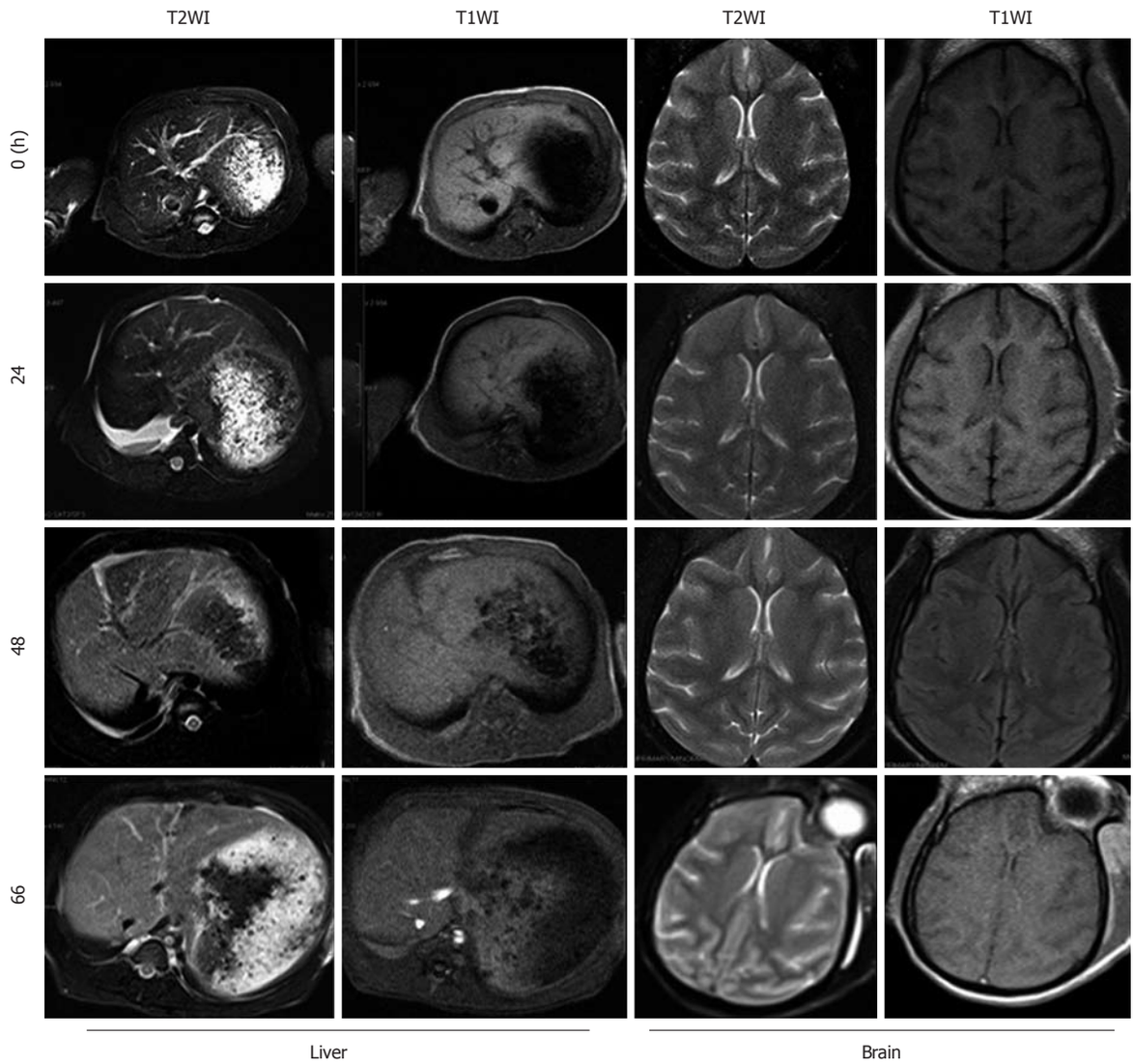

Figure 2 Magnetic resonance imaging of the liver and brain of *Macaca mulatta* model at different times. T2WI: T2-weighted imaging; T1WI: T1-weighted imaging.

died. At 24 h, MRI showed slight signals emerging in the left lobe of the liver, and Gd-enhanced T1-weighted imaging demonstrated homogeneous intensity. The signal began to increase heterogeneously in the parenchyma at 48 h, and became more heterogeneous and diffuse at 66 h, and the intensity decreased on a widespread basis in the hepatobiliary period (Figure 2). In ultrasonography, the echo of the liver parenchyma was slightly enhanced at 48 h, especially in the left lobe, and the contrast sonogram with SonoVue showed low perfusion. The hepatic artery resistance index increased. At 60 h, the echo was still enhanced slightly, but the portal vein velocity decreased and the hepatic artery resistance index increased further (Figure 3). Before 48 h, no predominant changes were found in brain MRI, but at 66 h, abnormal rhythmic signals emerged in the frontal lobe and both temporal lobes, which demonstrated hyperacute ischemia (Figure 2). We also scanned the thoracic and abdominal organs and no marked abnormality was found (data not shown).

Pathological changes

Core needle biopsies were performed at 12 h and 36 h after administration and the animals underwent necropsies after death. The main organs were evaluated, including the liver, kidneys, lungs, heart, brain, spleen, pancreas and lymph nodes, for macroscopic and histological changes.

H and E and oil red O staining showed that the liver developed severe steatosis at 36 h after toxin infusion, and in the necropsy liver, extensive parenchymal necrosis was found. The hepatic cord was dissociated, with disordered hepatocytes, and the hepatic sinusoid was extended with hyperemia. Vacuoles appeared in the cytoplasm, and patchy necrosis was found in the portal areas. In the non-necrotic areas, most hepatocytes were stained reddish-orange by oil red O. In the necropsy section, massive necrosis caused loss of almost all of the hepatocytes, and only the reticular structure remained, which is similar to the case in humans suffering from acute severe viral hepatitis (Figure 4).

With the naked eye, the livers appeared softer and smaller, with sharper and thinner edges, than the normal liver. The surface color was red and yellow, and it was a uniform yellow at the cut surface (Figure 4). Vascular dilatation and hyperemia were found on the surface of

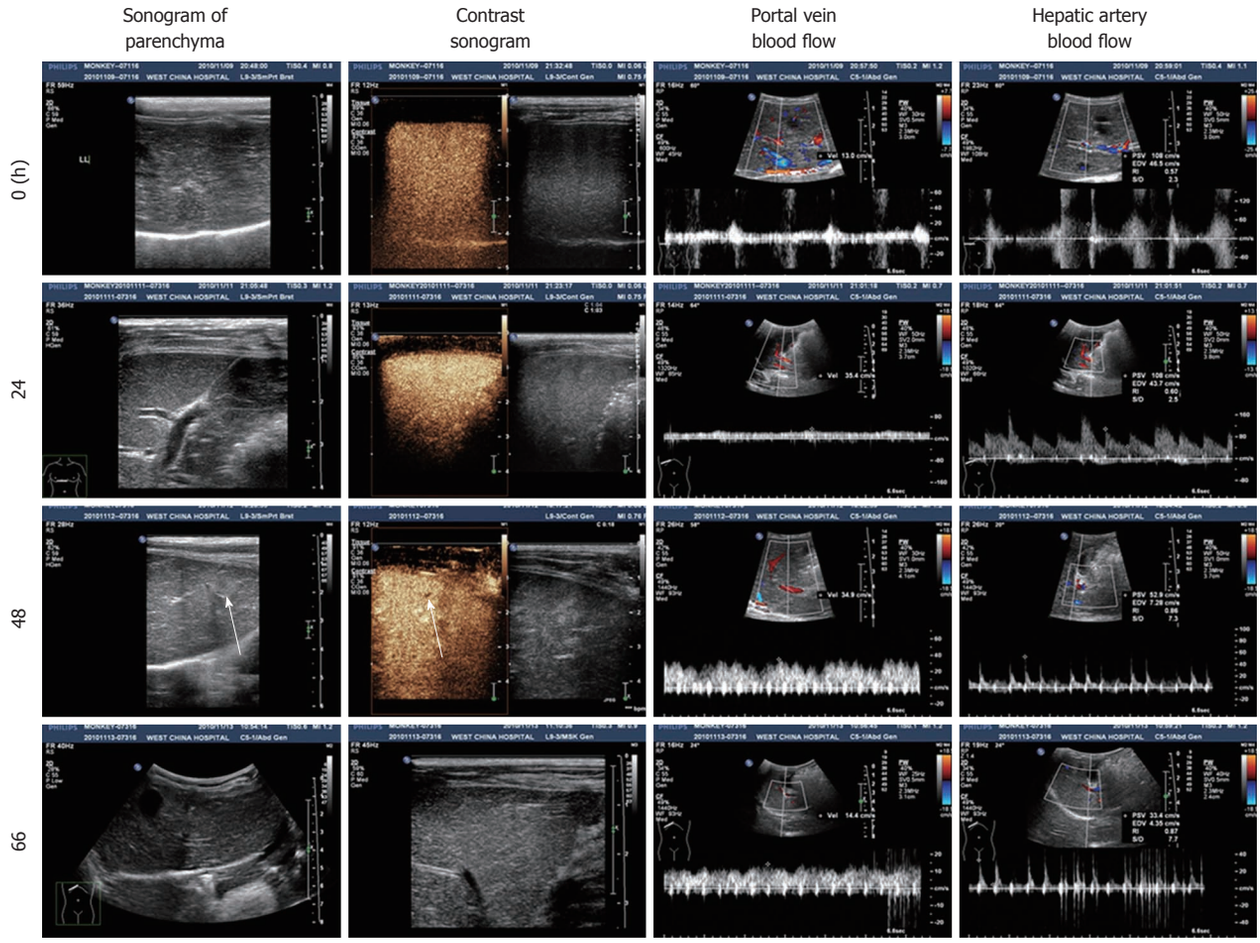

Figure 3 Liver sonogram of the *Macaca mulatta* model at different times. Arrows show a slightly enhanced echo and lower perfusion of SonoVue at 48 h.

the brain and cerebral edema was proved by widened gyri and shallowed sulci (Figure 5). Under microscopy, the superficial layer of the brain was loose, edematous and weakly stained (Figure 6).

Both kidneys showed hyperemia. The heart, spleen and pancreas did not display obvious gross changes (Figure 5). No peritoneal hyperemia, adhesions or edema in the peritoneal cavity was found. The lymph nodes located in the mesentery were enlarged and darkened. Under microscopy, they showed diffuse bleeding and lymphocytes were extensively reduced with much histiocyte proliferation. Arterioles in the lymphoid follicles were found to have hyaline degeneration. Both kidneys were also injured and demonstrated ectasia and hyperemia of the capillaries in the renal glomerulus and the stroma of the kidney tubules, and there was cellular swelling in the tubule epithelial cells. Only a wave-like change of the myocardium was found in the heart (Figure 6).

DISCUSSION

In this study, we have established, for the first time, a *Macaca mulatta* model of FHF, which satisfies all of the criteria for a large animal model of FHF proposed by Terblanche *et al*[10] and Fourneau *et al*[11], especially a conscious animal model for observation of the development of hepatic encephalopathy. All the clinical features and biochemical parameters, including liver enzymes, bilirubin as well as coagulation activity, confirmed that this model matched all the diagnostic criteria of human FHF. In addition, we dynamically examined and recorded the pathological and imaging changes in the liver and extrahepatic organs during disease progression.

Animal models of FHF are urgently needed to fully investigate the pathogenesis, progression, diagnosis and treatment of this serious disease clinically. Novel therapeutic strategies including hepatocyte transplantation, stem cell transplantation, tissue engineered liver and bioartificial liver support systems are under investigation. However, all the therapies need to be tested in an animal model before clinic use. Over the past 30 years, various animal models of FHF have been created with different methods[7,12,13], including models using rodents[15-18], dogs[19-23] and pigs[24,25]. However, differences in the metabolic and physiological properties in these distantly relative species restricted the application of the results to humans. As the closest relative to the human, the primate possesses much more similar metabolic and physiological properties, and a primate with FHF is considered to be the best large animal model for use in basic and pre-clin-

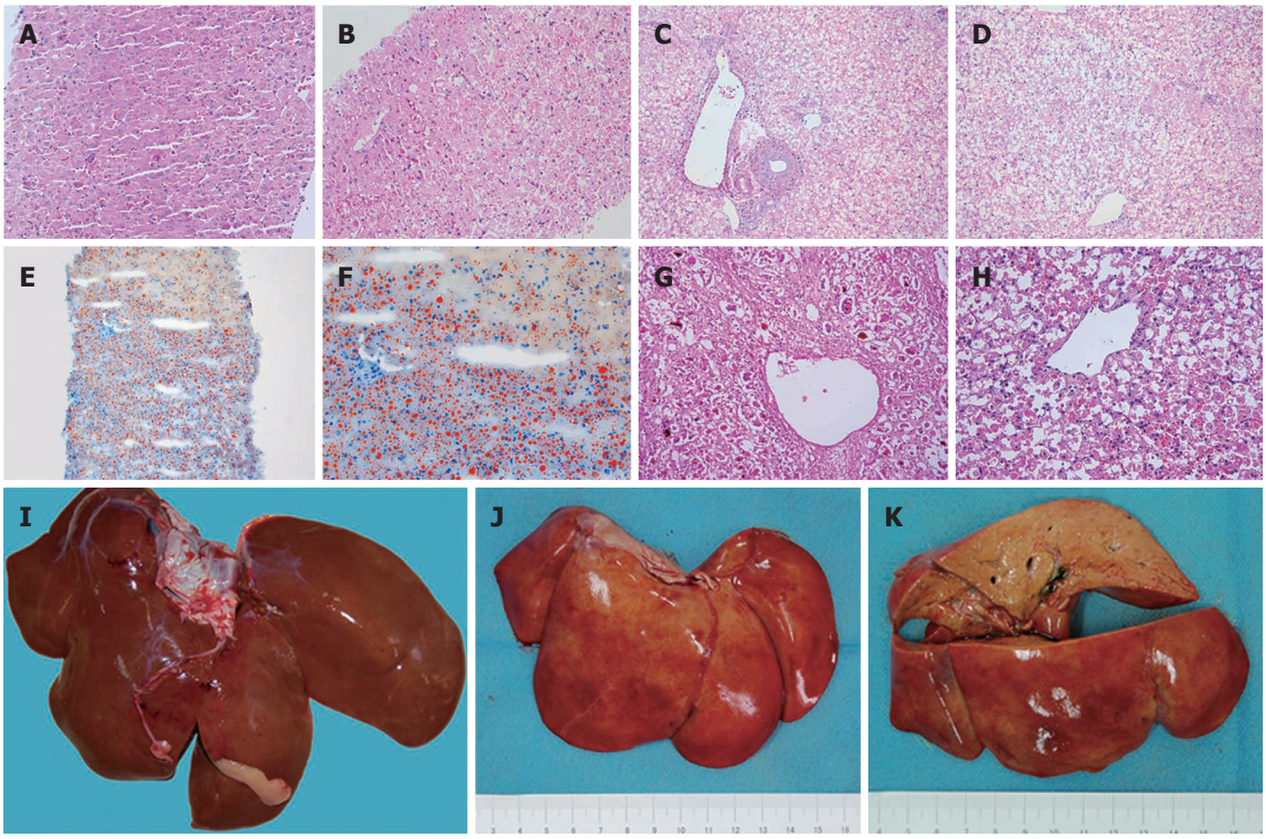

Figure 4 Histopathological changes of the liver. A: Tissue from needle biopsy at 12 h. Changes in organizational structure and cell morphology were not obvious [hematoxylin and eosin (H and E) stain, × 200]; B: Tissue from needle biopsy at 36 h. Vacuoles appeared in the hepatocellular cytoplasm (H and E stain, × 200); C, D: Tissue from necropsy after death at 70 h. Hepatocellular necrosis was distributed in the portal area (C) and central area (D) (H and E stain, × 200). Massive necrosis caused almost all of the hepatocytes to be lost, and only the support structure remained; E: Frozen tissue from needle biopsy at 36 h. The extensive reddish-orange color suggested serious fatty degeneration (oil red O stain, × 100); F: Frozen tissue from needle biopsy at 36 h. In the borderline between necrosis and steatosis, the reddish-orange color was obvious (oil red O stain, × 200); G, H: Comparison of pathological changes of fulminant hepatic failure in humans induced by viral hepatitis (G) and a *Macaca mulatta* model induced by α-amanitin and lipopolysaccharide (H) (H and E stain, × 200); I: Normal liver of *Macaca mulatta* for comparison; J: The surface view of the experimental liver on necropsy appeared red and yellow; K: The cut surface view of the experimental liver was uniformly yellow.

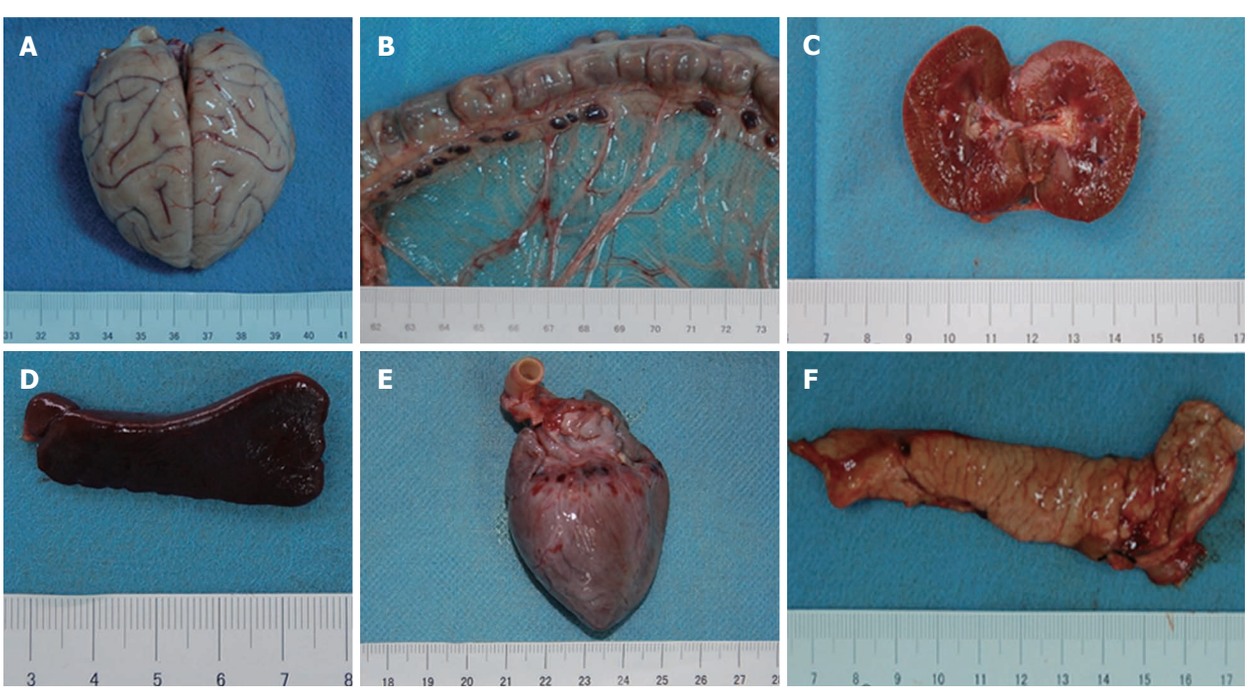

Figure 5 Other main organs on necropsy. A: The brain with cerebral edema; B: Mesenteric lymph nodes enlarged and beaded; Hyperemia of the kidney (C), spleen (D), heart (E), and pancreas (F) without obvious gross lesions.

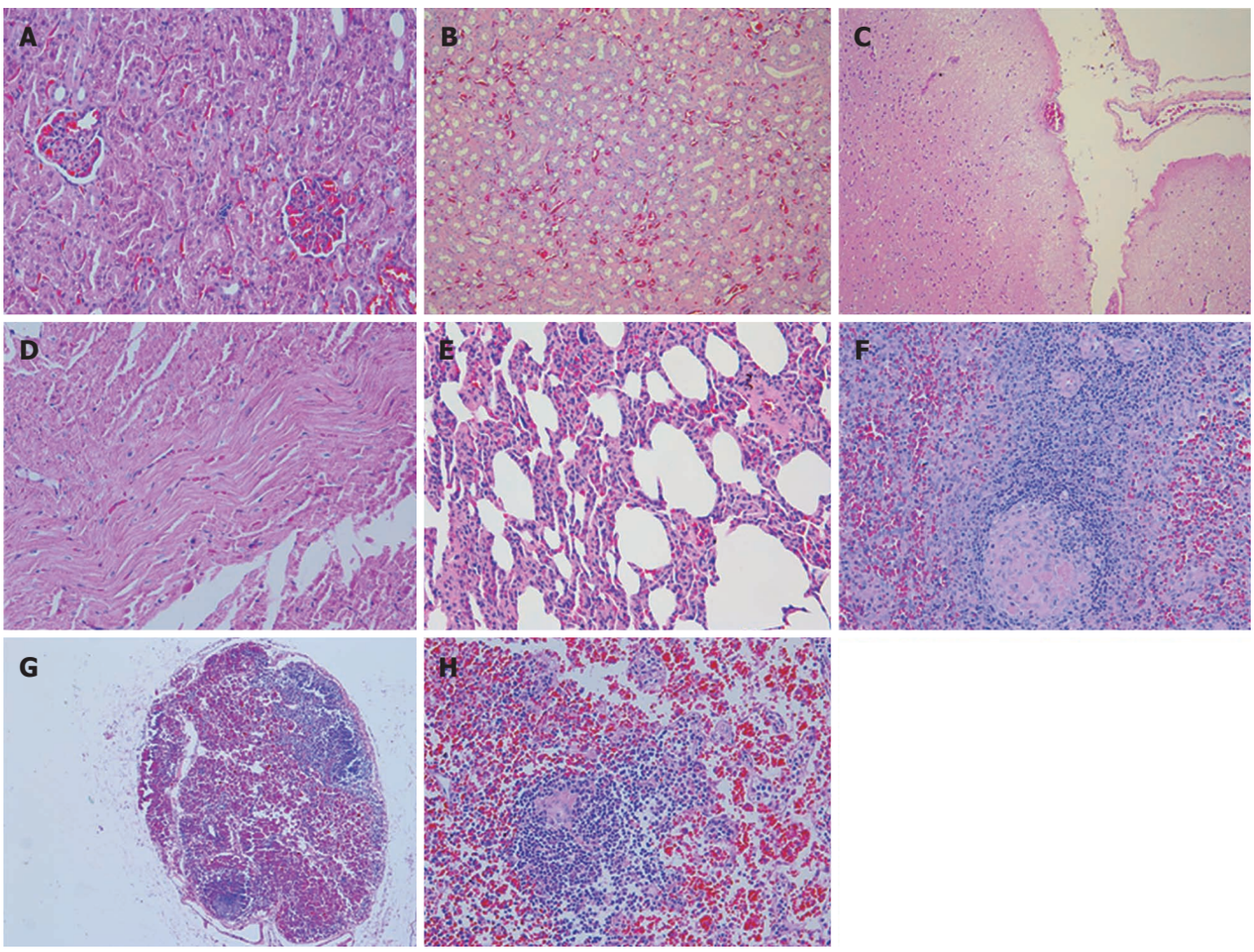

Figure 6 Histopathological changes of other organs. A: Ectasia and hyperemia of capillaries in the renal glomerulus [hematoxylin and eosin (H and E) stain, × 200]; B: Widespread hyperemia of capillaries in the renal stroma and cellular swelling in the tubular epithelial cells (H and E stain, × 100); C: Weak staining in the superficial layer of the brain suggest cerebral edema (H and E stain, × 100); D: Wave-like changes in the myocardium (H and E stain, × 200); E: Hyperemia of capillaries in the stroma and hyaline changes in the arterioles of the lung (H and E stain, × 200); F: Hyaline changes in the central artery of the spleen (H and E stain, × 200); G: Widespread reduction in lymphocytes and hemorrhage in the mesenteric lymph nodes (H and E stain, × 50); H: Hemorrhage and histiocyte proliferation in the mesenteric lymph nodes. Hyaline change appeared in the vessel wall of lymphoid follicles (H and E stain, × 200).

ical research. To the best of our knowledge, our *Macaca mulatta* model of FHF is the first large primate model in this field.

Methods used to induce the FHF models included surgical intervention and hepatotoxin administration. Total or partial hepatectomy and hepatic ischemic injury were widely used, but these relied on extensive surgical expertise and were influenced by individual variations in the animals. Hepatotoxins, which induce massive hepatic necrosis, are widely used in the establishment of a FHF model. Acetaminophen and galactosamine were the two most widely used hepatotoxins[17,19,22,26-31], and in some studies, carbon tetrachloride[32], thioacetamide[33,34], azoxymethane[35,36], concanavalin A[37] and amanita phalloides[38,39] were also employed. However, a lack of standardization, especially a lack of reproducibility, is the major disadvantage of models induced by acetaminophen[7,10]. The high costs restrict the application of galactosamine in large-scale models[40]. Amatoxin, a polypeptide extracted from a hypertoxic mushroom, has been used as a hepatotoxin in pigs with an endotoxin (LPS), and this model was reported to satisfy the criteria of near-universal mortality from highly reproducible hepatic failure, of mortality occurring in a defined time range, of damage specific to the liver, and of potential for recovery of the damaged liver[38,39]. In our preliminary experiment, LPS (2.25 μg/kg) and α-amanitin (0.225 mg/kg) were injected in a *Macaca mulatta* through a branch of the mesenteric vein by laparotomy at the dose converted from that used in pigs, which received LPS and α-amanitin at a dose of 1 μg/kg and 0.1 mg/kg, respectively[38,39]. However, the animal died within 8 h. We supposed that the direct and undiluted toxic effects to the liver might be the main cause of death and the surgery might promote the death of the *Macaca mulatta*. Thus, we reduced the dose of the two hepatotoxins to the level used in pigs, which was nearly half of our preliminary dose. We then improved the administration pathway by slow intraperitoneal infusion of the toxins diluted in 50 mL physiological saline instead of direct portal injection. Our results showed that the animals survived for at least 49 h and all the features of FHF were clearly presented. Most importantly, the

prolonged disease course ensures a wide therapeutic window. It is possible that intraperitoneal infusion of toxins could lead to severe peritonitis. Fortunately, we did not find any symptoms and signs of peritonitis, either in imaging examination or in necropsy.

Induced by α-amanitin and LPS, the hepatocytes presented severe steatosis first and massive necrosis at the end stage of FHF, which is similar to the case in humans suffering from acute severe viral hepatitis. Serum parameters, including hepatic enzymes, bilirubin, and coagulation activities, as well as serum ammonia, are often considered as the markers representing the level of damage of hepatocytes, since a biopsy is seldom carried out because of the restriction of invasive operation and bleeding. In fact, the hepatic injury during the course of FHF still needs direct confirmation. Our model offers an appropriate model to obtain real-time pathological changes in the hepatocytes, which makes it valuable for study of clinical features based on the pathological changes.

We also monitored the continuous alteration of the liver by imaging, and this is the first report on dynamic MRI of the liver during the course of FHF, either in patients or in an animal model. Combining the biopsy and the MRI, it is feasible to establish the diagnostic criteria of the damage level based on the MRI values, thus offering a non-invasive strategy to reveal the level of hepatic injury.

Hepatic encephalopathy is one of the most important clinical features and causes of death of FHF. Being consanguineous with humans, the *Macaca mulatta* presents similar behavior and consciousness to humans suffering from FHF. In addition, the brain volume and the similar gyrus outline make it suitable for MRI examination of the cerebral edema, ischemia or infarction. In our model, the progressing symptoms of listlessness, anorexia, grasping disability, mental indifference, drowsiness and coma are fully developed and thoroughly recorded. The behavior and conscious alterations, combined with the MRI and pathological changes, make it a valuable model for further investigation of the pathogenesis and pathophysiology of hepatic encephalopathy, which could not be reproduced in humans or in any other animals. As a noninvasive means of detection, MRI may be an appropriate strategy to monitor the progression of the hepatic encephalopathy. The first objective assessment of MR brain changes specific to hepatic encephalopathy was made in the early 1990s. It was noted that in patients with cirrhosis, the basal ganglia appeared hyperintense on T1-weighted MRI[41]. However, continuous data are still absent to reveal the correlation between the degree of encephalopathy and the MRI parameters during the course of FHF. With our model, it is feasible to obtain the dynamic imaging alterations during the whole course of hepatic encephalopathy.

It is necessary to rule out that animal death was induced by extrahepatic organ failure. In our experiment, the main extrahepatic organs were also evaluated. The increasing of CRE happened late, suggesting that the damage to the kidneys was a result of hepatorenal syndrome rather than direct toxin damage. The values of CK and LDH increased immediately after toxin administration, but no marked abnormality was found by echocardiogram and no myocardial necrosis was observed in histopathological examination. The cause of the increased CK and LDH still needs clarification. The lesion of the mesenteric lymph nodes might be associated with the toxins, which needs further study. We did not find any pathological evidences indicating cell injury of the lung and pancreas. Above all, we can conclude that the toxicity of intraperitoneal infusion of amatoxin and endotoxin is liver-specific and animal death is directly correlated with FHF and not extrahepatic organ failure.

We describe, for the first time, a *Macaca mulatta* model of fulminant hepatic failure, which satisfies all of the criteria for a large animal model of FHF[10,11]. With similar metabolic and physiological properties to humans, our primate animal model of FHF offers a valuable model to be used for investigation of the pathophysiology of FHF and for evaluation of potential medical therapies. Compared with other animals, primate models are more expensive, and the ethics of using such animals should be taken into consideration.

COMMENTS

Background

Fulminant hepatic failure (FHF) is an uncommon and challenging clinical disease characterized by sudden and severe hepatic injury and dysfunction, and it results in many different symptoms and complications with a high mortality. Previous reports have described different animal models of FHF induced by various methods, but all the established models did not entirely satisfy all the criteria.

Research frontiers

Animal models of FHF are urgently needed to fully investigate the pathogenesis, progression, diagnosis and treatment of this serious disease in the clinic. Recently, more studies have focused on large animal models and therapy for FHF. Novel therapeutic strategies including hepatocyte transplantation, stem cell transplantation, tissue engineered liver and BAL support systems are under investigation. Also, various animal models of FHF have been created with different methods, including models using rodents, dogs and pigs. However, differences in metabolic and physiological properties in these distantly relative species restricted the application of the results to humans. No primate animal model with FHF has been created before.

Innovations and breakthroughs

In this article, the authors describe, for the first time, a *Macaca mulatta* model of FHF, which satisfies all of the criteria for the large animal model of FHF. It is important that with the model, it is feasible to obtain dynamic imaging and pathologic alterations, and this is the first report about dynamic MRI of the liver during the course of FHF, either in patients or in animal models. Though lipopolysaccharide and α-amanitin were reported to induce a pig model of FHF, the main extrahepatic organs were not evaluated. In this article, the authors improved the administration pathway and evaluated the main extrahepatic organs.

Applications

The primate animal model of FHF offers a valuable model to be used for investigation of the mechanism of FHF and for evaluation of potential medical therapies.

Terminology

Macaca Mulatta: A species of the genus *MACACA* inhabiting India, China, and other parts of Asia. The species is used extensively in biomedical research and adapts very well to living with humans.

Peer review

The authors show an appropriate large primate model of fulminant hepatic failure. They described the rigid data of hepatic failure by biochemical results, imaging, and pathological changes. The primate animal model of fulminant hepatic failure has clinical benefit because of similar metabolic and physiological properties to human. This paper is an interesting and instructive manuscript. It is well written.

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S- Editor Sun H **L- Editor** Cant MR **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i5.445

World J Gastroenterol 2012 February 7; 18(5): 445-452
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Chronic fatigue is associated with increased disease-related worries and concerns in inflammatory bowel disease

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Supported by Oslo University Hospital Aker; Østfold Hospital Trust; and Ferring Pharmaceuticals
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Received: February 5, 2011 Revised: June 16, 2011
Accepted: June 23, 2011
Published online: February 7, 2012

Abstract

AIM: To investigate the impact of chronic fatigue on disease-related worries in inflammatory bowel disease (IBD) and the potential multicolinearity between subjective questionnaires.

METHODS: Patients in remission or with mild-to-moderate disease activity completed the fatigue questionnaire (FQ), the rating form of IBD patient concerns (RFIPC), the Short-Form 36 (SF-36), and IBD questionnaire (N-IBDQ). In addition, clinical and epidemiological data were obtained.

RESULTS: In total, 140 patients were included; of which 92 were diagnosed with ulcerative colitis and 48 with Crohn's disease. The mean age of patients with chronic fatigue was 44.2 years (SD = 15.8) and for non-fatigued patients was 44.7 years (SD = 16.0). Chronic fatigued patients had clinically significantly increased levels of disease-related worries, as measured by Cohen's *d* effect size. Worries about having an ostomy bag, loss of bowel control, and energy levels were most prominent in both chronic fatigued and non-chronic fatigued IBD patients. Variance inflation factor (VIF) and tolerance indicated that there were no problematic multicolinearity among the FQ, RFIPC, SF-36 and N-IBDQ responses (VIF < 5 and tolerance > 2).

CONCLUSION: Chronic fatigue is associated with increased levels of disease-related worries and concerns in IBD. Increased levels of worries were also associated with impaired health-related quality of life.

Key words: Fatigue; Worries; Health-related quality of life; Patient reported outcome; Inflammatory bowel disease

Peer reviewer: Wojciech Blonski, MD, PhD, University of Pennsylvania, GI Research-Ground Centrex, 3400 Spruce St, Philadelphia, PA 19104, United States

Jelsness-Jørgensen LP, Bernklev T, Henriksen M, Torp R, Moum B. Chronic fatigue is associated with increased disease-related worries and concerns in inflammatory bowel disease. *World J Gastroenterol* 2012; 18(5): 445-452 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i5/445.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i5.445

INTRODUCTION

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease (CD). Both UC and CD are chronic, recurrent diseases of the gastrointestinal tract, with symptoms that include abdominal pain, frequent bowel movements, and rectal bleeding[1,2]. Traditionally, these diseases have been defined as abnormalities in the structure and function of both organs and tissues[3]. However, given that IBD is associated with impaired health-related quality of life (HRQoL), increased fatigue, and increased worrying regarding the potential consequences of the disease[4-7], this definition seems too restrictive. The concept of illness might therefore be broader, embodying the subjective experiences of reduced health and bodily function[3].

Living with chronic illness can be challenging, and individual responses to these challenges vary widely[8]. Several HRQoL studies have found that many patients with IBD tend to do well[9]. However, there are subgroups of patients - such as patients with more severe disease or anxiety - who seem to be particularly affected[4,5,9]. It was recently reported that IBD patients with chronic fatigue (CF) (defined as an elevated fatigue level of a duration longer than six months) had clinically significant reductions in HRQoL, compared to patients without CF[10]. Thus, an important aspect of measuring subjective health is to identify patients who are most severely affected.

Both generic (i.e., irrespective of illness/condition) and disease-specific HRQoL questionnaires have been developed[11-13]. In addition, questionnaires such as the rating form of IBD patient concerns (RFIPC) and the fatigue questionnaire (FQ) are used to quantify other aspects of living with chronic illness[7,14]. We hypothesised that measuring aspects such as worries/concerns (RFIPC) and fatigue (FQ) might help us to identify subgroups of patients that tend to have a poorer outcome. Potentially, however, there might be areas of overlap between the various questionnaires, which is often referred to as multicolinearity. Multicolinearity refers to a situation wherein predictor variables in a regression model are strongly correlated and, consequently, that variables included in the model are closely related.

The primary aim of this study was to examine the impact of chronic fatigue on disease-related worries in IBD. In addition, we wanted to investigate whether the potential association between worries, fatigue and HRQoL in IBD exhibit multicollinearity.

MATERIALS AND METHODS

Subjects

Patients, who were over the age of 18 years, had IBD that was previously verified clinically, endoscopically, or histologically, and who were either in remission or with mild-to-moderate disease activity [defined as Simple Clinical Colitis Activity Index (SCCAI)[15] or Simple Crohn's Disease Activity Index (SCDAI)[16] score of less than 10], were eligible for inclusion in this study. Patients were excluded if they had cognitive impairment, were deemed unlikely to comply with the study procedures, or if they participated in another study. Participants were consecutively recruited from three outpatient clinics in South-Eastern Norway (the counties of Østfold and Hedmark) during routine follow-up visits. At each of the centre, a senior gastroenterologist was in charge of the study protocol. The inclusion period was from August 23, 2005 to January 29, 2007.

Clinical and sociodemographic data

Sociodemographic variables were gathered by interview, and data regarding clinical status and symptoms were obtained from laboratory tests, medical records and disease activity indices (SCCAI/SCDAI)[15,16]. In addition, we asked the patients to complete a symptom-based questionnaire that graded their self-perceived IBD symptoms during the previous 14 d, using the following categories; no symptoms, mild symptoms (did not interfere with everyday activities), moderate symptoms (interfered with everyday activities and may have resulted in sick leave), and severe symptoms (unable to perform everyday activities, on sick leave, or hospitalized)[5].

Each patient's phenotype was classified according to the Vienna Classification for CD patients, as the Montreal Classification did not exist when the study protocol was designed. The UC patients were classified into three subgroups: proctitis, left-sided colitis (with inflammation up to the splenic flexure), and extensive colitis (with inflammation beyond the splenic flexure).

The information regarding fatigue was collected with the FQ[14], the generic HRQoL with the Short-Form 36 (SF-36)[11], the disease specific HRQoL with the Norwegian version of the IBD questionnaire (N-IBDQ)[17], and disease-related worries and concerns with the RFIPC[7] (Table 1). The questionnaires were self-administered by the patients at the various centres, following a standardized procedure, which allowed the patients to fill out the questionnaires in the peace and quiet of the hospital's outpatient clinic.

Questionnaires

The RFIPC: The RFIPC is a disease-specific questionnaire that was developed by Drossman *et al*[7]. This questionnaire rates various important worries and concerns that are raised by IBD patients. The questionnaire consists of the 25 most frequently reported concerns reported by IBD patients, with every item framed in the same style: "Because of your condition, how concerned are you with…?" The responses were scored on a 100-mm horizontal visual analog scale. A score of 0-mm represents no worries/concerns, and a score of 100-mm represents the highest possible worries and concerns. The mean scores of all 25 items yields the "sum score". The RFIPC has been translated into Norwegian and validated (Jelsness-Jørgensen LP, Moum B, Bernklev T. Worries and concerns among inflammatory bowel disease patients followed prospectively over one year. Submitted:

Table 1 Main characteristics of the questionnaires used in this study

Questionnaire	Number of items and dimensions	Main characteristics
SF-36 Ware *et al*[11]	36 items, divided into 8 dimensions	Measure generic HRQoL Scale scores from 0-100, with higher scores indicating better HRQoL
N-IBDQ Bernlev *et al*[17]	32 items, divided into 5 dimensions	Measure disease-specific HRQoL Scale scores from 32-224, with higher scores indicating better HRQoL
FQ Chalder *et al*[14] Jelsness-Jørgensen *et al*[10]	11 items, divided into 2 dimensions The FQ contains 2 items asking about duration and extent of fatigue symptoms	Measure both physical and mental fatigue Scale scores from 0-33 with higher scores indicating higher levels of fatigue In addition scored as a dichotomized scale where original scores 0 + 1 = 0 and 2 + 3 = 1 Chronic fatigue defined as a score of ≥ 4 on the dichotomized scale and duration of fatigue symptoms ≥ 6 mo
RFIPC Drossman *et al*[7]	25 items, divided into 6 dimensions	Measure the 25 most frequently reported disease-related worries/concerns by IBD patients

IBD: Inflammatory bowel disease; SF-36: Short-Form 36; N-IBDQ: IBD questionnaire (Norwegian version); FQ: Fatigue questionnaire; RFIPC: Rating form of IBD patient concerns; HRQoL: Health-related quality of life.

Gastroenterol Res Pract).

Factor analysis of the Norwegian version of the RFIPC yielded six factors. F1: Impact of disease (e.g., financial difficulties/energy/loss of bowel control); F2: Expectancy (e.g., developing cancer/dying early); F3: Treatment (e.g., effects of medication); F4: Intimacy (e.g., ability to perform sexually); F5: Stigma (e.g., feeling dirty/smelly); and F6: Complications (e.g., undergoing surgery/ostomy bag placement).

FQ: The FQ was developed by Chalder *et al*[14] and consists of 11 items that are divided into two main dimensions: physical fatigue (PF), which contains seven items, and mental fatigue (MF), which contains four items. The available responses included four options: 0 = better than usual, 1 = no more than usual, 2 = worse than usual, and 3 = much worse than usual. A higher score reflects a higher level of fatigue. Combining the scores of PF and MF yields the total fatigue score, with a maximum possible scale score of 33. The scale scores of the FQ were also scored on a dichotomized scale (0 = better than usual and no more than usual; 1 = worse than usual and much worse than usual). In addition to measuring episodic fatigue (irrespective of the duration of symptoms), the FQ contains two questions regarding the duration and extent of fatigue symptoms. Based on the results of the original validation study, the Norwegian validation study, and general consensus[14,18,19], CF was defined as dichotomized scores ≥ 4 and duration > 6 mo. The FQ was recently validated for use in IBD[6].

SF-36: The SF-36 is a generic, self-administered questionnaire containing 36 questions[11] that are divided into eight multi-item scales consisting of: physical functioning, role limitations due to physical problems, bodily pain, general health, vitality, social functioning, role limitations due to emotional problems, and mental health (MH). For each question, the raw score was coded and transformed into a scale from 0 to 100, with 0 and 100 representing the lowest level and highest level of function, respectively. The SF-36 has been validated by others[20]. Missing data were treated following published recommendations: if data for half of the items within a scale or fewer were missing, they were replaced by the mean value of the respondent's completed items in the same scale in accordance with the SF-36 scoring algorithms[21].

N-IBDQ: The IBDQ is a disease-specific questionnaire that was developed by Irvine[12,13]. The original version consists of 32 items divided into four dimensions: bowel symptoms (e.g., loose stools or abdominal pain), systemic symptoms (e.g., fatigue or altered sleep patterns), social function (e.g., work attendance) and emotional function (e.g., anger or depression). The Norwegian validation study (N-IBDQ) revealed a five-dimensional structure: emotional function-1 (fatigue, energy), bowel function-1 (stool consistency and pattern), bowel funtion-2 (bowel pain and discomfort), social function (work attendance, cancelling social events) and emotional function-2 (worries)[17]. All of the responses were scored on a 7-point Likert Scale, with a score of 7 representing no problems and a score of 1 representing severe problems. This gives a possible score range of 32-224, with a higher score reflecting improved HRQoL[12,13,17].

Statistical analysis

To assess the characteristics of the patients, we used descriptive analyses and frequencies. A student's *t* test was used to evaluate the differences in the distribution of epidemiological and clinical variables between the diagnostic groups.

To test potential associations between the RFIPC, FQ, N-IBDQ and SF-36 questionnaires, both bivariate correlation analysis with spearman's ρ and linear regression analysis were used. In addition, the latter analysis was used to explore potential multicollinearity. A variance inflation factor (VIF) greater than 5 and a reciprocal tolerance value of less than 0.20 were defined as indicative of colinearity in accordance with published recommendations[22,23]. We chose to analyse the RFIPC sum score, the FQ and N-IBDQ total score as dependents only, to reduce the number of dimensions tested.

Table 2 Main clinical/sociodemographic characteristics *n* (%)

	UC (*n* = 92)	CD (*n* = 48)
Age, (yr), mean ± SD	46.9 ± 5.8	40.0 ± 15.0
Age range, (yr)	20-82	19-69
Gender		
Female	43 (47)	36 (75)
Male	49 (53)	12 (25)
Disease duration, (yr), mean ± SD	8.5 ± 9.5	9.2 ± 9.6
Educational level		
Second level, first stage (lower)	16 (17.4)	5 (10.4)
Second level, second stage (medium)	42 (45.7)	22 (45.8)
Third level (university)	34 (37.0)	21 (43.8)
UC extension		
Proctitis	27 (29.3)	
Left-sided colitis	23 (25.0)	
Extensive colitis	42 (45.7)	
CD extension		
L1 terminal ileum		11 (22.9)
L2 colon		17 (35.4)
L3 ileocolon		18 (37.6)
L4 upper GI		2 (4.1)
Perceived IBD symptom score		
No symptoms	23 (25.0)	9 (18.8)
Mild symptoms	41 (44.6)	22 (45.8)
Moderate symptoms	25 (27.2)	13 (27.1)
Severe symptoms	3 (3.2)	4 (8.3)

UC: Ulcerative colitis; CD: Crohn's disease; IBD: Inflammatory bowel disease; GI: Gastrointestinal.

To control for and eliminate the effect of potential confounding factors that are known to influence RFIPC scores, age, gender, and educational level were entered as covariates through univariate analysis of variance. Perceived IBD symptoms were hypothesized to affect the RFIPC scores and we therefore additionally chose to correct for this factor as well. The effect size was calculated with Cohen's *d*[24]. Operational definitions of 0.2, 0.5 and 0.8 were categorized as small, medium and large effect sizes, respectively[24].

All tests were 2-sided with a 5% significance level. All statistics were performed using the Predictive Analytics Software, PASW, version 18.0 (IBM Corporation, Route 100 Somers, NY 10589).

Ethical considerations

This study was performed in accordance with the principles of the Helsinki declaration and approval was obtained from the Regional Ethics committee and the Norwegian Data Inspectorate.

RESULTS

One hundred and forty-four patients who were diagnosed with either UC or CD gave their written informed consent for participation in the study. One patient was excluded due to severe disease activity at inclusion (SCDAI > 10), one patient withdrew from the study after a few weeks, and two patients were excluded from analysis due to incomplete responses to the questionnaires. A total of 140 patients provided complete data sets and were suitable for statistical analysis.

Epidemiological and clinical characteristics

The CD patients were significantly younger than the UC patients ($P = 0.014$). There were no significant differences in the perceived IBD symptoms or the duration of disease between the two diagnostic groups. Twenty of the 92 (22%) UC patients and 14 of the 48 (29%) CD patients were judged to have CF. There were no significant differences in age, gender, disease duration or educational level between CF and non-CF patients. However, the perceived IBD symptoms were more severe in CF than in non-CF patients ($P < 0.01$). The primary characteristics of the participants are presented in Table 2.

CF and RFIPC scores

CF was associated with significantly higher RFIPC scores in one of the six dimensions in CD and in five of the six dimensions in UC. In addition, the RFIPC sum score was significantly higher in UC patients with CF compared to those without CF (Table 2). Among the individual RFIPC items, the differences between UC patients with and without CF were most pronounced in worries regarding loss of bowel control, developing cancer, dying early, energy level, being a burden to others, having surgery, or an ostomy bag ($P < 0.01$ for all items). In addition, the scores for the following RFIPC items were significantly higher in CD patients with CF compared to those without CF: ability to achieve full potential, financial difficulties, and energy level ($P < 0.05$ for all items). When comparing RFIPC dimensional scores for UC patients with CF to CD patients with CF, the analysis revealed no statistical difference. Only one of the twenty-five individual RFIPC items (worries about pain and suffering) was significantly different between UC and CD patients with CF, with CD patients reporting more worries in this item ($P < 0.01$).

Effect size (Cohen's *d*) is a measure of the estimated magnitude of a relationship between two variables and is calculated by subtracting the mean dimensional RFIPC scores of CF patients from non-CF patients, then dividing by the common σ of both groups. The analysis revealed that the RFIPC factors that were statistically significant in CF *vs* non-CF patients - regardless of their IBD diagnosis - also produced a large Cohen's *d* ($d > 0.80$) (Table 3). The analysis further revealed that although they did not reach statistical significance, the numerical differences produced small-to- medium effect sizes. Among the CD group, the differences between CF and non-CF patients resulted in a medium effect size ($d > 0.50$) in the RFIPC sum score, whereas the effect size in the remaining five out of six dimensions were small ($d > 0.20$). Among the UC group, one out of the six RFIPC factors did not differ significantly between CF and non-CF patients, producing a small Cohen's *d* ($d > 0.20$).

After the mean dimensional RFIPC scores (Table 4)

Table 3 Cohen`s *d* effect size calculated from crude dimensional rating form of inflammatory bowel disease patient concerns scores and presence of chronic fatigue

RFIPC factors	CD no CF (*n* = 34)	CD CF (*n* = 14)	*P* value	Cohen`s *d*	UC no CF (*n* = 72)	UC CF (*n* = 20)	*P* value	Cohen`s *d*
Impact of disease	31.1 (20.6)	46.7 (17.7)	0.017	-0.81	25.6 (19.2)	45.7 (18.3)	< 0.001	-1.07
Expectancy	38.1 (30.5)	51.1 (23.1)	0.160	-0.48	32.6 (23.7)	55.3 (20.8)	< 0.001	-1.02
Treatment	27.5 (21.2)	38.0 (25.4)	0.146	-0.44	27.5 (22.0)	49.1 (22.5)	< 0.001	-0.97
Intimacy	20.3 (21.6)	28.3 (28.5)	0.299	-0.31	18.5 (18.9)	27.2 (20.1)	0.076	-0.44
Stigma	21.2 (24.3)	26.6 (20.2)	0.467	-0.24	17.3 (19.7)	35.0 (22.0)	0.001	-0.84
Complications	26.3 (23.6)	30.6 (18.6)	0.549	-0.20	21.2 (17.9)	35.7 (17.3)	0.002	-0.82
Sum score	29.2 (19.1)	39.6 (16.3)	0.082	-0.58	25.0 (15.9)	43.5 (14.5)	< 0.001	-1.21

Data are presented as mean (SD), calculated with independent samples *t* tests. RFIPC: Rating form of inflammatory bowel disease patient concerns; CD: Crohn's disease; UC: Ulcerative colitis; CF: Chronic fatigue. Cohen`s *d* effect size: small, *d* = 0.2; medium, *d* = 0.5; large, *d* = 0.8.

Table 4 Univariate analysis of mean rating form of inflammatory bowel disease patient concerns scores in non-chronic fatigue and chronic fatigue patients

	Mean RFIPC adjusted for age, gender and education		Mean RFIPC adjusted for age, gender, education and IBD symptoms	
	No CF mean	CF mean	No CF mean	CF mean
CD (*n* = 48)	(*n* = 34)	(*n* = 14)	(*n* = 34)	(*n* = 14)
Impact of disease	30.7	47.6[a]	32.2	44.0[a]
Expectancy	37.3	53.0	37.8	52.0
Treatment	27.5	38.0	27.2	39.0
Intimacy	19.7	30.0	20.6	27.8
Stigma	20.9	27.3	20.8	27.6
Complications	26.4	30.3	25.9	31.8
Sum score	28.8	40.3	29.4	39.0
UC (*n* = 92)	(*n* = 72)	(*n* = 20)	(*n* = 72)	(*n* = 20)
Impact of disease	25.7	45.3[c]	26.3	43.1[c]
Expectancy	33.0	53.8[b]	33.4	52.4[c]
Treatment	27.9	47.8[c]	28.5	45.6[c]
Intimacy	18.7	26.7	19.0	25.6[a]
Stigma	17.5	34.3[b]	17.9	33.1[c]
Complications	21.4	35.0[b]	21.7	34.0[b]
Sum score	25.2	42.8[c]	25.6	41.2[c]

RFIPC: Rating form of inflammatory bowel disease patient concerns; CD: Crohn's disease; UC: Ulcerative colitis; CF: Chronic fatigue; IBD: Inflammatory bowel disease. Significance levels are between patients reporting CF or not: [a]$P \leqslant 0.05$, [b]$P \leqslant 0.01$, [c]$P \leqslant 0.001$.

were adjusted for the covariates age, gender and education, statistically significant differences were reproduced in the same RFIPC factors as in the raw, unadjusted analysis for both the UC and CD patients. Furthermore, when controlling for the perceived IBD symptom score, the significance level increased in two of the six factors (expectancy/stigma) for the UC group. One RFIPC factor (intimacy) in the UC group changed from non-significant to significant. Among the CD patients, controlling for perceived IBD symptoms had only a minor effect.

The correlation between RFIPC sum score, total fatigue, N-IBDQ total score and subdimensions of the SF-36 questionnaire

In both the UC and CD patient groups, increased RFIPC sum scores were associated with higher fatigue levels (measured as total fatigue) and reduced HRQoL (Table 5).

Table 5 Correlation (Spearman's ρ) between rating form of inflammatory bowel disease patient concerns sum, fatigue questionnaire sum, Norwegian inflammatory bowel disease questionnaire total and Short Form 36 dimensions

	CD (*n* = 48) RFIPC sum score	UC (*n* = 92) RFIPC sum score
FQ		
TF	0.36	0.49
N-IBDQ		
IBDQ Total	-0.53	-0.51
SF-36		
PF	-0.34	-0.40
RP	-0.28	-0.48
BP	-0.22	-0.44
GH	-0.49	-0.43
VT	-0.32	-0.48
SF	-0.40	-0.57
RE	-0.12	-0.49
MH	-0.31	-0.50

RFIPC: Rating form of inflammatory bowel disease patient concerns; CD: Crohn's disease; UC: Ulcerative colitis; FQ: Fatigue questionnaire; TF: Total fatigue; N-IBDQ: Norwegian inflammatory bowel disease questionnaire; SF-36: Short Form 36; PF: Physical functioning; RP: Role physical; BP: Bodily pain; GH: General health; VT: Vitality; SF: Social functioning; RE: Role emotional; MH: Mental health.

The latter showed a negative Spearman's ρ both for N-IBDQ and the SF-36 subdimensions. In general, these associations were stronger for the UC group than for the CD group.

Multicolinearity

Testing for multicollinearity revealed satisfactory values of both the VIF and tolerance (Table 6). The VIF was below the limit of 5 and the tolerance above the crucial threshold of 0.2 for both the UC and CD group. In the CD group, the ranges for the VIF and tolerance were 1.6-4.9 and 0.21-0.61, respectively. In the UC group, the ranges of the VIF and tolerance were 1.9-4.1 and 0.24-0.54, respectively.

DISCUSSION

The ramifications of IBD on patients subjective health has been thoroughly studied, identifying both a sub-

Table 6 Linear regression analysis of the rating form of inflammatory bowel disease patient concerns sum score, Norwegian inflammatory bowel disease questionnaire total score, total fatigue, chronic fatigue, Short Form 36 subdimensions and calculation of multicolinearity

	Dependent	Independent	ß	*P* value	Tolerance	VIF
UC	RFIPC sum	N-IBDQ total	-0.70	< 0.001	0.24	4.1
	N-IBDQ total	RFIPC sum	0.29	< 0.001	0.58	1.7
		SF-36 BP	0.14	0.032	0.57	1.7
		SF-36 VT	0.17	0.019	0.50	2.0
		SF-36 SF	0.29	< 0.001	0.39	2.6
	TF	SF-36 VT	-0.25	0.031	0.49	2.0
	CF	RFIPC sum	0.34	0.018	0.46	2.2
CD	RFIPC sum	N-IBDQ total	-0.60	0.047	0.21	4.9
	N-IBDQ total	RFIPC sum	-0.17	0.047	0.71	1.4
		SF-36 SF	0.35	0.004	0.39	2.6
		SF-36 PF	0.22	0.033	0.49	2.0
		TF	-0.31	0.015	0.34	2.9

RFIPC: Rating form of inflammatory bowel disease patient concerns; CD: Crohn's disease; UC: Ulcerative colitis; TF: Total fatigue; CF: Chronic fatigue; N-IBDQ: Norwegian inflammatory bowel disease questionnaire; SF-36: Short Form 36; PF: Physical functioning; BP: Bodily pain; VT: Vitality; SF: Social functioning; VIF: Variance inflation factor.

group of patients with a worsening in HRQoL scores and other subgroups of patients with HRQoL scores that are comparable to the general population[4,5,9,15]. This division of patient responses to IBD can partly be explained by demographic differences and differences in clinical variables[4,5,9,15]. Recently, CF was reported to be at least twice as prevalent in IBD as in the general population, and CF has been reported to lead to clinically significant reductions in HRQoL[6,10].

In the present study, we hypothesized that chronic fatigue might influence the level of disease-related concerns in IBD patients, and we found significant differences in the RFIPC scores between UC patients with CF and UC patients without CF. Although only one factor was significantly higher for CD patients with CF compared to CD patients without CF, there was a tendency of elevated scores in all RFIPC factors. In addition, we calculated effect sizes according to Cohen[24], because statistical significant differences need not be of clinical importance and insignificant differences might be. As expected, the effect sizes were more pronounced in UC patients than in CD patients. The five RFIPC factors that were not significant in CD were, however, within Cohen's limits of small-to-medium effect sizes[24]. This finding indicates that there are clinically important differences between IBD patients with CF and IBD patients without CF among all of the RFIPC scales.

IBD is often characterized by periods of remission and exacerbation; therefore, potentially negative consequences of the disease may become the primary focus in daily living[1,2,5]. This has also been reported by Drossman[7]. Worries and concerns are aspects of subjective health that can lead to decreased well-being[7]. These worries may potentially be linked to situations in which the patient's expectations regarding physical and mental functioning in "normal life" do not match their experienced reality[8].

Within the published literature, there appears to be a pattern regarding which of the individual RFIPC items are rated by IBD patients as being most important[7,25-27]. Our results concur with previous studies in this regard. However, the presence of chronic fatigue seems to further increase worries in IBD patients. To our knowledge, this association has not been previously reported for IBD. Among CD patients, we found that CF was associated with increased worries regarding pain and suffering. Research has highlighted the negative association between body pain and CD[5] and recently it was reported that chronic fatigue further decreased body pain scores[10]. In addition, patients with CD and CF seem to negatively relate their perceived energy capacity with their ability to achieve full potential and experience financial difficulties. The latter may be related to their ability to work and provide a household income. Indeed, studies have found that patients who are on sick-leave because of their IBD have a significant deterioration in HRQoL[28].

The nature of worry is not clear; however, it is most often prospective and predominated by negative thinking[29]. In IBD patients, these processes are linked to potentially negative events that may or may not occur in the future, including requiring surgery or an ostomy bag[3,7,25-27]. IBD patients are reported to believe that stress influences the course of the disease[30]. However, this potential link between psychological stress and inflammatory exacerbations is the subject of debate[31-33]. Addressing the worries and concerns of patients might therefore have a potentially positive influence on both fatigue and HRQoL; on the other hand, Borgaonkar *et al*[34] found that providing disease-related information to IBD patients worsens their short-term HRQoL.

In the present study, we found that increased levels of worrying were associated with both increased fatigue levels and reduced HRQoL. When investigating the potential association between worries, fatigue, and HRQoL in IBD patients, it is natural to wonder which is the cause and which is the effect. Is increased worrying a result of chronic fatigue or vice versa? Does impaired HRQoL increase worries and fatigue, or is HRQoL exaggerated by worries? The cross-sectional design of our study makes it difficult to reach any final conclusions. Our hypothesis, which we regard as plausible, is that both reduced HRQoL and increased fatigue are secondary effects of increased disease-related worries and concerns. This theory has also been proposed by others[30]. Potentially, elevated levels of disease-related concerns may increase energy loss and thus manifest as increased fatigue and reduced HRQoL[35].

The RFIPC was developed just a few years after the IBDQ, which may explain why the questionnaire has received much less attention in the literature[7,12,36,37]. As shown by our study, employing a set of various questionnaires that measure subjective health increases the

likelihood of detecting subgroups of IBD patients who are at risk of coping less successfully[8]. Naturally, there is the potential risk that subdimensions of different questionnaires are in fact operationalizations of the same phenomena[22,23]. However, both the VIF and tolerance were within acceptable limits for both UC and CD patients, indicating that the questionnaires seem to measure different aspects of health perception. Given these results, we would argue that patient-reported outcome, rather than HRQoL, seems to be a more adequate definition in cases were one is interested in a wide variety of outcomes (e.g., worries, concerns, fatigue, HRQoL).

A limitation of the present study is that we did not include a specific questionnaire for measuring depression. This precludes the possibility of adjusting for depression as a potentially confounding variable for fatigue. Several reports have indicated a connection and overlap between fatigue and depression[7,19,38]. However, the MH dimension of the SF-36 questionnaire indicated that the patients in our study did not differ from the general population in this respect. Consequently, depression does not appear to be a particular problem in this patient population. Moreover, in the CD patient group there may be type 2 statistical errors, as we were not able to obtain the strong associations between levels of worries and fatigue, as was shown for UC patients.

In conclusion, we report that chronic fatigue is associated with clinically significantly increased levels of disease-related worries in both UC and CD patients. This study provides additional information to the complex nature of understanding how IBD patients perceive their own subjective health.

ACKNOWLEDGMENTS

The authors wish to thank Professor Trudie Chalder, Kings College London, for the permission to use the fatigue questionnaire (FQ) in this study; Professor Jon Håvard Loge, University of Oslo and Professor Stein Kaasa, University Hospital of Trondheim, for the permission to use the validated Norwegian version of FQ; and Professor Leiv Sandvik, University of Oslo, for the statistics. Hege Hølmo Johannessen is acknowledged for correcting the manuscript for grammatical errors. Furthermore, we wish to thank Marita Nilsen (Innlandet Hospital), Hilde Næs, Brita Olafsen and Margrethe Solheim (Østfold Hospital Trust) for their contribution in gathering the data for this study.

COMMENTS

Background

Fatigue is reported to be a prevalent symptom in chronic diseases. In inflammatory bowel disease (IBD), however, there has been a lack of studies with fatigue as the primary endpoint. Recent publications have revealed that the amount of patients reporting long-lasting, chronic fatigue is two to three times elevated in patients with IBD compared to the background population.

Research frontiers

Chronic fatigue has been reported to significantly reduce IBD patients' health-related quality of life. In this study, the authors demonstrate that chronic fatigue is a potential important contributor to increased disease-related worries and concerns.

Innovations and breakthroughs

Recent studies have highlighted the importance of fatigue as a subjective symptom in IBD. This is the first study to report that chronic, long-lasting fatigue symptoms is associated with increased levels of disease-related worries.

Applications

By adding further complexity to the understanding of subjective health experiences in IBD, this study might help clinicians to detect patients at risk of less successful coping, potentially enhancing the patient's quality of life, and also their course of disease.

Terminology

Chronic fatigue refers to fatigue symptoms of some intensity, which have a long-lasting duration of six months or more.

Peer review

This is a very interesting and novel original contribution analyzing the problem of chronic fatigue and its relationship with worries associated with IBD. The study is interesting, seems well done and well reported. It adds a valuable bit of information to the IBD literature.

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S- Editor Yang XC **L- Editor** Stewart GJ **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i5.453

World J Gastroenterol 2012 February 7; 18(5): 453-457
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Pneumatosis cystoides intestinalis: A single center experience

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Received: April 25, 2011 Revised: August 25, 2011
Accepted: August 31, 2011
Published online: February 7, 2012

Abstract

AIM: To share our experience of the management and outcomes of patients with pneumatosis cystoides intestinalis (PCI).

METHODS: The charts of seven patients who underwent surgery for PCI between 2001 and 2009 were reviewed retrospectively. Clinical features, diagnoses and surgical interventions of patients with PCI are discussed.

RESULTS: Seven patients with PCI (3 males, 4 females; mean age, 50 ± 16.1 years; range, 29-74 years) were analyzed. In three of the patients, abdominal pain was the only complaint, whereas additional vomiting and/or constipation occurred in four. Leukocytosis was detected in four patients, whereas it was within normal limits in three. Subdiaphragmatic free air was observed radiologically in four patients but not in three. Six of the patients underwent an applied laparotomy, whereas one underwent an applied explorative laparoscopy. PCI localized to the small intestine only was detected in four patients, whereas it was localized to the small intestine and the colon in three. Three patients underwent a partial small intestine resection and four did not after PCI was diagnosed. Five patients were diagnosed with secondary PCI and two with primary PCI when the surgical findings and medical history were assessed together. Gastric atony developed in one case only, as a complication during a postoperative follow-up of 5-14 d.

CONCLUSION: Although rare, PCI should be considered in the differential diagnosis of acute abdomen. Diagnostic laparoscopy and preoperative radiological tests, including computed tomography, play an important role in confirming the diagnosis.

Key words: Pneumatosis cystoides intestinalis; Peritoneal free air; Radiological tools; Diagnosis

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Arikanoglu Z, Aygen E, Camci C, Akbulut S, Basbug M, Dogru O, Cetinkaya Z, Kirkil C. **Pneumatosis cystoides** intestinalis: A single center experience. *World J Gastroenterol* 2012; 18(5): 453-457 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i5/453.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i5.453

INTRODUCTION

Pneumatosis cystoides intestinalis (PCI) is a relatively uncommon condition, characterized by the presence of multiple gas-filled cysts within the wall of the gastrointestinal tract[1-12]. The term "pneumatosis intestinalis" was first used by Duo Vernoi while observing autopsy specimens in 1730. The entity defined by Duo Vernoi is what we now know as primary PCI. The term "second-

ary PCI" was termed by Koss in 1952, who analyzed 213 pathological specimens and attributed 85% of the cases to a secondary disease[1,2].

One of the pathognomonic features of PCI is pneumoperitoneum without peritoneal irritation as a result of a cyst rupture. In contrast, air retention leading to acute abdominal findings may be seen in some cases[3].

PCI is a radiological or exploratory entity, not a disease, and the underlying causes are numerous. PCI may develop either after a benign procedure, such as endoscopy, or from an unknown cause (primary or idiopathic). In some cases, a more serious disease, such as secondary necrotizing enterocolitis, may be the cause. No clear consensus has yet been established, although many mechanical, bacterial, and pulmonary hypotheses have been proposed regarding the etiopathogenesis of PCI[4]. PCI usually does not lead to clinical findings and may disappear spontaneously in cases in which the primary disease is treated. Steroids, an elemental diet, hyperbaric oxygen, antibiotics, and surgery have been used as treatments. In this study, we describe seven PCI cases, which were diagnosed and treated at our clinic.

MATERIALS AND METHODS

Seven patients were admitted to Firat University Faculty of Medicine, Department of Surgery, Emergency Unit, between January 2001 and August 2009. Their medical records were evaluated retrospectively to obtain follow-up and clinical data, including age, sex, initial complaints, medical histories, white blood cell, abdominal and thoracic radiography, intraoperative findings, surgical intervention, duration of hospital stay, complications and follow-up time (Table 1). Preoperative tests were performed, including routine biochemistry and thoracic and abdominal radiography. Five of the patients had findings consistent with an acute abdomen and were operated on. Contrast-enhanced abdominal computed tomography (CT) was performed in one case due to vague abdominal findings. The CT findings were consistent with a rectal perforation. Abdominal ultrasonography (USG) was also used in one patient who had marked tenderness in the right upper quadrant. All patients operated on underwent emergent surgery, and all patients were diagnosed with PCI intraoperatively. Surgical team consensus was used to determine which patients would be resected after a laparotomy and/or laparoscopic exploration. The affected segment was resected in cases with suspicion of bowel perforation and ischemia, whereas no additional surgical intervention was performed in cases in which only PCI was detected. The primary or secondary nature of the PCI was determined by considering the medical history and preoperative findings. Cases with no underlying predisposing disease were considered primary or idiopathic PCI, whereas those accompanying some disease, such as appendicitis, Crohn's disease, pyloric stenosis, necrotizing enterocolitis, peptic ulcers, cystic fibrosis, or chronic obstructive lung disease, were regarded as secondary PCI. Follow-up time was determined from the time of surgery to the last visit to our outpatient clinic.

RESULTS

Data for seven patients with PCI (3 males, 4 females; age, 50 ± 16.1 years (mean ± SD); range, 29-74 years) were analyzed retrospectively. Of the patients who presented at the Emergency Unit, three had severe abdominal pain, two had abdominal pain and vomiting, and two had abdominal pain, vomiting, and constipation. Liver and renal function tests as well as electrolyte values were normal in all patients, while marked leukocytosis was detected in four. Exam findings in six of the patients were consistent with acute peritonitis, whereas no findings other than minimal tenderness were noted in one patient (female, aged 29 years). Subdiaphragmatic free air was detected on plain thoracic and abdominal radiographs in four patients. Abdominal USG used in one patient (female, aged 74 years), who had marked tenderness in the right upper quadrant, revealed cholecystitis together with an image consistent with a stone in the lower tip of the choledoch. An abdominal CT of a 29-year-old female patient with vague findings revealed free air extending into the retroperitoneum, indicating a rectal perforation. This patient was diagnosed with acute abdomen and was scheduled for urgent surgery. A PCI diagnosis was established in seven patients after a laparotomy in six and a laparoscopic exploration in one. An appearance consistent with PCI was observed in the small intestine of four patients and in the small intestine and colon of three (Figure 1A). A small intestinal perforation was observed in only one (female, aged 34 years) of these cases (Figure 1B). PCI was detected incidentally in a 74-year-old female patient who was scheduled for bile duct surgery (cholecystectomy and choledoch exploration only). Three patients underwent a partial small intestinal resection and anastomosis, while four had no additional surgical procedures after PCI was diagnosed. All patients were given 3 L/min oxygen during the first 3 postoperative days. During the 5-14 d clinical follow-up, a 48-year-old male developed gastric atony, whereas the remaining six patients were discharged with no complications. No additional complications were observed in any of the patients during the 15 ± 5.4 mo (range, 8-23 mo) follow-up. After considering the surgical findings and medical and surgical histories, five of these patients had secondary PCI and two had primary PCI.

DISCUSSION

PCI is a rare condition characterized by multilocular gas-filled cysts localized in the submucosa and subserosa of the gastrointestinal tract[5,13-17]. The term "pneumatosis intestinalis" was first used by Duo Vernoi during postmortem observations. A PCI diagnosis in surviving patients was first established by Hahn in 1899. A PCI diagnosis *via* preoperative radiological findings was first

Table 1 Demographic and clinical characteristics of the seven patients with pneumatosis cystoides intestinalis

No.	Age	Sex	Complaint	Medical history	WBC	Radiologic tools	Loc.	Etiology	Surgical intervention	Length of hospital stay (d)	Postoperative complication	Follow-up (mo)
1	29	F	AP + V	Endoscopy	11.1	X-ray, CT[4]	SB	Secondary	Ileal resection + anastomosis	7	No	14
2	48	M	AP + V + C[2]	Peptic ulcer perforation	NR	X-ray	SB	Secondary	Ileal resection + anastomosis	14	Gastric atony	21
3	71	M	AP + V + C[2]	CLL (CT[3])	35	X-ray	SB	Secondary	Laparatomy	5	No	8
4	74	F	AP	Normal	NR	X-ray, US	SB	Secondary	Cholecystectomy + choledocotomy + drainage	11	No	23
5	53	F	AP	Colonoscopy	NR	X-ray	SB + C[1]	Secondary	Laparatomy	8	No	18
6	34	F	AP + V	Normal	23	X-ray	SB + C[1]	Primary	Ileal resection + anastomosis	10	No	12
7	41	M	AP	Normal	16	X-ray	SB + C[1]	Primary	Laparoscopic exploration	7	No	9

WBC: White blood cell; SB: Small bowel; [1]C: Colon; AP: Abdominal pain; V: Vomiting; [2]C: Constipation; CLL: Chronic lymphocytic leukemia; [3]CT: Chemotherapy; NR: Normal range; [4]CT: Computed tomography; US: Ultrasonography.

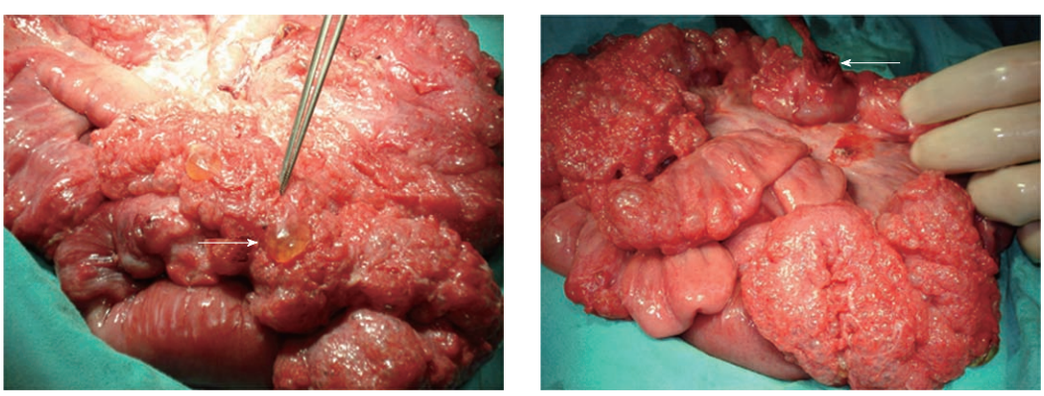

Figure 1 Intraoperative appearance of multiple air sacs in the small intestine. A: Multiple air sacs (white arrow); B: A perforation and multiple air sacs (white arrow).

described by Baumann-Schender in 1939. The condition originally described by Duo Vernoi is what we now consider primary PCI. The term "secondary PCI" was coined by Koss in 1952, who analyzed 213 pathological specimens and attributed 85% of the cases to a secondary disease[1-3].

Several hypotheses have been proposed regarding the development of PCI, although its pathogenesis is still controversial. Two main hypotheses regarding the fundamental pathogenesis of PCI are mechanical and bacterial[17]. The mechanical hypothesis postulates that PCI develops when defects in the mucosa, in combination with increased intraluminal pressure, allow gas to infiltrate the gastrointestinal (GI) tract wall. A subgroup of patients with severe pulmonary conditions may present with PCI arising from pulmonary causes, such as cough and rapid changes in intra-abdominal pressure. The bacterial hypothesis proposes that PCI develops when gas-producing bacteria gain entry into the GI tract wall and produce gas pockets. Much of the supporting evidence for these two hypotheses is derived from observational studies, and mechanical and bacterial mechanisms may occur simultaneously[4,6,7].

Although PCI may occur anywhere in the gastrointestinal tract, from the esophagus to the rectum, it is usually seen in the intestine. A previous study reported that 20%-51.6% of all PCI cases involve the small intestine, 36%-78% involve the colon, and 2%-22% include both the small intestine and colon. The small intestine was involved in 57.1% of the cases we presented here and 42.9% involved the small intestine and the colon[1,2,6,8].

PCI is not a disease but a clinical entity. The etiology can be classified by considering factors thought to play a role in its development. Based on this notion, PCI can be divided into primary and idiopathic (15%) or secondary (85%) type[9]. No identifiable underlying or predisposing factor is present in the primary or idiopathic type. However, numerous gastrointestinal diseases, including appendicitis, necrotizing enterocolitis, Crohn's disease, pyloric stenosis, ulcerative colitis, diverticular disease, necrotizing enterocolitis, gastroduodenal ulcer, and sigmoid volvulus, may accompany PCI as a secondary cause. PCI has also been reported as accompanying some non-gastrointestinal diseases, such as chronic obstructive pulmonary disease, collagen tissue diseases, acquired immune deficiency syndrome, and glucocorticoid use. PCI

cases secondary to surgical or endoscopic trauma have also been reported[10,11].

Lesions are usually localized to the left hemicolon or its mesentery or to the submucosal layer and are frequently characterized by segmentary involvement in the primary form of the disease. However, involvement is usually subserosal in the secondary form, and occurs in the stomach, small intestine, and right colon, usually in a generalized or segmented pattern[7].

The incidence of PCI is unknown, because it is usually asymptomatic. Symptoms, if any, are usually secondary to an underlying disease. Together with non-specific symptoms, such as abdominal discomfort, diarrhea, constipation, rectal bleeding, tenesmus, or loss of weight, severe complications, including volvulus, intestinal obstruction, tension pneumoperitoneum, bleeding, intussusception, and intestinal perforation may be seen in 3% of patients[18-23].

Radiological tools are important for diagnosing PCI. These include plain radiographs, USG, barium series, CT, CT-colonoscopy, magnetic resonance imaging and MRI-colonography, endoscopy, and colonoscopy[19,20]. X-ray is of great importance, because it is readily available in every emergency room. Cysts usually appear as radiolucent shadows, similar to a bunch of grapes, close to the intestinal lumen on radiographs. Free air underneath the diaphragm may be seen if these cysts perforate. An appearance of bulging into the lumen as a filling defect is seen on barium-colon radiographs[15,23]. Linear or spot-like hyperechoic images may be seen in the intestinal wall on USG. CT is the most useful method for diagnosing PCI and is important because it provides data on other abdominal pathologies. However, CT may not provide data on intestinal ischemia and necrosis[1,4,7,12]. The colonoscopic findings may be similar to multiple polyposis or collections of submucosal tumors, but subserous pneumatosis may go undetected[20]. A laparoscopic exploration is quite useful to confirm a PCI diagnosis, if the physical examination findings are suspicious, and particularly in cases that are not preoperatively diagnosed clearly using the above-mentioned radiological methods. Diagnostic laparoscopy provides the convenience of converting to open surgery as well as confirming the diagnosis.

When presence of such an entity is confirmed radiologically, gastroenterologic surgeons begin to feel annoyance. The answer to the question, "What should we do to these patients?" is correlated with the experience of each surgeon on that entity. The approach to a patient with PCI should be determined by evaluating the underlying causes and exam findings together. A specific treatment is not recommended in asymptomatic patients who are detected as having PCI radiologically and whose examination findings are negative. Conservative approaches, including nasogastric decompression, intestinal rest, antibiotic therapy and oxygen, are recommended for patients with positive examination findings and normal biochemical parameters who are confirmed radiologically to have no intestinal ischemia or perforation[24]. Applying 250 mmHg PO_2 pressure or 70% oxygen inhalation for 5 d or 2.5 atmospheres of hyperbaric oxygen pressure for 150 min/d for 3 consecutive days can lead to resolution of gas collection within a cyst[10,13,24,25]. An urgent laparotomy is necessary in cases of intestinal ischemia, obstruction, intestinal bleeding, or peritonitis[14,16]. Definitive surgery should be performed during a laparotomy if necrosis, perforation, or marked ischemia is observed in the intestine. Furthermore, no additional surgical procedures should be conducted unless other pathology is detected in addition to serosal or subserosal air cysts.

Consequently, clinical suspicion, physician experience, radiological tools, and team spirit are important in terms of the approach to PCI. When and how to treat these patients is the main issue to lower mortality and morbidity.

COMMENTS

Background

Pneumatosis cystoides intestinalis (PCI) is a pathologic condition defined as infiltration of gas into the wall of the gastrointestinal tract.

Research frontiers

The authors retrospectively reviewed the diagnosis and management of seven patients with pneumatosis cystoides intestinalis.

Innovations and breakthroughs

Clinical suspicion, physician experience, radiological tools and team spirit are important in terms of the approach to PCI. When and how to treat these patients is the main issue to lower mortality and morbidity.

Applications

According to authors' opinion, specific treatment is not recommended in asymptomatic patients who are detected to have PCI radiologically and whose examination findings are negative. However, laparotomy is necessary in cases of intestinal ischemia, obstruction, intestinal bleeding or peritonitis

Terminology

The primary and idiopathic or secondary nature of the PCI is determined by considering the medical history and by preoperative examination. Cases with no underlying predisposing disease are considered primary PCI, whereas those accompanying some disease, such as appendicitis, Crohn's disease, pyloric stenosis, necrotizing enterocolitis, peptic ulcers, cystic fibrosis or chronic obstructive lung disease, are regarded as secondary PCI.

Peer review

This is a well written report on a small series of a rare entity. It has some educational value in the presentation and the figures.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i5.458

World J Gastroenterol 2012 February 7; 18(5): 458-465
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Survival analysis of cholangiocarcinoma: A 10-year experience in Malaysia

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Received: February 2, 2011 Revised: April 26, 2011
Accepted: May 3, 2011
Published online: February 7, 2012

Abstract

AIM: To investigate the clinical features and survival of patients treated for cholangiocarcinoma in our institution and to analyze the factors affecting their survival.

METHODS: This retrospective cohort study assessed patients diagnosed with cholangiocarcinoma between January 1997 and December 2007 at the University Malaya Medical Centre in Malaysia. The clinical data and associated outcomes were collected using a structured proforma.

RESULTS: Of the 69 patients diagnosed with cholangiocarcinoma, 38 (55%) were male; mean patient age was 61 years. Twelve patients (17%) had intrahepatic, 38 (55%) had perihilar and 19 (28%) had distal tumors. Only 12 patients underwent curative surgery, including seven R0 resections. Only one patient died within 30 d after surgery. The overall median survival was 4 mo, whereas the median survival of R0 resected patients was 16 mo. The overall 1-, 2- and 3-year cumulative survival rates were 67%, 17% and 17%, respectively. Survival rates were significantly associated with curative resection (P = 0.002), intrahepatic tumor (P = 0.003), negative margin status (P = 0.013), early tumor stage (P = 0.016), higher tumor differentiation (P = 0.032) and absence of jaundice (P = 0.038). Multivariate analysis showed that tumor location was a significant independent predictor of patient survival.

CONCLUSION: Curative, margin-negative resection of early stage, well-differentiated intrahepatic tumors is associated with improved patient survival.

Key words: Cholangiocarcinoma; Bile duct tumor; Surgery; Malaysia

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Yusoff AR, Abdul Razak MM, Yoong BK, Vijeyasingam R, Siti ZM. Survival analysis of cholangiocarcinoma: A 10-year experience in Malaysia. *World J Gastroenterol* 2012; 18(5): 458-465 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i5/458.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i5.458

INTRODUCTION

Cholangiocarcinoma is a rare malignant neoplasm of biliary tract epithelium, accounting for less than 2% of all human malignancies[1]. Cholangiocarcinoma is the second most common primary hepatic malignancy after hepatocellular carcinoma (HCC)[2] and is associated with

poor patient outcomes. Over the past three decades, however, the worldwide incidence of and mortality from cholangiocarcinoma have steadily increased[3].

Cholangiocarcinoma is difficult to diagnose and is usually fatal, due to its late clinical presentation and the absence of effective non-surgical therapeutic modalities[4]. The incidence of cholangiocarcinoma peaks in patients aged 50-70 years, and there is a slight male predominance[5]. Most patients have unresectable disease at the time of diagnosis and usually die within 6-12 mo from the cancer cachexia, liver failure and biliary sepsis[6]. The 5-year survival rate is low (about 5%), and has remained virtually unchanged over the past 20 years[5,7]. Surgical resection remains the only hope for cure, and radical resection has improved outcomes, although it is also associated with high perioperative morbidity and mortality rates[8]. Since most patients with cholangiocarcinoma present with advanced disease, palliative stenting and chemoradiation are reserved for non-resectable patients, those with recurrence, and those who refuse surgical treatment[3].

Little is known about the survival of cholangiocarcinoma patients in Malaysia. We therefore analyzed the factors affecting survival of patients with cholangiocarcinoma treated at the University Malaya Medical Centre, Malaysia.

MATERIALS AND METHODS

This retrospective cohort study assessed patients diagnosed with cholangiocarcinoma and treated between January 1997 and December 2007 at our center, a tertiary referral center in Malaysia with a specialized hepatobiliary surgery unit and a gastroenterology unit. Patients with a diagnosis of cholangiocarcinoma confirmed histologically by tissue biopsy, and patients without a histologically confirmed diagnosis but with a strong provisional diagnosis by clinical examination, biochemical results and positive endoscopic or imaging [i.e., endoscopic retrograde cholangiopancreatography (ERCP)/magnetic resonance cholangiopancreatography (MRCP) or computed tomography (CT) of the abdomen] results[7] were included. Patients with HCC, tumor at the head of the pancreas and gallbladder carcinoma were excluded.

Combinations of ultrasonography, CT, MRCP, ERCP and percutaneous cholangiography (PTC) were used for tumor diagnosis and staging, and for assessment of resectability. Metastatic disease was evaluated by CT of the thorax, abdomen and pelvis and/or chest radiography (CXR).

Patients were classified into three groups based on the anatomic location of the primary lesion, specifically intrahepatic, perihilar and distal types as proposed by the guidelines for the diagnosis and treatment of cholangiocarcinoma[5]. Intrahepatic tumors were defined as those confined to the liver and not involving the extrahepatic biliary tree. Perihilar tumors were defined as those involving or requiring resection of the hepatic duct bifurcation and were typically located in the extrahepatic biliary tree proximal to the origin of the cystic duct. Distal tumors were defined as extrahepatic lesions located in the peripancreatic region[9].

Patients were staged according to the tumor-node-metastasis (TNM) system[6,10] and assessed for resectability. Variables assessed included therapeutic options (surgical or palliative treatment), operative data, and 30-d postoperative morbidity and mortality. Survival was measured from the date of first presentation to the date of death or last follow-up visit.

Statistical calculations were performed using SPSS version 13.0. Categorical variables were compared using χ^2 tests for association. One-way analysis of variance was used to compare continuous variables among the three groups of patients classified by tumor location. Results were presented as means ± SD, unless otherwise specified. Survival curves were calculated using the Kaplan-Meier method and compared using log-rank tests (Mantel-Cox). Cox proportional hazard models were used to calculate adjusted hazard ratios. A P value < 0.05 was considered significant.

RESULTS

Demography

Of the 69 patients included in this study, 12 (17%) had intrahepatic, 38 (55%) had perihilar and 19 (28%) had distal tumors. Thirty-eight patients were male (55%) and 31 (45%) were female (a male to female ratio, 1.2:1). Mean ± SD patient age was 61 ± 14.2 years (range, 18-91 years), although patients with intrahepatic tumors were younger than those with perihilar and distal tumors (P < 0.05). When subdivided ethnically, 42 patients (61%) were Chinese, 20 (29%) were Malay and 7 (10%) were Indian.

Risk factors

Of the 69 patients, 22 (29%) had a history of chronic cigarette smoking, 13 (19%) each had diabetes mellitus and regular alcohol intake, and 5 (7%) had chronic hepatitis B, with only 1 each (1.4%) having hepatolithiasis and choledochal cyst. None of our patients had other strong risk factors, such as inflammatory bowel disease, primary sclerosing cholangitis or liver fluke infestation.

Clinical evaluation

The most common symptoms among our patients were jaundice (78%), anorexia (57%), weight loss (52%), abdominal pain (44%), abdominal mass (44%), itchiness (25%), vomiting (9%) and fever (7%). Ninety-eight percent of the patients with extrahepatic cholangiocarcinoma were jaundiced (63% of perihilar tumor and 35% of distal tumor patients), whereas only 2% of intrahepatic patients was jaundiced (P < 0.001). No specific symptom was significantly related to any of the three types of cholangiocarcinoma. The median duration of complaints prior to medical consultation was 30 d (range 1-365 d). However, patients with intrahepatic lesions present somewhat later (P

Table 1 Laboratory data on admission and location of tumours

	Total (n = 69)	Intrahepatic (n = 12)	Perihilar (n = 38)	Distal (n = 19)	P value[1]
Total bilirubin (mmol/L)	178 (158)	45 (109)	216 (163)	188 (131)	0.003
Conjugated bilirubin (mmol/L)	140 (122)	31 (87)	170 (123)	151 (102)	0.002
INR	1.2 (0.7)	1.1 (0.1)	1.0 (0.1)	1.6 (1.1)	0.005

[1]Analysis of variance. Values in mean (SD); P value < 0.05 is significant. INR: International normalized ratio.

< 0.05) than those with perihilar or distal lesions.

Investigations

When we assessed laboratory variables in these patients, we found that total bilirubin concentration was significantly higher in patients with perihilar than in those with distal or intrahepatic lesions (P = 0.003) (Table 1). The international normalized ratio (INR) value was also predictive of lesion site, as it was higher in patients with distal than with perihilar or intrahepatic lesions (P = 0.005).

The tumor markers carcinoembryonic antigen, alpha-fetoprotein and carbohydrate antigen 19-9 were measured in about 50% of these patients, but none of them significantly correlated with tumor location.

Imaging

Ultrasound was the first line non-invasive imaging method used in 45 (65%) patients, detecting 50%-76% of obstructed biliary systems. Almost 91% of these patients underwent CT scans to further assess the extent of pathology, including liver masses, lymph nodes and the involvement of major vessels. We found that about 20% of the abdominal lesions were not detected by ultrasound alone, but were detected on CT scan. Only four patients underwent MRCP.

In 58 (84%) patients, ERCP was the first invasive method used to assess the obstructed biliary system. ERCP showed that 51 (74%) patients had strictures at various levels of their biliary trees. Plastic stents were inserted successfully into 44 (76%) of these patients to relieve their biliary obstructions, whereas the other seven (14%) failed in stent insertion; five later underwent PTC drainage. Cytology brushing of suspicious strictures was performed in 22 (38%) patients, with 10 (45%) being positive for cancer.

Preoperative staging for resectability was predicted based on ultrasound, CT scan, MRCP and CXR. None of these patients underwent staging laparoscopy because it was not a routine practice in our center.Based on the American Joint Committee on Cancer TNM staging system, 23 (33%) patients had stage 1 tumors, 7 (10%) had stage 2, 17 (25%) had stage 3 and 22 (32%) had stage 4. Stages 3 and 4 were considered unresectable.

Surgical treatment

Although preoperative staging indicated that 30 (43%) patients were candidates for surgical resection, only 22 (32%) patients underwent laparotomy with curative intent. Three patients (and their relatives) refused surgery, citing age as their primary concern (mean age 78 years), whereas five were excluded from surgery due to age (mean 71 years) and/or associated comorbidity. At laparotomy, 10 patients had extensive local disease and/or hepatic metastasis that precluded resection. The remaining 12 patients underwent potentially curative resection (resectability rate, 55%). All except one with evidence of biliary obstruction underwent preoperative biliary drainage either by endoscopic stenting or percutaneous transhepatic biliary drainage (PTBD). The type of surgery depended on the location of the tumor (Table 2). In general, intrahepatic tumors were treated by hepatic resection; perihilar lesions by excision of the extrahepatic biliary tree and lymph node dissection, with or without hepatic resection; and distal tumors by Whipple pancreaticoduodenectomy. Biliary reconstructions were mostly by Roux-ex-Y hepaticojejunostomy. All operations were performed by trained hepatobiliary surgeons.

Table 2 Types of surgical resection

Types of surgery	Patients (n = 12)
Left hemihepatectomy	2
Right hemihepatectomy	1
Segmental hepatic resections	2
Extrahepatic bile duct resection	5
Whipple's procedure	2

Surgical morbidity and mortality

Complications occurred in patients with all three tumor types, but the differences were not statistically significant. Three patients developed post-operative ileus, which resolved after conservative treatment. Two patients had intra-abdominal hemorrhage. One had undergone a Whipple procedure and was re-explored within 24 h because of portovenous bleeding; unfortunately, this patient died the next day. The second patient did not require intervention and was treated with blood transfusion and correction of coagulopathy. Three patients developed intra-abdominal collection, later complicated by abscesses, including one caused by bile leakage from the anastomosis. None of these patients, however, required surgical or percutaneous intervention. Two patients had surgical site infections, including one with anastomotic stenosis and the other with deep venous thrombosis (DVT) despite DVT prophylaxis. Only one patient died of complications within 30 postoperative days; hence the perioperative mortality rate was 8%.

Of the 10 patients who underwent surgery but were found to have advanced inoperable disease at laparotomy, 3 underwent a palliative bypass procedure, consisting of hepaticojejunostomy and gastrojejunostomy, whereas four underwent gastrojejunostomy alone. Two patients underwent cholecystectomy and biliary stent insertion, whereas one underwent only laparotomy and biopsy.

Table 3 Tumour histology, degree of differentiation, diameter, margin, perineural and lymph node involvements by tumour location *n* (%)

	Total (*n* = 12)	Intrahepatic (*n* = 3)	Perihilar (*n* = 6)	Distal (*n* = 3)	*P* value[1]
Tumour histology					
Adenocarcinoma	11 (92)	2 (18%)	6 (55%)	3 (27)	
Other	1 (8)	1 (100%)	0	0	
Degree of differentiation					
Well	5 (42)	2 (40%)	2 (40%)	1 (20)	
Moderate	6 (50)	1 (17%)	3 (50%)	2 (34)	
Poor	1 (8)	0	1 (100%)	0	
Tumour diameter, cm					
Size, mean ± SD	9.6 ± 15.2	27.1 ± 24.3	2.8 ± 2.2	5.7 ± 15.2	0.048[1]
Margin					
Negative	7 (58)	3 (43)	3 (43)	1 (14)	
Positive	5 (42)	0	3 (60)	2 (40)	
Lymph node involvement					
Negative	8 (67)	2 (25)	4 (50)	2 (25)	
Positive	4 (33)	1 (25)	2 (50)	1 (25)	
Perineural involvement					
Negative	5 (42)	3 (60)	2 (40)	0	
Positive	7 (58)	0	4 (57)	3 (43)	
Lymphovascular invasion					
Negative	7 (58)	2 (29)	4 (57)	1 (14)	
Positive	5 (42)	1 (20)	2 (40)	2 (40)	

[1]Analysis of variance. *P* value < 0.05 is significant.

Tumor characteristics

Of the 12 patients who underwent curative resection, 11 (92%) had adenocarcinoma and one had a papillary adenocarcinoma. The mean ± SD tumor diameter was 9.6 ± 15.2 cm (range, 0.5 cm-55 cm). Intrahepatic tumors were larger than perihilar and distal tumors ($P < 0.05$). Five (42%) patients had well-differentiated adenocarcinoma and the others had moderately or poorly differentiated adenocarcinoma. Seven (58%) patients had perineural involvement, 5 (42%) had lymphovascular invasion and 4 (33%) had regional lymph node metastases. Seven (58%) patients were resected with negative margins (R0 resection), whereas the other 5 (42%) had microscopically positive margins. Table 3 summarizes the characteristics of these tumors.

Palliation

Forty-seven (68%) patients did not undergo surgery but received palliative treatment, including 31 (66%) who underwent palliative biliary drainage by endoscopic stenting, 23 (49%) who underwent PTBD with or without concurrent stenting and 13 who underwent both. Sixteen patients had their stents changed on subsequent follow-up, of whom, nine had self-expanding metal stents. Three (6%) patients received palliative chemotherapy alone, using a variety of chemotherapeutic agents (5-fluorouracil, cisplatin, gemcitabine). Three (6%) patients were too ill and hence received best supportive care.

DISCUSSION

We have described our experience managing patients with cholangiocarcinoma, a rare type of tumor. The incidence of this tumor is likely increasing in Malaysia. The latest National Cancer Registry 2003-2005 Peninsular Malaysia has classified cholangiocarcinoma into the category of liver and gallbladder cancers rather than as a separate tumor. The incidence of liver cancer in Malaysia has been reported to be 3.6% for males and 1.2% for females, while the rates of gallbladder cancer were 0.8% and 0.7%, respectively. Morphologically, about 2.2% of these liver cancers and 33.9% of these gallbladder cancers were cholangiocarcinomas. The incidence of both liver and gallbladder cancers was higher for Chinese than for Malays and Indians.

Cholangiocarcinoma is best classified according to its anatomical location into intrahepatic, perihilar and distal tumors[9,11,12]. Most (40%-60%) tumors are perihilar or Klatskin tumors, with 20%-30% being distal and 10% being intrahepatic tumors[9,13]. Our findings were similar to these rates, in that 17% of tumors were intrahepatic, 54% were perihilar and 29% were distal.

The demographic characteristics of our patients were comparable to those of patients in other larger series. Mean patient age was 61 years (range, 18-91 years), while a review of 294 patients with cholangiocarcinoma by Nakeeb *et al*[9] showed a mean age of 62.2 years and a review by DeOliveira *et al*[13] of 564 patients showed a median age of 65 years. We found, however, that patients with intrahepatic tumors were significantly younger than those with perihilar or distal tumors. We observed a slight male predominance, with a male to female ratio of 1.2:1, similar to the ratios of 1.2:1[9] and 1.38:1 reported previously[13].

Of these patients, 32% presented in 1997-2002, whereas 68% presented in 2003-2007. We observed similar increases in the number of patients with primary tumors, suggesting that these higher rates may be due to improvement in tumor detection, ERCP proficiency, or increased awareness of the availability of local surgical expertise.

Only a few patients in our series had strong risk factors such as chronic hepatitis B, hepatolithiasis and choledochal cyst[14-16]. None had a history of primary sclerosing cholangitis[14] or liver fluke infestation[17], the latter of which is not endemic in Malaysia. Chronic cigarette smoking, regular alcohol intake and diabetes mellitus were among the risk factors[18,19] observed in our population, but none of these risk factors was associated with any particular tumor site.

The most common symptom observed in these patients was jaundice. Jaundice was significantly more common in patients with extrahepatic (i.e., perihilar and distal) than with intrahepatic cholangiocarcinoma ($P < 0.001$). This was confirmed by laboratory results showing that the concentration of bilirubin was significantly higher in patients with perihilar (extrahepatic) than in those with intrahepatic tumors ($P = 0.002$). Similar results have been reported previously[13,20]. We also found that INR was higher in patients with distal tumors ($P =$

Table 4 Univariate analysis of different variables in relation to survival

Variables	Survival rates (%)			*P* value
	1-year	2-year	3-year	
Jaundice				0.038
Absent	40	19	9	
Present	19	6	2	
Staging				0.016
Stage 1	43	17	9	
Stage 2	43	14	0	
Stage 3	12	4	0	
Stage 4	5	0	0	
Surgery (curative or palliative)				0.017
No	13	10	0	
Yes	45	9	9	
Curative resection				0.002
No	14	8	0	
Yes	67	17	17	
Type (among resected patient)				0.003
Intrahepatic	100	33	33	
Perihilar	67	17	17	
Distal	33	0	0	
Type (among all patient)				0.027
Intrahepatic	50	23	11	
Perihilar	18	6	3	
Distal	16	5	0	
Histology				0.032
Well	80	40	40	
Moderate and poor	57	0	0	
Margin involvement				0.013
Negative	86	29	29	
Positive	40	0	0	

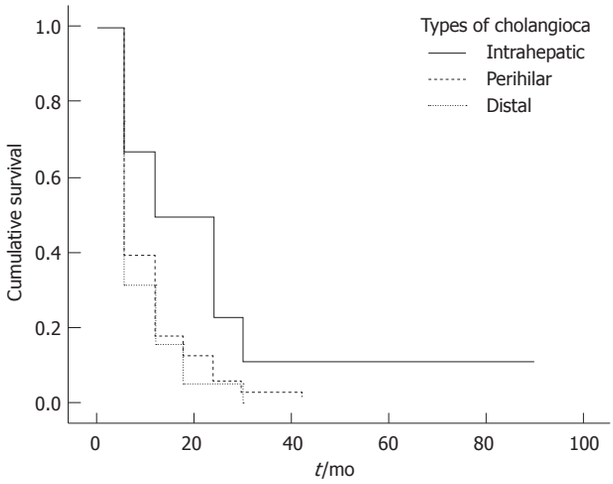

Figure 1 Overall survival by types of cholangiocarcinoma regardless of surgery or not, *P* = 0.027.

0.005). Obstructive jaundice may decrease the concentrations of vitamin K-dependent coagulation factors, resulting in aberrant coagulation profiles[5]. However, none of the other symptoms or blood parameters we assayed was significantly related to tumor location.

Many of our patients initially underwent ERCP, not MRCP. ERCP enables cytological brushing and can decompress the obstructed biliary system[6]. Although we found that the success rate of internal stent placement for drainage was high, PTBD may also play a role, especially when the endoscopic approach has failed. PTBD can also be used to visualize proximal biliary tumors and anatomy[5]. Failure of biliary decompression may result in an infected biliary system and further risks of liver failure and sepsis[21]. These risks may be prevented by decompression of an obstructed biliary system in patients with potentially resectable cholangiocarcinoma, although a series by Figueras *et al*[22] demonstrated no significant differences in morbidity and mortality between patients with and without preoperative biliary drainage. None of our patients who underwent hepatic resection developed postoperative liver failure.

Despite improved diagnostic methods and a relatively early presentation (median duration of symptoms, 30 d), only about one-third of our patients (32%) underwent surgery, and only 12 underwent curative resection. More than half of our patients (58%) were considered to have advanced unresectable disease, whereas the remaining patients were considered unsuitable for surgery because of comorbidity and/or advanced age, findings similar to those observed previously[7]. Our overall resectability, 55% (12/22), was similar to previously reported rates 18%-70%[23]. Postoperative complications were not associated with tumor location. In high volume centers with considerable experience, the operative mortality and morbidity rates varied from 6%-14% and 32%-65%, respectively[24-26]. Our 30-d postoperative mortality and morbidity rates were similar, 8% and 67%, respectively.

Patients who underwent surgical resection had a definite survival advantage over those who did not, confirming that surgical resection is the best treatment available for patients with cholangiocarcinoma and providing further evidence that potentially resectable patients should be referred early to a specialized surgical team[7,10]. The median survival time for the 12 patients who underwent curative resection was 16 mo, compared with 3 mo for the 57 patients who did not undergo curative resection (P = 0.002). The 1-, 2- and 3-year cumulative survival rates in patients who underwent resection were 67%, 17% and 17%, respectively, significantly higher than the 14%, 8% and 0%, respectively, in those who did not. Furthermore, complete surgical resection with histologically negative margins offers the best chance for cure and long-term survival[27-30].

Univariate log-rank analyses of tumor related variables in Table 4 showed that absence of jaundice (P = 0.038), tumor location (P = 0.027) (Figure 1)[17], curative resection (P = 0.002) (Figure 2)[31], early tumor stage (P = 0.016)[32], negative margin status (P = 0.013) (Figure 3)[17,32,33] and higher degree of tumor differentiation (P = 0.032) (Figure 4)[17] were significant predictors of longer survival. In contrast to previous reports, regional lymph node metastasis[28] and perineural invasion[34] were not associated with survival in resected patients. Moreover, we found that age, gender, race and risk factors were not predictors of survival.

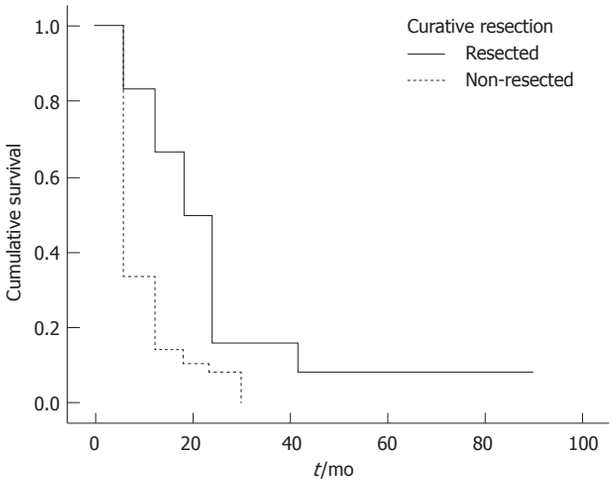

Figure 2 Cumulative survival curves after curative resection, *P* = 0.002.

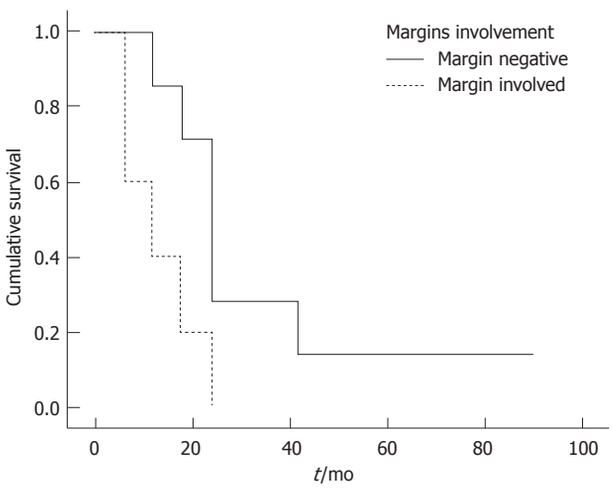

Figure 3 Cumulative survival curves after resection according to histologic margin clearance, *P* = 0.013.

Multivariate analysis showed that tumor location was the only independent predictor of long-term survival. Survival was significantly lower in patients with perihilar than in those with intrahepatic and distal tumors (HR = 0.016, 95% CI: 0.01-0.607; $P = 0.026$), in agreement with the findings of other series showing that patients with intrahepatic tumors had the best survival[17,2]. This could be explained by the fact that, compared with extrahepatic tumors, intrahepatic tumors are characterized by different epidemiology and tumor biology, younger age ($P < 0.05$), and lower rate of tumor negative margins[10,17].

For the majority of patients with cholangiocarcinoma who cannot undergo curative resection, palliative treatment to relieve jaundice, pruritus and cholangitis and to avoid liver failure becomes the priority. This can be achieved surgically *via* biliary-enteric bypass or stent placement *via* PTBD or ERCP[33]. We found however, that neither palliative surgical bypass nor biliary drainage had significant survival benefits, in agreement with previous findings by Prat *et al*[34] Both groups had a median survival of 3 mo.

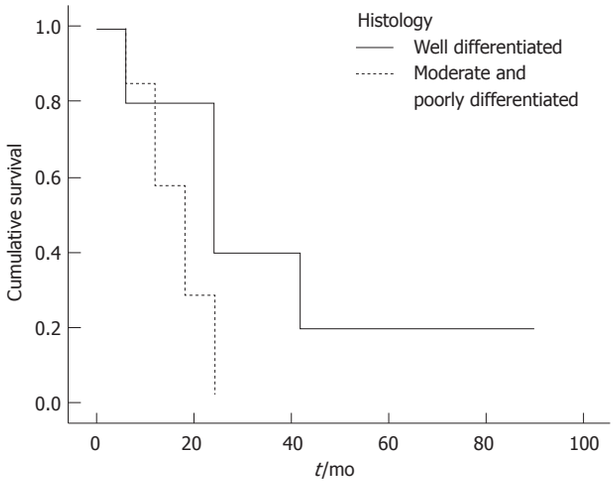

Figure 4 Overall survival according to degree of tumour differentiation, *P* = 0.032.

A novel palliative therapy for unresectable cholangiocarcinoma, photodynamic therapy (PDT), has shown promising benefits in terms of patient survival, cholestasis and quality of life[35]. PDT utilizes the intravenous photosensitizer sodium porfimer, which accumulates in tumor tissues. Upon illumination of the tumor bed by a specific endoscopic light, the porfimer becomes activated and forms oxygen free radicals, resulting in tumor necrosis[36].

The major limitation of our study was its involvement of patients at a single center. Therefore, our findings may not be representative of patients with cholangiocarcinoma throughout Malaysia.

The incidence of cholangiocarcinoma is increasing throughout Malaysia, as shown by the increase in the number of patients diagnosed per year throughout our study period. This may be due to an increased detection rate and to increases in referrals to our center. Curative surgical resection with clear histological margins of early stage well-differentiated intrahepatic tumors is associated with improved long-term survival. Further prospective randomized studies involving multiple centers are warranted.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Aqel Mohd Dhaher of Universiti Teknologi MARA and Dr. Azlan Darus of the University of Malaya for their assistance with the data analysis.

COMMENTS

Background

Cholangiocarcinoma is a rare malignant cancer of the bile duct with poor prognosis. This cancer is difficult to diagnose and often the patient presented late when curative surgical resection that can provide the chance for cure, is not feasible. Any person in his or her fifth decade with jaundice and significant

weight loss should raise a suspicion of this cancer. Despite its rarity, the incidence of this cancer has been steadily increasing worldwide.

Research frontiers

Many studies have concurred that early detection together with radical surgery will increase the survival period of these patients. A research article to be published in the *World Journal of Gastroenterology* has further emphasized the importance of early diagnosis and early referral to a specialized surgical centre with vast experience in the management of this cancer.

Innovations and breakthroughs

The authors have analysed various factors that affect the survival of their cholangiocarcinoma patients treated over a 10-year period. It was found that the survival period will improve if complete tumour excision is performed whereby the margins are tumour-free on histology. The outcome is also favourable if surgery is performed early when the abnormal cells are still at their early grade and have not spread elsewhere. Despite a range of palliative procedures available for inoperable cancer, none could surpass the result of a successful surgical treatment. These have further consolidated similar findings and recommendations of early aggressive surgery from other larger centres in the West and Far East.

Applications

The study also shows that cholangiocarcinoma is an important health problem in Malaysia. Although the increase in number of patient being treated may not truly reflect a true increase in incidence, this would mean more need to be done to address such problem. This study provides a framework for future studies in Malaysia and hopefully stimulates other groundbreaking research in this region especially concerning the epidemiology and pathophysiology of this fatal cancer.

Peer review

This 10-year retrospective review defines treatment modalities and survival statistics for cholangiocarcinoma in a Malaysian Referral Hospital. Not surprisingly, patients who underwent R-O resections had higher cumulative survival rates than patients palliated surgically, endoscopically or by percutaneous transhepatic biliary drainage. This manuscript is worthy of publication.

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S- Editor Sun H **L- Editor** Ma JY **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i5.466

World J Gastroenterol 2012 February 7; 18(5): 466-471
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Thalidomide-based multidisciplinary treatment for patients with advanced hepatocellular carcinoma: A retrospective analysis

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Received: March 23, 2011 Revised: May 27, 2011
Accepted: June 3, 2011
Published online: February 7, 2012

Abstract

AIM: To evaluate the efficacy of thalidomide in combination with other therapies to treat patients with advanced hepatocellular carcinoma (HCC).

METHODS: We performed a retrospective analysis of all patients with HCC who were treated with thalidomide for at least two months. The medical records of patients with HCC who were treated at our institution between April 2003 and March 2008 were reviewed. Image studies performed before and after treatment, tumor response, overall survival, and the decrease in α-fetoprotein (AFP) levels were evaluated.

RESULTS: A total of 53 patients with HCC received either 100 or 200 mg/d of thalidomide. The patient population consisted of 9 women and 44 men with a median age of 61 years. Thirty patients (56.6%) were classified as Child-Pugh A, and 12 patients (22.6%) were classified as Child-Pugh B. Twenty-six patients had portal vein thrombosis (49.1%), and 25 patients had extrahepatic metastasis (47.1%). The median duration of thalidomide treatment was 6.0 mo. Six of the 53 patients achieved a confirmed response (11.3%), one achieved a complete response (1.9%) and 5 achieved a partial response (9.4%). The disease control rate (CR + PR + SD) was 28.3% (95% CI: 17.8-42.4), and the median overall survival rate was 10.5 mo. The 1- and 2-year survival rates were 45% and 20%, respectively. Only one complete response patient showed an improved overall survival rate of 66.8 mo. Sixteen patients (30.2%) showed more than a 50% decrease in their serum AFP levels from baseline, indicating a better response rate (31.3%), disease control rate (43.8%), and overall survival time (20.7 mo). The therapy was well tolerated, and no significant toxicities were observed.

CONCLUSION: Thalidomide was found to be safe for advanced HCC patients, demonstrating anti-tumor activity including response, survival, and AFP decreases of greater than 50% from baseline.

Key words: Thalidomide; Hepatocellular carcinoma

Peer reviewer: Dr. Thomas Kietzmann, Professor, Department of Biochemistry, University of Oulu, Oulu 90014, Finland

Chen YY, Yen HH, Chou KC, Wu SS. Thalidomide-based multidisciplinary treatment for patients with advanced hepatocellular carcinoma: A retrospective analysis. *World J Gastroenterol* 2012; 18(5): 466-471 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i5/466.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i5.466

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer, the fifth most common malignancy worldwide (with over 700 000 new cases per year), and the third most common cause of cancer deaths[1]. In

Taiwan, HCC, which ranks second among the major types of cancer in the list of cancer-related mortalities, is responsible for approximately 7000 to 8000 deaths per year[2]. Unfortunately, most patients seek treatment when the disease is beyond curative treatment (surgery or percutaneous ablation), and palliative care is the only alternative. According to the Barcelona Clinic Liver Cancer (BCLC) staging classification[1] and treatment schedule, chemoembolization is the best option for intermediate-stage patients. However, for advanced-stage patients, no standard treatment was established until 2007. Systemic chemotherapy is generally ineffective and is associated with significant toxicity because hepatic function is often impaired by underlying cirrhosis that is often accompanied by hypersplenism and peripheral cytopenia[3]. Fortunately, after the positive results of the Study of Heart and Renal Protection (SHARP) trials[4], a new treatment, sorafenib, was approved for advanced-stage patients, which offers major improvements in overall survival and time to progression compared to placebo. There are many new modalities of treatment with more favorable therapeutic indices that are suitable for patients with advanced HCC. HCC is a hypervascular tumor that is one of the most antiangiogenic and angiogenesis-dependent tumors[5,6]. Consequently, it is reasonable to hypothesize that antiangiogenesis therapy may inhibit the growth of HCC. A number of antiangiogenic agents have been developed, including thalidomide, which is a glutamic acid derivative that was first described in 1953 when it was labeled as a sedative and anti-emetic agent. However, it was withdrawn from the European market 30 years ago because of its teratogenic effects[7]. Recently, oral thalidomide has been shown to inhibit basic fibroblast growth factor- and vascular endothelial growth factor-induced angiogenesis of cancer cells[8,9]. Studies published on the efficacy of thalidomide in advanced hepatocellular carcinoma have reported modest responses to therapy with acceptable toxicity[10-12]. Treatment of patients with HCC continues to present a major challenge. We retrospectively analyzed our records of HCC patients who received thalidomide in combination with other therapies to determine whether thalidomide was effective.

MATERIALS AND METHODS

Patients

Between April 2003 and March 2008, 53 patients with HCC were treated for at least two months with either 100 or 200 mg/d of thalidomide (50 mg/capsule, TTY Biopharm Co. Ltd., Taipei, Taiwan) at Changhua Christian Medical Center in Taiwan. HCC was diagnosed by histological examination and imaging findings. The diagnosis of HCC was confirmed by histological examination or the presence of all of the following criteria: (1) pathological diagnosis of HCC; (2) cirrhotic liver with a tumor size greater than 2 cm plus one dynamic image [computed tomography (CT) or magnetic resnane iamge (MRI)] or alpha fetoprotein (AFP) > 200 ng/mL; (3) cirrhotic liver with a tumor size of 1-2 cm plus two dynamic images (CT + MRI); and (4) non-cirrhotic liver greater than 2 cm plus one dynamic image (CT or MRI) and AFP > 200 ng/mL. The inclusion criteria were as follows: (1) advanced HCC (surgically unresectable); (2) failed previous local therapy, such as radiotherapy, hepatic arterial chemoembolization, radiofrequency ablation, or percutaneous interventional therapy; and (3) distant metastasis (lung, lymph node, or bone) that is not eligible for curative surgery and radiotherapy or locoregional therapy failure [e.g., transarterial chemoembolization (TACE), recurrence-free interval or percutaneous ethanol injection (PEI)]. All patients had bidimensionally measurable disease that was staged by the pathological tumor-node-metastasis (TNM) system, the Okuda system, and the BCLC parameters for HCC. The demographic data, details of the primary tumors, serum AFP levels, dates of recurrence, length of survival, and last follow-up dates were analyzed retrospectively. The responses to thalidomide were determined by CT performed according to the Response Evaluation Criteria in Solid Tumor Guidelines[13], and the AFP levels were also analyzed before and after thalidomide treatment. Overall survival was calculated from the date of the start of chemotherapy and analyzed by the Kaplan-Meier method. Follow-up data were obtained for all patients until the time of their death or the last follow-up.

RESULTS

Patient and demographic characteristics

A total of 53 patients with HCC were available for analysis, and their demographic characteristics are shown in Table 1. The patient population included 9 females (17.0%) and 44 males (83.0%) with a median age of 61 years (range, 29-88 years). Of the 53 patients, 10 had not received prior treatment or therapy. Pretreatment curative surgery had been performed on 12 patients (22.6%), transarterial embolization on 13 patients (24.5%), TACE on 16 patients (30.2%), radio frequency ablation on 10 patients (18.9%), and radiotherapy (RT) on 10 patients (18.9%). Twenty-six patients had portal vein thrombosis (49.1%), and 25 patients had extrahepatic metastasis (47.2%). The prevalence of hepatitis B was 56.5% (30/53), that of hepatitis C was 37.7% (20/53), and that of concomitant hepatitis was 1.9% (1/53). Of the 53 patients, most patients had TNM stage Ⅳ (45.3%), Okuda stage I (51.9%), and BCLC stage C (71.2%). There were 22 patients (41.5%) whose serum AFP levels were greater than 400 ng/mL above the baseline. The liver functions of the majority of patients were classified as Child-Pugh A (56.6%), and the median duration of treatment was 6.0 mo (range, 1.5-53.9 mo) (Table 1).

Efficacy

Of the 53 patients, one had a complete response (CR, 2.9%) to thalidomide, five had a partial response (PR, 9.4%) and nine were classified as stable disease (SD,

Table 1 The clinical characteristics of 53 patients with hepatocellular carcinoma

Characteristic	*n* (%)
Age, yr (median)	61 (range, 29-88)
Sex	
Male	44 (83.0)
Female	9 (17.0)
Type of hepatitis	
Hepatitis B	30 (56.6)
Hepatitis C	20 (37.7)
Hepatitis B + C	1 (1.9)
Child-Pugh classification	
A	30 (56.6)
B	12 (22.6)
C	1 (1.8)
TNM stage	
Ⅰ	0 (0)
Ⅱ	6 (11.2)
ⅢA	11 (20.8)
ⅢB	3 (5.7)
ⅢC	6 (11.2)
Ⅳ	24 (45.3)
Okuda stage	
Ⅰ	27 (51.9)
Ⅱ	16 (30.8)
Ⅲ	1 (1.9)
BCLC stage	
A	2 (3.9)
B	7 (13.5)
C	37 (71.2)
D	1 (1.9)
Extrahepatic metastasis	
Yes	25 (47.2)
No	28 (52.8)
Portal vein thrombosis	
Yes	26 (49.1)
No	26 (49.1)
Unknown	1 (1.8%)
Site of extrahepatic metastasis	
Lung	11 (21.2)
Bone	6 (11.5)
Brain	1 (1.9)
Others	7 (13.5)
Prior therapy	
Surgery	12 (22.6)
TACE	16 (30.2)
TAE	13 (24.5)
Radiation therapy	10 (18.9)
RFA	10 (18.9)
No therapy	10 (18.9)
Duration of treatment, mo	
Median	6.0 (range, 1.5-53.9)
AFP level	
> 400 ng/mL	31 (58.5)
< 400 ng/mL	22 (41.5)

BCLC: Barcelona Clinic Liver Cancer; TACE: Transarterial chemoembolization; TAE: Transarterial embolization; RFA: Radiofrequency ablation; AFP: Alpha fetoprotein; TNM: Tumor node metastasis.

17.0%). The remaining 38 patients had disease that continued to progress after the thalidomide treatments. The objective response rate was 11.3% (95% CI: 4.3-23.0), and the disease control rate (CR + PR + SD) was 28.3% (95% CI: 17.8-42.4). The median overall survival rate was 10.5 mo (95% CI: 6.9-23.3). The 1- and 2-year survival rates were 45% and 20%, respectively. Sixteen patients (30.2%) showed more than a 50% decrease in their serum AFP levels below the baseline and showed a better response rate (31.3%), disease control rate (43.8%), and overall survival time (20.7 mo) (Table 2, Figure 1). The prognostic factors for the response rate, disease control rate, and overall survival in HCC patients receiving thalidomide are listed in Table 3. Multivariate analysis showed that almost all of these patients qualified as having independent prognostic factors for the efficacy analysis. The only significant difference in the efficacy activity was an AFP decrease of > 50% after treatment. The median overall survival time of the patients who registered > 50% AFP decrease was 20.7 mo, with a response rate of 31.3% and a disease control rate of 43.8%. The median overall survival time of those patients with a < 50% AFP decrease was 7.1 mo, with a response rate of 2.7% and a disease control rate of 21.6% (Table 3, Figure 1). Table 4 is a comparison of the patients who responded and the patients whose disease progressed. Patients in the CR + PR + SD group had a significantly longer survival time (33.3 mo) than those in the progressive disease (PD) group (6.9 mo, $P < 0.003$) (Figure 1).

Table 2 Efficacy results of thalidomide

Overall objective response, *n* = 53, (%)	
CR	1 (2.9)
PR	5 (9.4)
SD	9 (17.0)
PD	38 (44.1)
Objective response rate	6 (11.3), 95% CI: 4.3-23.0
Disease control rate	15 (28.3), 95% CI: 17.8-42.4
Overall survival, mo	
Median	10.5, 95% CI: 6.9-23.3
1-year survival	(45)
2-year survival	(20)
A decrease in AFP > 50% after treatment	
Yes	16 (30.2)
No	37 (69.8)

CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; AFP: Alpha fetoprotein.

DISCUSSION

Thalidomide has been used in the treatment of advanced HCC patients. Hsu *et al*[10] reported an overall response rate of 6.3% with an overall survival time of 18.7 wk when an escalating dose (100-600 mg/d) of thalidomide was used for the treatment of advanced HCC. Patt *et al*[14] also showed a 5% overall response rate with a 6.8-mo overall survival time when a high dose (400-1000 mg/d) of thalidomide was used. In a phase Ⅱ study[12], high-dose (200-800 mg/d) single-agent thalidomide demonstrated a response rate of 3.9% with an overall survival time of 123 d. The first retrospective study to analyze the efficacy and tolerability of fixed low-dose thalidomide in the treatment of advanced HCC patients[15]

Table 3 Prognostic factors for efficacy analysis in hepatocellular carcinoma patients receiving thalidomide

Variables		*P* value
Overall response rate *n* (%)		
Child-Pugh classification		
A	3/30 (10.0)	1.000[1]
B and C	3/23 (13.0)	
Okuda staging		
Stage 1	2/27 (7.4)	0.344[1]
Stage 2	3/16 (18.8)	
AFP level		
> 400 ng/mL	1/31 (3.2)	0.071[1]
< 400 ng/mL	5/22 (22.7)	
A decrease in AFP > 50% after treatment		
Yes	5/16 (31.3)	0.007[1]
No	1/37 (2.7)	
Disease control rate *n* (%)		
Child-Pugh Classification		
A	6/30 (20.0)	0.218[1]
B and C	9/23 (39.1)	
Okuda staging		
Stage 1	6/27 (22.2)	0.719[1]
Stage 2	5/16 (31.3)	
AFP level		
> 400 ng/mL	7/31 (22.6)	0.357[1]
< 400 ng/mL	8/22 (36.4)	
A decrease in AFP > 50% after treatment		
Yes	7/16 (43.8)	0.071[1]
No	8/37 (21.6)	
Overall survival, mo		
Child-Pugh Classification		
A	8.8	0.922[2]
B and C	10.8	
Okuda staging		
Stage 1	22.2	0.075[2]
Stage 2	6.9	
AFP level		
> 400 ng/mL	10.8	0.679[2]
< 400 ng/mL	6.5	
A decrease in AFP > 50% after treatment		
Yes	20.7 (95% CI: 1.7-NA)	0.307[2]
No	7.1 (95% CI: 6.3-24.3)	

[1]*P* value was calculated by Fisher's exact test; [2]*P* value was calculated by Log-rank test. AFP: Alpha fetoprotein; NA: Not assessable.

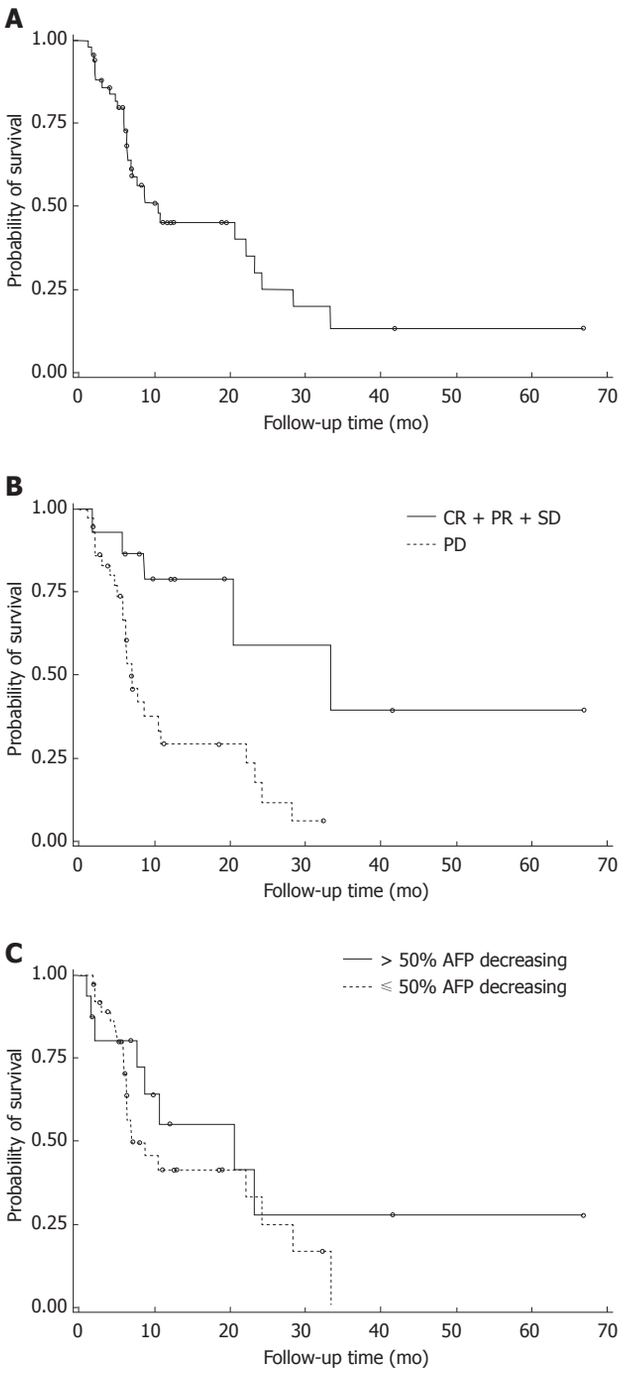

Figure 1 Kaplan-Meier analysis of the survival time in all advanced hepatocellular carcinoma patients (A), in the subgroup of disease stabilization (B), and in the subgroup of > 50% decrease in alpha fetoprotein (C). CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; AFP: Alpha fetoprotein.

showed that low-dose thalidomide has a comparable single-agent activity (response rate of 5%, with an overall survival time of 4.3 mo) but fewer treatment-related toxicities than high-dose thalidomide when treating advanced HCC patients. Patients treated with low-dose thalidomide have similar overall survival times compared to patients treated with chemotherapeutic agents, with a far better toxicity profile and less hematological toxicity (no grade 3/4 neutropenia or thrombocytopenia)[15,16]. The largest randomized phase Ⅲ trial for HCC (the SHARP trial) showed better progression free survival and overall survival times with sorafenib than with placebo[4]. The primary drug-related adverse events were dermatological (constitutional and hand-foot skin reactions) and gastrointestinal[4,17]. The toxicity of sorafenib is a serious problem because approximately 50% of the patients had to interrupt or stop their treatment because of sorafenib-induced toxicity. The tolerance of low-dose thalidomide in HCC patients may be worth further investigation.

The treatment of hepatoma with thalidomide appears to be feasible. A complete response was rare with thalidomide treatment of HCC; the PR rate was 5%-10%, and the SD rate was approximately 37%[10,12,14], depending on the duration of observation, cancer stage, and the definition of stability. In our study, one patient had complete remission; the PR rate was 9.4%, and the SD rate was 17%. One CR patient received thalidomide alone after a TACE therapy failure; the duration of the treatment

Table 4 Comparison of patients who responded and patients with progressive disease

Characteristic	CR	PR	SD	PD	CR + PR + SD	*P* value[a]
AFP level						0.357
> 400 ng/mL	0 (0)	1 (3.2)	6 (19.4)	24 (77.4)	7 (22.6)	
< 400 ng/mL	1 (4.6)	4 (18.2)	3 (13.6)	14 (63.6)	8 (36.4)	
A decrease in AFP > 50% after treatment	1 (6.3)	4 (25.0)	2 (12.5)	9 (56.3)	7 (43.8)	0.182
Overall survival, mo	66.8	NA	20.7 (95% CI: 20.7-33.3)	6.9 (95% CI: 6.3-10.8)	33.3 (95% CI: 20.7-NA)	0.003[b]

AFP: Alpha fetoprotein; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; NA: Not assessable. [a]$P < 0.05$ between the two groups (CR + PR + SD *vs* PD); [b]$P = 0.0003$ (less than 0.005) between progress disease and CR + PR + SD.

was 53.9 mo, the patient had no recurrence, and he is still alive (66.8 mo post-treatment). The most interesting finding was the AFP decrease from 11 005.3 ng/mL at diagnosis to < 20 ng/mL (Table 4). Of the 5 patients with partial responses, 2 had prior TACE treatments, 2 had RT, 1 had PEI and 1 had systemic therapy. The median survival time among these patients was 502 d (range, 248-1263 d), and 3 of them are still alive. The median survival time of patients with stable disease was 412 d (range, 60-1013 d).

Patients in the CR + PR + SD group had a significantly longer survival time (33.3 mo) than those in the PD group (6.9 mo, $P < 0.003$). Thalidomide may offer HCC stabilization and prolong survival, especially in patients with stabilization. Survival time should be the focus of future clinical trials of thalidomide therapy. In this study, we evaluated the clinical implication of the AFP tumor marker response in assessing the therapeutic effects of thalidomide in HCC. The results showed that the AFP response was an independent prognostic factor for the response rate, disease control rate, and overall survival time. We also identified patients with more than or less than a 50% decrease in serum AFP levels from the baseline, which made a significant difference in their response rates (31.3% *vs* 2.7%, $P = 0.007$). There was a better trend in the disease control rate (43.8%) and overall survival time (20.7 mo) when there was greater than a 50% AFP decrease (Table 3). The AFP response may correlate with the biological response and, consequently, predict the survival benefits of thalidomide in HCC.

In conclusion, thalidomide has shown modest clinical activity, including response and survival, and was safely administered to patients with advanced HCC. Because the present study is retrospective in nature with a relatively small number of patients, a larger, randomized phase Ⅱ/Ⅲ study is needed to clearly define the role of single-agent thalidomide in the treatment of HCC as an alternative to the expensive molecular-targeted therapies.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer, the fifth most common malignancy worldwide (with over 700 000 new cases per year), and the third most common cause of cancer deaths. However, for advanced-stage patients, no standard treatment was established until the positive result of the Study of Heart and Renal Protection study. However, there are many new modalities of treatment with more favorable therapeutic indices that are suitable for patients with advanced HCC.

Research frontiers

HCC is a hypervascular tumor that is one of the most antiangiogenic and angiogenesis-dependent tumors. Recently, thalidomide was shown to inhibit the angiogenesis of cancer cells and studies have reported modest responses to this therapy in advanced HCC. The authors retrospectively analyzed the records of HCC patients who received thalidomide in combination with other therapies to determine whether thalidomide was effective.

Innovations and breakthroughs

Studies published on the efficacy of thalidomide in advanced hepatocellular carcinoma have reported modest responses to the therapy with acceptable toxicity. Some of them highlighted the alpha fetoprotein (AFP) tumor marker response in assessing the therapeutic effects of thalidomide in HCC. In this study, the authors concluded thalidomide showed modest clinical activity, including response and survival, and was safely administered to patients with advanced HCC. Furthermore, they also identified patients with more than or less than a 50% decrease in serum AFP levels from the baseline, which made a significant difference in their response rates.

Applications

The results showed that the AFP response was an independent prognostic factor for the response rate, disease control rate, and overall survival time. The AFP response may correlate with the biological response and, consequently, predict the survival benefits of thalidomide in HCC.

Peer review

This is an interesting and well written manuscript summarising the effects of thalidomide on HCC patients in a retrospective study.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i5.472

World J Gastroenterol 2012 February 7; 18(5): 472-478
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

A pharmacodynamic model of portal hypertension in isolated perfused rat liver

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Supported by The Major State Creative New Drug Project, No. 2009ZX09502-017; Education Ministry Science Foundation of China, No. 108019
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Received: April 22, 2011 Revised: June 30, 2011
Accepted: July 7, 2011
Published online: February 7, 2012

Abstract

AIM: To develop a pharmacodynamic model of portal hypertension from chronic hepatitis.

METHODS: Pathological changes and collagen depositions were analyzed using morphometry to confirm CCl_4-induced chronic hepatitis. At d_0, d_{28}, d_{56} and d_{84} of the process, the portal perfused velocities (μL/min) in isolated rat livers were exactly controlled with a quantified pump. The pressure (mmHg) was monitored with a Physiological System. The geometric concentrations of phenylephrine or acetylcholine were added to a fixed volume (300 mL) of the circulating perfusate. The equation, the median effective concentration and its 95% confidence intervals of phenylephrine or acetylcholine were regressed with Prism-4 software in non-linear fit and various slopes. In the isolated perfused rat livers with chronic hepatitis, both median effective concentrations were defined as the pharmacodynamic model of portal hypertension.

RESULTS: At d_0, d_{28}, d_{56} and d_{84}, the equations of portal pressure potency from the concentrations of phenylephrine used to constrict the portal vein in isolated perfused rat livers were $Y = 0.1732 + 0.3970/[1 + 10^{(-4.3061-0.4407 X)}]$, $Y = -0.004934 + 0.12113/[1 + 10^{(-3.1247-0.3262 X)}]$, $Y = 0.0104 + 0.2643/[1 + 10^{(-8.8462-0.9579 X)}]$, and $Y = 0.01603 + 0.12107/[1 + 10^{(-5.1134-0.563 X)}]$; the median effective concentrations were 1.69×10^{-10} mol/L, 2.64×10^{-10} mol/L, 5.82×10^{-10} mol/L, and 8.24×10^{-10} mol/L, respectively. The equations from the concentrations of acetylcholine used to relax the portal vein were $Y = -0.4548 + 0.3274/[1 + 10^{(6.1538 + 0.5554 X)}]$, $Y = -0.05391 + 0.06424/[1 + 10^{(3.8541 + 0.3469 X)}]$, $Y = -0.2733 + 0.22978/[1 + 10^{(3.0472 + 0.3008 X)}]$, and $Y = -0.0559 + 0.053178/[1 + 10^{(5.6336 + 0.5883 X)}]$; the median effective concentrations were 8.40×10^{-10} mol/L, 7.73×10^{-12} mol/L, 5.98×10^{-11} mol/L, and 2.66×10^{-10} mol/L, respectively.

CONCLUSION: A pharmacodynamic model of portal hypertension in isolated perfused rat livers with chronic hepatitis was defined as the median effective concentrations of phenylephrine and acetylcholine.

Key words: Chronic hepatitis; Isolated portal perfused rat liver; Pharmacodynamic model; Portal hypertension

Peer reviewers: Dr. Orhan Sezgin, Professor, Gastroenteroloji Bilim Dalı, Mersin Üniversitesi Tıp Fakültesi, Mersin 33190, Turkey; Yoshiaki Iwasaki, MD, PhD, Associate Professor, Health Service Center, Okayama University, 2-1-1, Tsushima-Naka, Kita-ku, Okayama 700-8530, Japan

Zhang T, Xu XY, Zhou H, Zhao X, Song M, Zhang TT, Yin H, Li T, Li PT, Cai DY. A pharmacodynamic model of portal hypertension in isolated perfused rat liver. *World J Gastroenterol* 2012; 18(5): 472-478 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i5/472.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i5.472

INTRODUCTION

Patients with portal hypertension have significant mortality[1]. A lack of drugs[2] to treat this disease is derived from the failure to use the reversible mechanisms in its pathogenesis[3]. Being similar to amiloride, a candidate drug for portal hypertension[4], molecules from medical plants have been demonstrated to affect portal hypertension in rats[5-7] and in patients[8] with chronic hepatitis. The effect of these molecules on relaxation of the extrahepatic portal rings did not account for the efficacy of these therapies *in vivo*[9]. A novel mode of portal perfusion has been characterized with both controlled velocity and monitored pressure in the isolated portal perfused rat liver (IPPRL)[10-12]. With the primary velocity and preload at the various advanced stages of CCl_4-induced chronic hepatitis in rats, constriction with phenylephrine (PE) and relaxation with acetylcholine (Ach) were more sensitive than those reported previously[13,14]. With standardization of the IPPRL[15], both median effective concentrations of Ach and PE were defined as the pharmacodynamic model of portal hypertension. Both the controlled velocity and monitored pressure made the model sensitive enough for the basis of systems biology in portal regulation[16].

MATERIALS AND METHODS

Materials

Thirty two healthy male Wistar rats weighing 200-220 g were supplied by the Animal Center of the Chinese Academy of Medical Sciences. Standard rodent pellets for rats were prepared by Beijing Scientific Animal Feedstuff Company. The study was approved by the Animal Study Committee of the Chinese Academy of Medical Sciences. All experimental procedures were performed in accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics, National Health Department of China. All rats were maintained in a temperature-controlled room (25.0 ℃ ± 0.2 ℃) in the SPF laboratory, with a 12-h/12-h light/dark photoperiod and 45% ± 2% humidity. The rats were fed standard rodent pellets and allowed free access to tap water throughout the experiment.

Carbon tetrachloride (CCl_4, MW 153.84, CAS 56-23-5), Olive oil (CAS 8001-25-0) and Heparin sodium (MW 12 000, CAS 9041-08-1) were purchased from Sinopharm Chemical Reagent Company to induce chronic hepatic hepatitis or for anticoagulation.

As the perfusate in portal perfusion, Krebs-Henseleit solution consisted of KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaCl 118, and Glucose 11.0 mmol/L in the final concentration at pH 7.35-7.45 equilibrated with 95% O_2-5% CO_2 and warmed to 37.8 ℃ before being added to the livers.

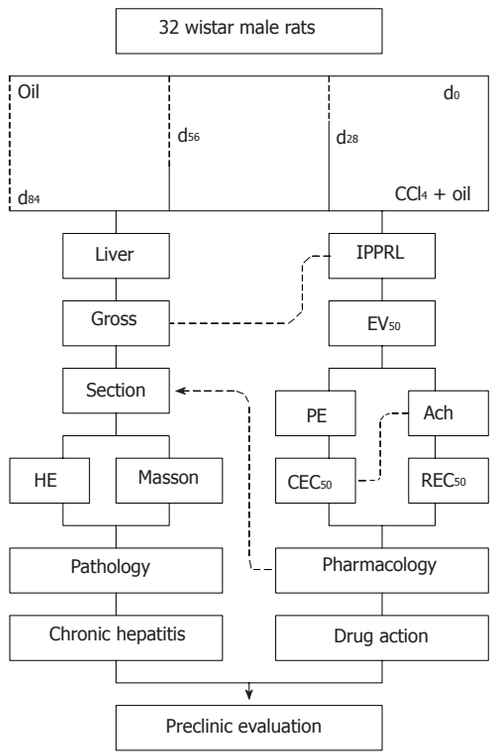

Figure 1 Group design of pharmacological model of portal hypertension. Oil indicated was administered subcutaneously with 3 mL/kg olive oil twice weekly for 84 d, as was CCl_4 + Oil 40% (v/v) CCl_4 in olive oil; IPPRL: Isolated portal perfused rat livers; EV_{50}: Median effective velocity; PE: Phenylephrine; Ach: Acetylcholine; CEC_{50}: Median effective concentration of PE constriction; REC_{50}: Median effective concentration of Ach relaxation; HE: Hematoxylin and eosin.

Phenylephrine hydrochloride (PE, MW 203.67, CAS 61-76-7) and Acetylcholine chloride (Ach, MW 181.67, CAS 60-31-1) were purchased from Sigma (United States) as the α_1-adrenoceptor and M_3-cholinoceptor agonist, respectively, used to elevate or reduce portal pressure.

Experimental design

Male Wistar rats were randomly divided into four groups. In four rats, PE was used to constrict the portal vein and in the other rats Ach was used to relax the portal vein in each group. Group 1 was the vehicle control without CCl_4 In this group, rats were subcutaneously administered 3 mL/kg olive oil twice weekly for 84 d. Groups 2, 3 and 4 were model groups with CCl_4-induced chronic hepatitis, the rats in these groups were subcutaneously administered the same volume of a mixture of 40% (v/v) CCl_4 in olive oil twice weekly for 28 d, 56 d and 84 d, beginning at d_{58}, d_{28}, and d_0, respectively (Figure 1). Forty-eight hours after the last CCl_4 injection, rats were anesthetized with 50 mg/kg pentobarbital sodium subcutaneously; a midline incision was made to expose the liver and its vessels. The hepatic artery, portal vein and hepatic vein were canalized. The remaining blood in the IPPRLs

Table 1 Hepatic pathological changes in model rats (mean ± SE, n_1 = 8)

Advanced	Lobule ratio	Collagen ratio
d_0	0.38 ± 0.05	0.0000700 ± 0.0001180
d_{28}	0.33 ± 0.04[b]	0.0019658 ± 0.0024864[b]
d_{56}	0.11 ± 0.04[b,d]	0.0043315 ± 0.0048768[b,d]
d_{84}	0.06 ± 0.01[b,d,f]	0.0143996 ± 0.0143860[b,d,f]

Lobule ratio is the average percentage obtained from ten random fields using Image-Pro Plus v 5.1 software, and each field had a ratio of lobule area per total analyzed field area in hematoxylin-eosin stained sections under a Digital Pathology System at × 20 magnification from isolated portal perfused rat livers. Collagen ratio is the average percentage obtained from ten random fields using Image-Pro Plus v 5.1 software, and each field had a ratio of collagen area per total analyzed field area in Masson-stained sections under a Digital Pathology System at × 40 magnification from isolated portal perfused rat livers. a and b denote significant ($^bP < 0.01$) differences between those at d_0 and at d_{28}, d_{56}, or d_{84}. c and d denote significant ($^cP < 0.05$ and $^dP < 0.01$) differences at d_{28} and at d_{56}, or d_{84}. e and f denote significant ($^eP < 0.05$ and $^fP < 0.01$) differences at d_{56} and at d_{84}.

was eliminated using Krebs-Henseleit perfusate through the hepatic artery. When portal perfusion was complete, a small portion of the liver was removed for pathological examination following fixation with 40 g/L formaldehyde solution and subsequent embedding in paraffin.

Protocol for portal perfusion

When CCl_4-induced chronic hepatitis was complete, eight rats from each group were randomized into two subgroups, one for PE constriction and the other for Ach relaxation of the portal vein. Each IPPRL was instrumented for portal pressure measurement.

Each IPPRL was perfused in a recirculation at a fixed temperature of 37.8 ℃ and equilibrated with 95% O_2-5% CO_2 mixed gas (Beijing Specialized Mixed Gas Institute), portal velocity was precisely controlled by a quantified BTO_1 pump (Beijing Yidaxk Technical Company), and 3935.50, 4720.63, 4753.35, and 5164.16 (μL/min) at d_0, d_{28}, d_{56}, and d_{84}, were chosen, respectively, the equation of portal perfusion median velocity (Y) from the day (x) of chronic hepatitis was Y = 13.28x + 4085 (r = 0.935, P < 0.01)[12].

The portal pressure of perfusion (mmHg) was continuously monitored and recorded with a strain-gauge transducer connected to the portal inflow cannula 6 cm proximal to the perfusion cannula with BL-420S Physiological Systems (Taimeng Instruments, Chengdu) according to a previously published method[10-14]. The global viability of livers was assessed by gross appearance and perfusate stable pH.

Pharmacodynamic actions

Perfusions were performed in the recirculating system containing 300 mL Krebs-Henseleit solution. Each preparation was allowed to stabilize for 15 min. The flow rate during each individual perfusion was maintained at a constant rate equalized to the portal perfusion median velocity at d_0, d_{28}, d_{56}, and d_{84}, respectively, the average portal pressure during this condition had been designated as the baseline. With a fixed volume of the recirculating perfusate in the portal perfusion system, cumulative geometric concentrations of PE (10^{-12}-10^{-6} mol/L) were added to elevate portal pressure.

After the median effective concentration of PE to constrict the portal vein was added, cumulative geometric concentrations of Ach (10^{-13}-10^{-7} mol/L) were added to reduce portal pressure.

Concentration-response curves were obtained following the addition of PE and Ach, and the changes in intrahepatic resistance expressed as the percentage increase or decrease in perfusion pressure from baseline in the various portal perfused velocities were obtained.

Pathological changes due to chronic hepatitis

To observe pathological changes after portal perfusion, a portion of the left liver lobe (40 mg) from each liver was fixed in 40 g/L formaldehyde solution for 48 h, embedded in paraffin, sectioned (6 μm), and stained with hematoxylin-eosin and Masson according to standard procedures.

Images were acquired with a Nano Zoomer Digital Pathology system (Hamamatsu, Japan), at a low magnification (× 20); all the compartments of the liver were analyzed. At high magnification (× 40), the collagen density in the liver section was quantified using a computerized image analysis system (Image-Pro Plus v 5.1). The density of collagen in blinded specimens was expressed as a percentage (the ratio of collagen area per total analyzed field area). The average of the score taken from ten random fields was used to generate a single score for each IPPRL.

Statistical analysis

All primary data are presented as means ± SE for each dosage in each group. Statistical significance was calculated using Student's t test between groups, P < 0.05 was significant. (1) Dose-effect relationship: The equation, the median effective concentration and its 95% confidence intervals of PE or Ach were calculated by regression analysis using Graph-Pad Prism 4 in non-linear fit and various slopes, to express the dose-effect relationship; and (2) Time-effect relationship: The median effective concentrations of PE or Ach were calculated by linear regression analysis with the duration (0 d, 28 d, 56 d, and 84 d) of chronic hepatitis, to express the time-effect relationship of pathological conditions affecting the portal response to both molecules.

RESULTS

Pathological changes due to chronic hepatitis

Lobule or pseudo-lobule ratio: Hepatic tissues were clear in hematoxylin and eosin-stained sections. When compared with those in control rats at d_0, the lobule ratios (Table 1) in the model rats at d_{28}, d_{56} and d_{84} were significantly decreased by 4.04%, 70.22%, and 83.82%, respectively (P < 0.01). When compared with those at d_{28}, the pseudo-lobule ratios in the model rats at d_{56} and d_{84} were significantly decreased by 65.35% and 81.00%, respectively (P < 0.01). In addition,

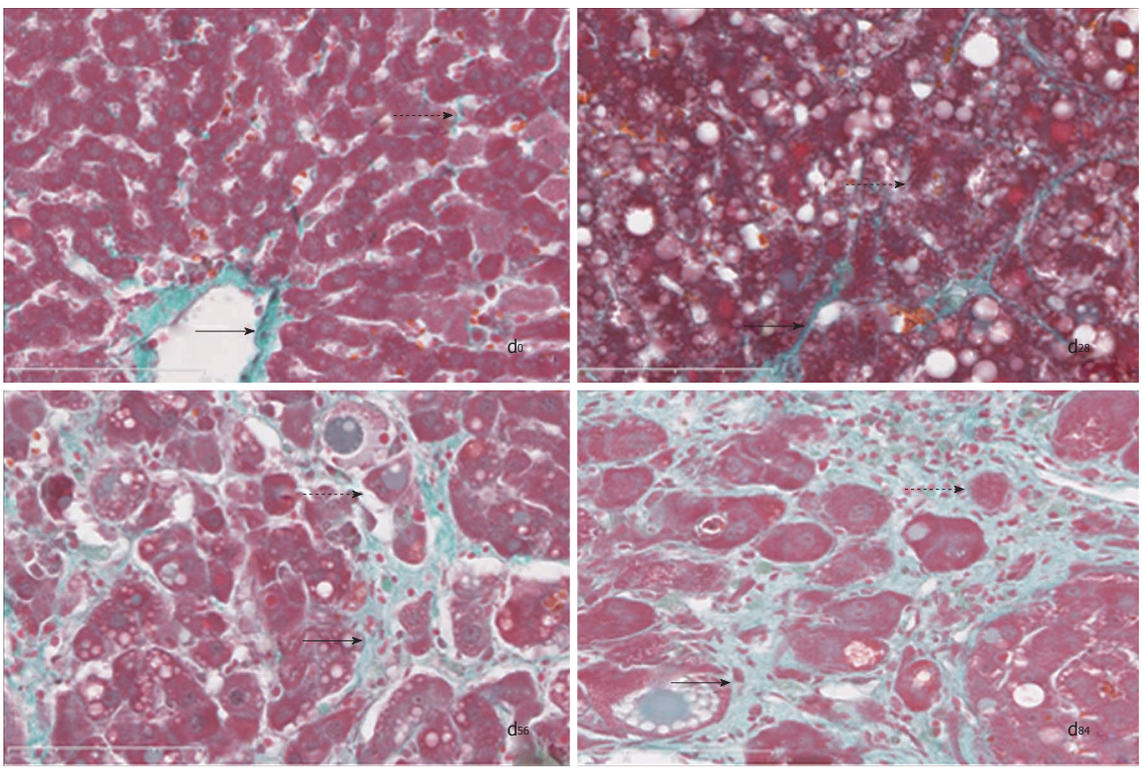

Figure 2 Collagen distributions in rat model livers (Masson × 40). Normal hepatic structure (d_0) was demonstrated in control rats administered 3 mL/kg olive oil subcutaneously twice weekly for 84 d. Hepatic degeneration (d_{28}), fibrosis (d_{56}) and cirrhosis (d_{84}) were seen in the chronic hepatitis model rats administered 3 mL/kg 40% (v/v) CCl_4 in olive oil subcutaneously twice weekly for 28 d, 56 d, and 84 d, respectively. There was less collagen mainly around the central veins (solid arrow) and the portal areas, with some staining noise signals in lobules (dashed arrow) in normal hepatic structure (d_0). The collagen fiber bundles (solid arrow) had partly spread into the lobules from the portal areas, the hepatic cells had obvious watery and fatty degeneration (dashed arrow) and had not been isolated by collagen during hepatic degeneration (d_{28}). The collagen fiber bundles (solid arrow) had completely separated some of the lobules to form many pseudo-lobules, several hepatic cells with obvious fatty degeneration (dashed arrow) had been completely isolated by collagen in hepatic fibrosis (d_{56}). The collagen fiber bundles (solid arrow) had limited smaller pseudo-lobules, some single hepatic cells (dashed arrow) were completely isolated by collagen in hepatic cirrhosis (d_{84}).

these ratios were significantly decreased by 45.67% at d_{84} compared with those at d_{56} ($P < 0.01$).

Hepatic collagen distribution: Hepatic histological changes in Masson-stained sections showed collagen depositions along with CCl_4-induced chronic hepatitis (Figure 2). (1) Normal structure at d_0: The histological structure in control rats showed normal hepatic architecture with some fatty degeneration and less collagen located at the lobules; (2) Degeneration at d_{28}: The pathological changes in the model rats at d_{28} showed mainly hepatic fatty degeneration and cellular swelling, collagen was deposited around the center veins, thus the enlarged hepatic cords severely narrowed the hepatic sinusoid; (3) Hepatic fibrosis at d_{56}: The pathological changes in the model rats at d_{56} showed more collagen deposited in the lobules, thus the enlarged hepatic cords led to significant widening of the hepatic sinusoid; and the collagen in interlobular area extended into the lobules, some separating the lobules completely, therefore the direction of the circulating blood did not change in the hepatic sinusoid of the lobules; and (4) Hepatic cirrhosis at d_{84}: The pathological changes in the model rats at d_{84} showed extensive collagen deposited in the lobules, which were all pseudo-lobules instead of normal lobules, thus the direction of the circulating blood had completely changed in the hepatic sinusoid.

Deposited collagen ratio: Compared with the control rats (Table 1), the collagen ratio in the model rats at 28 d, 56 d and 84 d was significantly increased by 2707.65%, 60 860.51%, and 20 466.49%, respectively ($P < 0.01$). Compared with the model rats at 28 d, the collagen ratio in the model rats at 56 d and 84 d was significantly increased by 120.34% and 632.52%, respectively ($P < 0.01$). The collagen ratio in the model rats at 84 d increased by 232.44% compared to that at 56 d ($P < 0.01$).

Pharmacodynamic actions on the portal vein

Phenylephrine elevated portal pressure: Geometric concentrations of PE to activate the α_1 receptor were added to the recirculating perfusate to elevate perfused portal pressure (Table 2 and Figure 3). The equation, the median effective concentration of PE and its 95% confidence intervals were regressed: (1) dose-effect at d_0: The data showed that the equation of PE was $Y = 0.1732 + 0.3970/[1 + 10^{(-4.3061-0.4407\ x)}]$ ($r = 0.9701$, $P < 0.01$); the median effective concentration with its 95% confidence intervals was 1.69×10^{-10} (4.9769×10^{-12} - 5.7599×10^{-9}) mol/L; (2) dose-effect at d_{28}: The data showed that the equation of PE was $Y = -0.004934 + 0.121134/[1 +$

Table 2 Phenylephrine elevates portal pressure (mean ± SE, n_i = 4)

Log [PE (mol/L)]	d_0	d_{28}	d_{56}	d_{84}
-12	0.210 ± 0.19	0.013 ± 0.02	0.013 ± 0.02	0.019 ± 0.03
-11	0.260 ± 0.16	0.025 ± 0.03	0.017 ± 0.02	0.024 ± 0.04
-10	0.360 ± 0.18	0.044 ± 0.06	0.046 ± 0.06	0.047 ± 0.07
-9	0.420 ± 0.24	0.072 ± 0.09	0.182 ± 0.25	0.076 ± 0.09
-8	0.550 ± 0.37	0.090 ± 0.12	0.247 ± 0.27	0.119 ± 0.10
-7	0.520 ± 0.37	0.093 ± 0.12	0.269 ± 0.27	0.123 ± 0.11
-6	0.570 ± 0.24	0.113 ± 0.13	0.286 ± 0.28	0.138 ± 0.12

Primary data were used to calculate the dose-effect relationship between phenylephrine and portal vein constriction in the isolated portal perfused rat livers without chronic hepatitis, where rats were administered 3 mL/kg olive oil subcutaneously twice per week for 84 d (control rats) (d_0). Rats with chronic hepatitis at three stages, hepatic degeneration (d_{28}), fibrosis (d_{56}) and cirrhosis (d_{84}), were administered 3 mL/kg 40% (v/v) CCl_4 in olive oil subcutaneously twice per week for 28 d, 56 d and 84 d, respectively. PE: Phenylephrine.

Table 3 Acetylcholine reduces portal pressure (mean ± SE, n_i = 4)

Log [Ach (mol/L)]	d_0	d_{28}	d_{56}	d_{84}
-13	-0.16 ± 0.12	-0.002 ± 0.01	-0.076 ± 0.08	-0.001 ± 0.01
-12	-0.19 ± 0.10	-0.009 ± 0.01	-0.091 ± 0.10	-0.006 ± 0.01
-11	-0.31 ± 0.07	-0.025 ± 0.01	-0.125 ± 0.15	-0.012 ± 0.01
-10	-0.39 ± 0.08	-0.035 ± 0.18	-0.176 ± 0.23	-0.019 ± 0.01
-9	-0.42 ± 0.08	-0.043 ± 0.04	-0.203 ± 0.26	-0.041 ± 0.02
-8	-0.44 ± 0.12	-0.049 ± 0.05	-0.225 ± 0.26	-0.050 ± 0.03
-7	-0.47 ± 0.14	-0.052 ± 0.07	-0.256 ± 0.28	-0.054 ± 0.03

Primary data were used to calculate the dose-effect relationship between acetylcholine and portal vein relaxation in the isolated portal perfused rat livers without chronic hepatitis, where rats were administered 3 mL/kg olive oil subcutaneously twice per week for 84 d (control rats) (d_0). Rats with chronic hepatitis at three stages, hepatic degeneration (d_{28}), fibrosis (d_{56}) and cirrhosis (d_{84}), were administered 3 mL/kg 40% (v/v) CCl_4 in olive oil subcutaneously twice per week for 28 d, 56 d and 84 d, respectively. Ach: Acetylcholine.

$10^{(-3.1247-0.3262\ x)}$] (r = 0.9937, P < 0.01); the median effective concentration with its 95% confidence intervals was 2.64×10^{-10} (7.1864×10^{-12} - 9.6834×10^{-9}) mol/L; (3) dose-effect at d_{56}: The data showed that the equation of PE was $Y = 0.0104 + 0.2643/[1 + 10^{(-8.8462-0.9579\ x)}]$ (r = 0.9980, P < 0.01); the median effective concentration with its 95% confidence intervals was 5.82×10^{-10} (3.0691×10^{-10} - 1.1031×10^{-9}) mol/L; (4) dose-effect at d_{84}: The data showed that the equation of PE was $Y = 0.01603 + 0.12107/[1 + 10^{(-5.1134-0.563\ x)}]$ (r = 0.9963, P < 0.01); the median effective concentration with its 95% confidence intervals was 8.24×10^{-10} (2.2476×10^{-10} - 3.0207×10^{-9}) mol/L; and (5) time-effect: The linear regression equation was $Y = 0.081\ x + 1.173$ (r = 0.981, P < 0.01) between the median effective concentrations of PE (1.69, 2.64, 5.82 and 8.24) $\times 10^{-10}$ mol/L and the durations (0 d, 28 d, 56 d and 84 d) of chronic hepatic hepatitis.

Acetylcholine reduced portal pressure: Geometric concentrations of Ach to activate the M_3 receptor were added to the circulating perfusate to reduce perfused portal pressure. The equation, the median effective concentration of Ach and its 95% confidence intervals of Ach were regressed (Table 3 and Figure 4): (1) dose-effect at d_0: The data showed that the equation of Ach was $Y = -0.4548 + 0.3274/[1 + 10^{(6.1538\ +\ 0.5554\ x)}]$ (r = 0.9950, P < 0.01); the median effective concentration with its 95% confidence intervals was 8.40×10^{-10} (1.3263×10^{-12} - 5.3240×10^{-11}) mol/L; (2) dose-effect at d_{28}: The data showed that the equation of Ach was $Y = -0.05391 + 0.06424/[1 + 10^{(3.8541\ +\ 0.3469\ x)}]$ (r = 0.9982, P < 0.01); the median effective concentration with its 95% confidence intervals was 7.73×10^{-12} (7.3614×10^{-13} - 8.1095 ×

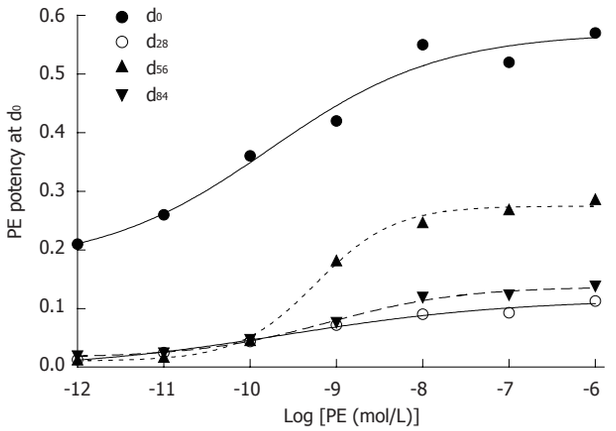

Figure 3 Phenylephrine elevates portal pressure (mean ± SE, n_i = 4). The dose-effect relationship between phenylephrine and portal vein constriction in isolated portal perfused rat livers without chronic hepatitis, where rats were administered 3 mL/kg olive oil subcutaneously twice per week for 84 d (control rats) (d_0). Rats with chronic hepatitis at three stages, hepatic degeneration (d_{28}), fibrosis (d_{56}) and cirrhosis (d_{84}), were administered 3 mL/kg 40% (v/v) CCl_4 in olive oil subcutaneously twice per week for 28 d, 56 d and 84 d, respectively. PE: Phenylephrine.

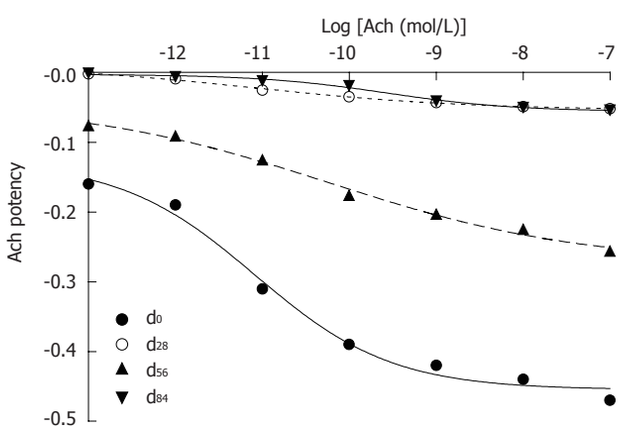

Figure 4 Acetylcholine reduces portal pressure (mean ± SE, n_i = 4). The dose-effect relationship between Acetylcholine and portal vein relaxation in isolated portal perfused rat livers without chronic hepatitis, where rats were administered 3 mL/kg olive oil subcutaneously twice per week for 84 d (control rats) (d_0). Rats with chronic hepatitis at three stages, hepatic degeneration (d_{28}), fibrosis (d_{56}) and cirrhosis (d_{84}), were administered 3 mL/kg 40% (v/v) CCl_4 in olive oil subcutaneously twice per week for 28 d, 56 d and 84 d, respectively. Ach: Acetylcholine.

10^{-11}) mol/L; (3) dose-effect at d_{56}: The data showed that the equation of Ach was Y = -0.2733 + 0.22978/[1 + $10^{(3.0472 + 0.3008\ x)}$] ($r$ = 0.9964, P < 0.01); the median effective concentration with its 95% confidence intervals was 5.98 × 10^{-11} (4.2797 × 10^{-12} - 8.3556 × 10^{-11}) mol/L; (4) dose-effect at d_{84}: The data showed that the equation of Ach was Y = -0.0559 + 0.053178/[1 + $10^{(5.6336 + 0.5883\ x)}$] ($r$ = 0.9956, P < 0.01); the median effective concentration with its 95% confidence intervals was 2.66 × 10^{-10} (6.5887 × 10^{-11} - 1.0701 × 10^{-9}) mol/L; and (5) time-effect: The linear regression equation was Y = 0.046 X -1.470 (r = 0.945, P < 0.05) between the median effective concentrations of Ach (0.0773, 0.598 and 2.66) × 10^{-10} mol/L and the durations (28 d, 56 d and 84 d) of chronic hepatitis.

DISCUSSION

Patients with portal hypertension have significant morbidity and mortality[1] without special drugs[2] based on the reversible pathogenesis of this disease[3]. Some candidate drugs from chemicals and medical plants have demonstrated effects on portal hypertension in animal experiments and in clinical trials[4-8]. Data from the extrahepatic portal rings failed to account for these effects[9]. Consequently, sensitive portal perfusion for intra-hepatic portal resistance has been developed with both controlled velocity and monitored pressure in IPPRLs[10-14]. The pharmacodynamic model of portal hypertension has further been defined as the median effective concentrations of Ach and PE in the IPPRLs at various stages of CCl_4-induced chronic hepatitis.

At d_0, d_{28}, d_{56}, and d_{84} in CCl_4-induced chronic hepatitis, there were similar portal pressure potency equations with various coefficients due to the concentrations of PE and Ach in the IPPRLs. The median effective concentrations of PE increased geometrically during the process, suggesting that the function of portal smooth muscle cells gradually decreased. A similar effect was noted with the median effective concentrations of Ach in advanced stages, which suggested that portal endothelia were gradually damaged. During portal perfusion with both controlled pressure and monitored velocity, as reported previously, the effective range of PE and Ach concentrations was from 10^{-3} mol/L to 10^{-8} mol/L[15,16]. In this novel model of portal perfusion with both controlled velocity and monitored pressure, the effective range was from 10^{-6} mol/L to 10^{-12} mol/L, which indicated that this novel mode was more sensitive than the previous mode by 10^3-10^6 times in IPPRLs.

Hepatocyte injuries originate from the free radicals of CCl_4 metabolites[3]. Amiloride reduced intra-hepatic portal resistance through inhibition of the Rho kinase pathway in hepatic stellate cells[4]. Glycyrrhizinate and Salvianolic acid B are representative molecules from medical plants used for portal hypertension in rats[5-7] and patients[8] with chronic hepatitis, however, their biomolecular mechanisms are not yet clear. PE, as a α_1-adrenoceptor agonist, constricts vascular smooth muscle[13] and Ach, as a M_3-cholinoceptor agonist in endothelia, relaxes vascular smooth muscle[14]. Due to these mechanisms in IPPRLs, the median perfused velocity in portal pressure has been defined as the primary flow rate of portal perfusion in this novel mode, the median effective concentration of PE for elevating portal pressure as the preload, the median effective concentration of Ach for reducing the portal pressure as the positive action at the classic stages of the pathological process in chronic hepatitis.

The pharmacodynamic model of portal hypertension has been defined as both the median effective concentrations of PE and Ach in the IPPRLs with advanced chronic hepatitis in this study. This model may be used to evaluate the preclinical effects of candidate drugs for the treatment of portal hypertension. Both controlled velocity and monitored pressure[10-12] made this model more sensitive than previous models[13-14]. Based on the standardization[15] of the IPPRL, this sensitive model is considered the basis of systems biology for portal regulation in an isolated setting[16].

COMMENTS

Background

Portal hypertension results in significant mortality without the administration of special drugs. Candidate drugs for this condition require serious pre-clinical evaluation using suitable methods.

Research frontiers

The recently identified reversible pathogenesis of portal hypertension may allow the development of new drugs for this disease. Candidate drugs derived from medical plants used in Chinese medical practices have confirmed its reversible pathogenesis. A sensitive pressure transducer was used here for exploiting the reversible pathogenesis as the pharmacological models. The optimal conditions for each step of the procedure can be defined as an available model.

Innovations and breakthroughs

Reversible portal hypertension was replicated in the advanced stages of chronic hepatitis in rats using CCl_4. A pharmacological model was developed using the median primary velocity of perfused flow as the anatomical preload, median effective concentrations of phenylephrine to constrict portal veins as the physiological preload, and the median effective concentrations of acetylcholine to relax portal veins in IPPRLs with chronic hepatitis.

Applications

This novel pharmacological model can be used to evaluate candidate drugs for the treatment of portal hypertension.

Terminology

This novel mode of portal perfusion is characterized by both controlled velocity and monitored pressure in the isolated portal perfused rat livers. The controlled velocity creates the optimal conditions for research purposes, and the monitored pressure gives exact data from vascular smooth muscle or endothelia.

Peer review

The authors investigated to develop a pharmacodynamic model for portal hypertension from chronic hepatitis. They have developed a pharmacodynamic model for portal hypertension in rats with chronic hepatitis and demonstrated that the model had been defined as the median effective concentrations of phenylephrine and acetylcholine. The results are clear and informative for the study on portal hypertension.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i5.479

World J Gastroenterol 2012 February 7; 18(5): 479-488
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Safety assessment of *Bifidobacterium longum* JDM301 based on complete genome sequences

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Supported by The National Key Program for Infectious Diseases of China, No. 2008ZX10004 and 2009ZX10004; the Program of Shanghai Subject Chief Scientist, No. 09XD1402700; the Program of Shanghai Research and Development, No. 10JC1408200; and a China Partnering Award from the Biotechnology and Biological Sciences Research Council, United Kingdom
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Received: May 25, 2011 Revised: July 31, 2011
Accepted: August 7, 2011
Published online: February 7, 2012

Abstract

AIM: To assess the safety of *Bifidobacterium longum* (*B. longum*) JDM301 based on complete genome sequences.

METHODS: The complete genome sequences of JDM301 were determined using the GS 20 system. Putative virulence factors, putative antibiotic resistance genes and genes encoding enzymes responsible for harmful metabolites were identified by blast with virulence factors database, antibiotic resistance genes database and genes associated with harmful metabolites in previous reports. Minimum inhibitory concentration of 16 common antimicrobial agents was evaluated by *E*-test.

RESULTS: JDM301 was shown to contain 36 genes associated with antibiotic resistance, 5 enzymes related to harmful metabolites and 162 nonspecific virulence factors mainly associated with transcriptional regulation, adhesion, sugar and amino acid transport. *B. longum* JDM301 was intrinsically resistant to ciprofloxacin, amikacin, gentamicin and streptomycin and susceptible to vancomycin, amoxicillin, cephalothin, chloramphenicol, erythromycin, ampicillin, cefotaxime, rifampicin, imipenem and trimethoprim-sulphamethoxazol. JDM301 was moderately resistant to bacitracin, while an earlier study showed that bifidobacteria were susceptible to this antibiotic. A tetracycline resistance gene with the risk of transfer was found in JDM301, which needs to be experimentally validated.

CONCLUSION: The safety assessment of JDM301 using information derived from complete bacterial genome will contribute to a wider and deeper insight into the safety of probiotic bacteria.

Key words: *Bifidobacterium longum*; Safety assessment; Genome; Antibiotic resistance; Harmful metabolite; Virulence factor

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Wei YX, Zhang ZY, Liu C, Malakar PK, Guo XK. Safety assessment of *Bifidobacterium longum* JDM301 based on complete genome sequences. *World J Gastroenterol* 2012; 18(5): 479-488 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i5/479.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i5.479

INTRODUCTION

Bifidobacteria spp are high-GC content, Gram-positive bacteria which belong to the *Actinobacteria* branch and these species naturally colonize the gastrointestinal tract (GIT) of mammals, birds and insects[1]. Scientists have determined the major probiotic properties of *Bifidobacteria* spp isolated from the human intestine and these properties include the strengthening of the intestinal barrier, modulation of the immune response and antagonism of pathogens[2].

Bifidobacterium spp has been reported to possess various glycosyl hydrolases (GH) and these hydrolases metabolize plant- or milk-derived oligosaccharides including nondigestible ones such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS)[3,4]. The capability to utilize nondigestible oligosaccharides confers a competitive advantage to *Bifidobacterium* spp in the human gut.

Bifidobacterium longum (*B. longum*) and various other bifidobacteria strains are often added to probiotic products in combination with other lactic acid bacteria (LAB). Through their long and safe history of application, LAB have acquired the status of "Generally Regarded As Safe" (GRAS), but the safety of *Bifidobacteria* and other LAB strains selected for probiotics still need to be carefully evaluated. The key safety aspects for use of bifidobacteria and other LAB strains in probiotics include antibiotic resistance, production of harmful metabolites and the potential for virulence. Antibiotic resistance in potential probiotic strains is not considered a risk factor unless resistance is transferred to pathogens or it renders the *probiotic untreatable* in very rare cases of infection[5]. Biogenic amines, D-lactic acid, azoreductases and nitroreductases produced by bifidobacteria and other LAB strains are potential health hazards[6,7] and the safety of some of these compounds have been evaluated[8]. Virulence genes may be present in commensal bacteria and absence of virulence in these bacteria needs to be proved on a case by case basis.

Probiotic agents are widely used in the food and drug industry and as more commercial probiotic products are being introduced in the market, it is timely to reassess the safety of these probiotic products using the latest technology. Information from the complete genome sequences of *Bifidobacteria* will provide additional insight into the genetic basis for their safety. We sequenced the complete genome sequences of *B. longum* JDM301 (GenBank accession number CP002010), a commercial strain used widely in China with several probiotic functions, for this purpose[9].

The aim of the present work was to assess the safety of *B. longum* JDM301 based on complete genome sequences. The criteria used were the potential to transfer antibiotic resistance to pathogens, the potential for production of harmful metabolites and the potential for virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions

JDM301 was isolated from commercial probiotic product and identified using a sequence analysis of its 16S rRNA gene. De Man-Rogosa-Sharpe (MRS) broth (Difco) supplemented with 0.05% L-cysteine·HCl (Sigma) was used for cultivating JDM301. Cultures were incubated at 37 ℃ under anaerobic conditions.

Genome sequencing and assembly

We determined the complete genome sequence of JDM301 at the Chinese National Human Genome Center in Shanghai using the GS 20 system (454 Life Science Corporation, Branford, Connecticut). A total of 192 888 reads with an average length of 210 bps were assembled into 112 contigs by the 454 assembly tool. The order of most large contigs, which were larger than 500 bp, was determined through the basic local alignment search tool (BLAST) analysis with the reference strain *B. longum* ATCC15697 (GenBank accession number CP001095) and the others were arranged by multiplex polymerase chain reaction (PCR). Gap closure was carried out by sequencing gap-spanning PCR products or clones using ABI 3730 xl DNA sequencers. Primer design and sequence assembly were performed by the Phred/Phrap/Consed software package[9]. The locations of low-quality sequences in genome were verified by directly resequencing the PCR products spanning the low-quality sequences using the ABI 3730 xl DNA sequencers.

Statistical analysis

The genome sequences of *Bifidobacteria* except JDM301 were retrieved from GenBank at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/)[10]. Potential open reading frames (ORF) were identified using Glimmer[11] and ZCURVE[12] 1.0 using default settings. Clusters of orthologous group (COG) functional categories were used for functional classification of all genes in the genome sequences of JDM301 and the COGs. A BLAST analysis of the translations with GenBank's nonredundant database was performed, which was followed by manual curation. The best matches were chosen for preliminary product assignments. Insertion sequences (IS) elements, prophage sequences and clustered regularly interspaced short palindromic repeats (CRISPR) were identified by IS finder (http://www-is.biotoul.fr/is.html), Prophage Finder[13] and CRISPRFinder (http://crispr.u-psud.fr/crispr/)[14] respectively. Putative orthologues were determined by Omics Explorer (http://omics.biosino.org:14000/kweb/about.jsp) using default values. Ribosomal RNA genes were detected on the basis of BLASTN searches and transfer RNA genes were identified using tRNAscan-SE[15]. The atlas of genome was drawn using GenomeViz1.1[16]. Putative virulence factors and putative antibiotic resistance genes were identified by blast with virulence factors database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm)[17] and antibiotic resistance genes database (ARDB) (http://ardb.cbcb.umd.edu/)[18] respectively.

Antibiotic susceptibility

Minimum inhibitory concentration (MIC) of 16 common

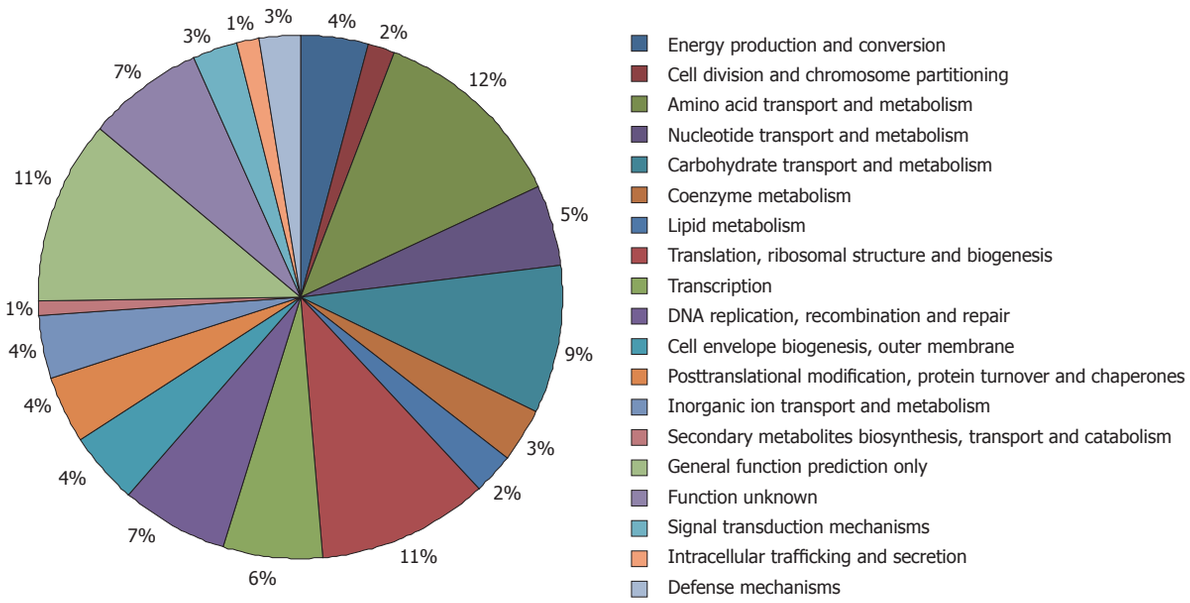

Figure 1 Functional distribution of *Bifidobacterium longum* core proteins. A total of 1265 proteins were conserved in all four *Bifidobacterium longum* (*B. longum*) strains (*B. longum* JDM301, *B. longum* NCC2705, *B. longum* DJO10A and *B. longum* ATCC15697), representing the "core" genome of *B. longum*.

antimicrobial agents was evaluated by *E*-test (AB Biodisk, Solna, Sweden) including amoxicillin (0.016-256 mg/L), amikacin (0.016-256 mg/L), ampicillin (0.016-256 mg/L), bacitracin (0.016-256 mg/L), cephalothin (0.016-256 mg/L), ciprofloxacin (0.002-32 mg/L), cefotaxime (0.016-256 mg/L), chloramphenicol (0.016-256 mg/L), erythromycin (0.016-256 mg/L), gentamicin (0.016-256 mg/L), imipenem (0.002-32 mg/L), rifampicin (0.016-256 mg/L), streptomycin (0.016-256 mg/L), tetracycline (0.016-256 mg/L), trimethoprim-sulphamethoxazol (0.002-32 mg/L), and vancomycin (0.016-256 mg/L). Tests were done with MRS agar supplemented with 0.05% L-cysteine·HCl (Sigma) and were conducted in triplicate for each antibiotics. Cultures sub-inoculated into the MRS agar supplemented with 0.05% L-cysteine·HCl were incubated anaerobically at 37 ℃ for 24 h.

RESULTS

Comparative genomic analysis of Bifidobacteria

The predicted proteins of *B. longum* JDM301 were functionally categorized. The functional distribution of genes assigned to clusters of orthologous groups of proteins was relatively similar to the other *Bifidobacteria*, e.g., *B. longum* and *B. adolescentis* in the GIT and *B. dentium* in the oral cavity[3,4,19]. The top four functional categories in *B. longum* JDM301, namely, carbohydrate transport and metabolism, amino acid transport and metabolism, were identical with other *Bifidobacteria*[20].

Putative orthologues among *B. longum* strains were determined in a comparative study (Figure 1). Overall, 1265 proteins were conserved in all four *B. longum* strains (*B. longum* JDM301, *B. longum* NCC2705, *B. longum* DJO10A and *B. longum* ATCC15697). These proteins represent the "core" genome of *B. longum*, whereas 219 proteins are unique to *B. longum* JDM301. The most common functional distributions of the core proteins were these involved in housekeeping functions including amino acid transport and metabolism, translation, ribosomal structure and biogenesis, carbohydrate transport and metabolism and DNA replication, recombination and repair. Twenty-one percent of the core proteins were dedicated to carbohydrate and amino acid transport and metabolism, indicating the important roles of these proteins in *Bifidobacteria*.

Stability of the genome of B. longum JDM301

Horizontal gene transfer (HGT) events are responsible for introduction of alien genes, which may reinforce the adaptation of bacteria in their specific niches. Genes on plasmids, bacteriophages, genomic islands and IS are sensitive to HGT[21]. Twelve phage-related fragments were identified in the genome of *B. longum* JDM301[9], but no complete prophages were found. The JDM301 chromosome also possesses 15 complete or disrupted IS elements[9]. The number of IS element in JDM301 is relatively smaller than the other sequenced *B. longum* spp[3,4]. Another set of genes disseminated by HGT in *Bifidobacteria* is the CRISPR-related system. No CRISPR was discovered in the genome.

One complete type Ⅱ restriction-modification (R-M) system and one type Ⅲ R-M system were present in the genome of JDM301. A complete and incomplete type Ⅰ R-M system was also identified in this genome. Two complete type Ⅱ R-M systems and one type Ⅰ R-M system were present in the genome of *B. longum* NCC2705, while one complete type Ⅱ R-M system and type Ⅰ R-M system were found in *B. longum* DJO10A.

Antibiotic resistance determinants

The antibiotic resistance genes in JDM301 were identified

Table 1 Putative antibiotic resistance genes identified in the genome of *Bifidobacterium longum* JDM301

Antibiotics	Antibiotic resistance genes	Product name
Bacitracin	*BLJ_1636*	ABC transporter-related protein
	BLJ_0984	ABC transporter-related protein
	BLJ_0923	ABC transporter-related protein
	BLJ_1055	Undecaprenyl pyrophosphate phosphatase
	BLJ_1119	Bacitracin transport ATP-binding protein bcrA
Vancomycin	*BLJ_0853*	VanU
	BLJ_1764	Dehydrogenase VanH
	BLJ_1084	Sensor protein vanSB
	BLJ_0707	VanSD5
	BLJ_0343	Histidine kinase VanSc3
	BLJ_0287	D-Ala: D-Lac ligase VanD
Multiple drugs	*BLJ_1090*	ATP-binding protein
	BLJ_1650	Lsa
	BLJ_1437	LmrB
	BLJ_0618	Multidrug export protein MepA
	BLJ_0769	Efflux transporter, RND family, MFP subunit
	BLJ_0181	Multidrug efflux protein QacB
	BLJ_1062	Multidrug export protein MepA
Chloramphenicol	*BLJ_1672*	Chloramphenicol resistance protein
	BLJ_1322	Chloramphenicol resistance protein
Thiostrepton	*BLJ_0885*	Thiostrepton-resistance methylase
Penicillin	*BLJ_1301*	Penicillin binding protein
Kasugamycin	*BLJ_2030*	S-adenosylmethionine-6-N', N'-adenosyl (rRNA) dimethyltransferase
Tetracycline	*BLJ_0814*	Tetracycline-resistance determinant tetV
	BLJ_1245	TetW
	BLJ_0594	Tetracycline resistance protein
	BLJ_1401	TetQ
Carbomycin	*BLJ_1625*	Carbomycin resistance protein
Sulfonamide	*BLJ_1629*	Dihydropteroate synthase
Tetracenomycin C	*BLJ_1624*	Tetracenomycin C efflux protein
Trimethoprim	*BLJ_1657*	dihydrofolate reductase
Macrolide	*BLJ_0925*	Macrolide-efflux protein
	BLJ_1936	Macrolide-efflux protein
	BLJ_0819	Macrolide-efflux protein
	BLJ_0042	Macrolide-efflux protein
	BLJ_1154	Macrolide-efflux protein variant

ABC: ATP-binding cassette; RND: Resistance-nodulation-cell division.

using ARDB (E < 1e-2, coverage > 70%)[18]. Homologs of the antibiotic resistance determinants for vancomycin, methicillin, tetracycline, chloramphenicol and trimethoprim were found in the genome of JDM301 (Table 1) and 6 putative resistance genes for vancomycin. *B. longum* JDM301 also possessed 5 putative bacitracin efflux pumps, 5 homologs of macrolide efflux proteins. Additionally, 7 putative multidrug resistance efflux pumps belonging to an ATP-binding cassette (ABC)-type transport system, a major facilitator superfamily transporter and resistance-nodulation-cell division (RND) family were found in the genome. The genome of *B. longum* JDM301 also contains 4 tetracycline resistance genes encoding for TetV, TetW, TetPB and TetQ. The gene for TetW shows a strong difference in G + C content (53.0%) compared to the average value of *B. longum* JDM301 (59.8%) genome and it is flanked by genes encoding for integrases, indicating that this region may have been acquired by HGT.

Table 2 Minimum inhibitory concentration values of 16 antibiotics for *Bifidobacterium longum* JDM301

Antibiotics	Minimum inhibitory concentration (mg/L)
Ciprofloxacin	> 32
Amikacin	> 256
Gentamicin	> 256
Bacitracin	26.67
Streptomycin	170.67
Vancomycin	0.9
Amoxicillin	0.064
Cephalothin	1.33
Chloramphenicol	0.25
Erythromycin	0.04
Ampicillin	0.058
Cefotaxime	0.19
Rifampicin	0.074
Tetracycline	8
Imipenem	0.19
Trimethoprim-sulphamethoxazol	1.83

The antibiotic susceptibility of *B. longum* JDM301 to 16 antibiotics was determined by an *E*-test to probe the *in silico* analyses of the complete genome sequence. The results of the *E*-test are summarized in Table 2. The breakpoints for determining susceptibility were determined using accepted protocols[22-25]. *B. longum* JDM301 showed a high resistance to ciprofloxacin, amikacin and gentamicin, moderate resistance to streptomycin and bacitracin and were sensitive to tetracycline, vancomycin, amoxicillin, cephalothin, chloramphenicol, erythromycin, ampicillin, cefotaxime, rifampicin, imipenem and an antimicrobial compound, trimethoprim-sulphamethoxazol.

Putative enzymes for harmful metabolites

Genes encoding enzymes responsible for harmful metabolites, including beta-glucosidase (GS), arylsulphatase (AS), beta-glucuronidase (GN), nitroreductase (NR), azoreductase (AR), D-lactate dehydrogenase (DLD), amino acid decarboxylase (AD) and conjugated bile salt hydrolase (CBSH) were searched for in the genome of *B. longum* JDM301. Two GS genes (BLJ_1280, BLJ_1540) and one CBSH gene (BLJ_0948) were found in the chromosome of *B. longum* JDM301. Homologs of DLD (BLJ_1306, BLJ_1436) and NR (BLJ_1980) were also discovered in the genome. Enzymes involved in putatively harmful metabolites, AR, GN, AD and AS were not found in JDM301 genome.

Putative virulence factors

Published reports of rare infections involving *Lactobacilli* or *Bifidobacteria* are available and the potential virulence of *Lactobacilli* or *Bifidobacteria* used as probiotics should be assessed[5]. Putative virulence genes of *B. longum* JDM301 were determined by BLAST analysis of the VFDB[17]. A total of 141 homologs of virulence factors were identified in the genome of JDM301, including 28 sugar-binding transcriptional regulators, 20 genes associated

Table 3 Putative virulence factors identified in the genome of *Bifidobacterium longum* JDM301

Query	Identity	Subject	Predicted functions
BLJ_1089	24.9	VFG0934	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase
BLJ_1835	26.36	VFG0934	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase
BLJ_0323	29.3	VFG0934	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase
BLJ_1476	22.11	VFG2378	6 kDa early secretory antigenic target esxA
BLJ_0992	32.98	VFG0869	AatC ATB binding protein of ABC transporter
BLJ_1080	34.81	VFG0869	AatC ATB binding protein of ABC transporter
BLJ_1968	37.3	VFG0869	AatC ATB binding protein of ABC transporter
BLJ_0770	37.43	VFG0869	AatC ATB binding protein of ABC transporter
BLJ_0026	35.71	VFG1404	ahpC
BLJ_0136	28.73	VFG2218	ATPase VirB11 homolog
BLJ_0880	24.18	VFG1042	ATP-binding protein FecE
BLJ_0787	47.92	VFG0077	ATP-dependent Clp protease proteolytic subunit
BLJ_0786	53.8	VFG0077	ATP-dependent Clp protease proteolytic subunit
BLJ_0948	37.66	VFG2162	Bile salt hydrolase
BLJ_1243	22.97	VFG2242	Conjugal transfer protein trag
BLJ_0551	26.54	VFG1108	Conserved hypothetical protein
BLJ_1951	29.85	VFG1269	Cyclolysin secretion ATP-binding protein
BLJ_1925	32.31	VFG1269	Cyclolysin secretion ATP-binding protein
BLJ_1863	45.5	VFG0079	Endopeptidase Clp ATP-binding chain C
BLJ_1465	56.77	VFG0079	Endopeptidase Clp ATP-binding chain C
BLJ_0713	30.12	VFG0925	Ferric enterobactin transport ATP-binding protein fepC
BLJ_1872	25.51	VFG2225	GDP-mannose 4,6-dehydratase
BLJ_1324	32.49	VFG1399	glnA1
BLJ_0624	62.11	VFG1399	glnA1
BLJ_1834	29.47	VFG0313	Glucose/galactose transporter
BLJ_1926	30.02	VFG1557	HlyB protein
BLJ_1477	56.12	VFG1855	Hsp60, 60K heat shock protein HtpB
BLJ_0064	26.21	VFG1397	hspX
BLJ_1444	40.85	VFG1563	Hypothetical protein
BLJ_1606	27.78	VFG1593	Hypothetical protein
BLJ_1640	30.81	VFG1593	Hypothetical protein
BLJ_0011	22.16	VFG1604	Hypothetical protein
BLJ_1513	26.3	VFG1604	Hypothetical protein
BLJ_1846	27.67	VFG1604	Hypothetical protein
BLJ_0337	44.25	VFG1630	Hypothetical protein
BLJ_0336	44.38	VFG1630	Hypothetical protein
BLJ_1500	23.53	VFG1963	Hypothetical protein Cj1435c
BLJ_1169	24.64	VFG1390	Hypothetical protein Rv0981
BLJ_0708	36.8	VFG1390	Hypothetical protein Rv0981
BLJ_0802	28.83	VFG1824	Hypothetical protein Rv3133c
BLJ_1357	30.46	VFG1824	Hypothetical protein Rv3133c
BLJ_1113	32.41	VFG1824	Hypothetical protein Rv3133c
BLJ_0835	32.42	VFG1824	Hypothetical protein Rv3133c
BLJ_0859	27.93	VFG1206	Iron(Ⅲ) ABC transporter, ATP-binding protein
BLJ_0348	28.13	VFG1206	Iron(Ⅲ) ABC transporter, ATP-binding protein
BLJ_0530	29.29	VFG1206	Iron(Ⅲ) ABC transporter, ATP-binding protein
BLJ_2016	35.81	VFG1206	Iron(Ⅲ) ABC transporter, ATP-binding protein
BLJ_1875	36.19	VFG1627	IS100 transposase; transposase ORFA
BLJ_1249	37.55	VFG1627	IS100 transposase; transposase ORFA
BLJ_1252	39.22	VFG1627	IS100 transposase; transposase ORFA
BLJ_0930	42.29	VFG1627	IS100 transposase; transposase ORFA
BLJ_1966	30.68	VFG1485	L7045
BLJ_1850	59.7	VFG1411	leuD
BLJ_0379	39.24	VFG0320	Lipopolysaccharide core biosynthesis protein (kdtB)
BLJ_1549	22.02	VFG1817	mbtA
BLJ_1204	25.8	VFG0574	Mg<up>2+</up> transport protein
BLJ_2010	30.62	VFG0574	Mg<up>2+</up> transport protein
BLJ_1270	28.62	VFG1116	N-acetylglucosamine-6-phosphate deacetylase
BLJ_1832	21.89	VFG1109	N-acetylneuraminate lyase, putative
BLJ_0490	25.95	VFG1109	N-acetylneuraminate lyase, putative
BLJ_0021	26.83	VFG0307	Neutrophil activating protein (bacterioferritin)
BLJ_1889	24.14	VFG2227	O-antigen export system permease protein
BLJ_1251	26.05	VFG1461	ORF A protein
BLJ_0214	30.5	VFG0594	Pathogenicity island encoded protein: SPI3
BLJ_0159	33.25	VFG0594	Pathogenicity island encoded protein: SPI3
BLJ_1474	57.32	VFG1386	phoP
BLJ_1703	25.65	VFG2220	Phosphoglucomutase
BLJ_0497	28.35	VFG2362	Phosphomannomutase
BLJ_1137	25.1	VFG1983	ABC-type amino-acid transporter periplasmic solute-binding protein
BLJ_0508	25.93	VFG1983	ABC-type amino-acid transporter periplasmic solute-binding protein
BLJ_1453	29.27	VFG1983	ABC-type amino-acid transporter periplasmic solute-binding protein
BLJ_0408	38.22	VFG2059	ATP-binding component of ABC transporter
BLJ_0480	27.04	VFG2061	Phosphoprotein phosphatase
BLJ_0805	28.09	VFG1384	proC
BLJ_1396	31.06	VFG1384	proC
BLJ_0584	26.09	VFG1387	purC
BLJ_1772	22.28	VFG0480	Putative amino acid permease
BLJ_0538	25.17	VFG0480	Putative amino acid permease
BLJ_1329	24.42	VFG1965	Putative aminotransferase
BLJ_0025	30.45	VFG2301	Putative carbonic anhydrase
BLJ_0922	23.51	VFG0031	Putative glycosyl transferase
BLJ_1670	38.88	VFG1668	Putative lysil-tRNA synthetase LysU
BLJ_0563	25	VFG1498	Putative periplasmic solute binding protein
BLJ_1171	28.48	VFG0483	Putative regulatory protein, deoR family
BLJ_1517	29.25	VFG0483	Putative regulatory protein, deoR family
BLJ_0344	37.02	VFG1702	Putative response regulator
BLJ_0040	27.91	VFG1746	Putative two-component response regulator
BLJ_0740	29.13	VFG1746	Putative two-component response regulator
BLJ_1105	24.49	VFG0168	Pyochelin biosynthesis protein PchD
BLJ_0409	25.56	VFG0168	Pyochelin biosynthesis protein PchD
BLJ_0720	41.04	VFG0479	Pyruvate kinase I (formerly F), fructose stimulated
BLJ_1163	55.32	VFG1826	relA
BLJ_0995	25.84	VFG1889	Response regulator GacA
BLJ_1679	28.89	VFG1889	Response regulator GacA
BLJ_1083	40.89	VFG0473	Response regulator in two-component regulatory system with BasS
BLJ_1273	26.57	VFG1115	ROK family protein
BLJ_1620	26.62	VFG1115	ROK family protein
BLJ_1622	31.35	VFG1115	ROK family protein
BLJ_1796	27.31	VFG0526	Salmonella iron transporter: fur regulated
BLJ_0662	29.06	VFG0526	Salmonella iron transporter: fur regulated

BLJ_0712	25.4	VFG0528	Salmonella iron transporter: fur regulated
BLJ_1174	51.39	VFG1405	sigA
BLJ_1258	41.15	VFG1412	sigH
BLJ_1342	33.11	VFG2161	Signal peptidase Ⅱ
BLJ_0906	21.73	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1923	22.38	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1360	22.88	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1421	23.24	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0611	23.31	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1836	23.32	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0459	23.33	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1278	23.43	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1998	23.51	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1522	23.6	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0109	23.63	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0418	23.69	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0118	23.7	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0520	23.85	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1976	24.27	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0099	24.31	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1605	24.34	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0132	24.53	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1912	24.58	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0912	24.69	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0318	24.71	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1515	24.93	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1933	25	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1718	25	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1997	25.36	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0400	25.37	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0515	27.08	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1321	28.21	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1232	29.83	VFG1028	Tn21 integrase IntI1
BLJ_1160	43.1	VFG2168	Transcriptional regulator, Cro/CI family
BLJ_0747	28.98	VFG1122	Transposase ORFAB, subunit B
BLJ_1180	43.64	VFG1398	trpD
BLJ_1871	39.62	VFG1967	UDP-galactopyranose mutase
BLJ_1644	39	VFG2361	UDP-glucose 4-epimerase
BLJ_1680	54.49	VFG2361	UDP-glucose 4-epimerase
BLJ_1891	52.63	VFG0963	UDP-glucose 6-dehydrogenase
BLJ_0697	46.15	VFG1414	whiB3

MIC: Minimum inhibitory concentration; ABC: ATP-binding cassette.

Table 4 Putative genes associated with adhesion identified in the genome of *Bifidobacterium longum* JDM301

Locus_tag	Pfam number	Product name
BLJ_1932	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0112	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1284	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1420	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0131	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1604	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1686	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1964	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1994	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1996	pfam01547	Family 1 extracellular solute-binding protein
BLJ_2001	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0288	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0321	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0345	pfam01547	phosphate ABC transporter periplasmic phosphate-binding protein
BLJ_0414	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0522	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0523	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0524	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0012	pfam07174	Hypothetical protein BLJ_0012
BLJ_1801	pfam05738	LPXTG-motif protein cell wall anchor domain-containing protein
BLJ_0140	pfam07811	TadE family protein

with iron, amino acid and sugar transport, 5 transposases, and 2 glutamine synthetase related to plasminogen (Plg)-binding (Table 3).

Although the ability to adhere to the intestinal wall has been one of the selection criteria for probiotics and also a characteristic of commensal bacteria in the intestine, adhesion is also considered to be a significant step in the initial pathogen infections[26]. Thus, predicted proteins for adhesion of JDM301 were also included in the analysis of virulence. A total of 21 predicted proteins for adhesion were identified in JDM301 (Table 4). A large number of predicted surface and extracellular proteins were identified in JDM301, which may be involved in the bacterium-host interaction as in other LAB[27]. A total of 217 proteins with probable Sec-type signal peptides were identified by the tool, Signal P[28]. The genome of JDM301 also harbors 18 copies of extracellular solute-binding protein (SBP, pfam01547) which is predicted to bind oligosaccharides (SBP family 1) as a component of the ABC transporter complex.

DISCUSSION

As more probiotic strains are used in the food and drug industry, more attentions should be paid to the safety of strains used as probiotics. Thus, the safety of LAB used as probiotics need to be reassessed using the latest technology. *B. longum* JDM301, is a commercial probiotic strain used in many probiotic products sold in China. Analysis of the genome of JDM301 reveals several potential risk factors needing further experimental validation, including a tetracycline resistance gene (*tetW*) with the risk of transfer, and the genes associated with harmful metabolites.

Bifidobacteria were considered free of phage infection until prophage-like elements were identified in the genomes of *B. longum* NCC2705, *B. longum* DJO10A and *B. breve* UCC2003[29]. Absence of complete prophages is important for the stability of genomes and for industrial applications of probiotic bacteria[21,30]. Absence of complete prophages and scarcity of IS element may play important roles in promoting genome stability of JDM301[31]. Another set of genes disseminated by HGT in *Bifidobacteria* is the CRISPR-related system (CASS), which is involved in defense against phages and plasmids[32]. No CRISPR was discovered in the genome. R-M systems are diverse and widespread in nature and they are considered as barriers to HGT, e.g., in transformation and phage infection[33]. The diversity of R-M systems in *B. longum* JDM301 may be significant to the stability of genome and its use in industry compared with the other two *B. longum* strains.

B. longum JDM301 was not resistant to tetracycline as the minimum inhibitory concentration (8.0 mg/L) was not higher than the breakpoint value (8.0 mg/L)[34]. However, the MIC for *B. longum* strains ranges from 0.5 to 2 mg/L in a report[35]. Thus, further experiments may be needed to determine the microbiological breakpoint. The *tetW* (BLJ_1245) gene encodes for a ribosomal protection protein and *tetW* genes were responsible for acquired tetracycline resistance in human *B. longum* strains[36]. The rest of the tetracycline resistance genes found in *B. longum* JDM301 were *tetV* (BLJ_0814), *tetQ* (BLJ_1401) and *tetPB* (BLJ_0594). The gene *tetV* encodes for a tetracycline efflux pump and the genes *tetQ* and *tetPB* encode for ribosomal protection proteins. Further experiments are needed to confirm whether the *tetW* gene in the chromosome of *B. longum* JDM301 is a transferable antibiotic resistance determinant and responsible for resistance to tetracycline in human *B. longum* strains.

The MIC of *B. longum* JDM301 to bacitracin was 26.7 mg/L, which indicated a moderate resistance. A previous report[25] indicated that *B. longum* strains were susceptible to bacitracin. A total of 7 putative bacitracin resistance genes were identified, including 6 genes encoding for ABC transporters and 1 for an uncharacterized bacitracin resistance protein. These genes may be responsible for the resistance to bacitracin.

The resistances to ciprofloxacin, amikacin, gentamicin and streptomycin and susceptibility of JDM301 to vancomycin, amoxicillin, cephalothin, chloramphenicol, erythromycin, ampicillin, cefotaxime, rifampicin, imipenem and an antimicrobial compound, trimethoprim-sulphamethoxazol were consistent with reported findings[22-25,36]. However, there are discrepancies between the phenotype and the genotype. *B. longum* JDM301 was sensitive to vancomycin and chloramphenicol but the genome contained vancomycin and chloramphenicol resistance genes. Further analysis will be needed to determine this discrepancy.

Several cases of D-lactic acidosis associated with consumption of LAB in patients with short bowel syndrome were reported[37,38], implying that bacteria used as probiotics should be screened for the ability to generate D-lactate. In this study, two homologs of DLD genes were identified in the genome of JDM301. Since there were no reported cases of D-lactic acidosis caused by bifidobacteria[37-39], the activities of these homologous DLDs in bifidobacteria may be low so that the amount of lactate produced is insufficient to cause D-lactic acidosis.

Although biogenic amines (BA) play an important physiological role in mammals, a high amount of BA in the diet may have a variety of toxic effects[40]. The main BA contained in food and beverage includes histamine, tyramine, putrescine, and cadaverine, some of which are associated with toxicological characteristics of food poisoning[41]. The decarboxylase activities of histidine, tyrosine and ornithine were reported in lactobacilli and the capabilities might be strain-dependent rather than species-dependent[42]. Therefore, BA production, especially thylamine and tyramine, must be carefully evaluated for individual strains.

Bacterial enzymes, such as GN, GS, NR, AR and AS, play important roles in the metabolism of carcinogens and other toxicants in the intestine. Homologs of GS are common in sequenced *Bifidobacteria* genomes where GS and GN facilitate the absorption of a variety of toxicants and may contribute to the development of colon cancer. The link between *Bifidobacteria* and the genotoxic enzyme activities of intestinal microflora has been reported[43,44], with *Bifidobacteria* inhibiting the activity of some genotoxic enzymes[45]. NR activity is common in oral bacteria and it plays an important role in bacterial nitrate reduction. Although NR activities have been reported in *Bifidobacteria*, the activity of this enzyme is lower than the NR activity of other gut bacteria[6].

CBSH mediates microbial bile tolerance and enhances microbial survival in the intestine[46]. Metagenomic analyses demonstrated that CBSH activity is enriched in the human gut microbiome, and has the potential to greatly influence host physiology[46]. In *Bifidobacterium* spp. and *Lactobacillus* spp., CBSH activity is also common and nearly all *Bifidobacteria* species and strains have bile salt hydrolase activities[47]. However, bile salt hydrolase activity releases free bile acids which are harmful to the human body and may act as mutagens[48,49]. Recommendations have been made for absence of bile salt transformation capacity in bacteria added to food[50]. However, it is noteworthy that the evidence for harmful effects is inconclusive so far and bile salt deconjugation activity may play a role in reducing human serum cholesterol[51]. Given the huge CBSH pool in intestinal microflora, the CBSH activities of the small number of additional bacteria consumed as probiotics can be ignored[48].

Putative genes for Plg-binding proteins, DnaK (BLJ_0123) and glutamine synthetase (BLJ_0624 and BLJ_1324) were found in the JDM301 genome, where these proteins play a role in the interaction with human epithelial cells. The protein DnaK has been shown to be present on the surface of pathogens, such as *Neisseria meningitides*[52]. The glutamine synthetases BLJ_0624 and BLJ_1324 had a 62.11% and 32.49% similarity to the glutamine synthetases in *Mycobacterium tuberculosis* H37 Rv. In

the presence of Plg activators, Plg binding to the bacterial surface is converted to plasmin, which is a broad-spectrum serine protease involved in degradation of fibrin and noncollagenous proteins of extracellular matrices and activates latent procollagenases[53]. It is believed that the capability to intervene with the Plg/plasmin system of a host is a strategy for host colonization and bacterial metastasis shared by several pathogens and commensals of the human intestinal tract[53,54]. The plasminogen-dependent proteolytic activity of *B. lactis* BI07 and *B. longum* was shown to be dose-dependent[55,56].

A homolog (BLJ_0880, 24.18% identity) of a gene encoding a component in ferric dicitrate uptake system (Fec) of *Shigella flexneri* serotype 2a, FecE, was identified in the genome of JDM301. As an iron uptake system, Fec is critical for bacterial survival and plays an important role in bacterial virulence[57]. In addition, BLJ_1105 and BLJ_0409 proteins associated with iron acquisition in JDM301 were 24.49% and 25.56% similar to pyochelin biosynthesis protein in *Pseudomonas aeruginosa,* and BLJ_0712, BLJ_1796 and BLJ_0662 proteins were 25.4%, 27.31 and 29.06% similar to iron transporters of *Salmonella enterica*.

The human pathogen, *Helicobacter pylori*, produces a neutrophil activating protein (NAP) which activate human leukocytes and induces an inflammation, which facilitates the growth of the pathogen[58]. A homolog (BLJ_0021; 26.83% identity) of the gene encoding a NAP was identified in the genome of JDM301.

In JDM301, BLJ_0012 encodes a protein harboring fibronectin-binding motif (Pfam number 07174) that allows mycobacteria to bind to fibronectin in the extracellular matrix and may mediate the adhesion of JDM301 to its host[59]. A potential protein for *Bifidobacteria* adhesion to intestinal cells is the putative LPXTG-motif protein with collagen binding motifs (Cna_B, pfam05738) encoded by BLJ_1801, which shows a 34% identity to a predicted fimbrial subunit in the genome of *B. dentium* Bd1. This protein may be involved in the recognition of and adhesion to mucosal epithelial cell surfaces[19]. Its homologous proteins were also identified in the genome sequences of both *B. longum* NCC2705 and *B. longum* DJO10A genomes[3,60]. *B. longum* subsp. *infantis* 15697, *B. longum* NCC2705 and *B. adolescentis* contains 21, 10 and 11 copies of extracellular solute-binding protein, respectively[3,4]. Comparably, the SBP family 1 proteins are more abundant in JDM301 than the three other *Bifidobacteria* strains due to the genome size.

Finally, JDM301 encodes a number of proteases and peptidase that may contribute to virulence owing their ability to degrade host proteins for bacterial nutrition sources[61]. However, not all the genes associated with virulence have been known until now. Thus, despite the evaluation based on the whole genome sequences, it is recommended that the rat endocarditis and the immunocompromised mouse model should be used for *in vivo* assessment of safety for the low pathogenicity of LAB[48].

Recently, there has been more interest in using probiotic products to promote health and treat diseases. Probiotics have been investigated in clinical trials, such as treatment for diarrhea, D-lactic acidosis, necrotizing enterocolitis, inflammatory bowel disease and so on[39,62-64]. The mechanisms by which probiotics exert their effects are still obscure, which may include modification of gut pH, antagonism of pathogens, modulation of immunity as well as supplements of some nutrients[65]. However, safety issues of probiotics have been discussed in many reports[5,48]. There are reported cases of infections associated with probiotic strains[5]. Although the strain is safe based on phenotype, the information derived from complete bacterial genome sequences reveals some putative unfavorable genes, such as genes encoding for Plg-binding proteins, proteases and genes associated with production of D-lactate. In addition, patients are generally more susceptive to infection and harmful metabolites, such as D-lactate than healthy persons. Thus, the biosafety of probiotics, especially strains used in therapy, must be assessed more carefully and comprehensively.

In conclusion, this study compared the genome of JDM301 with other *Bifidobacteria* and assessed the genomic stability, the potential for antibiotic resistance, the potential for virulence and the potential production of harmful metabolites of this strain. The core genome of *B. longum* is composed of 1265 genes, and 219 genes are unique in JDM301. Our data showed putative virulence genes in the genomes of JDM301 as well as putative genes associated with production of harmful metabolites. In addition, a potentially transferable antibiotic resistance gene was detected in the chromosome of JDM301, which needs to be experimentally validated. This assessment provides information on potential risk factors, which should be further evaluated experimentally, e.g., *in vivo* assessment using animal models.

ACKNOWLEDGMENTS

The authors thank Dr. Hua-Jun Zheng from Shanghai-MOST Key Laboratory of Health and Disease Genomics, Chinese National Human Genome Center at Shanghai for kindly providing us assistance in data analysis.

COMMENTS

Background

Bifidobacterium longum JDM301 is a commercial strain used widely in China with several probiotic functions. Recently, there has been more interest in using probiotic products to promote health and treat diseases. As model probiotic bacteria, *Bifidobacteria* are often added to probiotic products in combination with other lactic acid bacteria. The biosafety of probiotic bacteria is attracting more attentions with its enlarged applications. As more commercial probiotic products are being introduced in the market, it is necessary to reassess the safety of these probiotic products using the latest technology.

Research frontiers

With a long and safe history of application, lactic acid bacteria have acquired the status of "Generally Regarded As Safe". However, published reports of rare infections involving *Lactobacilli* or *Bifidobacteria* are available. The strains selected as probiotics are needed to be assessed carefully and comprehensively. This study may contribute to a better biosafety assessment of probiotic bacteria.

Innovations and breakthroughs

This is the first study to assess the biosafety of probiotic bacteria based on

complete genome sequences. Through bioinformatics analysis of the genome sequences, the authors found that although the strain was safe based on phenotype, the information derived from complete bacterial genome sequences revealed some putative unfavourable genes that should be paid attention to.

Applications

The study provides a comprehensive assessment on potential risk factors of a probiotic strain based on complete genome sequences. The information related to biosafety derived from the genome of JDM301 will contribute to a wider and deeper insight into the safety of probiotic bacteria.

Peer review

This is a very nice and comprehensive study assessing the genomic stability, potential of antibiotic resistance, virulence and production of harmful metabolites. This adds valuable information to current knowledge about probiotics.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i5.489

World J Gastroenterol 2012 February 7; 18(5): 489-490
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Potential implications of *Helicobacter pylori*-related neutrophil-activating protein

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Received: June 14, 2011 Revised: August 18, 2011
Accepted: August 27, 2011
Published online: February 7, 2012

Abstract

Helicobacter pylori (*H. pylori*) virulence factors promote the release of various chemoattractants/inflammatory mediators, including mainly the neutrophil-attractant chemokine interleukin-8 and neutrophil-activating protein (NAP), involved in *H. pylori*-induced gastric pathologies. Co-administration of Chios mastic gum (CMG), which inhibits *H. pylori* NAP, with an *H. pylori* eradication regimen might add clinical benefits against *H. pylori*-related gastric pathologies, but possibly not CMG as main therapy. Although *H. pylori* NAP and other *H. pylori*-related cytotoxins [i.e., vaculating cytotoxin (VacA)] appear to play a major role in generating and maintaining the *H. pylori*-associated gastric inflammatory response and *H. pylori* NAP is a promising vaccine candidate against *H. pylori* infection (*H. pylori*-I), concerns regarding its potential drawbacks, particularly neurogenic ones, due to possible cross-mimicry, should be considered. Possible cross-mimicry between *H. pylori* NAP and/or bacterial aquaporin (AQP) and neural tissues may be associated with the anti-AQP-4 antibody-related neural damage in multiple sclerosis (MS)/neuromyelitis optica patients. Moreover, the sequence homology found between *H. pylori* VacA and human Na+/K+-ATPase A subunit suggests that antibodies to VacA involve ion channels in abaxonal Schwann cell plasmalemma resulting in demyelination in some patients. A series of factors have been implicated in inducing blood-brain barrier (BBB) disruption, including inflammatory mediators (e.g., cytokines and chemokines induced by *H. pylori*-I) and oxidative stress. BBB disruption permits access of AQP4-specific antibodies and T lymphocytes to the central nervous system, thereby playing a major role in multiple sclerosis pathogenesis. Relative studies show a strong association between *H. pylori*-I and MS. *H. pylori*-I induces humoral and cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with components of nerves, thereby contributing and perpetuating neural tissue damage. Finally, *H. pylori* NAP also plays a possible pathogenetic role in both gastric and colon oncogenesis.

Key words: *Helicobacter pylori*; Neutrophil-activating protein; Chios mastic gum; Cross-mimicry; Multiple sclerosis; Demyelination; Gastric carcinogenesis

Peer reviewers: Francesco Franceschi, MD, PhD, Assistant Professor, Internal Medicine, Catholic University of Rome, Gemelli Hospital, Largo A. Gemelli, 8, 00168 Rome, Italy; Dr. Orhan Sezgin, Professor, Gastroenteroloji Bilim Dalı, Mersin Üniversitesi Tıp Fakültesi, 33190 Mersin, Turkey; Da-Jun Deng, Professor, Department of Cancer Etiology, Peking University School of Oncology, 1 Da-Hong-Luo-Chang Street, Western District, Beijing 100034, China

Kountouras J, Zavos C, Deretzi G, Gavalas E, Chatzopoulos D, Katsinelos P, Tsiaousi E, Gagalis S, Polyzos SA, Venizelos I. Potential implications of *Helicobacter pylori*-related neutrophil-activating protein. *World J Gastroenterol* 2012; 18(5): 489-490 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i5/489.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i5.489

TO THE EDITOR

In their recent paper published in this journal, Choli-Papadopoulou *et al*[1] consider the development of new drugs targeting *Helicobacter pylori* (*H. pylori*) neutrophil-activating protein (NAP) and this raises some concerns.

With reference to a study[2] focusing on *H. pylori* NAP-mediated neutrophil activation before and 2 mo after *per os* administration of Chios mastic gum (CMG), the authors claimed that "these results indicate a substantial down-regulation of the innate cellular immune effectors, which, according to unpublished clinical data in the context of this study, are accompanied by a significant clinical improvement of the patients' complaints (dyspepsia, epigastric discomfort, distention)"[1]. However, such clinical benefits cannot be deduced from this study[2] and, as mentioned, relative clinical data on CMG as treatment for *H. pylori* and peptic ulcer are controversial[2]. Although *H. pylori* virulence factors promote the release of various chemoattractants/inflammatory mediators including mainly the neutrophil-attractant chemokine interleukin-8 and *H. pylori* NAP involved in *H. pylori*-induced gastric pathologies[3], our clinical experience suggests that only co-administration of CMG with an *H. pylori* eradication regimen might add clinical benefits against *H. pylori*-related gastric pathologies, but possibly not CMG as main therapy, as the authors claimed[1,2]. In particular, co-administration of CMG might be a potential therapy to reduce damage of gastric mucosa induced by *H. pylori* NAP. However, large-scale relative prospective studies are needed to elucidate this field.

The authors, further considering data on the safety and immunogenicity of a vaccine comprising *H. pylori*-induced vaculating cytotoxin (VacA), cytotoxin associated gene and *H. pylori* NAP, suggested that the obtained neutrophil activation by the C-terminal region of *H. pylori* NAP opens new pathways for drug design directed at *H. pylori* inflammation[1]. In particular, both VacA and *H. pylori* NAP play a major role in generating and maintaining the *H. pylori*-associated gastric inflammatory response, and *H. pylori* NAP is a promising vaccine candidate against *H. pylori* infection (*H. pylori*-I). However, concerns regarding potential drawbacks of *H. pylori* NAP, particularly neurogenic ones, should be considered. For instance, possible cross-mimicry between *H. pylori* NAP and/or bacterial aquaporin (AQP) and neural tissues may be associated with the anti-AQP-4 antibody-related neural damage in multiple sclerosis (MS)/neuromyelitis optica (NMO) patients. In this regard, by using histology, the practical gold standard for the diagnosis of *H. pylori*-I, we have shown a strong association between *H. pylori*-I and MS[4]. *H. pylori*-I induces humoral and cellular immune responses that, owing to the sharing of homologous epitopes (molecular mimicry), cross-react with components of nerves, thereby contributing and perpetuating neural tissue damage[4]. In this respect, *H. pylori* NAP, as a virulence factor, recruits leukocytes from the vascular lumen, and activates neutrophils, monocytes and mast cells, as mentioned by the authors. Besides, the sequence homology found between *H. pylori* VacA and human Na+/K+-ATPase A subunit suggests that antibodies to VacA involve ion channels in abaxonal Schwann cell plasmalemma resulting in demyelination in some patients[5]. Moreover, VacA exhibits chemotactic activities to the bone marrow-derived mast cells (BMDMCs) and induces BMDMCs to produce pro-inflammatory cytokines[5]. A series of factors have been implicated in inducing blood-brain barrier (BBB) disruption, including the aforementioned inflammatory mediators (e.g., cytokines and chemokines induced by *H. pylori*-I) and oxidative stress. BBB disruption permits access of AQP4-specific antibodies and T lymphocytes to the central nervous system, thereby playing a major role in MS/NMO pathogenesis[6]. Therefore, *H. pylori* NAP and *HP*-I itself, by inducing several mediators, may influence MS/NMO (including relapsing type) pathophysiology, thereby raising possible concerns regarding even the C-terminal region of *H. pylori* NAP use as a candidate vaccine. Accordingly, relative studies are also needed to clarify the aforementioned concerns.

Finally, the possible *H. pylori* NAP pathogenetic role in gastric carcinogenesis, mentioned by the authors, may also apply to colon oncogenesis[2,7].

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
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www.wjgnet.com

World J Gastroenterol 2012 February 7; 18(5): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
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www.wjgnet.com

World J Gastroenterol 2012 February 7; 18(5): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
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San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
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Orlando, FL 32808, United States

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April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
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Prague, Czech

April 18-20, 2012
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Barcelona, Spain

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Internal Medicine 2012
New Orleans, LA 70166, United States

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Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
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Kiev, Ukraine

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
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www.wjgnet.com

World J Gastroenterol 2012 February 7; 18(5): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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Name of journal

World Journal of Gastroenterology

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

Editorial office
World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
Ocean International Center,
No. 62 Dongsihuan Zhonglu,
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
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Indexed and abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifer, and Directory of Open Access Journals. ISI, Thomson Reuters, 2010 Impact Factor: 2.240 (35/71 Gastroenterology and Hepatology).

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World Journal of Gastroenterology®

Volume 18 Number 6
February 14, 2012

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ISSN 1007-9327

ISSN 1007-9327 CN 14-1219/R Local Post Offices Code No. 82-261

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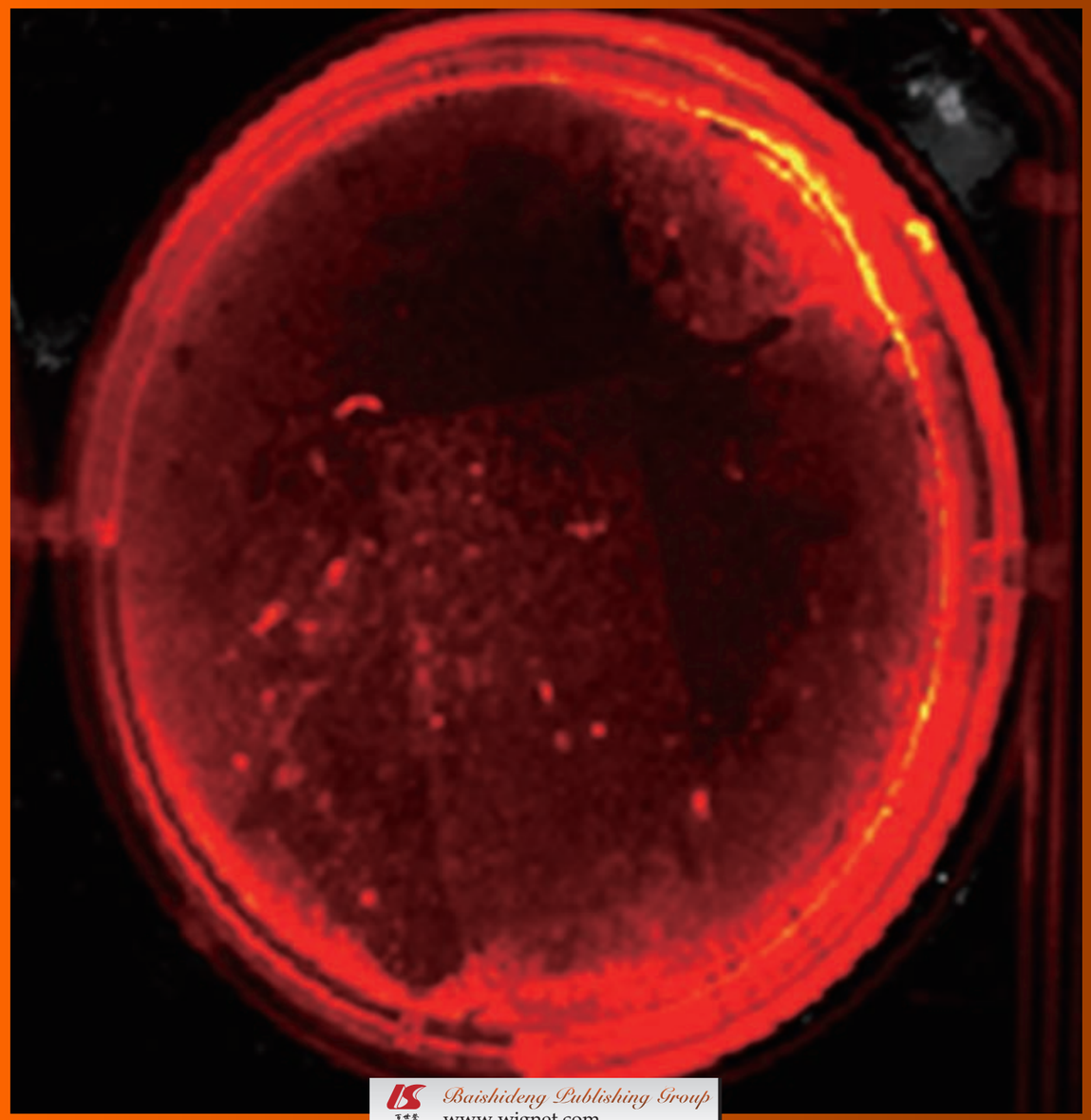
World J Gastroenterol 2012 February 14; 18(6): 491-588



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World Journal of Gastroenterology
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NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
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PRINT SUBSCRIPTION
RMB 300 Yuan for each issue, RMB 14400 Yuan for one year.

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INSTRUCTIONS TO AUTHORS
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World J Gastroenterol 2012 February 14; 18(6): 491-497
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Endoscopic ultrasound-guided biliary drainage

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Telephone: +1-352-2739400 Fax: +1-352-3923618
Received: February 3, 2011 Revised: April 15, 2011
Accepted: April 22, 2011
Published online: February 14, 2012

Abstract

Endoscopic ultrasound (EUS)-guided biliary drainage has emerged as a minimally invasive alternative to percutaneous and surgical interventions for patients with biliary obstruction who had failed endoscopic retrograde cholangiopancreatography (ERCP). EUS-guided biliary drainage has become feasible due to the development of large channel curvilinear therapeutic echo-endoscopes and the use of real-time ultrasound and fluoroscopy imaging in addition to standard ERCP devices and techniques. EUS-guided biliary drainage is an attractive option because of its minimally invasive, single step procedure which provides internal biliary decompression. Multiple investigators have reported high success and low complication rates. Unfortunately, high quality prospective data are still lacking. We provide detailed review of the use of EUS for biliary drainage from the perspective of practicing endoscopists with specific focus on the technical aspects of the procedure.

Key words: Bile duct; Biliary obstruction; Biliary drainage; Endoscopic ultrasound; Endoscopic ultrasound-guided biliary drainage

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Chavalitdhamrong D, Draganov PV. Endoscopic ultrasound-guided biliary drainage. *World J Gastroenterol* 2012; 18(6): 491-497
Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i6/491.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i6.491

INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) has become the procedure of choice for the management of biliary obstruction[1]. If ERCP fails to achieve biliary drainage, more invasive options are usually considered. These include percutaneous transhepatic biliary drainage (PTC) and surgical intervention but they have been associated with a higher risk of complications and prolonged hospital stay[2]. Recently, endoscopic ultrasound (EUS)-guided biliary drainage has been introduced as an alternative for patients who had failed ERCP. EUS was developed in the early 1980s to overcome the limitations of abdominal ultrasonography and computed tomography for pancreatic imaging[3]. Since its introduction, EUS has become a very valuable imaging tool to visualize the gastrointestinal luminal wall and its adjacent structures. The proximity of the EUS probe to the area of interest not only provides high resolution images but also allows for real time EUS-guided fine needle aspiration (FNA) tissue sampling. The high quality imaging combined with FNA capability has made EUS an indispensable diagnostic modality. EUS has also been utilized as a therapeutic tool but until recently its role has been confined mostly to injection of various substances[4,5]. The availability of larger channel echo-endoscopes has allowed the broadening of the therapeutic applications of EUS by combining the advantages of real-time ultrasound and fluoroscopy imaging with the use of ERCP based devices and tech-

niques. As a result of these developments and combination of technologies, EUS-guided biliary drainage has become possible.

EUS-guided diagnostic cholangiography was first reported by Wiersema *et al*[6] in 1996 and the first EUS-guided biliary drainage was reported in 2001 by Giovannini *et al*[7] by creating a choledochoduodenal fistula using a needle knife followed by transduodenal stenting in a patient with pancreatic cancer. These initial reports were followed by the description of technique modifications and expanding indications including EUS-guided hepaticogastrostomy with stent placement[8], transduodenal EUS-rendezvous biliary access[9,10] and EUS-guided biliary therapy of choledocholithiasis with the creation of a neopapilla[11]. Since then a number of series have reported variations on these techniques[12-31] including the recent description of a placement of a fully covered metal stent as a route for interventional endoscopic procedures in the bile duct[32]. A published large case series (≥ 10 cases) on EUS-guided biliary drainage is shown in Table 1.

The purpose of this manuscript is to provide a detailed review of the use of EUS for biliary drainage from the perspective of practicing endoscopists with specific focus on the technical aspects of the procedure. We performed a Medline search using the key words EUS, biliary drainage, biliary obstruction, therapeutic EUS and interventional EUS. In addition we manually reviewed the references of the identified review papers.

INDICATIONS FOR EUS-GUIDED BILIARY DRAINAGE

ERCP guided stent placement has become the procedure of choice for biliary decompression but it can be occasionally associated with difficulty gaining access to the obstructed biliary tract. In a recent prospective study, first attempt ERCP failed in 25% of patients with obstructive jaundice due to pancreatic cancer[33]. The two main reasons for failed ERCP are failure to cannulate the bile duct or inability to reach the papilla due to duodenal stenosis or post surgical changes. For patients with accessible papillae, most endoscopists will consider a second attempt at ERCP to gain access to the bile duct. Traditionally, patients who had ultimately failed ERCP have been offered PTC or surgical biliary decompression but these approaches are associated with a higher rate of complications and prolonged hospital stay[2]. Lately, EUS-guided biliary drainage emerged as a less invasive alternative for biliary drainage. EUS-guided decompression is not only minimally invasive but it is a single step procedure that provides more physiological internal bile drainage with improved patient comfort and decreased risk for fluid and electrolyte disturbances. Furthermore, EUS-guided biliary drainage using transgastric puncture of the intrahepatic duct or the common bile duct is feasible in patients with inaccessible papillae due to duodenal obstruction, surgically altered anatomy or hilar block due to cholangiocarcinoma or gallbladder cancer[11]. Finally, EUS-guided biliary drainage may be safer than PTC since the bile duct is accessed under real-time EUS guidance using Doppler to avoid blood vessels in the needle path[34-36]. The patient preparation for EUS-guided biliary drainage is not different from the standard preparation for ERCP as the patient's position is prone on the fluoroscopy table. All patients should receive periprocedural antibiotics. General anesthesia can be considered but the majority of cases can be done with monitored sedation. Contraindications include coagulopathy and severe hemodynamic instability.

INSTRUMENTATION

Both EUS and fluoroscopy equipment should be available in the procedure room. The use of a therapeutic channel (3.7 mm on the Olympus and 3.8 mm on the Pentax linear echo-endoscopes) is recommended. It is possible to use the diagnostic channel (2.8 mm) linear echo-endoscope to perform the puncture and wire insertion into the bile duct and then exchange the scope over the wire with the therapeutic channel ERCP scope but this maneuver is more complex and the bile duct access can be easily lost during the scope exchange.

Both 22 and 19 gauge EUS needles can be used for the initial puncture of the bile duct. In general, the use of a 19 gauge needle will allow the passage of 0.035 inch wire but the stiffness of the 19 gauge needle may provide a challenge to advance the needle tip to the desired target (i.e., bile duct). The 22 gauge needle provides better flexibility but allows passage of only 0.018 inch guidewire. The new blunt-tip Access Needle (Cook Endoscopy, Winston-Salem, NC, United States) is now available for initial puncture. This needle allows smooth guidewire manipulation and prevents guidewire damage as seen with the standard sharp tip needles. The EUS needle can be preloaded with the guidewire and pre-flushed with contrast using a three way Ⅳ adaptor so that contrast can be injected without removing the guidewire during its manipulation. Besides the standard ERCP devices, an over-the-wire needle-knife sphincterotome or Cystotome (Cook Endoscopy, Winston-Salem, NC, United States) should be available along with choices of plastic (straight or pigtail) and self-expandable metallic stents (uncovered, partially covered or fully covered) of varying calibers and lengths[35,37,38]. A list of the typical devices that are needed to perform EUS-guided biliary drainage is presented in Table 2.

TECHNIQUES

The reported success rate of EUS-guided biliary drainage ranges from 73% to 97%[14,19,22,30,31,39,40]. EUS-guided biliary drainage should be as effective as transpapillary biliary drainage once the stent is successfully placed. Simultaneous tissue diagnosis and staging can be provided in the same setting. Several approaches to access the biliary tree

Table 1 Published large case series on endoscopic ultrasound-guided biliary drainage

Authors	Yr	Number of cases	Technical success (%)	Complication (number of cases)
Kahaleh *et al*[14]	2006	23	91	Bile leak (1), pneumoperitoneum (2), bleeding (1)
Bories *et al*[31]	2007	11	91	Biloma (1), cholangitis (1), stent occlusion (1)
Will *et al*[22]	2007	12	90	Pain (1), cholangitis (1)
Shami *et al*[34]	2007	23	91	Bile leak (1), pneumoperitoneum (2)
Park *et al*[38]	2009	14	100	Stent migration (1)
Maranki *et al*[27]	2009	49	84	Biliary peritonitis (1), pain (1), pneumoperitoneum (4), aspiration pneumonia (1), bleeding (1)
Kim *et al*[19]	2010	15	100	None

Table 2 Devices for endoscopic ultrasound-guided biliary drainage (in addition to standard endoscopic retrograde cholangiopancreatography devices)

Therapeutic EUS scope
19 and 22 gauge standard EUS-FNA needles
19 gauge access EUS needle (Cook Endoscopy)
Standard length guide wires (450 cm)
0.035 inch
0.018 inch
Over-the-wire needle-knife sphincterotome
Cystotome (Cook Endoscopy)
Biliary dilation balloons
Three way IV adaptor with needleless cap
Stents
Plastic stent
Pigtail biliary stent
Straight biliary stent
Self expandable metal stents (uncovered, partially covered and fully covered)

EUS: Endoscopic ultrasound; FNA: Fine needle aspiration.

and provide biliary drainage have been described either *via* the duodenum or the stomach into the common bile duct or hepatic duct, respectively. EUS-guided biliary drainage can be divided into three principal methods[41]. (1) EUS-ERCP rendezvous technique. With this technique the EUS is used solely to puncture the obstructed duct and pass a guidewire antegrade through the papilla for subsequent rendezvous by ERCP[30]; (2) Stent placement *via* the EUS endoscope across the site of biliary obstruction in antegrade fashion in which EUS is used to create a temporary fistula *via* a transgastric or transduodenal access to allow stent placement; and (3) Transluminal drainage in which EUS is used to create a permanent fistula and subsequent stent placement is performed across that fistula between bile duct and bowel wall. In this approach the site of biliary obstruction is not traversed by a stent but rather an alternative tract for bile drainage is created by creation of a bilioenteric fistula.

EUS access to the biliary tree is first gained under real time guidance using the EUS needle. Contrast is then injected *via* the needle under fluoroscopic visualization to obtain a cholangiogram followed by insertion of a guidewire into the biliary tree. The use of 0.035 inch guidewire (requires 19-gauge needle for initial access) is preferred because it may facilitate traversing of strictures and stent placement. The created fistula can then be enlarged with an ERCP cannula, tapered biliary dilators, needle-knife, cystotome, or biliary balloon dilator and in some cases may require the use of several devices[34]. Once the fistula tract is enlarged, the type of stent placement is decided based on the obstruction site (whether the stricture is accessed in antegrade or retrograde fashion and whether the stricture has been traversed with the guidewire). When the site of obstruction cannot be traversed, then transluminal stenting may be performed with the end of the stent lying within the biliary tree proximal to the obstruction and the other end of the stent lying in the stomach or duodenum. Stent placement can also be done through an existing duodenal metal stent in combined biliary and duodenal obstruction situations[42]. Combined duodenal stent placement and EUS-guided biliary drainage can be used for malignant duodenal obstruction with biliary stricture[43].

Bile duct access

Bile duct access depends on the level of obstruction and whether the ampulla is endoscopically accessible.

Transhepatic approach (hepaticogastrostomy): The echo-endoscope is positioned within the proximal stomach (cardia or proximal body) and oriented along the lesser curvature of the stomach or more posterior position to visualize the dilated left intrahepatic bile ducts. Color Doppler is used to rule out overlying vasculature before inserting the needle into the identified dilated intrahepatic bile duct branch. Bile is aspirated and contrast is instilled to confirm placement. Several options exist based on the level of obstruction, success of traversing the obstruction with guidewire and availability of endoscopic assess to the papilla. (1) A guidewire is advanced in an antegrade fashion through the EUS needle and manipulated across the biliary obstruction and into the duodenum under fluoroscopic and endosonographic guidance. Several loops of wire should be created to reduce the risk of wire dislodgement. If access to the second portion of the duodenum is feasible the procedure can be completed with the rendezvous technique with standard ERCP scope; (2) If the stricture is traversed with the guidewire and the duodenum is not accessible, a stent can be inserted across the biliary stenosis in antegrade fashion. To allow passage of the stent, the fistula tract should be enlarged as previously described; and (3) If the guidewire cannot be

advanced across the obstruction, a transluminal drainage approach can be utilized by deploying a stent that bridges the fistulous tract (the distal end of the stent is in the dilated intrahepatic duct within the left lobe and the proximal end of the stent is in the cardia of the stomach).

Extrahepatic approach (transcholedochal or choledochoduodenostomy): The echo-endoscope is placed in the distal antrum or duodenum, permitting imaging of the common bile duct. Color Doppler is used to rule out overlying vasculature. Bile duct puncture is carried out with an EUS needle under endosonographic control. The bile duct typically is entered in its midportion (superior to the intrapancreatic portion and inferior to the hilum) then several options are available depending on the site of obstruction: (1) for distal (intrapancreatic portion) main bile duct obstruction, a stent can be deployed bridging the created fistulous tract and therefore creating choledochoduodenostomy proximal to the obstruction; (2) alternatively, an attempt to manipulate the wire and traverse the stricture in antegrade fashion can be made and then the procedure can be completed with the duodenoscope by rendezvous technique; and (3) if the obstruction is proximal to the entry point in the bile duct (e.g., hilum), the stricture should be traversed in retrograde fashion and then stented.

Stent placement

Enlargement of the fistulous tract is usually necessary before stent placement. Stent insertion may be performed in an antegrade fashion *via* the echo-endoscope or as a rendezvous procedure with ERCP depending on the transpapillary accessibility and the ease of stent deployment. Rendezvous drainage requires endoscopic access to the region of the papilla, while direct transluminal stent placement entirely by EUS does not.

Antegrade: The stent is placed using the echo-endoscope in an antegrade fashion. Stent placement can be transluminal (transgastric or transduodenal). An attempt should be made to traverse the stricture followed by stent placement with the stent bridging the stricture. Stent placement creating bilioenteric anastomosis is an option if trans-stricture stenting is unsuccessful. If feasible, placement of the stent across the site of obstruction is the preferred approach.

Rendezvous (retrograde): This technique is for ERCP access *via* the native papilla or surgical anastomosis by EUS-guided placement of a guidewire and subsequent access by ERCP. EUS-guided rendezvous was first reported for pancreatic duct access in 2001 and for both biliary and pancreatic ductal drainage in 2004[9]. The bile duct is accessed proximal (superior) to the obstruction (transgastric or transduodenal). The wire is used to transverse the stricture in antegrade fashion and then curled in the duodenum. The echo-endoscope is removed carefully while leaving the wire in place. A duodenoscope is advanced to the duodenum and the wire exiting the papilla is grasped with a snare and withdrawn through the accessory channel. The procedure is then completed with standard ERCP techniques.

It is believed that placement of a longer stent may diminish the risk of bile leak. In our institution, we favor the use of a fully covered metal stent but there are no studies evaluating the performance among different stents.

Exchanging the occluded stent

An occluded stent can be removed by using snare or a Dormia basket through the duodenoscope. The fistulous tract can then be recannulated, followed by new stent placement[44]. If the stent has not been *in situ* for 2-3 wk and there are concerns for the maturity of the fistula, the guidewire should be inserted into the bile duct through or alongside the occluded stent by ERCP catheter before removing the stent[44]. If a metal stent was used at the initial procedure, a plastic stent can be inserted *via* the occluded metal stent. EUS-guided stent placement can be an option for biliary diversion for an occluded biliary metal stent after a failed reinterventional ERCP[45].

Technical tips: The extrahepatic bile duct is very close to the portal vein in the first and second portion of the duodenum and the intrahepatic bile duct is close to the intrahepatic portal vein. Therefore, the puncture should be done very carefully especially in patients with mild dilatation of the hepatic bile duct. Angling the needle combined with gradual retraction and re-advancement can help the guidewire advance and traverse the obstruction[46]. The use of the EchoTip Ultra Access Needle (Cook Endoscopy, Winston-Salem, NC, United States) may significantly facilitate wire manipulation once bile duct access is achieved. The Access Needle has a sharp beveled stylet that protrudes beyond the blunt needle tip to facilitate easy puncture. Once the tip of the needle reaches the bile duct, the stylet is then removed. The blunt needle tip allows for smooth wire manipulation including advancement and withdrawal and prevents "pilling" of the hydrophilic wire coating as it frequently happens with standard sharp tip EUS needle. In addition, fluoroscopic techniques allowing imaging at different angles may facilitate wire passage. Care should be taken to limit the volume of the contrast injection to help maintain the visualization of the targeted area.

RESULTS OF EUS-GUIDED BILIARY DRAINAGE

The reported success rate of EUS-guided biliary drainage ranges from 73% to 97%[14,19,22,30,31,39,40]. EUS-guided biliary drainage should be as effective as transpapillary biliary drainage once the stent is successfully placed. Simultaneous tissue diagnosis and staging can be provided in the same setting. EUS-guided biliary drainage appears

to carry significant advantages over PTC and surgical biliary decompression and can be considered as a first step in patients who had failed ERCP[34-36]. It should be emphasized that the perceived advantages of EUS-guided biliary drainage over PTC or surgery have not been evaluated in prospective randomized studies and therefore the final choice of therapeutic modality should be based on local expertise and patient preferences.

COMPLICATIONS

The reported complication rates vary from 4% to 21%[14,38,40,47]. Internal drainage eliminates skin infection and maintenance issues of external drainage. Pancreatitis is a complication associated with prior failed ERCP and not attributed to EUS biliary drainage because of the lack of influence on the pancreatic duct[19].

The most common complications are biliary leakage and pneumoperitoneum (perforation into the peritoneal cavity or retroperitoneum, depending on the path of the access)[7,13,14,21,26,27]. The pneumoperitoneum is an early complication and usually self-limited[38]. Biliary leakage may occur predominantly with extrahepatic duct puncture, transluminal drainage, and larger hole[14,20,26,27]. Confirmation of the absence of bile leakage can be done using contrast medium on fluoroscopy. Focal bile peritonitis tends not to occur if the stent is promptly placed after dilation of the fistula[13,25]. Other common complications include abdominal pain and cholangitis[31]. Other reported complications include bacteremia, bleeding, biloma, ileus, aspiration pneumonia, cholecystitis, duodenal perforation and cardiopulmonary failure[11,14,19,31]. The use of color Doppler ultrasound to detect vascular structures can decrease the risk of bleeding[13,25]. No mortality has been reported to date. When comparing drainage using a stent across a fistula *vs* the rendezvous technique, the rendezvous drainage is less likely to lead to perforation, leakage, bleeding, or peritonitis which is attributed to the more physiological approach[41].

Late complications are stent migration and occlusion[31,35]. To date, there have been no long term follow-up reports on stent patency in a large number of patients. A case series of five cases reported by Yamao *et al*[48] using 7-8.5-Fr plastic stents *via* EUS-guided choledochoduodenostomy had an average stent patency of 211.8 d. The relatively long patency time of stent is believed to be due to the stent placement being far from the obstructing tumor[44].

LIMITATIONS

Advanced or complex strictures may not be traversed by EUS-guided approach and hence the percutaneous or surgical approach should remain an option.

The plastic stents' size is limited by the working channel of the linear echo-endoscope. Furthermore, the therapeutic EUS scopes channel is smaller than the therapeutic ERCP scope channel (4.2 mm *vs* 3.8 mm). Although a 10-Fr plastic stent can be deployed *via* the therapeutic EUS scope, it can be technically challenging due to significant friction in the relatively narrow channel. Finally, simultaneous passage of two wires into the bile duct as a safety measure to preserve access follow by 10-Fr stent placement over one of the guidewires is impossible with the current EUS therapeutic channel size.

These EUS drainage techniques have exciting potential but are performed mostly in tertiary referral centers by expert therapeutic endoscopists. Only endoscopists who are skilled at both ERCP and EUS should perform this procedure to avoid potential serious complications. Additionally, trained hepato-biliary surgeons and interventional radiologists should be available in the event of failure or complication.

At present, there are no dedicated devices for EUS-guided biliary drainage. The standard EUS and ERCP devices are being used for this purpose.

Although many case series have been reported, firm conclusions are limited due to great variations among studies in the endoscopic approaches applied, procedural goals, technical and clinical endpoints, definitions of success and duration and extent of follow-up. Furthermore, we lack data from well designed prospective randomized controlled studies comparing different therapeutic approaches.

We do not have a firm understanding on the exact rate of EUS guided biliary drainage complications as it is well recognized that underreporting can occur in retrospective case series.

CONCLUSION

EUS-guided biliary drainage has provided a new and promising route for biliary decompression. It has been reported to be feasible, highly successful and safe. It provides significant advantages over PTC and surgical biliary drainage and has become the procedure of choice in patients with obstructive jaundice who had failed ERCP in many institutions. The procedure is technically complex and confined to major referral centers. Despite the perceived advantages of EUS-guided drainage over PTC or surgery, well-designed, prospective, comparative studies are lacking. Therefore the choice of therapeutic modality in patients with biliary obstruction who had failed ERCP should be based on local expertise and patient preference.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i6.498

World J Gastroenterol 2012 February 14; 18(6): 498-506
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Targeted systemic therapies for hepatocellular carcinoma: Clinical perspectives, challenges and implications

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Received: February 21, 2011 Revised: June 6, 2011
Accepted: June 13, 2011
Published online: February 14, 2012

Abstract

Hepatocellular carcinoma (HCC) is a lethal disease in most patients, due to its aggressive course and a lack of effective systemic therapies for advanced disease. Surgical resection and liver transplantation remain the only curative options for a small subset of patients. Few patients with HCC are diagnosed early enough to be eligible for curative treatment. Angiogenesis inhibition is a natural therapeutic target for all solid tumors, but particularly for the highly vascularized HCC tumors. With the approval of the targeted agent sorafenib, there are now additional options for patients with HCC. Although sorafenib does produce some improvement in survival in HCC patients, the responses are not durable. In addition, there are significant dermatologic, gastrointestinal, and metabolic toxicities, and, as importantly, there is still limited knowledge of its usefulness in special subpopulations with HCC. Other angiogenesis inhibitors are in development to treat HCC both in the first-line setting and for use following sorafenib failure; the furthest in development is brivanib, a dual fibroblast growth factor pathway and vascular endothelial growth factor receptor inhibitor. Additional agents with antiangiogenic properties also in phase Ⅱ and Ⅲ development for the treatment of patients with HCC include bevacizumab, ramucirumab, ABT-869, everolimus and ARQ 197.

Key words: Hepatocellular carcinoma; Angiogenesis; Vascular endothelial growth factor; Fibroblast growth factor; Sorafenib; Tumor response; Brivanib

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Frenette C, Gish R. Targeted systemic therapies for hepatocellular carcinoma: Clinical perspectives, challenges and implications. *World J Gastroenterol* 2012; 18(6): 498-506 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i6/498.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i6.498

INTRODUCTION

Primary liver cancer, including hepatoma and hepatocellular carcinoma (HCC) is diagnosed in more than 560 000 people worldwide each year[1], including more than 24 000 Americans[2]. HCC accounts for up to 90% of all primary liver cancers[3]. HCC can be treated curatively with surgical resection or liver transplantation if diagnosed at an early stage; however, since most HCC patients present with advanced disease, only 15% are eligible for curative treatments[4]. Even for patients undergoing surgical resection, recurrence rates may be as high as 50% after 2 years and 76% by 10 years[5,6]. Patients meeting the Milan criteria who undergo liver transplantation can achieve a 5-year cancer-free survival rate greater than 60%[7].

As most patients with HCC are diagnosed with advanced disease, they generally have a poor prognosis, with median survival times of less than 1 year[3]. This is due, at least in part, to the absence of effective systemic therapies. Systemic therapies examined in the past, including both cytotoxic and hormonal agents, have provided limited or no benefit for these patients[6]. In late 2007, the angiogenesis inhibitor sorafenib was approved for use

in advanced HCC based on an improvement in survival compared with placebo[8,9]. While initial responses are observed, over time a loss of efficacy is apparent that may be due to "resistance" *via* escape/compensatory mechanisms. Like other angiogenesis inhibitors, sorafenib also has known class side effects, including skin-related toxicities, hypertension, proteinuria, diarrhea, and an increased risk for thromboembolism and bleeding events[10-12]. While most are manageable, certain rare events can be life-threatening (i.e., gastrointestinal perforation, fatal hemoptysis, thromboembolic events). Thus, the balance between risk and benefit in every clinical setting is an integral part of the differentiation and evaluation of targeted agents.

RATIONALE FOR ANGIOGENESIS INHIBITION IN HCC

Angiogenesis is a ubiquitous process that is required for tumor growth[13,14]. Angiogenic processes are also indirectly involved in tumor invasion and metastasis through the secretion of matrix-degrading proteinases by vascular endothelial cells[15] and the ability of tumor cells to travel to distant sites *via* the vascular network[16].

Proangiogenic factors are attractive therapeutic targets because they stimulate cancer formation, growth, and proliferation *via* angiogenesis using a number of distinct mechanisms. Established proangiogenic factors and their receptor signaling pathways include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2, platelet-derived growth factor (PDGF), angiogenin, and angiopoietin-2 (Ang-2)[17]. Other mediators, such as c-MET and mTOR, although not directly related to new vessel formation, affect angiogenesis *via* influences on downstream signaling.

HCC tumors are generally hypervascularized[18], suggesting that they may be especially vulnerable to angiogenesis inhibition. Several endogenous proangiogenic factors are expressed in HCC[19-22], and evidence indicates they play a role in HCC pathogenesis. For instance, serum VEGF levels increase with advancing HCC stages, being highest in patients with metastatic disease[23]. Elevated VEGF levels after locoregional therapy also are associated with poor prognosis and diminished response to therapy[24,25]. Expression of the proangiogenic factor FGF-2, the target of newer agents, is also elevated in patients with HCC[22] and its expression in HCC correlates with tumor microvessel density[26] and postoperative recurrence rate[27]. Tumor angiogenin expression correlates with microvascular density in patients with HCC, and high serum angiogenin levels are associated with decreased survival at 5 years[28]. Finally, mRNA angiopoietin expression level (*via* Ang-2/Ang-1 ratio) is positively correlated with tumor portal vein invasion, diameter, microvascular density, and poor prognosis[29]. Taken together, this evidence provides a strong rationale for targeting angiogenesis and related proangiogenic factors to provide more effective therapies for the treatment of HCC.

CHALLENGES AND LIMITATIONS OF SORAFENIB IN TREATING HCC

Sorafenib was the first systemic targeted therapy to be approved by the US Food and Drug Administration for patients with unresectable HCC[30], based on a 2.8-mo survival advantage over best supportive care (BSC) [hazard ratio (HR) 0.69, $P = 0.00058$] in the Sorafenib Hepatocellular carcinoma Assessment Randomized Protocol (SHARP) trial[31]. Sorafenib, which is also approved for advanced renal cell carcinoma (RCC)[30] inhibits the following receptor tyrosine kinases: VEGFR-2, VEGFR-3, PDGFR-β, c-KIT, and Flt-3[32]. It also binds to the serine-threonine kinases Raf, MEK, and ERK[32,33]. The VEGFR and PDGFR pathways and Raf-1 have all been implicated in the pathogenesis of HCC[34,35], providing a rationale for sorafenib activity in HCC.

Although sorafenib represents a much needed treatment option for patients with HCC, it also produces toxicities that may significantly affect patients' quality of life. High rates of dermatologic side effects are commonly reported with sorafenib, the most clinically significant being hand-foot skin reaction (HFSR)[36]. HFSR typically develops in the first few weeks of therapy, with painful hyperkeratotic lesions on the palms and soles that are surrounded by a ring of erythema localized on areas of pressure or flexure[37]. A meta-analysis examining the incidence of HFSR in phase Ⅱ and Ⅲ trials across solid tumors reported an incidence of 33.8% with sorafenib use, 8.9% of which were grade 3[38]. In the SHARP trial, sorafenib-associated HFSR had an overall incidence of 21% and a grade 3 incidence of 8%[9]. Of particular concern, HSFR appears to be more prevalent in Asian patients, the population most affected worldwide by HCC. A phase Ⅲ trial conducted solely in Asian patients with HCC reported a doubling of the overall incidence of HFSR compared with the SHARP trial[8,9]. Similarly, a phase Ⅲ trial of sorafenib monotherapy following transarterial chemoembolization in Asian patients reported an HFSR incidence of 82%[39].

Bleeding events, albeit rare, are also a toxicity of sorafenib and other angiogenesis inhibitors. In a meta-analysis of 2109 patients enrolled in sorafenib clinical trials or expanded-access programs, the relative risk of bleeding events was 1.86 ($P < 0.001$) and the incidence of grade 3 or higher bleeding events was 2.2%[40]. It is notable that patients with HCC have not demonstrated an increased incidence of bleeding events with sorafenib; for example, in the SHARP trial, grade 3/4 bleeding events were 1% in the sorafenib arm.

By design, SHARP was conducted in the Americas, Europe, and Australasia, and thus generated limited data in Asian patients. Patients included in SHARP had preserved liver function and were of mostly Child-Pugh (C-P) A status[9]. In order to gain additional data, a randomized phase Ⅲ trial in Asian patients with advanced HCC was completed, but again C-P B and C patients were excluded[8,39]. The overall trend of response to sorafenib from

this Asian population was similar to those of SHARP, with time to progression (TTP) and overall survival (OS) improved compared with placebo, and similar treatment effects (HRs of 0.68-0.69). However, median OS appeared to be shorter (6.2 mo) *vs* SHARP (10.7 mo), most likely due to more advanced disease based on performance status, number of tumor sites, and presence of lung metastases compared with SHARP[8,9]. A subsequent single-arm, phase Ⅱ trial was conducted in Asian patients with worse prognosis, including C-P B and C status (29%) and portal vein thrombosis (43%)[41]. In this trial, 26% of patients derived clinical benefit from sorafenib. Again, the incidence of toxicities was higher than in SHARP, with grade 3/4 diarrhea occurring in 20% and grade 3/4 HFSR in 16% of patients. As the majority of HCC cases are found in Asia[42], the development of safe and more effective therapies for this population represents a significant unmet need.

Hence, while the sorafenib trials have provided valuable information in patients with preserved liver function[8,9,41], determining efficacy and safety in the substantial portion of patients with advanced HCC remains a challenge. Other recent phase Ⅱ trials are beginning to provide preliminary evidence of sorafenib safety and efficacy in patients with more advanced disease. A trial of sorafenib monotherapy in 59 patients with unresectable HCC, including 39% with C-P B status and 17% of C-P C status, showed promising activity regardless of disease stage and liver function[43]. Responses for patients of C-P B status were similar to those from SHARP. Median OS, however, declined with more advanced C-P status, most likely due to underlying cirrhosis, and because drug toxicities are more prevalent with compromised liver function, causing liver-related or systemic complications that lead to early treatment discontinuations. In a second phase Ⅱ trial, pharmacokinetic profiles of sorafenib were similar in both C-P A and B subgroups, while median TTP and OS appeared shorter and adverse events related to poorer liver function were more frequent in C-P B patients[44]. Finally, another single-arm, phase Ⅱ trial in 51 Asian patients, including 15 (29%) with C-P B/C status, found no significant differences between C-P B/C status patients and C-P A patients in disease control rate, median OS, grade 3/4 hematologic toxicities, or grade 3/4 nonhematologic toxicities[41].

Despite initial responses to sorafenib, and similar to other targeted agents, most HCC patients experience a loss of efficacy. Furthermore, across clinical trials, 20%-38% of patients discontinued sorafenib due to adverse events[8,9,41]. Similar to what has been reported with bevacizumab, some data indicate that patients who discontinue sorafenib therapy may experience "rebound," whereby symptom and tumor progression develops rapidly upon discontinuation[45]. While this accelerated growth effect appears to be temporarily curtailed with re-initiation of therapy, insensitivity to treatment returns quickly. No effective second-line treatment options currently exist outside of clinical trials for patients who are resistant or refractory to and/or intolerant of sorafenib.

THE FAILED PROMISE OF SUNITINIB

Next to sorafenib, sunitinib is the most studied multitargeted tyrosine kinase inhibitor. Like sorafenib, sunitinib is an inhibitor of VEGFR and PDGFR and is currently indicated for the treatment of RCC, as well as for gastrointestinal stromal tumors[46]. While early indications were that sunitinib would have efficacy in HCC, the phase Ⅲ SUN 1170 trial comparing sunitinib with sorafenib in patients with advanced HCC was discontinued due to increased serious sunitinib-related adverse events and the improbability of achieving noninferior efficacy[47]. As a result, sunitinib is no longer in development for the treatment of HCC.

CHALLENGES IN ASSESSING TUMOR RESPONSE

The 2 traditional imaging criteria widely used for measuring tumor responses to treatment are Response Evaluation Criteria In Solid Tumors (RECIST)[48], which are used primarily in the United States, and the World Health Organization (WHO) criteria[49], which are used internationally. With the advent of targeted therapies that are cytostatic, and in particular regarding HCC tumors, these traditional criteria are limited in their usefulness to assess treatment response because (1) cirrhotic livers may not remodel around a necrotic tumor; (2) HCC tumors are occasionally diffuse and infiltrative in cirrhotic livers; (3) arterial phase enhancement of premalignant dysplastic nodules can be mistaken for progression; and (4) cytostatic targeted agents alter tumor vascularity without affecting tumor size[50]. Both criteria have been updated in the past decade to account for HCC tumor viability in order to appropriately evaluate the extent of tumor necrosis and/or viability to quantify treatment response. The European Association for the Study of the Liver updated the WHO criteria in 2000[51], and in 2008 the American Association for the Study of Liver Diseases updated RECIST[52] and then further clarified the modified criteria in 2010[53]. Specific differences between these current criteria-modified WHO (mWHO) and modified RECIST (mRECIST)-for treatment response in HCC are listed in Table 1.

As an example of the variability in response assessment that can arise according to which of the 2 criteria is used, Finn *et al*[54] retrospectively compared response assessment by the necrosis-adjusted mRECIST (mRECIST has not yet been validated) for HCC with the prospective use of mWHO criteria in 101 patients with HCC who received brivanib, an antiangiogenic targeted agent. They found that mWHO criteria underreported treatment benefit compared with mRECIST for HCC, with 31 patients classified as having progressive disease under the mWHO criteria but stable disease or partial response under mRECIST. This suggests that these patients may have been prematurely discontinued from treatment while still deriving benefit. These discrepancies can be explained by differences in the methodologies of mRECIST and

Table 1 Response assessment by modified World Health Organization criteria and modified Response Evaluation Criteria in Solid Tumors

Parameter	Modified WHO	Modified RECIST
Type of assessment	Spiral CT	Spiral CT or dynamic MRI
Frequency of assessment	⩾ 4 wk	6-8 wk
Measurement of tumor volume	Bidimensional measurement	Unidimensional measurement
Tumor necrosis measurement	Reduction in viable tumor area using contrast-enhanced radiological imaging	Reduction in viable tumor area using contrast-enhanced radiological imaging
Viable tumor definition	Enhanced areas inside treatment lesions	Uptake of contrast agent in the arterial phase
Complete response	Complete disappearance of tumor enhancement determined by 2 observations ⩾ 4 wk apart	Disappearance of any intratumoral arterial enhancement in all target lesions
Partial response	> 50% reduction in total area of tumor enhancement determined by 2 observations ⩾ 4 wk apart	⩾ 30% decrease in the sum of diameters of viable target lesions, taking as reference the baseline sum of the diameters of target lesions
Stable disease	Insufficient shrinkage to qualify for partial response and insufficient increase to qualify for progressive disease	Any cases that do not qualify for either partial response or progressive disease
Progressive disease	> 25% increase in total area of tumor enhancement or the appearance of new lesions	⩾ 20% increase in the sum of the diameters of viable target lesions, taking as reference the smallest sum of the diameters of viable target lesions recorded since the treatment started or the appearance of 1 or more new lesions

CT: Computed tomography; MRI: Magnetic resonance imaging; RECIST: Response Evaluation Criteria in Solid Tumors; WHO: World Health Organization.

Table 2 Agents with antiangiogenic properties in development for the treatment of hepatocellular carcinoma

Agent	Therapeutic target	Phase of study
Brivanib	VEGFR, FGFR	Ⅲ
Bevacizumab	VEGF	Ⅱ
Ramucirumab	VEGFR-2	Ⅲ
ABT-869	VEGFR-1, VEGFR-2, PDGFR-β, c-KIT, Flt-3	Ⅲ
Everolimus	mTOR	Ⅲ
ARQ 197	c-MET	Ⅱ

PDGFR: Platelet-derived growth factor receptor; VEGFR: Vascular endothelial growth factor receptor.

mWHO, which employ different measurements and calculations of tumor size, thereby producing distinct interpretations of treatment response. In addition, there are differences in how progression is defined, with cytologic confirmation of ascites required with mRECIST and small new lesions (< 1 cm) unlikely representing progression with the mRECIST. While not conclusive, these results suggest that treatment response based on assessment criteria vary widely and indicate the need for further clinical validation.

LOSS OF EFFICACY TO ANTIANGIOGENIC THERAPY: ESCAPE AND RESISTANCE

Clinical trials of antiangiogenic agents have shown that most patients with advanced tumors eventually experience progression, including those who initially respond to treatment[8,9,55-57]. Recent evidence suggests that relapse during treatment with antiangiogenic agents occurs due to VEGF inhibition-driven hypoxia, which induces upregulation of alternate proangiogenic signals such as FGF, which overrides the VEGFR inhibition[58]. This was demonstrated preclinically in murine tumors that initially responded to treatment with an anti-VEGFR-2 antibody, but relapsed after 2 wk, showing higher levels of other proangiogenic signals, including FGF-1 and FGF-2, than untreated tumors[59]. Moreover, blockage of FGF signaling in this model slowed tumor growth and attenuated its revascularization during the relapse phase. Clinically, this has been observed in patients with HCC[26,27,60] and, more recently, in glioblastoma patients treated with the pan-VEGFR inhibitor AZD2171, in whom increased plasma levels of FGF were detected upon relapse[61]. Another study showed that approximately half of patients with metastatic colorectal cancer who received bevacizumab plus chemotherapy had more than a 5-fold increase in either placental growth factor or FGF prior to progression[62]. Patients with late-stage breast cancer have been reported to express a large number of proangiogenic factors, including FGF-2, in contrast to earlier stage lesions, which primarily express VEGF[58,63]. Taken together, these data support the hypothesis that tumor progression during inhibition of angiogenesis may be facilitated *via* activation of compensatory proangiogenic and tumorigenic mechanisms.

COMPOUNDS IN DEVELOPMENT FOR TREATMENT OF HCC

Several compounds in development stand to address the challenges and limitations of targeted therapy in the treatment of HCC. These are discussed in the following sections and summarized in Table 2.

Brivanib

Brivanib is currently in phase Ⅲ trials in HCC. It is distinct from both sorafenib and sunitinib in that it is an oral, selective, dual inhibitor of the FGF and VEGF signalling pathways[64,65]. Since FGF signaling may contribute to acquired "resistance," or compensatory signaling,

during anti-VEGFR therapy[58], the simultaneous inhibition of these[64,65] 2 pathways by brivanib may both delay initial progression in response to antiangiogenic therapy (as first-line treatment) and successfully treat tumors that have already progressed during anti-VEGFR therapy (as second-line treatment). With respect to its potential as first-line therapy, brivanib has delayed initial progression compared with sorafenib in preclinical studies[66]. It has also shown specific inhibitory activity in patient-derived HCC xenografts implanted in mice[67]. Clinically, brivanib has demonstrated a disease control rate of 51%, a median TTP of 2.8 mo, and an OS of 10 mo as first-line monotherapy in a phase Ⅱ trial of predominantly Asian patients with HCC[68]. A retrospective analysis using mRECIST for HCC criteria also demonstrated an objective response rate of 25%, with 9% complete responses[68]. Brivanib was also associated with a low incidence of grade 3/4 adverse events, including hypertension (10.9%), diarrhea (3.6%), and HFSR (1.8%)[68]. Due to its unique mechanism of action and favorable safety profile, brivanib is currently under phase Ⅲ investigation as first-line therapy *vs* sorafenib in patients with advanced HCC (BRISK-FL). As a potential second-line agent following antiangiogenic therapy, brivanib has demonstrated activity against xenograft tumors that were nonresponsive to bevacizumab[69]. Allen *et al*[66] also used a mouse model of pancreatic neuroendocrine cancer to show that brivanib administered after sorafenib failure could delay tumor growth modestly, despite showing evidence of revascularization. In a phase Ⅱ trial of brivanib in patients with HCC who had been treated with sorafenib, brivanib produced a median TTP of 2.7 mo and an OS of 9.8 mo[70]; in a retrospective analysis of paired TTP, at least 40% of patients had longer TTP with brivanib than with prior sorafenib[71]. Brivanib is currently under investigation in 2 second-line phase Ⅲ trials-1 in Asian patients following sorafenib failure (progression or intolerance; BRISK-APS), and another similar trial that is enrolling an ethnically unselected patient population (BRISK-PS).

Bevacizumab

The anti-VEGF monoclonal antibody bevacizumab was the first angiogenesis inhibitor to be approved as an antineoplastic agent[72]. Bevacizumab has shown activity in phase Ⅱ HCC testing in combination with chemotherapy[72-75], with the epidermal growth factor receptor inhibitor erlotinib[76], and as monotherapy[77]. Despite initial safety concerns, particularly gastrointestinal bleeding and thrombosis, phase Ⅱ trials in HCC have shown toxicities to be manageable[78]. New bevacizumab combinations are under investigation in ongoing phase Ⅱ HCC trials, including combination with sorafenib, everolimus, temsirolimus, chemoembolization, and hepatic arterial infusion of floxuridine and dexamethasone.

Ramucirumab

The monoclonal antibody ramucirumab is a specific inhibitor of VEGFR-2[79]. A phase Ⅱ study of 42 patients with advanced HCC and primarily well-preserved liver function (75% C-P A status) showed that first-line ramucirumab monotherapy produced a disease control rate of 50% and a median progression-free survival (PFS) of 4.3 mo[80]. This positive study prompted the initiation of the phase Ⅲ REACH trial in HCC, which is comparing ramucirumab/supportive care with placebo/supportive care for second-line treatment after sorafenib.

ABT-869

ABT-869 is a multitargeted tyrosine kinase inhibitor that inhibits multiple members of the VEGFR and PDGFR families[81]. In a xenograft model of HCC, ABT-869 significantly reduced tumor burden, either alone or in combination with rapamycin[82]. Interim phase Ⅱ results in patients with advanced HCC showed a median TTP of 3.7 mo with ABT-869 treatment and a safety profile consistent with angiogenesis inhibition[83]. ABT-869 is in phase Ⅲ testing as a first-line treatment for advanced HCC versus sorafenib.

INHIBITION OF OTHER ANGIOGENIC AND TUMORIGENIC PATHWAYS

mTOR inhibitors: Everolimus and sirolimus

mTOR inhibitors are not traditionally considered direct angiogenesis inhibitors; rather, they have well-known immunosuppressive properties. In fact, 2 of these agents, sirolimus and everolimus, are used to prevent rejection in organ transplant recipients[84]. mTOR inhibitors also have antineoplastic properties, *via* mTOR regulation of tumor proliferation and metabolism[85]. MTOR indirectly modulates angiogenesis through regulation of VEGF expression and translation of proteins involved in angiogenesis[86]. Clinically, there is growing evidence to suggest that mTOR inhibitors may reduce *de novo* malignant growth[87] and recurrence in the liver post-transplant[88]. In patients with advanced HCC, everolimus produced a median PFS of 3.8 mo and a disease control rate of 44% in phase Ⅰ/Ⅱ testing[81]. Consequently, the ongoing phase Ⅲ EVOLVE-1 trial has been initiated to compare everolimus with BSC in patients with HCC who progressed on or after sorafenib or who were intolerant to sorafenib.

ARQ 197

Similar to everolimus, ARQ 197 has antiangiogenic properties, but is not considered an angiogenesis inhibitor. ARQ 197 is an inhibitor of the oncogene c-MET, which stimulates tumor growth, invasion, metastasis, and angiogenesis *via* binding of its ligand, hepatocyte growth factor[89]. In phase I testing in cirrhotic patients with HCC, ARQ 197 demonstrated some activity and was well tolerated, with serious adverse events that were primarily hematologic[90]. ARQ 197 is currently in phase Ⅱ testing in second-line advanced HCC.

CONCLUSION

Unmet needs for HCC remain, despite the availability of

sorafenib. Indeed, sorafenib has some significant limitations, including modest, transient benefits, and toxicity challenges; and its use in patients with more advanced liver disease and in Asian patients has not yet been fully defined. The development of newer targeted therapies that inhibit angiogenesis simultaneously with inhibition of other key proangiogenic factors in HCC, such as FGFR or c-MET signaling, is providing further insights into the underlying pathogenesis of HCC tumors. Compounds that directly block angiogenesis and tumorigenesis *via* dual inhibition of FGFR and VEGFR, such as brivanib, and other compounds that indirectly modulate angiogenesis, such as mTOR inhibitors, are providing novel mechanisms that exploit critical pathways in HCC tumor progression and may have the potential to improve clinical outcomes both as monotherapy and in the case of escape from sorafenib. In the coming years, a number of phase Ⅲ clinical trials examining these angiogenesis inhibitors will be mature, providing a better picture of the clinical utility and treatment options for patients with HCC.

ACKNOWLEDGMENTS

We thank Lena Prisco, PhD, and Mark English, PhD, of PAREXEL and Bristol-Myers Squibb for their editorial assistance with this paper.

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S- Editor Gou SX **L- Editor** O'Neill M **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i6.507

World J Gastroenterol 2012 February 14; 18(6): 507-516
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Dynamic tracking of stem cells in an acute liver failure model

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Supported by Citadel Capital Scholarship Foundation, Egypt; and Dr. Leslie Borthwick/Ms. Anita Holme, Charitable Research Fund East and North Herts NHS TrustHertfordshire, United Kingdom
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Received: June 1, 2011 Revised: September 2, 2011
Accepted: October 28, 2011
Published online: February 14, 2012

Abstract

AIM: To investigate a dual labeling technique, which would enable real-time monitoring of transplanted embryonic stem cell (ESC) kinetics, as well as long-term tracking.

METHODS: Liver damage was induced in C57/BL6 male mice (n = 40) by acetaminophen (APAP) 300 mg/kg administered intraperitoneally. Green fluorescence protein (GFP) positive C57/BL6 mouse ESCs were stained with the near-infrared fluorescent lipophilic tracer 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide (DiR) immediately before transplantation into the spleen. Each of the animals in the cell therapy group (n = 20) received 5 × 10^6 ESCs 4 h following treatment with APAP. The control group (n = 20) received the vehicle only. The distribution and dynamics of the cells were monitored in real-time with the IVIS Lumina-2 at 30 min post transplantation, then at 3, 12, 24, 48 and 72 h, and after one and 2 wk. Immunohistochemical examination of liver tissue was used to identify expression of GFP and albumin. Plasma alanine aminotransferase (ALT) was measured as an indication of liver damage.

RESULTS: DiR-stained ESCs were easily tracked with the IVIS using the indocyanine green filter due to its high background passband with minimal background autofluorescence. The transplanted cells were confined inside the spleen at 30 min post-transplantation, gradually moved into the splenic vein, and were detectable in parts of the liver at the 3 h time-point. Within 24 h of transplantation, homing of almost 90% of cells was confirmed in the liver. On day three, however, the DiR signal started to fade out, and *ex vivo* IVIS imaging of different organs allowed signal detection at time-points when the signal could not be detected by in vivo imaging, and confirmed that the highest photon emission was in the liver (P < 0.0001). At 2 wk, the DiRsignal was no longer detectable *in vivo*; however, immunohistochemistry analysis of constitutively-expressed GFP was used to provide an insight into the distribution of the cells. GFP +ve cells were detected in tissue sections resembling hepatocytes and were dispersed throughout the hepatic parenchyma, with the presence of a larger number of GFP +ve cells incorporated within the sinusoidal endothelial lining. Very faint albumin expression was detected in the transplanted GFP +ve cells at 72 h; however at 2 wk, few cells that were positive for GFP were also strongly positive for albumin. There was a significant improvement in serum levels of ALT, albumin and bilirubin in both groups at 2 wk when compared with the 72 h time-point. In the cell therapy group, serum ALT was significantly (P = 0.016) lower and albumin (P = 0.009) was significantly higher when compared with the control group at the 2 wk time-point;

however there was no difference in mortality between the two groups.

CONCLUSION: Dual labeling is an easy to use and cheap method for longitudinal monitoring of distribution, survival and engraftment of transplanted cells, and could be used for cell therapy models.

Key words: Cell transplantation; Cell tracking; Embryonic stem cells; Acute liver failure; Liver regeneration

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Ezzat T, Dhar DK, Malago M, Olde Damink SWM. Dynamic tracking of stem cells in an acute liver failure model. *World J Gastroenterol* 2012; 18(6): 507-516 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i6/507.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i6.507

INTRODUCTION

Acute liver failure (ALF) is a life-threatening condition which often progresses rapidly to hepatic encephalopathy and multi-organ failure, leading to death within days. Viral hepatitis is the most common cause of ALF worldwide, with hepatitis B virus responsible for about 70% of cases[1]. ALF following hepatic resections is rare, occurring in only 2.6% of patients. However, it produces significant morbidity and mortality[2]. Another causative factor, which is more commonly encountered in the Western world, is acetaminophen (APAP) overdose, which leads to excessive production of its active metabolite N-acetyl-p-benzoquinone imine in the liver, causing depletion of the glutathione stores. This is ultimately followed by centrilobular hepatic necrosis and loss of functioning hepatocytes[3]. APAP-induced liver toxicity is of significant importance because of its continued increase in incidence; it is currently the most common cause of ALF in the Western world[4].

Several specific therapies have been designed to treat APAP-induced ALF. These have been in the form of drugs, such as N-acetyl cysteine being used to replenish glutathione stores in APAP-induced ALF[5], or bioartificial devices to remove toxic metabolites and provide temporary support until native hepatocytes regenerate or as a bridge to liver transplantation (LTx)[6]. LTx is the definitive therapy in patients not responding to conservative therapies which can only maintain the patients until a liver graft becomes available with moderate hypothermia as a bridge to LTx[7,8]. Although the patient's survival after LTx for ALF is lower than that for chronic liver diseases, it is still highest among all other treatment options, with a long-term survival rate of about 70%[9]. However, LTx may not always be feasible due to the severe shortage of donors. One alternative to LTx would be the use of pluripotent or multipotent cells to support the regeneration of the native liver.

So far, only a limited number of experimental studies used terminally differentiated hepatocytes to treat ALF with variable outcomes[10,11]. Hepatocytes seem to be the optimum cell type to replenish the damaged liver; however, they need to be isolated from the donor livers. Also, they cannot be cultured for indefinite periods *in vitro*, and multiple hepatocyte transplantations may be required to achieve a satisfactory regenerative drive[12].

These problems can be bypassed by the use of pluripotent or multipotent cells of different sources such as bone marrow, umbilical veins, fatty tissue or embryonic stem cells (ESCs). These cells have been used in the experimental set up to deliver genes to the liver in models with metabolic diseases[13], and also used to treat chronic liver diseases such as cirrhosis[14]. In the present study, we used undifferentiated ESCs in a mouse model of ALF to evaluate the cell kinetics. The ideal way of tracking transplanted cells used to repopulate damaged livers and improve their function remains to be elucidated. Optimizing cell therapies in patients would require an accurate, highly sensitive and non-invasive means to properly assess cell survival, biodistribution and fate in the same patient over time. Cell labeling offers the advantage of imaging distinct cell populations *in vivo* and investigating the efficacy of these therapies using non-invasive imaging techniques.

In this study, we wanted to evaluate a dual labeling technique, which enabled real-time monitoring of the kinetics of the transplanted ESCs as well as long-term tracking of the cells. A unique dual labeling of the cells was performed using a fluorescence dye and, for long-term tracking, a lentivirus mediated and constitutively expressed green fluorescence protein (GFP).

MATERIALS AND METHODS

Animals and experimental design

Forty male C57/BL6 mice (Charles River laboratories) at 5-6 wk of age with an average weight between 20-25 g were used for this study. All the surgical and experimental procedures were carried out according to the guidelines set by the University College London institutional procedures. All animals were acclimatized for 7 d prior to the experiments. The animals were allocated into 2 groups. Group 1 (n = 20) with cell therapy and group 2 (n = 20) with vehicle only. The group size was not powered for a mortality study. All animals received a single dose of APAP 300 mg/kg administered intraperitoneally[15]. To ensure adequate dissolution, APAP was sonicated over-

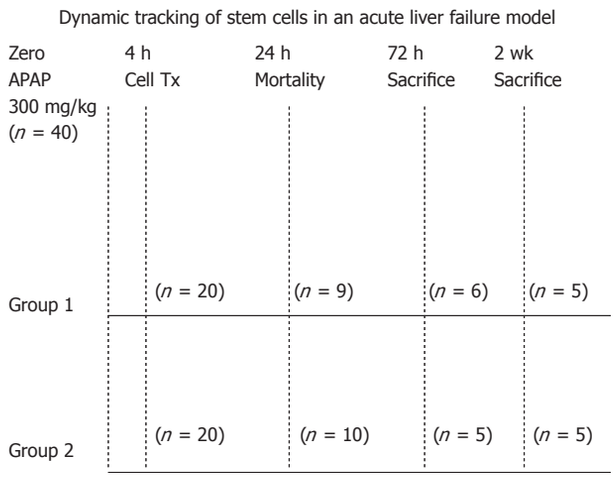

Figure 1 Experimental animal design. Forty mice were treated with acetaminophen (APAP). The animals were then divided into 2 groups. The cell therapy group = Group 1 with cell transplantation and the control group = Group 2. During the first 24 h, 19/40 animals died due to the effect of APAP. Animals from both groups were then killed at 72 h when the signal started to decay and at two weeks when the signal could no longer be detected *in vivo*.

night in a water bath at 42 ℃ and the temperature was maintained until injection time. APAP administration was preceded by a subcutaneous injection of 10% dextrose in order to prevent mortality from severe hypoglycemia[16]. The SC injection of 10% dextrose was repeated every 6 h during the first day of the experiment. At selected time points, the animals were killed by exsanguination (Figure 1). Time points were selected to have an optimal evaluation of ESC cell homing in organs using *ex vivo* imaging and for immunohistochemical (IHC) studies.

Embryonic stem cell line and culture conditions

Undifferentiated C57/BL6 ESCs constitutively expressing GFP (Millipore, Co Durham, United Kingdom) were maintained in the undifferentiated state using MilliTrace mouse ESC expansion medium (Millipore, Co Durham, United Kingdom), supplemented with 15% fetal bovine serum and leukemia inhibiting factor. Puromycin was added to the medium (0.5 μg/mL) for selective growth of GFP-positive cells. Cells were cultured on 0.1% gelatin coated T-75 cell culture flasks until 80% confluence was reached. The undifferentiated state of the ESCs was confirmed by alkaline phosphatase expression in more than 90% of the ESC colonies. Cell fixation was performed with 4% paraformaldehyde for 2 min followed by incubation with a mixture of fast red violet, naphthol AS-BI phosphate solution and water in a 2:1:1 ratio in a dark room for 15 min.

Dual labeling protocol

In order to achieve dual labeling of the GFP expressing ESCs for the purpose of *in vivo* tracking, the cells were labeled with the fluorescent lipophilic tracer 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide (DiR) (Invitrogen Ltd, United Kingdom). The excitation/emission spectrum of DiR is in the near infrared range (excitation 750 nm and emission 782 nm). After preparation of the stock solution, 5 mL of cell-labeling solution was directly applied to 1 mL cell suspension, mixed and incubated at 37 ℃ for 20 min. The labeled suspension was centrifuged at 1500 rpm for 5 min and the cell pellet was resuspended in warm fresh medium. This procedure was repeated twice to ensure complete removal of any unbound dye.

Cell preparation and transplantation

Following labeling with DiR, 5×10^6 cells were used per transplantation, which would be equivalent to approximately 7.5%-8% of the host liver hepatocytes[17]. The cells were suspended in 100 μL of sterile phosphate buffer solution (PBS) in a syringe that was kept on ice until the time of transplantation. Cell transplantation was performed 4 h following treatment with APAP. Under adequate anesthesia and strict aseptic technique, a 1 cm incision was made in the left flank of the abdomen and the spleen was delivered outside the incision. The cells were injected into the spleen over a period of 5 min to prevent any loss of the cells due to overflow. Following injection, the needle was removed and fibrin glue applied to the injection site to prevent bleeding and cell loss. After confirming hemostasis, the spleen was replaced back into the abdominal cavity and the abdominal wall closed in a single layer. The same procedure was performed for the control group with PBS only.

Tracking of the transplanted cells

The mice were anesthetized with inhaled isoflurane and placed into the *In vivo* Imaging System (IVIS Lumina 2) chamber (Caliper Life Sciences, Cheshire, United Kingdom) and images were acquired using the CCD camera at 30 min post-transplantation, then at 3, 12, 24, 48 and 72 h, and after one and 2 wk. Following the sacrificing of the animals in groups at predetermined time periods, the spleen, liver, lung and kidney were placed inside the IVIS and images were acquired of the regions of interest to quantify the cell uptake in different organs. Three fluorescence filters [GFP, tricarbocyanine 5.5 and indocyanine green (ICG)] were used to identify the filter with the least background autofluorescence Data analysis was performed using the Living Image™ Software 3.0 (Caliper Life Sciences, Cheshire, United Kingdom).

Green fluorescence protein fluorescence

A Nikon fluorescence microscope (E501) was used for direct detection of GFP+ cells using the fluorescein isothiocyanate filter on frozen tissue sections.

Blood and tissue sampling

Following the culling the animals at 72 h and 2 wk, blood was collected by puncturing the suprahepatic vena cava, preserved on ice, then centrifuged to collect serum for further analysis of liver functions. Tissue samples of the liver and spleen were also collected and preserved for hematoxylin and eosin (HE) staining and IHC.

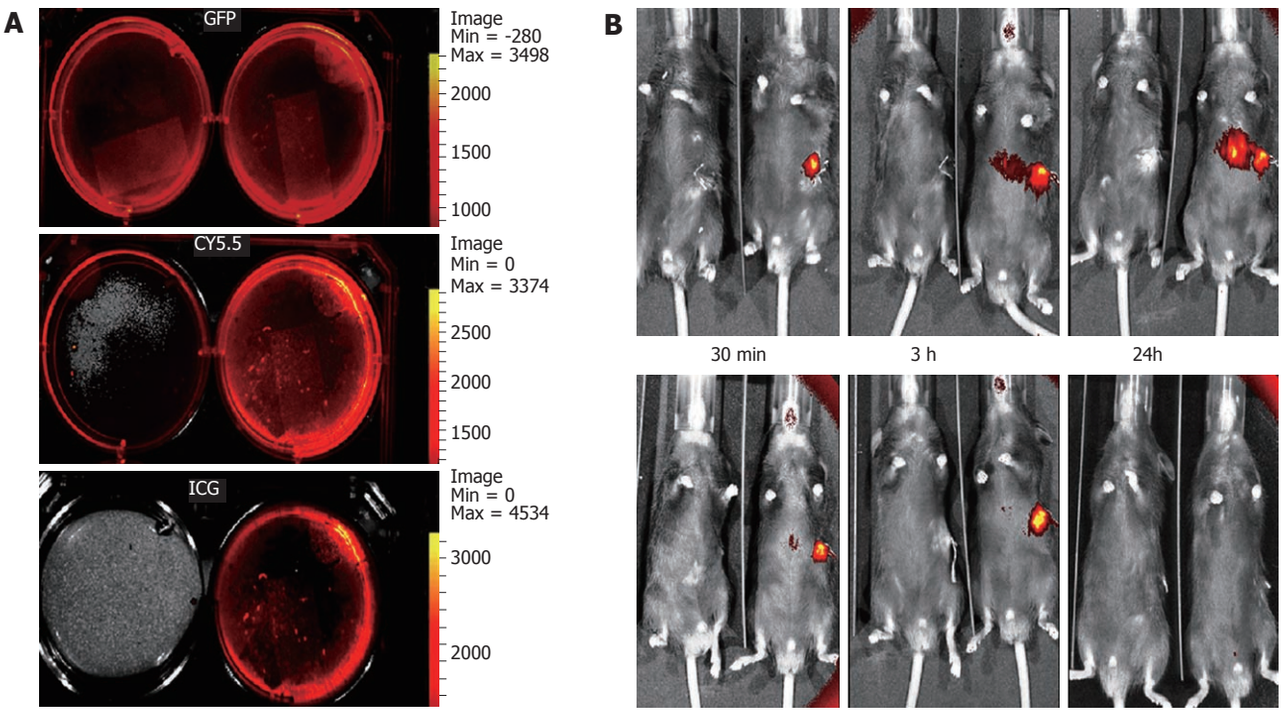

Figure 2 Labeling and tracking of the fluorescent embryonic stem cell following acetaminophen administration. IVIS images of green fluorescence protein (GFP) +ve cultured embryonic stem cells (ESCs) without 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide (DiR) staining (LT) and with DiR staining (RT) showing background fluorescence using the GFP and tricarbocyanine 5.5 filters, but not with the indocyanine green (ICG) filter (A). Images of a pair of mice (B), one from the cell therapy group-RT and the other from the control group-LT, were compared using IVIS. At 30 min following transplantation, a strong signal could only be detected from the spleen where the cells were injected. Between 3 and 24 h following the cell transplantation, the signal started to intensify between the spleen and the liver, which is most probably the splenic vein owing to its tortuous course. A strong signal was detected in the liver over 24 h post-transplantation, which faded out by the 72 h time-period. After one week, the signal could not be detected in the liver, but was still strong and was detectable over the spleen, which had completely disappeared by 2 wk.

Measurement of liver function tests

Measurement of hepatic enzyme and serum albumin levels were performed using the COBAS Integra 400 biochemistry analyzer (Roche) for measurement of plasma alanine aminotransferase (ALT), albumin, bilirubin and urea.

Immunohistochemistry

Sections of both the liver and spleen were preserved in 10% buffered formalin for HE staining and IHC studies. The IHC study was performed using the streptavidin ABC duet kit (Dako, Cambridgeshire, United Kingdom) on 5 μm thick paraffin embedded tissue sections. Briefly, sections were dewaxed in xylene, rehydrated in graded alcohol and rinsed with PBS. Antigen retrieval was performed by heating in citrate buffer solution at 95 ℃ for 10 min. Endogenous peroxidase activity was blocked by incubating the slides with 3% hydrogen peroxide for 20 min. The sections were incubated at 4 ℃ overnight with primary antibodies; polyclonal rabbit anti-GFP (1:10, Millipore), and anti-albumin antibody (1:1000, Abcam). Streptavidin-peroxidase labeled secondary antibody was applied for 30 min at room temperature. Between steps, slides were rinsed with PBS solution. Color was developed with the chromogen 3,3'-diaminobenzidine (Dako, Cambridgeshire, United Kingdom), and counterstained with Mayer's hematoxylin solution.

Statistical analysis

Continuous variables were expressed as mean ± SD. Differences were compared by *t*-test or Mann-Whitney *U* test as appropriate. For comparison of multiple groups, one-way ANOVA with Scheffe's *post-hoc* test was used. Differences in survival were analyzed by Kaplan Meier and a log rank test was used for significance. Statistical significance was taken as $P < 0.05$. All statistical analyses were done using the SPSS software for Windows 14.0 (SPSS, Inc., Chicago, IL).

RESULTS

In vivo detection of the transplanted cells

IVIS proved to be effective in precisely identifying the cells labeled with DiR in the liver and spleen at different time-points. This was possible only by using the ICG filter due to its high background passband of 665-695 nm with minimal background fluorescence (Figure 2A). At 30 min post-transplantation, the cells were confined inside the spleen and then gradually moved into the splenic vein and part of the liver at 3 h time point. By 24 h, the cells spread out over almost all areas of the liver and a residual signal was received over the spleen as well. The signal of DiR emitted from the liver faded out at 72 h. No signal could be detected in any other organs (Figure 2B).

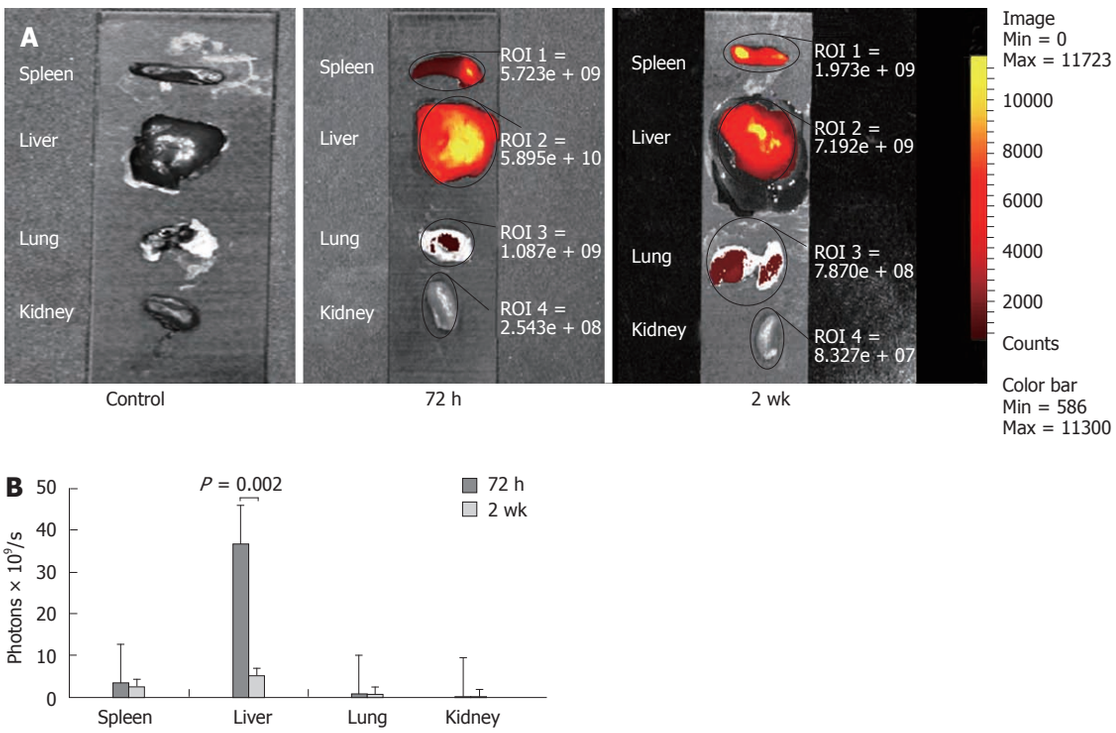

Figure 3 ***Ex vivo*** **images showing the distribution of fluorescent cells in different organs as detected by the indocyanine green filter of the IVIS.** A signal was detected at the site of injection in the spleen; however, the highest signal was noticed in the center of the liver at 72 h. which faded out by the 2 wk time-point. A weak fluorescent signal was also detected in the lungs at 72 h and 2 wk, but not seen in kidneys at any time-points (A). A graph showing the highest uptake of cells in the liver at all time-points with a significant drop at 2 wk (B).

Ex vivo imaging

A very clear fluorescent signal was detectable from both the liver and spleen when the organs were taken out and imaged directly with the IVIS. Eliminating the barrier effect of the anterior abdominal wall allowed signal detection at time-points when the signal could not be detected by *in vivo* imaging. Similar to *in vivo* imaging, the ICG filter was the most accurate filter to localize the cells with no autofluorescence from the organs. A weak signal was also detectable in the lungs and kidneys, in contrast to the *in vivo* imaging (Figure 3A). DiR uptake in the liver was significantly higher than the sum of uptake in all other organs at 72 h ($P < 0.0001$). At 2 wk, the amount of photons emitted from the liver decreased significantly from $3.67 \times 10^{10} \pm 1.7 \times 10^{10}$ photons/s at 72 h to $5.18 \times 10^{9} \pm 1.58 \times 10^{9}$ ($P = 0.002$). However, in the liver, the emission was still significantly higher than the spleen ($P = 0.002$), lungs ($P < 0.0001$) and kidneys ($P < 0.0001$) (Figure 3B).

Engraftment of the transplanted cells in the liver

Frozen liver sections, using fluorescent microscopy, were studied to validate that the signal detected using the IVIS was definitely emitted from the engrafted cells and not from the released fluorescent dye DiR following cell death. GFP fluorescence from the transplanted cells was easily detectable by direct fluorescence microscopy at 72 h, however, it became much weaker at 2 wk and, therefore, we relied on IHC detection of GFP positive cells. At 72 h, discrete GFP +ve colonies were located under the liver capsule and around the central veins (Figure 4A). GFP +ve cells were detected using IHC staining with anti-GFP +ve antibody. GFP +ve cells resembling hepatocytes were dispersed throughout the hepatic parenchyma with the presence of a larger number of GFP +ve cells incorporated within the sinusoidal endothelial lining (Figure 4E). GFP +ve cells could still be detected after 2 wk in the spleen, mainly around the central arteriole and the trabeculae (Figure 4F).

Effect of embryonic stem cell therapy on acetaminophen-induced liver injury

All mortality occurred during the first 24 h following APAP administration with 10/40 (25%) mice dying in the control group and 9/40 (22.5%) in the cell therapy group. According to the Kaplan Meier analysis, there was no significant difference in survival between the control and cell therapy groups ($P = 0.755$, log rank test). All animals surviving the initial 24 h lived until the time of sacrifice.

Liver damage was detected at 72 h, most prominent in the centrilobular regions with hepatocyte necrosis and secondary infiltration of inflammatory cells in both the cell therapy and control groups (Figure 4C and D). Improvement in liver histology was noticed both in both groups at 2 wk, with less inflammatory cell infiltration in the treatment group (Figure 4G and H).

In order to identify the nature of the transplanted cells, we stained with albumin antibody, which revealed very faint albumin expression in the GFP +ve cells at 72

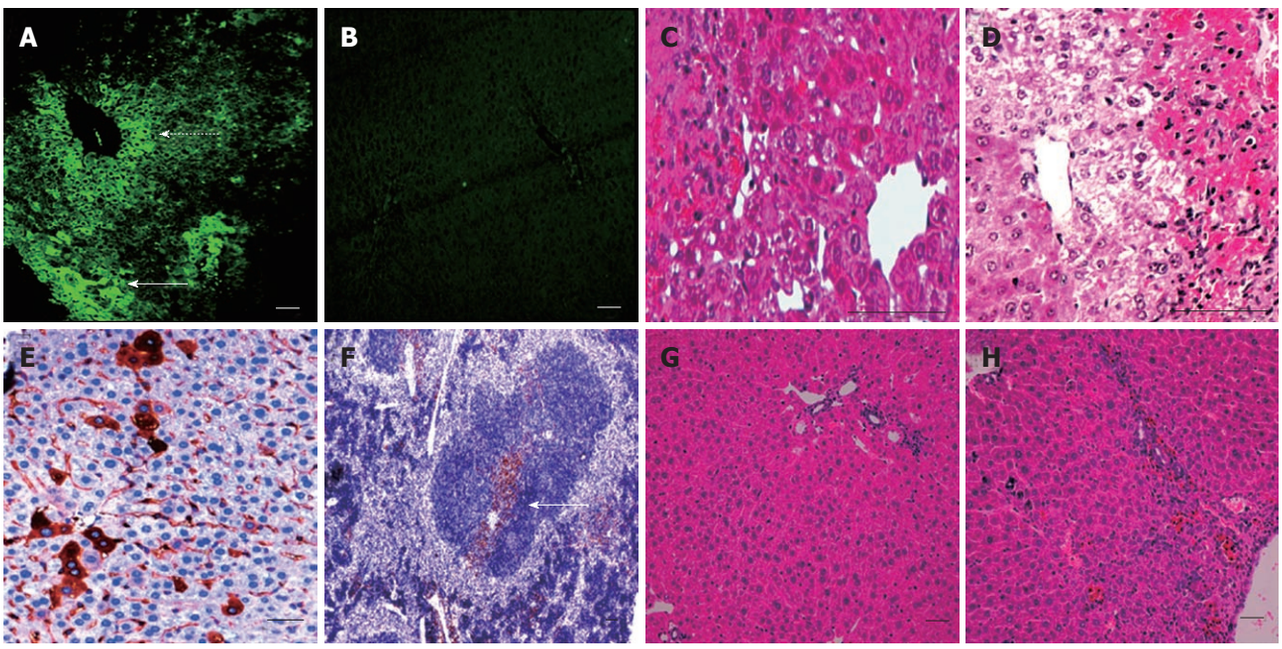

Figure 4 Embryonic stem cell liver engraftment following acetaminophen induced damage. A and B: Green fluorescence protein (GFP) +ve cells were present under the liver capsule (a-solid arrow) and around the central veins of the liver at 72 h, as seen under direct fluorescence (a-dashed arrow) with no fluorescence detected in the control group (B); C and D: The characteristic pattern of acetaminophen-induced liver damage after 72 h mainly affected the centrilobular portions of the liver, with marked damage of the pericentral hepatocytes in both the cell therapy and control groups, although pericentral vacuolation was more evident in the control group; E: At 2 wk, the GFP +ve cells could be detected using IHC in the hepatic parenchyma and within the sinusoidal lining; F: Localized colonies of GFP +ve cells were also detected in the spleen at 2 wk; G and H: After 2 wk the liver recovered in both groups, with the liver sections from the cell therapy group (G) showing less inflammatory cells and congestion than in the control group (H) (scale bar = 200 μ).

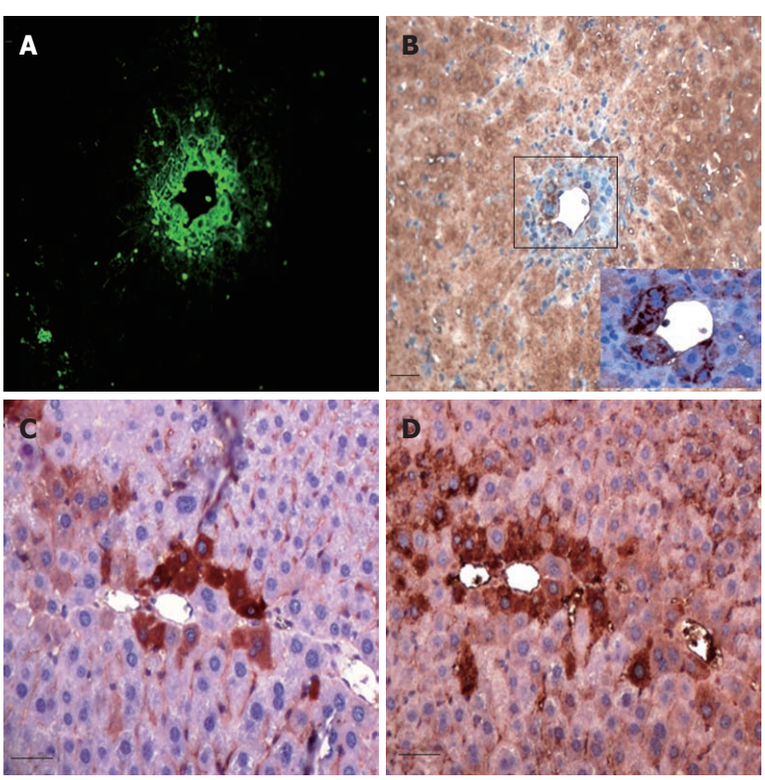

Figure 5 Serial sections examined for Green fluorescence protein and albumin expressions. Green fluorescence protein (GFP) +ve transplanted cells (A) showed very faint albumin expression at the cell periphery of dividing cells at 72 h (B), whereas areas stained with anti-GFP antibody (C) were positive for albumin (D) at 2 wk following cell therapy (scale bar = 100 μ).

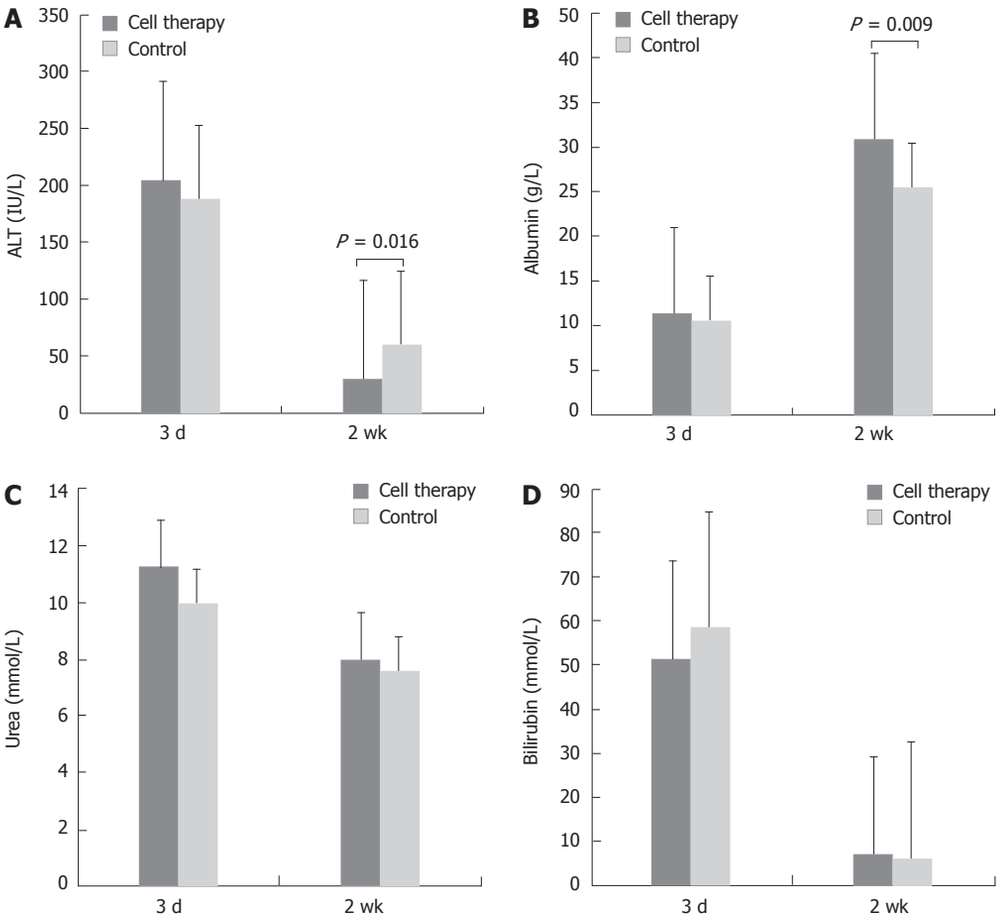

Figure 6 Serum levels of alanine aminotransferase, albumin, bilirubin and urea are shown (*n* = 21). A: The alanine aminotransferase (ALT) level significantly dropped in both groups at 2 wk when compared with the 72 h. When compared with the 72 h, there was a significant reduction in serum ALT level at 2 wk in the embryonic stem cell treatment group, but not in the control group; B: The albumin level also improved in both groups with the level being significantly higher in the cell therapy group when compared with the control group at 2 wk; C: The drop in urea levels was not significant in either group; D: Bilirubin levels dropped significantly from 72 h to 2 wk, with no significant differences between groups at similar time-points.

h (Figure 5A and B). However, when examined on serial sections, few cells that were positive for GFP were also strongly positive for albumin at 2 wk (Figure 5C and D).

There was a significant improvement in serum levels of ALT, albumin and bilirubin in both groups at 2 wk when compared with the 72 h time-point (Figure 6). Cell therapy itself had no effect on any of these parameters at the 72 h time point when compared with the control group. However, at 2 wk, serum ALT was significantly lower in the cell therapy group when compared to the control group (ALT: 29.58 ± 2.19 *vs* 60.38 ± 29.22, cell therapy *vs* control group respectively, Mann *U* Whitney test $P = 0.016$, *t*-test $P = 0.07$) (Figure 6A). The serum albumin level was also significantly higher in the cell therapy group (Albumin: 30.8 ± 1.27 *vs* 25.54 ± 2.29, cell therapy *vs* control group, respectively, $P = 0.009$) (Figure 6B). No significant changes occurred in serum bilirubin or urea levels between either group at 2 wk (Figure 6C and D).

DISCUSSION

In the present study we developed a novel model in which we were able to monitor both the immediate and late kinetics of transplanted ESC cells. In this way, we were able to track the cells over days in real-time after the injection and had a better understanding about the dynamics of the cells in an experimental model of ALF without the need to sacrifice animals.

In the present model, we used APAP to induce ALF. So far, most of the existing animal models look into the role of cell therapy on liver regeneration following partial hepatectomy or hepatocellular damage induced by CCl4[18-20]. In these models, the roles of cell therapy were not studied in a pathophysiological milieu reciprocating the clinical scenario of ALF[21]. For example, following liver resection, the liver bed reduces drastically and resection itself mobilizes the CD 133+ subset of cells from bone marrow into circulation, which may have played an independent role in liver regeneration[22]. Moreover, CCl4-induced liver damage builds up over time and does not produce a similar profound effect to that of ALF[21]. In this study, we used an ALF model induced by APAP at a relatively moderate dose of 300 mg/kg, and transplanted the undifferentiated ESCs 4 h following induction of injury so that most of the APAP would have been me-

tabolized and would not interfere with ESCs survival[15]. In order to track the cells *in vivo*, we used a reproducible imaging system for continuous real-time monitoring of the injected cells following transplantation. The IVIS is a relatively new optical imaging system that is being widely used in the fields of oncology and immunology, allowing non-invasive cell detection and migration[23]. Near infrared dyes have been previously used for monitoring cells *in vivo* with improved quality of signal detection by increasing the contrast between the signal and the background, which is comprised mainly of water, oxyhemoglobin and deoxyhemoglobin[24]. This has made it possible to track labeled cells located in deep-seated organs with minimal background fluorescence. To achieve that goal, we labeled the ESCs with a near infrared lipophilic tracer dye (DiR), which has the advantage of very low cell toxicity and laterally diffuses within the cell membrane, hence staining the whole cell. Very recently, Chois *et al*[25] used luciferase-transfected fetal hepatocyte cells for repopulating the liver in a monocrotaline-injected mice model and nicely showed the uptake of the cells by a CCD camera generated images. The advantages of the imaging we used in this study lies with the simplicity of the method. This technique is relatively cheap and easy to use without any necessity for genetic manipulation of the cells. There is also no necessity to inject any extra dye for visualization of the cells, which might interfere with body chemistry and prolong the anesthetic duration for the animals. The cells could be clearly seen migrating from the spleen to the liver (Figure 2). The signal intensity in the liver was highest between the second and third days post-transplantation, possibly because cell proliferation is highest at that time[26]. A rapid drop in the signal intensity was noticed after the third day and continued to fade until it completely disappeared at 2 wk post-transplantation. Similar to the DiR dye we used, the signal from firefly luciferase also dropped on day 10 following hepatocyte transplantation[25]. However, visualization and quantification of the transplanted cells was still possible at 2 wk by DiR imaging when the organs were extracted and imaged directly under the IVIS.

Since the ESCs were transfected with GFP, it was possible to observe the distribution of the transplanted cells on tissue sections directly under the fluorescent microscope or by immunohistochemistry using the anti-GFP antibody. Engraftment of the ESCs was confirmed on liver tissue sections in the post-transplantation period by direct visualization of GFP +ve cells, which appeared in the close vicinity of the central veins where the main brunt of the damage had occurred. The homed pericentral cells subsequently proliferated and spread throughout the liver parenchyma. The mechanism through which the ESCs integrate into the liver plate depends primarily on the disruption of the sinusoidal endothelial cells[27]. Although APAP overdose characteristically affects the centrilobular regions of the liver, causing extensive necrosis, there is also a direct toxic effect on the sinusoidal endothelial cells through glutathione depletion[28] and thus this may have played a role in the uptake of cells into the liver parenchyma, particularly since GFP +ve cells were also detected in the sinusoidal endothelial lining at 2 wk.

ESC transplantation did not improve either survival or liver functions at 72 h. At 2 wk some transplanted cells expressed albumin and morphologically resembled hepatocytes. There was an increase in serum albumin production at 2 wk in the cell therapy group when compared to the control group. It is unlikely that the differentiation of transplanted cells into hepatocytes could explain the improved liver functions at 2 wk in the cell therapy group, since the cells were few and we did not perform additional studies to confirm differentiation as this was not the purpose of the study. The paracrine effect of the transplanted cells, rather than direct differentiation, seems to be a more possible cause of improved liver functions. Mesenchymal stem cell conditioned medium has been shown to improve liver functions following induction of ALF in rats[29]. Proteins released from human undifferentiated ESCs were also shown to improve cardiomyocytes function[30].

ESC therapy has been riddled with several problems including allogenic rejection, ethical concerns and, most importantly, chances of malignant transformation of ESCs. Although Moriya *et al*[31] did not detect any teratoma formation following transplantation of undifferentiated ESCs in a liver cirrhosis model in mice; the transformation of undifferentiated ESCs into teratomas is very likely after a period of time. We used undifferentiated ESCs in this study to develop a suitable model to study the kinetics of transplanted cells in ALF; however, to study the therapeutic effect of ESCs in ALF, ideally we should have used differentiated ESCs, pluripotent stem cells or primary hepatocytes. Differentiation of ESCs *in vitro* into hepatocytes has been extensively studied by several groups and could be achieved by formation of embryoid bodies[32], or directly by adding growth factors to the ESCs in monolayer cultures[33]. However, full maturation of hepatocytes is not complete without the influence of the intrinsic environment[34]. Several studies have been conducted to simulate the inductive microenvironment *in vitro*, either through co-cultures with hepatocytes[35] or using conditioned media in *in vitro* cultures[36].

We conclude that serial longitudinal tracking of transplanted cells in a mouse model of ALF is possible using IVIS and that dual labeling would allow for both immediate and long-term tracking of the transplanted cells, which would in turn allow us to better understand their differentiation and fate.

COMMENTS

Background

Acute liver failure is a life-threatening condition. Several therapies have been designed to treat this condition with mixed results. Stem cells represent a promising therapeutic modality; however, in order to optimize cell therapy, it would be necessary to develop an accurate cell tracking method.

Research frontiers

We developed a novel imaging system for stem cell tracking using a dual

labeling fluorescent protocol. It was possible to track the cell dynamics *in vivo* without killing the animals, giving an insight into the cell biodistribution in different organs. This would allow real-time monitoring in the early phase following development of acute liver failure which would be very beneficial in optimizing cell therapy.

Innovations and breakthroughs

Other methods of cell monitoring rely either on early or late tracking of cells. Through dual labeling we could take advantage of the near infrared cell label, which is very efficient in localizing the cells *in vivo*, but has a short half-life. The authors could then track the green fluorescence protein label, which is not efficient for *in vivo* cell tracking, but could be used for localizing the cells in tissue sections either directly or indirectly even after a long period of time.

Application

Understanding cell dynamics in the acute phase of liver failure would allow for the optimizing of cell therapies according to the time-points when the cells engraft in the liver and could then serve as an alternative therapeutic option to liver transplantation.

Peer review

This is an interesting study about some non-invasive methods for tracking stem cells to evaluate the cell engraftment following acute liver failure by acetaminophen. The authors developed an interesting and novel model for *in vivo* tracking of embryonic stem cells, based in stained green fluorescence protein embryonic stem cells with the IVIS Lumina-2. Immunohistochemistry for albumin and green fluorescence protein were used to confirm liver embryonic stem cell nesting.

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S- Editor Lv S **L- Editor** Rutherford A **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i6.517

World J Gastroenterol 2012 February 14; 18(6): 517-521
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Seroprevalence of *Helicobacter pylori* in female Vietnamese immigrants to Korea

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Received: February 7, 2011 Revised: July 3, 2011
Accepted: July 11, 2011
Published online: February 14, 2012

Abstract

AIM: To investigate the seroprevalence of *Helicobacter pylori* (*H. pylori*) and its relationship to nutritional factors in female Vietnamese immigrants to Korea.

METHODS: A total of 390 female immigrants from Vietnam and 206 Korean male spouses participated in the study. Blood samples from 321 female immigrants and 201 Korean male spouses were analyzed for *H. pylori* antibodies. Data on age, sex, alcohol consumption, smoking status, dietary nutritional factors and gastrointestinal symptoms were collected using questionnaires. The daily intakes of the following nutrients were estimated: energy, protein, niacin, lipid, fiber, calcium, iron, sodium, potassium, zinc, folate, cholesterol, and vitamins A, B1, B2, B6, C and E.

RESULTS: The prevalence of *H. pylori* positivity was lower in the immigrants than in age-matched Korean females (55.7% *vs* 71.4%, respectively; $P < 0.0001$) and the domestic population of Vietnam. The prevalence of *H. pylori* positivity among married couples was 31.7% for both spouses. There were no statistically significant differences in the incidence of smoking, amount of alcohol consumed, or nutritional factors between the *H. pylori*-positive and negative groups.

CONCLUSION: The prevalence of *H. pylori* positivity was lower among female Vietnamese immigrants than among Korean females. Nutritional factors did not differ between the *H. pylori*-positive and negative groups.

Key words: Vietnam; Immigration; South Korea; *Helicobacter pylori*; Diet

Peer reviewer: Markus Raderer, Professor, Department of Internal Medicine I, Division of Oncology, Medical University Vienna, Waehringer Guertel 18-20, Vienna, A-1090, Austria

Baik SJ, Yi SY, Park HS, Park BH. Seroprevalence of *Helicobacter pylori* in female Vietnamese immigrants to Korea. *World J Gastroenterol* 2012; 18(6): 517-521 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i6/517.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i6.517

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is a major cause of gastric diseases such as chronic gastritis, peptic ulcer disease, and gastric malignancy[1]. *H. pylori* infection can be transmitted from mouth to mouth between family members[2]. Immigrants could be a source of *H. pylori* transmission.

In Korea, immigration has increased since 2000, and the number of marriages between young immigrant females and Korean males has increased rapidly. Official records show that there were 35 142 marriages between

immigrant females and Korean males in 2009[3]. Female Vietnamese immigrants are involved in 20.6% of marriages between immigrants and Koreans and are the second most common group of immigrant newlyweds after Chinese females. The number of marriages between immigrant Vietnamese females and Korean males increased by 42.5% in 2009[3]. Although the increase in the number of immigrants may have affected the prevalence of infectious and inherited diseases in Korea, a study on *H. pylori* infection of immigrants has not been conducted.

We conducted a serological study on the prevalence of *H. pylori* positivity and compared the prevalence of *H. pylori* infection among female Vietnamese immigrants with that of Korean females. We also evaluated the relationship between *H. pylori* infection and nutritional factors in female Vietnamese immigrants.

MATERIALS AND METHODS

Subjects

This survey involved 399 asymptomatic female Vietnamese immigrants and was conducted from March 2006 to August 2007 at the Ewha Mokdong University Hospital. In total, 321 immigrant females and 202 Korean male spouses were enrolled for serological testing for *H. pylori* antibodies. A total of 202 married couples were involved. Questionnaires were completed by the immigrant females with the help of a translator. Demographic data (age, sex, and past history of gastrointestinal disease) and lifestyle data (smoking, alcohol consumption, and dietary intakes) were obtained using the questionnaire. The same questionnaire was completed by the Korean spouses.

Serological analysis

A 5-mL blood sample was collected after an 8-h fast and centrifuged for 10 min at 3000 *g*, and the serum stored at -70 ℃. *H. pylori* infection was determined according to the presence of serum *H. pylori* immunoglobulin (IgG) antibodies using an enzyme-linked immunosorbent assay (ELISA) kit, Genedia® (Noksymja, Seoul, South Korea). The sensitivity and specificity of this assay were reported to be 93.2% and 83.5%, respectively[4]. The prevalence of *H. pylori* infection among the immigrants was compared with that determined from a national survey of *H. pylori* in Koreans. Associations between *H. pylori* prevalence and gastrointestinal symptoms, smoking, exercise, and alcohol consumption were analyzed.

Dietary survey

Trained dietitians interviewed the subjects using a quantitative food frequency questionnaire. The subjects were asked to recall their usual dietary intakes of foods. The energy and nutrient content of each food item were estimated using the Korean Foods and Nutrients Database[5]. The daily intakes of vitamins in the form of supplements were calculated according to their frequency of consumption, dosage, and vitamin content. The nutritional factors assessed were energy, protein, lipid, sugar, fiber, calcium, iron, sodium, potassium, zinc, folate, cholesterol, and vitamins A, B1, B2, B6, C and E. Associations between *H. pylori* prevalence and nutritional factors were analyzed.

Statistical analysis

The statistical significance of the difference in the prevalence of *H. pylori* between female Vietnamese immigrants and Korean females was evaluated. All data were analyzed using SAS version 9.1 (SAS institute, Cary, NC, United States) and SPSS version 10.0 (SPSS Inc, United States). A P value of less than 0.05 was considered significant.

RESULTS

General characteristics and H. pylori positivity in female Vietnamese immigrants

The mean age of female Vietnamese immigrants was 24.7 ± 6.4 years in the *H. pylori*-positive group and 25.0 ± 6.3 years in the *H. pylori*-negative group. The age of the female Vietnamese immigrants ranged from 18 to 60 years, and 40% had gastrointestinal symptoms. The most common symptom was epigastric pain (19.0%), followed by constipation (17.0%).

The incidence of *H. pylori* positivity was 55.7% among the 321 female Vietnamese immigrants, 57.7% among those aged 11-20 years, 57.8% among those aged 21-30 years, and 48.5% among those aged 31-50 years. *H. pylori* positivity did not increase markedly with age. The prevalence of *H. pylori* positivity was lower among female Vietnamese immigrants of all ages than among Korean females (55.7% *vs* 71.4%, respectively; $P < 0.0001$) and among Korean females aged 21-30 years (57.8% *vs* 85.2%, respectively; $P = 0.006$) and older than 30 years (48.6% *vs* 75.6%, respectively; $P < 0.0001$) (Table 1). The prevalence of H. pylori positivity was lower in female Vietnamese immigrants than in the corresponding age groups for Korean females.

Influence of lifestyle factors on H. pylori positivity

We analyzed whether *H. pylori* positivity was affected by smoking, exercise, history of gastrointestinal disease, or gastrointestinal symptoms (Table 2). The *H. pylori*-positive group had a lower incidence of history of gastrointestinal disease than that of the *H. pylori*-negative group (8.1% *vs* 15.1%, respectively; $P = 0.042$). Direct or indirect smoking, alcohol consumption, and frequency of exercise had no effect on *H. pylori* positivity.

H. pylori positivity in female Vietnamese immigrants and their spouses

The prevalence of *H. pylori* positivity among the male spouses of the immigrants was 64.3%. In the female Vietnamese immigrants, *H. pylori* positivity was highest at 21-30 years of age (57.8%), whereas in the Korean male spouses, it was highest at 31-50 years of age (64.8%) (Table 3). The mean *H. pylori* positivity for both spouses among the 202 married couples was 31.7%.

H. pylori positivity and its relationship to nutrition

None of the nutritional factors examined had a statisti-

Table 1 ***Helicobacter pylori*** **positivity according to age** ***n*** **(%)**

Age (yr)	Korean Females	Female Vietnamese immigrants	*P* value
11-20	10 (47.6)	23 (57.5)	0.462
21-30	23 (85.2)	122 (57.8)	0.006
31-50	167 (75.2)	34 (48.6)	< 0.0001
Total	200/280 (71.4)	179/321 (55.7)	< 0.0001

Table 2 Seropositivity/negativity for ***Helicobacter pylori*** **of female Vietnamese immigrants** ***n*** **(%)**

Characteristics (*n*)	*H. pylori* positive	*H. pylori* negative	*P* value
Smoking (*n* = 338)			
None	171 (98.8)	165 (100)	0.261
Yes	2 (1.2)	0 (0)	
Alcohol drink (*n* = 323)			
None	151 (93.2)	152 (94.4)	0.178
Yes	11 (6.8)	9 (5.6)	
Exercise (*n* = 338)			
None	124 (71.3)	125 (76.2)	0.301
Yes	50 (28.7)	39 (23.8)	
Gastrointestinal disease (*n* = 338)			
None	159 (91.9)	140 (84.9)	0.042
Yes	14 (8.1)	25 (15.1)	

Table 3 ***Helicobacter pylori*** **positivity in female Vietnamese immigrants and their male Korean spouses** ***n*** **(%)**

Age	Female Vietnamese immigrants (*n* = 321)	Spouses (*n* = 199)
11-20	23 (57.3)	0 (0)
21-30	122 (57.8)	1 (50)
31-50	34 (48.6)	127 (63.8)
Total	179 (55.7)	128 (64.3)

cally significant effect on *H. pylori* positivity among female Vietnamese immigrants (Table 4). The consumption of vitamin C and vitamin E was higher in the *H. pylori*-positive group than in the *H. pylori*-negative group, but the difference was not statistically significant. Among the male spouses, fiber consumption was higher in the *H. pylori*-positive group than in the *H. pylori*-negative group ($P = 0.01$). The mean sodium intake was 3378.9 mg in the *H. pylori*-positive group. The mean sodium intake of the Korean spouses (4661.2 mg) was higher than that of the female Vietnamese immigrants.

DISCUSSION

The most important environmental factors implicated in the pathogenesis of gastric cancer are diet and *H. pylori* infection[1,6]. *H. pylori* infection is also correlated with gastrointestinal diseases such as gastric and duodenal ulcerations[7]. As immigration to Korea is increasing, *H. pylori*-infected immigrants could spread these diseases among the Korean community. Until now, no information has been available on the prevalence of *H. pylori* infection among female Vietnamese immigrants.

Detection of *H. pylori* antibody in serum is the simplest method of evaluating whether *H. pylori* infection is present. *H. pylori* IgG has been used as a prevalence index in epidemiological studies. Although the *H. pylori* IgG method has lower sensitivity and specificity for detecting *H. pylori* infection than the C*ampylobacter*-like organism (CLO) test, it is useful for the screening of asymptomatic patients and for health checkups[8]. The *H. pylori* IgG ELISA used in this study had 93.2% sensitivity and 83.5% specificity among Koreans[4], but its sensitivity and specificity differ among countries[9]. Vietnam and Korea are both in East Asia, and *H. pylori* variants in both countries have the same CagA3' motif (CAGTF/CAGJR, CAGJF/CAGTR)[10].

In Korea, the prevalence of *H. pylori* positivity was 46.6% among asymptomatic patients in 2000[4] and 62.4% among Korean females older than 16 years[4]. This estimate is lower than that reported by Song et al. in 1997 for a study involving 477 Korean females (72.5%)[11]. In the present study, the incidence of *H. pylori* positivity among the female Vietnamese immigrants was 55.7%, which is much lower than that recorded among Korean females (71.4%) ($P < 0.0001$) in the Korean national survey. The prevalence of *H. pylori* positivity was also much lower in immigrant females aged 21-30 years than in the corresponding age group for Korean females.

In Korea, the prevalence of *H. pylori* positivity in the domestic population increased with age and was highest (74%) at 30-40 years of age[4], but in female Vietnamese immigrants, it was higher at 20 years of age than at 30. In developing countries, the prevalence of *H. pylori* infection increased between 10 and 20 years of age and remained constant at about 80% thereafter[12]. In our study, the prevalence of *H. pylori* positivity among female Vietnamese immigrants did not increase between 10 and 20 years of age. The *H. pylori* positivity of the immigrants was lower than that of age-matched Korean females and that of the domestic population of Vietnam, which was 74.6%[13]. The prevalence of *H. pylori* infection showed differences between Vietnamese cities and was 78.8% in Hanoi, the capital of Vietnam, but in rural areas such as Hatay, it was as low as 69.2%[13]. The prevalence of H. pylori infection of female population was 79.4% in Hanoi and 72.8% in Hatay[13]. The region of origin of the female Vietnamese immigrants could have influenced the results of our study, but these data were not collected. The lower prevalence of *H. pylori* infection among female Vietnamese immigrants compared with that among Korean females was probably due to differences in race and local region of immigration origin.

The prevalence of simultaneous *H. pylori* positivity in female Vietnamese immigrants and their Korean male spouses was 31.7%, which is lower than that published for Korea (77%-88%)[14]. This discrepancy may have been because of differences in the sample populations used as the published data involved only 26 CLO-positive patients and their spouses. In a study of a Western population, *H. pylori* positivity was 83.3% among spouses with

Table 4 Analysis of the association between *Helicobacter pylori* positivity and nutritional factors in female Vietnamese immigrants and their male spouses

	Female Vietnamese immigrants			Male spouses		
	H. pylori positive (n = 172)	*H. pylori* negative (n = 164)	*P* value	*H. pylori* positive (n = 125)	*H. pylori* negative (n = 72)	*P* value
Calorie (kcal)	1410.4 (677.3)	1430.96 (409.6)	0.739	1802.2 (573.7)	1691.5 (603.4)	0.202
Plant protein (g)	27.4 (1.8)	28.8 (10.4)	0.253	38.6 (15.6)	35.6 (10.7)	0.109
Animal protein (g)	31.9 (89.5)	26.8 (16.1)	0.464	31.9	39.8	0.061
Total protein (g)	59.3 (92.3)	55.6 (20.1)	0.615	70.5 (27.4)	75.4 (38.0)	0.448
Plant lipid (g)	15.1(10.8)	15.0 (9.9)	0.949	21.5 (15.3)	25.2 (48.8)	0.535
Animal lipid (g)	17.3 (14.9)	18.6(14.2)	0.392	22.7 (17.5)	22.8 (16.5)	0.967
Total lipid (g)	33.4 (20.5)	33.6 (17.9)	0.539	44.2 (24.4)	48.0 (23.5)	0.191
Sugar (g)	221.7 (73.1)	228.1 (62.7)	0.393	275.7 (81.6)	271.9 (101.4)	0.786
Fiber (g)	14.8 (7.3)	15.1 (6.5)	0.659	21.4 (8.5)	18.6 (6.7)	0.011
Calcium (mg)	381.7 (324.8)	400.9 (240.2)	0.539	520.7 (256.5)	684.6 (1075.9)	0.207
Iron (mg)	9.4 (4.6)	9.9 (3.7)	0.282	14.8 (11.1)	90.3 (462.)	0.170
Sodium (mg)	3378.9 (3272.2)	3333.7 (1458.2)	0.869	4661.2 (1700.7)	4801.6 (2086.3)	0.609
Potassium (mg)	2212.8 (3104.0)	2041.6 (822.2)	0.486	2654 (1002.2)	2537.2 (946.6)	0.413
Zinc (mg)	8.5 (23.1)	6.9 (2.4)	0.367	9.2 (5.5)	9.3 (6.2)	0.963
Vitamin A (μg RE)	404.8 (384.0)	441.8 (358.9)	0.363	693.7 (526.6)	771.7 (839.1)	0.478
Vitamin B1 (mg)	1.0 (0.5)	1.0 (0.4)	0.662	1.1 (0.58)	1.2 (0.6)	0.341
Vitamin B2 (mg)	0.8 (0.6)	0.8 (0.4)	0.591	1.0 (0.5)	1.4 (2.3)	0.136
Vitamin B6 (mg)	1.5 (0.9)	1.5 (0.7)	0.626	2.0 (0.9)	4.0 (14.0)	0.219
Niacin (mg NE)	12.9 (18.2)	12.2 (4.9)	0.615	16.0 (7.0)	26.0 (50.6)	0.099
Vitamin C (mg)	127.0 (116.5)	123.0 (136.5)	0.770	123.6 (100.5)	99.2 (85.8)	0.086
Folate (μg DFE)	187.7 (155.8)	173.8 (88.2)	0.314	260.0 (137.6)	240.6 (137.9)	0.340
Vitamin E (mg ATE)	10.2 (20.6)	9.3 (5.7)	0.550	13.4 (8.9)	11.4 (6.5)	0.068
Cholesterol (mg)	330.5 (1393.0)	218.3 (214.0)	0.298	264.9 (191.0)	319.3 (299.9)	0.173

Data are presented as mean (SD). $^{1}P < 0.05$, *Helicobacter pylori* (*H. pylori*) positive group *vs Helicobacter pylori* negative group in Korean male spouses.

H. pylori-positive partners and 28.5% among spouses with *H. pylori*-negative partners[14].

Smoking and alcohol consumption were not significantly related to *H. pylori* positivity in our study, which is in agreement with the results of another study[15].

The *H. pylori*-negative group had a higher incidence of history of gastrointestinal disease than the *H. pylori*-positive group. The association between gastrointestinal disease and *H. pylori* infection is controversial[16]. The lower incidence of history of gastric disease among *H. pylori*-positive immigrant females should be verified using an objective endoscopic method. The incidence of gastrointestinal symptoms and *H. pylori* positivity was 60% and was similar in both *H. pylori*-positive and negative groups.

There were no statistically significant differences in the intakes of energy, protein, lipid, sugar, fiber or vitamins between the *H. pylori*-positive and negative groups. Another report showed that *H. pylori* positivity is higher among people who ingest roasted food more than twice daily than in people who ingest roasted food once daily or less[15]. However, the consumption of spices, dairy products, and fresh fruit and vegetables was not related to *H. pylori* positivity[15].

Nutritional factors were not related to *H. pylori* positivity among the female Vietnamese immigrants. The mean sodium intake was 3378.9 mg in the *H. pylori*-positive group. The mean sodium intake of the Korean spouses (4661.2 mg) was higher than that of the female Vietnamese immigrants. Koreans have a relatively high sodium intake, and patients with gastric cancer have a higher sodium intake than healthy subjects[17]. The female Vietnamese immigrants consumed less salt than the Koreans. Tsugane[18] reported that the incidence of gastric cancer among Japanese immigrants in the United States could be explained by the extent to which migrants continued to maintain a high consumption of salt. The incidence of gastric cancer among Vietnamese immigrants needs to be investigated.

Fiber intake was higher in the male spouses of the *H. pylori*-positive group ($P = 0.01$), and there was no statistically significant difference in vitamin C intake ($P = 0.086$). It has been reported that *H. pylori*-positive subjects had a low concentration of vitamin C in gastric juice and that vitamin C levels increased after eradication of *H. pylori*[19]. *H. pylori* positivity and the severity of gastritis were associated with the concentration of vitamin C level in gastric juice[19]. There was no significant relationship between *H. pylori* positivity and vitamin C intake in this study. Other studies have stated that the consumption of vitamin C increases serum and gastric juice concentrations of vitamin C, resulting in a lower prevalence of gastric cancer when combined with *H. pylori* eradication[20]. An analysis of dietary micronutrients (vitamin C, vitamin E, carotenoids, fiber, flavonoids, and selenium) commonly considered protective against gastric cancer yielded conflicting results[6]. *H. pylori* positivity was not affected by smoking, alcohol, or nutritional factors.

In conclusion, the prevalence of *H. pylori* positivity among the female Vietnamese immigrants was lower than that among Korean females, and nutritional fac-

tors showed no significant difference between the *H. pylori*-positive and negative groups. More studies on the transmission of *H. pylori* infection in immigrants are warranted.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection is correlated with gastrointestinal diseases such as gastric cancer, gastric or duodenal ulcerations. As immigration to Korea is increasing, a difference in the prevalence of *H. pylori* infection between immigrants and the native population could affect the spread or prevalence of infectious disease.

Research frontiers

The prevalence of *H. pylori* positivity among female Vietnamese immigrants was lower than among Korean females, and nutritional factors showed no significant difference between *H. pylori*-positive and negative groups.

Innovations and breakthroughs

There have been few studies on the health or disease status of immigrants. Tsugane reported that the incidence of gastric cancer among Japanese immigrants in the United States could be explained by the extent to which migrants continued to maintain a high consumption of salt. There has been no report on the health of Vietnamese immigrants in Korea. The prevalence of *H. pylori* positivity was different between female Vietnamese immigrants and Korean females. Also, there were differences in the prevalence of *H. pylori* infection within Vietnam. The prevalence was lower in the female Vietnamese immigrants than in Korean females and the domestic female population of Vietnam. Nutritional factors did not show statistical differences related to *H. pylori* positivity.

Applications

H. pylori infection could be a source of infectious disease in family members. Investigation of *H. pylori* infection in immigrants could lead to further knowledge of gastric diseases such as gastric cancer or ulceration.

Peer review

This is an interesting comparison between two apparently relatively similar cohorts of females, who nevertheless have different infection rates with *H. pylori*. Interestingly, dietary factors do not appear to play a role-at least in adults. In view of this, it would be interesting to offer some speculation on the mode of infection with *H. pylori* in these cohorts of women!

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S- Editor Tian L **L- Editor** Cant MR **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i6.522

World J Gastroenterol 2012 February 14; 18(6): 522-531
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Hyperpolarization-activated cyclic nucleotide-gated cation channel subtypes differentially modulate the excitability of murine small intestinal afferents

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Supported by Science and Technology Commission of Shanghai Municipality, No. 10ZR1417300; Educational Commission of Shanghai Municipality, No. 10ZZ69
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Received: April 2, 2011 Revised: June 21, 2011
Accepted: June 28, 2011
Published online: February 14, 2012

Abstract

AIM: To assess the role of hyperpolarization-activated cyclic nucleotide-gated cation (HCN) channels in regulating the excitability of vagal and spinal gut afferents.

METHODS: The mechanosensory response of mesenteric afferent activity was measured in an *ex vivo* murine jejunum preparation. HCN channel activity was recorded through voltage and current clamp in acutely dissociated dorsal root ganglia (DRG) and nodose ganglia (NG) neurons retrogradely labeled from the small intestine through injection of a fluorescent marker (DiI). The isoforms of HCN channels expressed in DRG and NG neurons were examined by immunohistochemistry.

RESULTS: Ramp distension of the small intestine evoked biphasic increases in the afferent nerve activity, reflecting the activation of low- and high-threshold fibers. HCN blocker CsCl (5 mmol/L) preferentially inhibited the responses of low-threshold fibers to distension and showed no significant effects on the high-threshold responses. The effect of CsCl was mimicked by the more selective HCN blocker ZD7288 (10 μmol/L). In 71.4% of DiI labeled DRG neurons (n = 20) and 90.9% of DiI labeled NG neurons (n = 10), an inward current (I_h current) was evoked by hyperpolarization pulses which was fully eliminated by extracellular CsCl. In neurons expressing I_h current, a typical "sag" was observed upon injection of hyperpolarizing current pulses in current-clamp recordings. CsCl abolished the sag entirely. In some DiI labeled DRG neurons, the I_h current was potentiated by 8-Br-cAMP, which had no effect on the I_h current of DiI labeled NG neurons. Immunohistochemistry revealed differential expression of HCN isoforms in vagal and spinal afferents, and HCN_2 and HCN_3 seemed to be the dominant isoform in DRG and NG, respectively.

CONCLUSION: HCNs differentially regulate the excitability of vagal and spinal afferent of murine small intestine.

Key words: Hyperpolarization-activated cyclic nucleotide-gated cation; Vagal afferent; Spinal afferent; Gastrointestinal tract; CsCl

Peer reviewer: Rubén Ciria, MD, PhD, Hepatobiliary Surgery and Liver Transplantation Unit, Hospital Universitario Reina Sofía, Cordoba 14004, Spain

Wang YP, Sun BY, Li Q, Dong L, Zhang GH, Grundy D, Rong WF. Hyperpolarization-activated cyclic nucleotide-gated cation channel subtypes differentially modulate the excitability of murine small intestinal afferents. *World J Gastroenterol* 2012; 18(6): 522-531 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i6/522.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i6.522

INTRODUCTION

The gastrointestinal tract is innervated by vagal and spinal afferent nerves, whose cell bodies lie in the dorsal root ganglion (DRG) and nodose ganglion (NG), respectively[1]. Previous studies have shown that vagal and spinal afferents differ in their responsiveness to a variety of stimuli and hence may play different roles in gastrointestinal (GI) physiology and pathophysiology. The mesenteric nerves (consisting of both vagal and spinal afferents) of the small intestine exhibited biphasic increases in afferent activity in response to ramp distension in the rat *in vivo* and in the *ex vivo* mouse or rat jejunum preparations, suggesting the presence of low- and high-threshold mechanoreceptors[2,3]. Booth *et al*[3] demonstrated that the low-threshold response in rats was markedly reduced following chronic vagotomy whereas the high-threshold response remained unaltered. These data were consistent with the notion that vagal mechanoreceptors are primarily low-threshold fibers that convey innocuous signals in the GI tract and contribute to the control of satiety and food intake as well as reflex regulation of motility, secretion and absorption[1]. The spinal afferents, on the other hand, are mainly composed of high-threshold and wide dynamic range fibers and may therefore encode nociceptive signals in the GI tract. There has been extensive evidence suggesting that altered sensitivity of vagal and spinal afferents may underlie some of the debilitating symptoms such as bloating and pain seen in functional GI diseases[4]. However, the molecular mechanisms of sensory dysfunction remain poorly understood.

Previous studies have identified a number of ion channels and G-protein coupled receptors that are involved in sensory transduction and modulation of the excitability of primary afferents including those innervating the GI tract. In mammals, the hyperpolarization-activated cyclic nucleotide-gated cation (HCN) channel family consists of 4 cation channels, named HCN_{1-4}. In cells expressing these channels, hyperpolarization of the membrane potential would activate HCN channels, resulting in a slow inward current. The kinetics of these channel activity are regulated by cyclic adenosine 3',5'-monophosphate (cAMP). Because these channels are activated near the resting membrane potential, they can modulate the membrane excitability. Several groups have explored the possible role of HCN channels in sensory processing. It has been shown that HCN channels are expressed in DRG and nodose neurons[5,6]. Importantly, it has been reported that HCN channels were up-regulated in DRG neurons following nerve injury and neuropathic pain was reversed by HCN blockers, suggesting that HCN channels have an excitatory influence on sensory neurons and may represent a potential therapeutic target in pain management. However, another study demonstrated that in nodose neurons and aortic baroreceptors, HCN blockers reduced the threshold for activation, indicating that HCN channels have an inhibitory influence on the excitability of nodose neurons and baroreceptors[5,7].

Matsuyoshi *et al*[8] examined the expression of HCN channels in bladder afferent neurons. Among HCN-1, HCN-2 and HCN-4, positive staining with HCN-2 antibodies was found in approximately 60% of small- and medium-sized bladder afferent neurons. However, the amplitude and current density of hyperpolarization-activated current (I_h) was significantly larger in medium-sized bladder afferent neurons than in small-sized bladder neurons, suggesting that I_h currents could control the excitability of mechanoceptive Aδ-fiber bladder afferent neurons[9]. HCN channels are also localized in enteric nervous system[10]. Linden and colleagues[11] found that the enhancement of afterhyperpolarization neuronal excitability in inflamed guinea pig colon involves an increase in I_h current. However, there has been little information regarding the potential role of HCN channels in the function of extrinsic afferents of the GI tract.

In the present study, we investigated the effect of HCN channel blockers on the mechanosensory responses of mesenteric afferent nerves of the murine jejunum *in vitro* and compared the I_h current in vagal and spinal primary afferent neurons retrogradely labeled from the small intestine.

MATERIALS AND METHODS

Animals

Male Kunming mice weighing 20-25 g were purchased from Shanghai Jiaotong University School of Medicine. The mice were allowed free access to normal laboratory food and tap water and were kept under conditions of constant temperature and humidity with a 12-h light/dark cycle. Mesenteric afferent nerve recording experiments were performed on 6 mice for CsCl and 3 mice for ZD-7288. Immunohistochemistry was performed on 3 DiI labeled mice and 3 as control. All procedures involving use of animals were approved by the Institutional Animal Care and Use Committee at Shanghai Jiaotong University.

Tissue preparation and afferent nerve recording

Mice were deeply anaesthetized with pentobarbital (80 mg/kg, ip) and then killed by cervical dislocation. A midline laparotomy was performed and sections of jejunum were rapidly removed[2]. One of the jejunum segments was placed in a recording chamber (10 mL) and superfused with oxygenated Krebs solution (composition in mmol/L: NaCl 113, KCl 5.9, $CaCl_2$ 1.25, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, Glucose 11.5). Chamber temperature was kept at 34 ℃. The jejunum was cannulated at each end to allow intraluminal infusion (0.1 mL/min) and ramp distension of the gut. A branch of the mesenteric nerve was dissected and the electrical activity was recorded using a suction electrode connected to a Neurolog headstage (NL100, Digitimer Ltd., United Kingdom). The signal was amplified (NL104), filtered (NL 215, bandpass 300-3000Hz), digitized (Micro1401, CED, United Kingdom) and then captured by a computer with the Spike2 software (version 5.14, Cambridge Electronic Design, United Kingdom). The jejunum segment was distended

gradually to an intraluminal pressure of 60 mmHg by closing the outflow port to observe the mechanosensitivity of mesenteric nerves and this was repeated at an interval of 15 min. The effect of HCN channel blockers (5 mmol/L CsCl or 10 μmol/L ZD7288) on the mechanosensory responses was tested by switching the bath and intraluminal solution to one that contains one of the antagonists.

Retrograde labeling of DRG and NG neurons innervating the jejunum

DRG and NG neurons were retrogradely labeled by injecting the fluorescent dye, DiI (Molecular Probes, Eugene, OR)[12] into the gut wall. Briefly, mice were anaesthetized with 1% pentobarbital (40 mg/kg, ip). Under sterile conditions, a midline laparotomy was performed to expose the jejunum and 25 μL DiI (0.1% in DMSO) was injected at 15-20 sites into the smooth muscle layer. Incisions were sutured in layers and mice were allowed to recover for 1-2 wk to permit DiI to be transported to the cell soma.

Dissociation of neurons and patch-clamp recording

Mice were killed by an overdose of pentobarbital (100 mg/kg, ip). T_7-L_2 DRG and NG were quickly isolated under a dissecting microscope and transferred into ice cold Hanks' solution. The ganglia were minced with fine spring scissors after removing the connective tissue. The minced ganglia were placed in Hank's solution containing type Ⅱ collagenase (3 mg/mL) and trypsin (2.5 mg/mL) and incubated at 37 ℃ for 50 min, then washed 2-3 times in DMEM with 10% fetal calf serum, 100 U penicillin and 100 μg streptomycin. Cells were then dispersed by gentle titration with fire-polished Pasteur pipettes. The cell suspension was plated onto 35-mm culture dish coverslips precoated with 0.01% poly-lysine and incubated in 5% CO_2 at 37 ℃ overnight.

For patch-clamp recording, one of the coverslips was placed in the recording chamber (1 mL) and the cells were continuously perfused with extracellular solution (in mmol/L: NaCl 130, KCl 6, $MgCl_2$ 1.2, $CaCl_2$ 2.5, HEPES 10, Glucose 10, $BaCl_2$ 1) at room temperature (21-25 ℃). We added 1 mmol/L Ba^{2+} in the extracellular solution in order to block the inward-rectifier potassium current upon applying the hyperpolarizing voltage steps[13,14]. Whole cell recordings of the DiI-labelled cells were conducted using electrodes with a resistance of about 3-5 MΩ when filled with the internal solution (composition in mmol/L: K-gluconate 140, $MgCl_2$ 2, EGTA 1.1, HEPES 10).

Immunohistochemistry

Mice were killed by an overdose of pentobarbital and then perfused with saline followed by 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer with a pH of 7.4. T_7-L_2 DRG and NG were isolated and post-fixed in the same solution at 4 ℃ overnight and then transferred into 30% sucrose for 24 h at 4 ℃. The ganglia were then embedded in an Optimal Cutting Temperature compound and serially sectioned (10 μm) using a cryostat for immunofluorescent staining. Briefly, sections were treated with phosphate-buffered saline (PBS) containing 1% normal goat serum and 1% TritonX-100 at room temperature for 30 min twice followed by incubation with one of the primary antibodies at 4 ℃ overnight. The sections were rinsed in PBS for four times and then incubated with the secondary antibody at room temperature for 1 h. The primary antibodies used were rabbit polyclonal antibodies: 1:200 anti-HCN_1 (ab65706, Abcam), 1:200 anti-HCN_2 (ab65704, Abcam), 1:200 anti-HCN_3 (ab65705, Abcam) and 1:1000 anti-HCN_4 (ab65703, Abcam). The secondary antibody was a goat anti-rabbit Alexa Fluor 488 (Molecular Probes, Invitrogen) diluted in 10% NGS /PBS/Triton solution (1:1000).

Statistical analysis

Numerical data were expressed as mean ± SE. Statistical analysis of the data was performed using SPSS version 13.0 or Graphpad Prism version 5 (Graphpad software, San Diego, United States). The effect of CsCl on low- and high- threshold afferent responses were analyzed by paired Student's *t* test. The pressure-afferent nerve response curves were analyzed using linear regression and then compared using an analysis of covariance (ANCOVA). Amplitude, density and time constants of I_h current were compared between DRG and NG neurons by Wilcoxon rank sum test. Mean voltages of half activation ($V_{1/2}$) and slope factors (k) of I_h were acquired from the curves fitted by Boltzmann equation in Origin 6.0 (OriginLab, Northampton, Massachusetts, United States). The percentages of HCN-positive cells in DRG and NG were compared by the χ^2 test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of HCN channel blockers on the mechanosensory responses of small intestinal afferent nerves

Ramp distension of the jejunum segments typically evoked biphasic increases in mesenteric afferent nerve discharge (Figure 1), which is consistent with previous reports[2,15]. The first phase was a rapid increase in afferent activity when the intraluminal pressure started to rise and the nerve activity reached the first peak at an intraluminal pressure of approximately 5 mmHg and then plateaued or increased slowly as the intraluminal pressure continued to rise. The second phase was an accelerated increase in nerve discharge starting at an intraluminal pressure of 20 mmHg. This pattern of ramp distension-evoked afferent responses was due to the activation of three functional populations of afferent fibers, namely low-threshold fibers, wide dynamic range fibers and high-threshold fibers[2]. As is exemplified in Figure 1A, simultaneous bath and intraluminal application of the HCN channel blocker, CsCl (5 mmol/L), attenuated the initial phase of the nerve responses to ramp distension but seemed to render the second phase of the mechanosensory response unaffected. Figure 1B shows the average pressure-afferent response curves of the mesenteric nerves ($n = 6$) under the

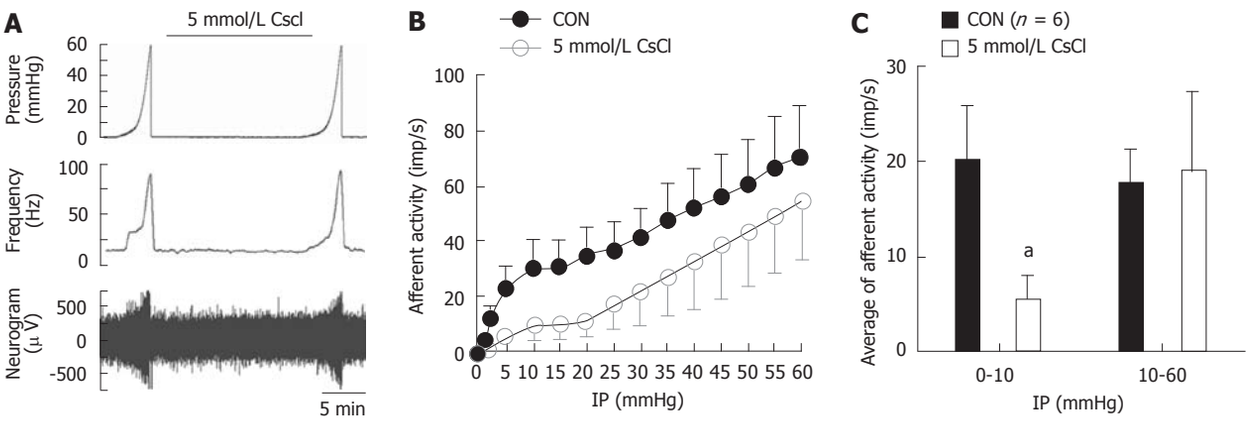

Figure 1 Effects of CsCl on the mechenosensory activity of small intestinal afferents. A: Original recording of the mesenteric afferent nerve activity in response to distension in an *ex vivo* jejunum preparation; B: The pressure-afferent nerve response curves in control and in the presence of hyperpolarization-activated cyclic nucleotide-gated cation blocker, 5 mmol/L CsCl; C: Bar graph showing the low- and high- threshold mechanosensory responses with or without the presence of 5 mmol/L CsCl. $^{a}P < 0.05$.

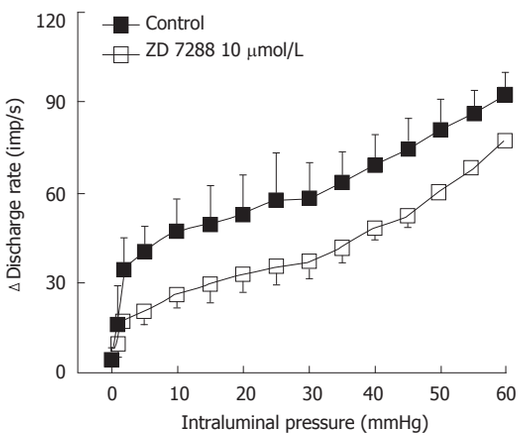

Figure 2 Effects of ZD-7288 on the mechanisensory responses of mesenteric afferent nerves. Note that the slopes of the average pressure-response curves in the pressure range of 10 mmHg - 60 mmHg were apparently similar with or without the hyperpolarization-activated cyclic nucleotide-gated cation channel blocker ZD-7288 (10 μmol/L). Data were pooled from 3 preparations.

control condition and in the presence of CsCl (5 mmol/L) (Figure 1B). For simplicity, each curve is divided into two parts (intraluminal pressure range, 0-10 mmHg and 10-60 mmHg) and analyzed using linear regression, reflecting the mechanosensitivity of low- and high-threshold fibers, respectively. Under the control condition, the initial part of the pressure-response curve had a slope of (2.95 ± 0.73) imp/s per mmHg and in the presence of 5 mmol/L CsCl, the slope was (0.90 ± 0.37) imp/s per mmHg, which was significantly smaller compared with the control curve ($P < 0.05$, ANCOVA, Figure 1B). The slope of the second part of the pressure-response curve (10-60 mmHg) in the presence of 5 mmol/L CsCl was not significantly different from that of the control curve [(0.86 ± 0.24) imp/s per mmHg *vs* (0.95 ± 0.27) imp/s per mmHg, $P > 0.05$, ANCOVA, Figure 1B]. Another way of addressing the responses of low- and high-threshold fibers is to calculate the changes in afferent discharge rate when the intraluminal pressure increased from the baseline to 10 mmHg (low-threshold response) and from 10 to 60 mmHg (high-threshold response). As shown in Figure 1C, 5 mmol/L CsCl attenuated the low-threshold response but rendered the high threshold response unaltered [0-10 mmHg: (19.91 ± 5.92) imp/s *vs* (5.65 ± 2.34) imp/s , paired *t* test, $t = 3.29$, $P < 0.05$; 10-60 mmHg: (17.48 ± 3.70) imp/s *vs* (18.83 ± 8.55) imp/s, paired *t* test, $P > 0.05$, Figure1C].

To confirm that CsCl-induced inhibition of the low-threshold mechanosensory response is due to the blockade of HCN channels, we observed the effects of ZD-7288, a more selective HCN channel blocker, on the responses of mesenteric nerves to ramp distension in 3 jejunum preparations. Indeed, 10 μmol/L ZD7288 mimicked the effects of CsCl in that it attenuated the low-threshold responses, but not significantly affected the high-threshold responses (Figure 2). These data clearly demonstrate that under the normal conditions, HCN channels exert a tonic facilitatory effect on the low-threshold mechanoreceptors but not on high-threshold mechanoreceptors.

Hyperpolarization-activated current in vagal and spinal primary afferent neurons projecting to the small intestine

We focused on DRG and NG neurons retrogradely labeled from the small intestine by patch-clamp recording of I_h current. I_h current was present in 71.4% (20/28) of DiI labeled DRG neurons (mean diameter, 35.67 ± 0.65 μm; mean capacitance, 45.83 ± 5.13 pF) and 90.9% (10/11) of NG neurons (mean diameter, 29.63 ± 1.20 μm; mean capacitance, 25.84 ± 2.60 pF). Figure 3 shows an example of DiI labeled DRG neurons viewed under the inverted fluorescence microscope, as well as the current and voltage traces obtained by whole cell recording. Under voltage-clamp recording, hyperpolarization pulses activated inward currents which were virtually abolished by 5 mmol/L CsCl. Under current-clamp recording, hyperpolarizing currents elicited hyperpolarization of the membrane potential which exhibited a "sag" (depolarization) that was also prevented by 5 mmol/L CsCl. These results show that both vagal and spinal primary afferent

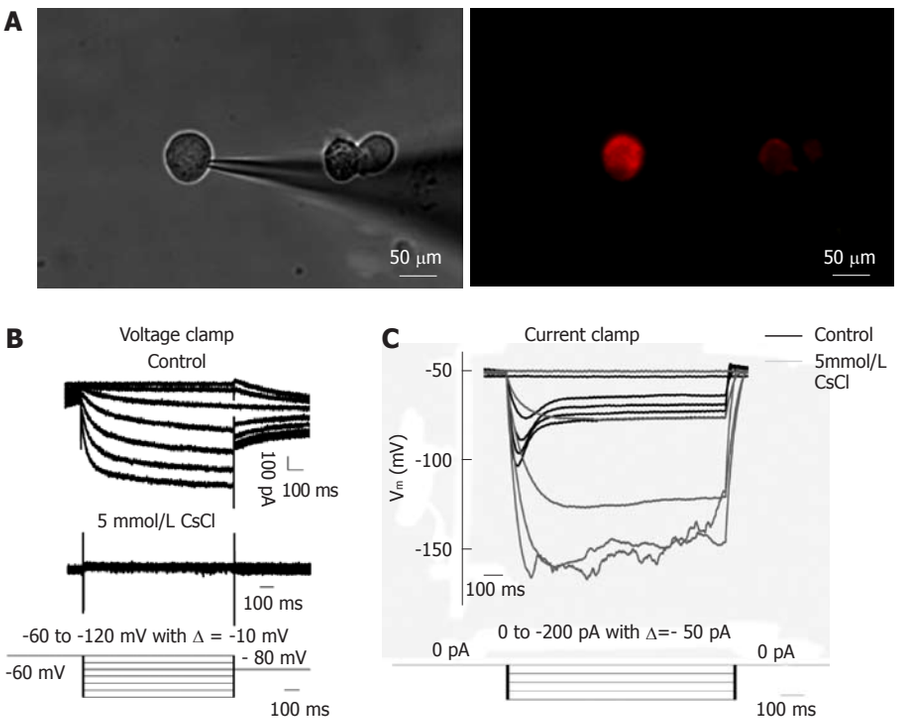

Figure 3 I_h recorded under whole-cell voltage and current clamp from DiI labeled dissociated dorsal root ganglia and nodose ganglia neurons innervating the jejunum. A: DiI labeled dissociated dorsal root ganglia neurons; B: Representative current traces of I_h current (top trace); Current recorded in the presence of 5 mmol/L CsCl (middle trace); voltage clamp protocal: I_h was induced from a holding potential of -60 mV in 1 s pulses from -60 mV to -120 mV in steps of 10 mV, followed by a final step to -80 mV to record the tail current (bottom trace); C: Voltage response to test current pulse before (black) and after (grey) application of 5 mmol/L CsCl (top trace); bottom trace shows the current clamp protocol, i.e., hyperpolarizing current pulses ranging from 0 pA to -200 pA in steps of 50 pA. Note that the current elicited an instantaneous hyperpolarization that was followed by depolarization (named "sag") of membrane potential. DRG: Dorsal root ganglia; NG: Nodose ganglia.

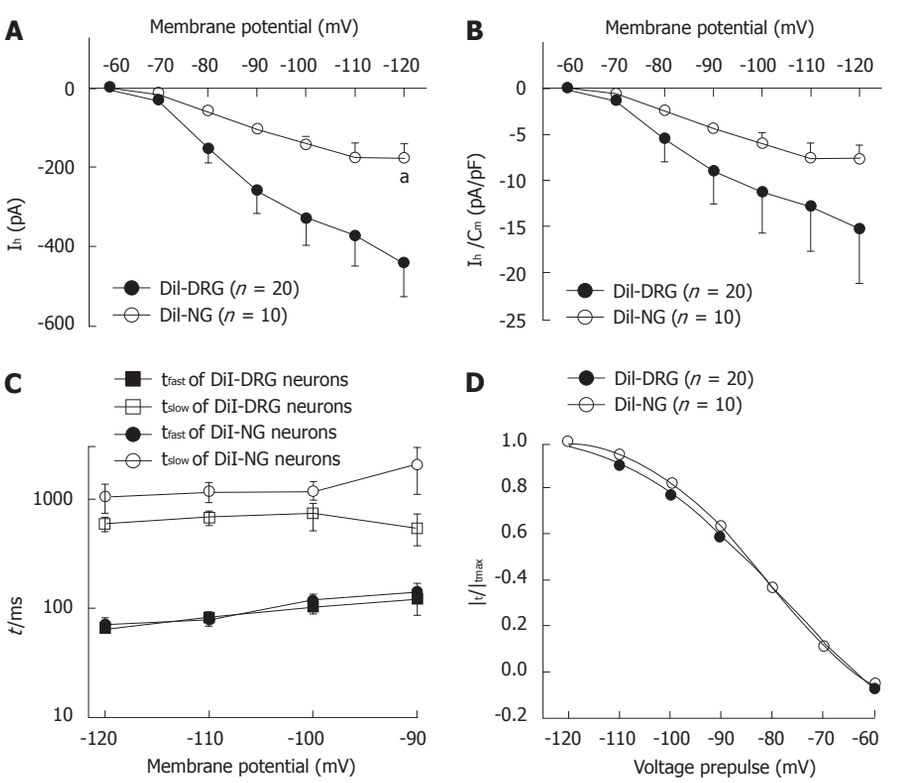

Figure 4 I_h steady-state parameters and activation kinetics in DiI-labeled DRG and NG neurons. A: I_h current-voltage relationship between DiI-labeled dissociated dorsal root ganglia (DRG) and nodose ganglia neurons (NG); B: I_h current density-voltage relationship between DiI-labeled DRG and NG neurons; C: The time constant of I_h current. I_h current traces were fitted with two exponentials according to the following equation: $I_h(t) = Af \exp(-t/\tau f) + As \exp(-t/\tau s)$, where $I_h(t)$ is the amplitude of the current at time t and Af and As are the initial amplitudes of the fast (τf) and slow (τs) activation time constant components, respectively. Time constants were obtained by fitting currents using pCLAMP; D: I_h current activation curves. Normalized activation curves were obtained from tail currents at -80 mV and fitted by Boltzmann function: $I_h/I_h(max) = 1/\{1+ \exp[(Vm - V_{1/2})/k]\}$, where I_h is the peak amplitude of the tail current recorded immediately after the pre-pulse, $I_h(max)$ is the maximal current recorded after the maximal prepulse of -120 mV, V_m is the membrane potential, $V_{1/2}$ is the membrane potential at which I_h conductance is half-activated, and k is a slope factor of the curve. Data were expressed as mean ± SE. [a]$P < 0.05$ *vs* DiI-labeled DRG neurons.

neurons projecting to the small intestine express functional HCN channels, which seemed to exert a facilitatory effect on the afferent excitability.

Hyperpolarization-activated currents in DiI-labeled DRG neurons were generally of larger amplitude than in NG neurons [-120 mV: (-439.08 ± 89.56) pA for DRG neurons and (-176.37 ± 36.96) pA for NG neurons, Wilcoxon rank sum test, $P < 0.05$, Figure 4A], although when normalized to the membrane capacitance, the difference in current density was not statistically significant between spinal and vagal afferents [-120 mV: (-15.22 ± 5.93) pA/pF for DRG neurons and (-7.64 ± 1.67) pA/pF for NG neurons, Wilcoxon rank sum test, $P > 0.05$, Figure 4B]. We compared the kinetics of I_h currents in these two populations. The time course of I_h activation was fitted by a two-exponential function (Figure 4C). There was no significant difference in the fast and slow time constant between DiI-labeled DRG and NG neurons [-120mV: (65.81 ± 6.89)

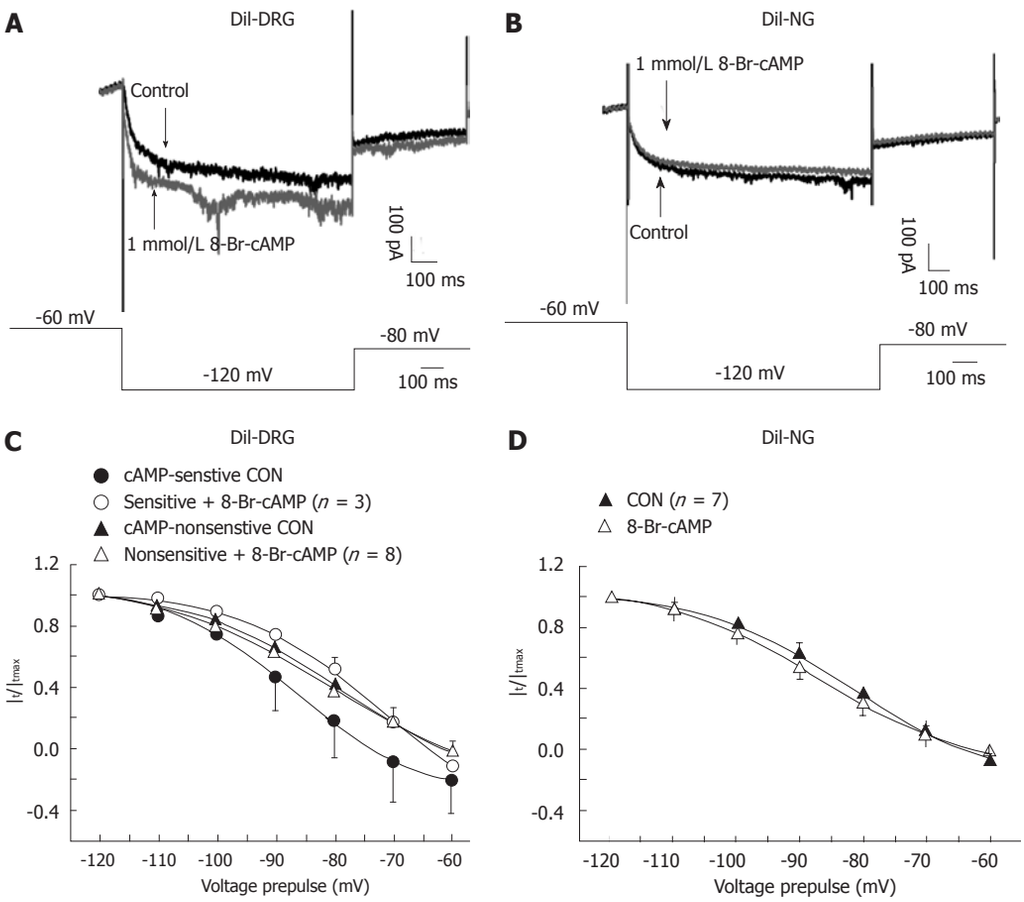

Figure 5 Effects of 8-Br-cAMP on the activation curves of I_h current in DiI-labeled dissociated dorsal root ganglia and nodose ganglia neurons. A and B: Overdraw of the I_h current traces elicited by a hyperpolarizing pulse (-120 mV) in control and in the presence of 8-Br-cAMP in DiI-labeled dissociated dorsal root ganglia (DRG) (A) and nodose neurons (B); C: Activation curves of cAMP-sensitive and cAMP-insensitive DiI-labeled DRG neurons before (black block symbols) and after treatment with 8-Br-cAMP (empty symbols); D: Activation curves of DiI-labeled nodose ganglia neurons before (black block triangle) and after treatment with 8-Br-cAMP (empty triangle). Data were expressed as mean ± SE.

ms and (590.57 ± 88.19) ms for DRG neurons, (70.12 ± 5.10) ms and (1044.54 ± 307.80) ms for NG neurons, respectively]. The activation parameters of I_h was $V_{1/2}$ (-77.86 ± 3.38) mV, slope factor 16.06 ± 2.96 for DiI-labeled DRG neurons and $V_{1/2}$ -81.31 ± 0.41 mV, slope factor 11.84 ± 0.40 for DiI-labeled NG neurons (Figure 4D).

We applied 8-Br-cAMP (a membrane permeable cAMP) to test the sensitivity of I_h currents in spinal and vagal afferent neurons to cAMP. In 3 of 11 DiI-labeled DRG neurons, I_h currents were augmented by 8-Br-cAMP, as is shown in Figure 5A. In these neurons, 8-Br-cAMP (1 mmol/L) caused a 14 mV depolarizing shift in the midpoint of voltage activation without marked effect on the slope factor [$V_{1/2}$ from (-86.49 ± 1.14) mV to (-72.16 ± 3.44) mV and slope factor from 10.59 ± 1.31 to 12.07 ± 2.04, Figure 5A and C]. I_h currents in the other 8 DRG neurons tested were not sensitive to 8-Br-cAMP [$V_{1/2}$ from (-79.45 ± 1.70) mV to (-80.43 ± 2.07) mV and slope factor from 12.17 ± 1.52 to 14.00 ± 2.03, Figure 5C]. Seven NG neurons were tested, but the I_h currents in these neurons were not altered in the presence of 8-Br-cAMP [$V_{1/2}$ from (-81.95 ± 0.78) mV to (-86.84 ± 0.29) mV and slope factor from 11.57 ± 0.78 to 11.98 ± 0.36, Figure 5B and D]. These data suggest that different isoforms of HCN channels may exist in subpopulations of spinal and vagal primary afferent neurons innervating the small intestine.

Immunohistochemical staining of HCN isoforms in DRG and NG neurons

There was no apparent immunofluorescent staining for HCN_1 in DRG or NG sections. HCN_2 immunostaining was detected in both ganglia. In DRG sections, 12.3% of DRG neurons and numerous afferent fibers were stained for HCN_2. In NG sections, HCN_2 immunostaining was less extensive in that only 2.3% of NG neurons ($P < 0.05$, χ^2 test, compared with DRG) and fewer fibers were stained for HCN_2. Weak HCN_3 immunoreactivity was detected in some DRG neurons. In contrast, moderate staining for HCN_3 appeared to be present in the majority of NG neurons. HCN_4 immunoreactivity was not detected in NG sections but was present in a minority of DRG neurons (Figure 6).

To determine the subtypes of HCN channels expressed in intestinal primary afferent neurons, we further observed the immunoreactivity of HCN_{1-4} in DRG and NG neurons retrogradely labeled from the small intestine

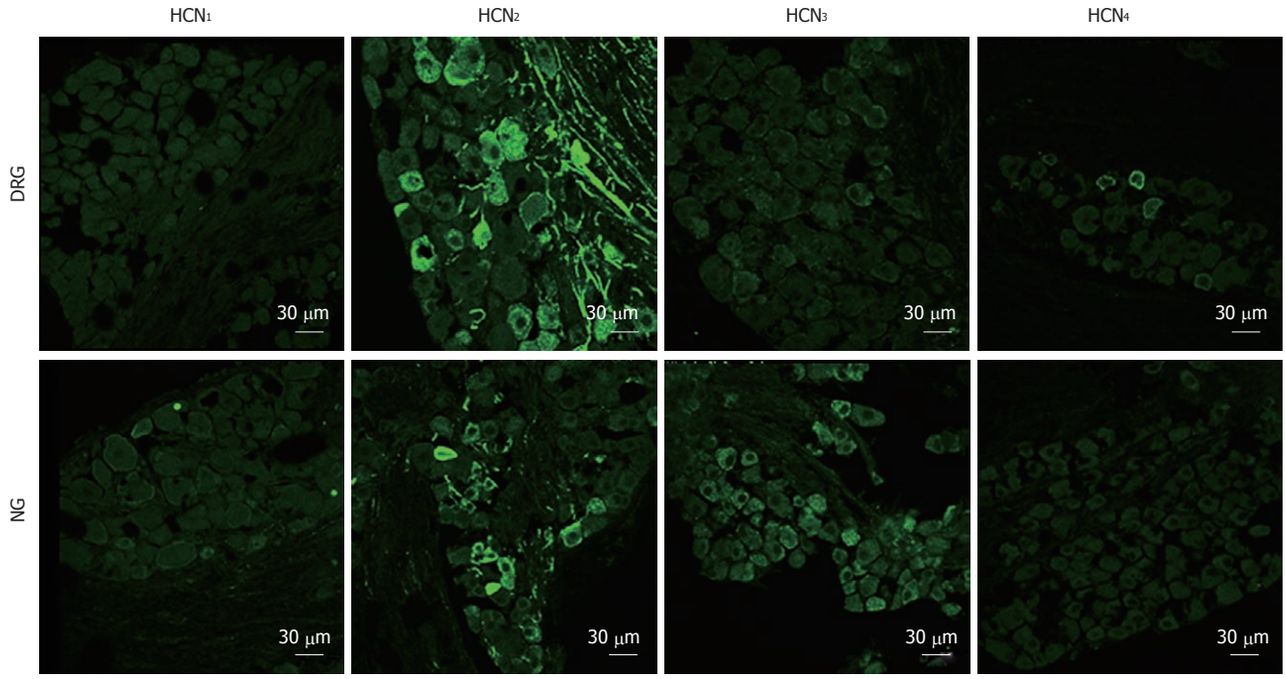

Figure 6 Immunostaining for hyperpolarization-activated cyclic nucleotide-gated cation isoforms in dorsal root ganglia and nodose ganglia sections. Hyperpolarization-activated cyclic nucleotide-gated cation channel 1 (HCN_1) immunoreactivity was not detectable in dorsal root ganglia (DRG) and nodose ganglia (NG) neurons. HCN_2 immunoreactivity was prominent in DRG (cell bodies and fibers) and was also present in some NG neurons and fibers. HCN_3 immunoreactivity was profuse in NG sections but was weaker in DRG sections. HCN_4 immunoreactivity was present in a minority of DRG neurons and was absent in NG.

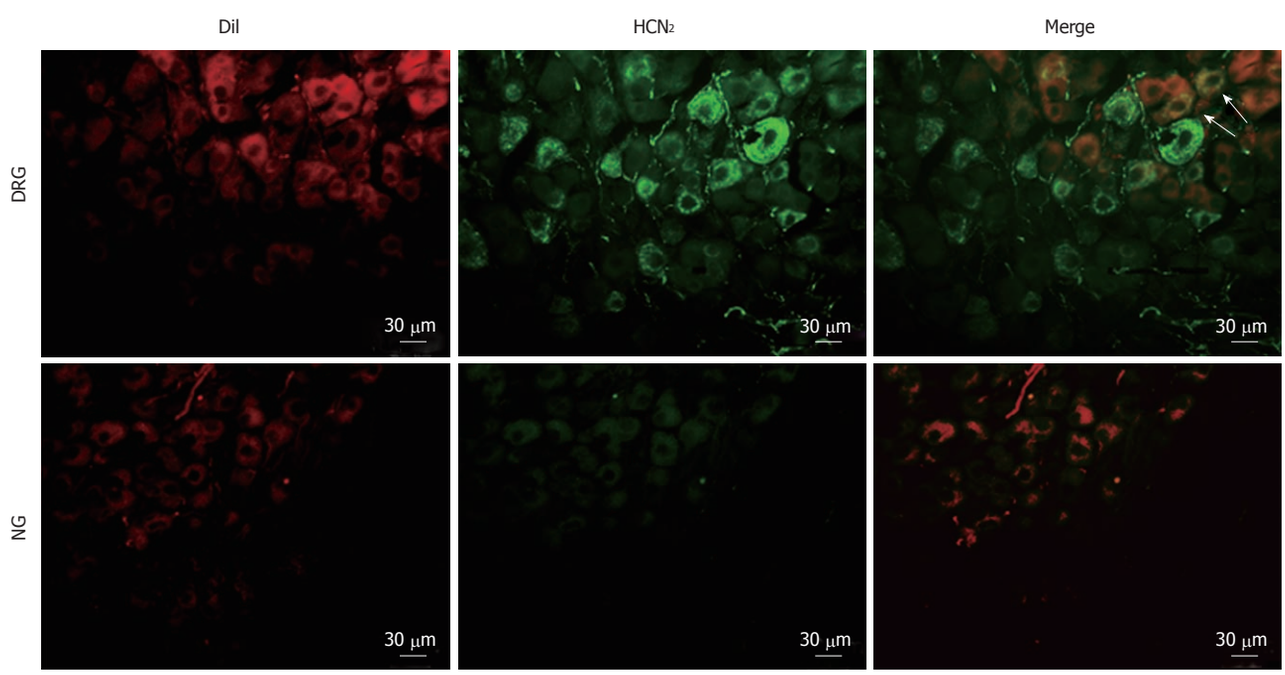

Figure 7 Immunostaining for hyperpolarization-activated cyclic nucleotide-gated cation channel 2 in intestinal primary afferent neurons. Spinal and vagal afferent neurons innervating the small intestine were labeled *via* injection of DiI (red) into the gut wall. Arrows indicate positive hyperpolarization-activated cyclic nucleotide-gated cation channel 2 (HCN_2) staining in DiI-labeled dorsal root ganglia neurons. DRG: Dissociated dorsal root ganglia; **NG**: Nodose ganglia.

via injection of DiI into the gut wall. As expected, HCN_1 immunoreactivity was not detected in any of the DiI-labeled DRG or NG neurons. HCN_2 immunoreactivity was present in some DiI-labeled DRG neurons but was not detected in DiI-labeled NG neurons (Figure 7). On the other hand, moderate HCN_3 immunoreactivity was apparently present in most DiI-labeled NG neurons. DiI-labeled DRG neurons, however, were only weakly immunoreactive to HCN_3 (Figure 8). HCN_4 immunoreactivity was not detected in DiI-labeled DRG or NG neurons.

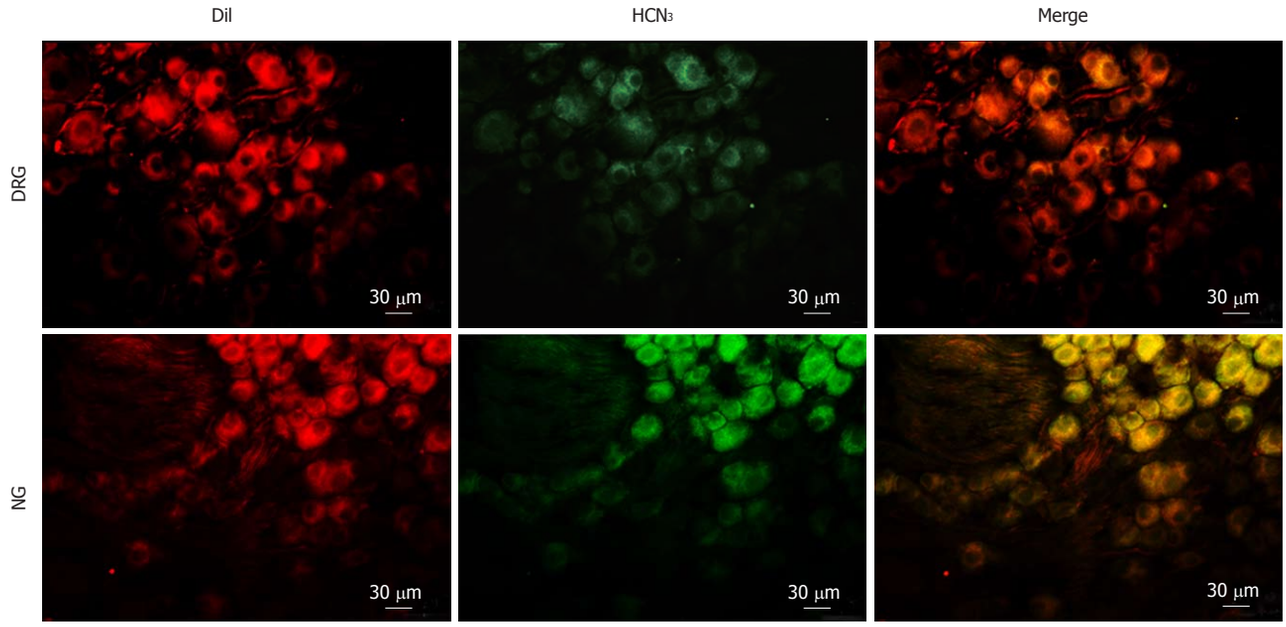

Figure 8 Immunostaining for hyperpolarization-activated cyclic nucleotide-gated cation channel 3 in intestinal primary afferent neurons. Intestinal afferents were labeled *via* injecting DiI into the gut wall. Note that weak hyperpolarization-activated cyclic nucleotide-gated cation channel 3 (HCN_3) immunoreactivity (green) was present in some DiI-labeled (red) dorsal root ganglia neurons. In contrast, most DiI-labeled nodose ganglia neurons were moderately stained for HCN_3.

DISCUSSION

Extrinsic afferent nerves play a major role in the control of GI function. Altered vagal and spinal afferent nerve sensitivity has been implicated in the pathophysiology of GI symptoms such as bloating, discomfort and pain. Previous studies have shown that there exist major differences in vagal and spinal primary afferent neurons in their sensitivity to mechanical and chemical stimulations. Delineating the molecular mechanisms that control the excitability of spinal and vagal afferents of the GI tract may potentially offer novel therapeutic targets. The present study provided evidence that HCN channels differentially regulate the excitability of spinal and vagal afferents innervating the small intestine.

In the first series of the study, we tested the effects of HCN channel blocker, CsCl, on the mechanosensory responses of mesenteric nerves of the jejunum. Consistent with previous reports, ramp distension of the jejunum evoked biphasic increases in mesenteric nerve discharge, which was due to the activation of three major types of afferent fibers: low-threshold fibers, wide dynamic range fibers and high-threshold fibers[2]. CsCl at 5 mmol/L was found to attenuate the first phase of the increase in afferent discharge (pressure rose by 10 mmHg from the baseline) and showed no significant effect on the second phase of the response (pressure rose from 10 to 60 mmHg). CsCl is a relatively weak HCN channel blocker and was non-selective among different HCN isoforms. We choose to test 5 mmol/L CsCl since this was the concentration used in many previous studies[7,16,17] and we noted in pilot experiments that the amplitude of action potential spikes was not altered, suggesting that conduction of nerve signals was not impaired in the presence of 5 mmol/L CsCl. To further exclude the possible off-target effects of 5 mmol/L CsCl, we tested a more potent HCN channel blocker ZD7288 (10 μmol/L) and found that it had a similar effect on the mechanosensory responses of mesenteric nerves. Thus, we could be relatively confident that the inhibitory effects of 5 mmol/L CsCl on the mechanosensory responses were due to blockade of HCN channels and that this set of data show certain HCN isoforms that exert a tonic facilitatory action on the excitability of low-threshold afferent fibers.

Booth *et al*[3] showed in rats that the low-threshold responses of mesenteric nerves to ramp distension of the jejunum diminished whilst the high- threshold responses remained intact following chronic vagotomy. Therefore, in the second series of the experiments, we tested the hypothesis that there were differences in the expression of functional HCN channels in vagal *vs* spinal primary afferent neurons. To this end, we conducted patch clamp recording of I_h in acutely dissociated DRG and NG neurons. I_h currents of varying amplitudes could be elicited by hyperpolarizing voltage pulses in approximately half of DRG and NG neurons. We then focused on DiI-labeled (intestinal) spinal and vagal sensory neurons. I_h current was present in 71.4% of DRG neurons and 90.9% of NG neurons. The I_h currents of DRG neurons were generally of greater amplitude than those of NG neurons, but the kinetics (fast and slow time constants and activation curves) of I_h currents in these two populations were not significantly different. At the present, subtype selective HCN blockers are not available, but different subtypes of HCN channels are known to show distinct cAMP sensitivity. HCN_2 and HCN_4 are strongly modulated by cAMP, whilst HCN_1 and HCN_3 show minimal or no sensitivity to cAMP[18]. We therefore tested the

sensitivity of I_h currents to cAMP in DiI-labeled DRG and NG neurons. In 3 of 11 DRG neurons tested, the I_h currents showed a significant sensitivity to 8-Br-cAMP with a significant depolarizing shift of the haft activation voltage. In the other 8 DRG neurons and all (7/7) DiI-labeled NG neurons, I_h currents were not altered by 8-Br-cAMP. Admittedly, the number of cells included in this study was small. Nevertheless, this set of data does suggest that subpopulations of spinal afferents of the small intestine might express cAMP-sensitive (HCN_2 or HCN_4) and cAMP-insensitive HCN isoforms (HCN_1 or HCN_3), whilst vagal afferents of the small intestine appeared to express the cAMP-insensitive HCN channels only.

In the final series of experiments, we detected the expression of HCN isoforms in spinal and vagal primary afferent neurons by means of immunohistochemistry. HCN_1 was not detected in DRG or NG sections. Intense HCN_2 immunoreactivity was present in 12.3% of unidentified DRG and 2.3% unidentified NG neurons, respectively. Conversely, HCN_3 was more abundant in NG than in DRG. HCN_4 was seen in a minority of DRG neurons but not in NG neurons. Furthermore, we found that DRG neurons retrogradely labeled from the small intestine were partially immunoreactive to HCN_2 and weakly stained for HCN_3, whereas NG neurons retrogradely labeled from the small intestine were virtually all immunoreactive to HCN_3 but not to HCN_2. Combined with the patch-clamp and the afferent nerve recording data, these results would suggest that the vagal primary afferent nerves of the small intestine express cAMP-insensitive HCN_3 channels. Since HCN blockers, CsCl and ZD7288, inhibited the low-threshold (i.e., vagal) mechanosensory responses of mesenteric nerves, it appears likely that HCN_3 channels exert a tonic facilitatory action on the excitability of vagal afferents.

The expression of HCN isoforms in sensory neurons has been investigated in several previous studies. For spinal primary afferent neurons, Tu *et al*[19] reported presence of HCN_1 immunoreactivity in large- and medium-sized neurons, HCN_2 in proportions of neurons of all sizes and absence of HCN_4 immunoreactivity in rat DRG (L_4-L_5). The distribution of immunoreactivity for HCN isoforms within the DRG found in the present study was consistent with the findings of Matsuyoshi *et al*[8], who demonstrated that HCN_2 was the dominant isoform in L_6-S_1 rat DRG. In that study, HCN_2 immunoreactivity was detected in 46.9%, 21.1% and 4.5% of small-, medium- and large-sized neurons, respectively; and there was no apparent staining for HCN_1 and scarce staining for HCN_4. For vagal afferent neurons, Tu *et al*[5] detected HCN_1, HCN_3, and HCN_4 in A-fiber neurons and HCN_2, HCN_3 and HCN_4 in C-fiber neurons in NG of rats using a double-staining technique. They further demonstrated that over-expression of HCN_{1-3} channels in nodose neurons contributed to the decreased excitability of vagal afferent neurons in diabetic rats. Li *et al*[7] also reported that over-expression of HCN_1 and HCN_2 was associated with the blunted excitability of A fiber aortic baroreceptor neurons in type-1 diabete rats. In the current study, most NG neurons were moderately stained for HCN_3 and minorities of NG neurons were stained for HCN_2, whilst HCN_1 and HCN_4 immunoreactivity was hardly detectable. It is likely that there exist subtle species differences in the distribution of HCN isoforms in vagal afferent neurons and that expression of HCN isoforms in vagal afferent neurons may be altered under certain pathological conditions.

Momin and colleagues demonstrated that I_h current had an important influence on action potential generation in sensory neurons[20]. We noted that CsCl and ZD7288 inhibited the low-threshold (vagal) mechanosenosry responses without significant effect on the high-threshold (presumably spinal) responses, although the patch-clamp and immunohistochemical data both showed that functional HCN channels were present in some spinal afferent neurons. It appears likely that under normal conditions, HCN channels expressed on spinal afferent neurons only have minimal effect on the excitability of afferent terminals. However, this does not preclude the possible contribution of HCN channels to the hyper-excitability of spinal afferents seen in pathological conditions such as inflammatory and non-inflammatory bowel diseases. In this regard, it is particularly interesting to note that a proportion of spinal afferents appeared to express HCN_2, which is sensitive to modulation by cAMP, a key intracellular second messenger for a variety of inflammatory mediators[18,21]. Conceivably, spinal afferents expressing HCN_2 may become hypersensitive due to the increased availability of inflammatory mediators such as bradykinin, ATP, 5-HT and prostaglandin E_2[22].

In summary, the data obtained in this study suggest that HCN_2 and HCN_3 are differentially expressed in vagal *vs* spinal primary afferents innervating the small intestine. HCN_2 is the major HCN isoforms in spinal afferents and HCN_3 is more abundant in vagal afferent neurons and seems to exert a tonic facilitatory modulation on the excitability of afferent terminals.

COMMENTS

Background

The gastrointestinal (GI) tract is innervated by vagal and spinal afferent nerves, which play important roles in the control of GI function. Altered vagal and spinal afferent nerve sensitivity has been implicated in the pathophysiology of GI symptoms such as bloating, discomfort and pain, but the molecular mechanisms are still not clear. Hyperpolarization-activated cyclic nucleotide-gated cation (HCN) channels underlie the pacemaker current in the heart and are also known to regulate CNS neuronal excitability. The current study investigated the possible role of HCN channels in the control of small intestinal afferent nerve sensitivity.

Research frontiers

Previous studies implicated HCN channels in neuropathic pain and in control of arterial baroreceptor and bladder afferent sensitivity. Linden and colleagues reported that enhanced I_h current contributes to the increased excitability of a subpopulation of enteric neurons in inflamed guinea pig colon. Until recently, little information is available regarding the potential role of HCN channels in the function of extrinsic afferents of the GI tract.

Innovations and breakthroughs

As reported in this study, HCN_2 and HCN_3 are differentially expressed in vagal *vs* spinal primary afferents innervating the small intestine. HCN_2 is the major

HCN isoform in spinal afferents whereas HCN_3 is more abundant in vagal afferent neurons. HCN_3 seems to exert a tonic facilitatory action on the excitability of vagal afferent terminals.

Applications

The current findings suggest that drugs targeting HCN_2 or HCN_3 may potentially reverse dysfunction of vagal and spinal afferents seen in functional and inflammatory GI diseases.

Terminology

HCN is a hyperpolarization-activated cyclic nucleotide-gated cation channel which mediates I_h current.

Peer review

The design of the study is correct and the findings are important, as these channels may redirect further research against bowel symptoms to other targeted drugs.

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S- Editor Wu X **L- Editor** Ma JY **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i6.532

World J Gastroenterol 2012 February 14; 18(6): 532-540
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Epigenetic inactivation of secreted frizzled-related protein 2 in esophageal squamous cell carcinoma

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Supported by National Natural Science Foundation of China, No. 81050016; Research Fund for the Doctoral Program of Higher Education of China, No. 200800250003

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Received: March 10, 2011 Revised: September 22, 2011
Accepted: October 28, 2011
Published online: February 14, 2012

Abstract

AIM: To investigate the expression and methylation status of the secreted frizzled-related protein 2 (SFRP2) in esophageal squamous cell carcinoma (ESCC) and explore its role in ESCC carcinogenesis.

METHODS: Seven ESCC cell lines (KYSE 30, KYSE150, KYSE410, KYSE510, EC109, EC9706 and TE-1) and one immortalized human esophageal epithelial cell line (Het-1A), 20 ESCC tissue samples and 20 paired adjacent non-tumor esophageal epithelial tissues were analyzed in this study. Reverse-transcription polymerase chain reaction (RT-PCR) was employed to investigate the expression of SFRP2 in cell lines, primary ESCC tumor tissue, and paired adjacent normal tissue. Methylation status was evaluated by methylation-specific PCR and bisulfite sequencing. The correlation between expression and promoter methylation of the *SFRP2* gene was confirmed with treatment of 5-aza-2′-deoxycytidine. To assess the potential role of SFRP2 in ESCC, we established stable SFRP2-transfected cells and examined them with regard to cell proliferation, colony formation, apoptosis and cell cycle *in vivo* and *in vitro*.

RESULTS: SFRP2 mRNA was expressed in the immortalized normal esophageal epithelial cell line but not in seven ESCC cell lines. By methylation-specific PCR, complete methylation was detected in three cell lines with silenced SFRP2 expression, and extensive methylation was observed in the other four ESCC cell lines. 5-aza-2′-deoxycytidine could restore the expression of SFRP2 mRNA in the three ESCC cell lines lacking SFRP2 expression. SFRP2 mRNA expression was obviously lower in primary ESCC tissue than in adjacent normal tissue (0.939 ± 0.398 *vs* 1.51 ± 0.399, $P < 0.01$). SFRP2 methylation was higher in tumor tissue than in paired normal tissue (95% *vs* 65%, $P < 0.05$). The DNA methylation status of the SFRP2 correlated inversely with the SFRP2 expression. To assess the potential role of SFRP2 in ESCC, we established stable SFRP2 transfectants and control counterparts by introducing pcDNA3.1/v5 hisA -SFRP2 or pcDNA3.1/v5 hisA -empty vector into KYSE30 cells lacking SFRP2 expression. After transfection, the forced-expression of SFRP2 was confirmed by the RT-PCR. In comparison with the control groups, stably-expressed SFRP2 in KYSE 30 cells significantly reduced colony formation *in vitro* (47.17% ± 15.61% *vs* 17% ± 3.6%, $P = 0.031$) and tumor growth in nude mice (917.86 ± 249.35 mm^3 *vs* 337.23 ± 124.43 mm^3, $P < 0.05$). Using flow cytometry analysis, we found a significantly higher number of early apoptotic cells in SFRP2-transfected cells than in the control cells ($P = 0.025$). The mean cell number in the S and G2-M phases of the cell cycle was also significantly lower in SFRP2-transfected KYSE30 cells compared with mock transfected counterparts.

CONCLUSION: Silencing of SFRP2 expression through promoter hypermethylation may be a factor in ESCC carcinogenesis through loss of its tumor-suppressive activity.

Key words: Esophageal squamous cell carcinoma; Secreted frizzled-related protein 2; Methylation; Tumor suppressor gene; Wnt signaling pathway

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Hao XW, Zhu ST, He YL, Li-P, Wang YJ, Zhang ST. Epigenetic inactivation of secreted frizzled-related protein 2 in esophageal squamous cell carcinoma.*World J Gastroenterol* 2012; 18(6): 532-540 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i6/532.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i6.532

INTRODUCTION

Esophageal cancer is the sixth leading cancer-related cause of death worldwide. Although esophageal adenocarcinoma is the most rapidly increasing cancer in Western countries, esophageal squamous cell carcinoma (ESCC) remains the predominant histological subtype in China[1]. Despite recent improvements in its diagnosis and treatment, the prognosis for patients with ESCC is still unsatisfactory[2]. Among the reasons cited for this situation are the lack of understanding of the carcinogenic mechanism of ESCC, and the lack of sensitive and specific molecular markers to detect these cancers at an early stage.

In addition to genetic changes, epigenetic modifications and in particular DNA methylation, are recognized as common molecular alteration in human tumors. Functional inactivation of tumor suppressor genes (*TSG*) through promoter methylation has been shown to be involved in the pathogenesis of various cancers, including ESCC[3-7]. Importantly, DNA methylation changes have been reported to occur not only in advanced cancers, but also in premalignant lesions[8,9]. DNA methylation changes are, therefore, potentially good early indicators for the clinical application of sensitive cancer detection.

Aberrant promoter methylation of secreted frizzled-related protein (*SFRP*) genes, a new group of tumor suppressor genes, has been documented in several human malignancies such as colorectal cancer[10], gastric cancer[11], and hepatocellular carcinoma[12]. SFRPs are a family of secreted glycoproteins that act as negative regulators of the Wnt signaling pathway. Every member in this family of genes contains a frizzled-like cysteine-rich domain through which they either interact with Wnt proteins to prevent them from binding to Fz proteins, or form nonfunctional complexes with Fz and then block the Wnt signaling pathway[13,14]. As the Wnt pathway plays a crucial role in various human carcinogenesis[15,16], its aberrant activation by epigenetic inactivation of SFRPs may induce tumorigenesis.

The aberrant hypermethylation of the SFRP2 promoter has been reported to be a good molecular marker in gastric and colorectal cancer[17,18], suggesting that SFRP2 is a tumor suppressor. However, other reports indicate that SFRP2 promotes tumor progression in glioma[19], renal cancer cells[20], and decreases apoptosis in breast cancer cells[21]. In spite of these studies, to our knowledge there have not yet been any reports describing the significance of epigenetic inactivation of the *SFRP2* gene in ESCC progression and its potential as a diagnostic and therapeutic target**.** We therefore analyzed the methylation and expression status, as well as the function, of this gene in ESCC.

Here, we first determine SFRP2 methylation and its expression level in 7 ESCC cell lines and 20 paired primary ESCC tissues. We then explore the functional significance of methylation-induced silencing of SFRP2 expression in ESCC cell lines both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell lines and cell culture

A total of seven ESCC cell lines (KYSE 30, KYSE150, KYSE410, KYSE510, EC109, EC9706 and TE-1) were maintained in our laboratory. One human immortalized normal esophageal epithelial cell line (Het-1A), which was used as a "normal" control for ESCC cell lines, was purchased from the American Type Culture Collection (Manassas, VA, United States). ESCC cell lines were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, United States) and antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin) at 5% CO2, 37 ℃, and 95% humidity. Het-1A cells were cultured in bronchial epithelial basal medium with growth supplements (Clonetics, San Diego, CA, United States).

Human esophageal tissue samples

Paired specimens of human ESCC and adjacent noncancerous esophageal squamous epithelium (n = 20) were obtained from patients who underwent resection for ESCC without chemotherapy or radiation therapy at the Beijing Friendship Hospital. Samples were stored in liquid nitrogen. All subjects gave informed consent for obtaining the study materials. The study was approved by the Ethics Committee of Beijing Friendship Hospital.

RNA extraction and reverse-transcription polymerase chain reaction

Total RNA was extracted from the 20 pairs of human tissue and eight cell lines by Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR), 2 μg of RNA was reversely transcribed using Superscript Ⅱ reverse transcriptase according to the manufacturer's protocol (Invitrogen).

Table 1 List of primer sequences

Primer	Forward primer (5'-3')	Reverse primer(5'-3')
RT-PCR		
SFRP2	5′-GATGATGACAACGACATAATGGAAACG -3′	5′-GAGTGTGCTTGGGGAACGGGAGCT-3′
GAPDH	5′-CCCTTCATTGACCTCAACTACATGG-3′	5′-CATGGTGGTGAAGACGCCAG-3′
SFRP2(CDS)	5′-CCAAGCTTATGCTGCAGGGCCCTGGCTCGC-3′	5′-CGGAATTCCTAGCACTGCAGCTTGCGGATGCTG-3′
MSP		
SFRP2-M	5′-GGGTCGGAGTTTTTCGGAGTTGCGC-3′	5′-CCGCTCTCTTCGCTAAATACGACTCG-3′
SFRP2-U	5′-TTTTGGGTTGGAGTTTTTTGGAGTTGTGT-3′	5′-AACCCACTCTCTTCACTAAATACAACTCA-3′
BSP		
SFRP2	5′-AAAAAGGTTAAGAAAATTTTGGT-3′	5′-AACCAAAACCCTACAACATC-3′

SFRP2: Secreted frizzled-related protein 2; GAPDH: Glyceraldehyde-3-phosohate dehydrogenase; MSP: Methylation-specific.

The mRNA expression levels of the SFRP2 were determined by conventional RT-PCR with Taq polymerase (Takara, Dalian, China). Glyceraldehyde-3-phosohate dehydrogenase was used as an internal control of RNA integrity. The RT-PCR procedure consisted of 35 cycles with an annealing temperature of 56 ℃. The primers used are listed in Table 1.

Isolation and bisulfite modification of genomic DNA

Genomic DNA was obtained from esophageal tissues and cell lines by standard phenol-chloroform extraction. Genomic DNA was treated with sodium bisulfite using a Zymo DNA Modification kit (Zymo Research, Orange, CA). Bisulfite induces deamination of unmethylated cytosines, converting unmethylated CpG sites to UpG without modifying methylated sites. This allows their differentiation by methylation-specific PCR, or sequencing.

Demethylation with the DNA demethylating agent 5-aza-2-deoxycytidine

Three human ESCC cell lines (TE-1, KYSE-30 and KYSE -510) were treated with DNA demethylating agent 5-Aza-dC by addition of fresh medium containing 5-Aza-dC (10 μmol/L, Sigma) every day for 3 consecutive days. Cells were harvested for DNA and RNA extractions. Control cells received no drug treatment.

Methylation-specific polymerase chain reaction

The methylation status of the SFRP2 in esophageal tissues and cell lines was determined by MSP. Briefly, bisulfite-treated DNA was used as a template to amplify MSP using primers specific for methylated and unmethylated sequences of the gene. The PCR reaction was set up using 0.5 U hot-start Taq-polymerase (Takara) per reaction in a total volume of 20 μL and run for 40 cycles with an annealing temperature of 60 ℃. MSP primers are listed in Table 1.

Bisulfite genomic sequencing

For bisulfite genomic sequencing, a fragment of the SFRP2 promoter CpG islands that contained 34 CpG sites was amplified from the bisulfite-treated DNA using specific primers (Table 1). The PCR procedure consisted of 35 cycles with an annealing temperature of 54 ℃. The PCR products were then cloned into the pEasy-T1 vector (Transgene, Beijing, China). Eight to ten colonies were randomly chosen and sequenced.

Cloning of SFRP2 and construction of expression vector

Expression vectors containing the human full-length cDNA fragment of SFRP2 were generated by PCR cloning. Primers are listed in Table 1. Amplified PCR products were cloned into pEasy-T1 vector (Transgene), and the fragments verified by sequencing, then cut using *Eco*RI^{HF} and *Hin*dⅢ, and ligated into the *Eco*RI^{HF}/*Hin*dⅢ sites of pcDNA3.1/V5-HisA vector (Invitrogen).

Cell cycle analysis

Cell cycle analysis was carried out by flow cytometry. KYSE30 cells were transfected with 4 μg of pcDNA3.1/V5-HisA-SFRP2-expression vector or pcDNA3.1 V5-HisA empty vector using Lipofectamine2000 (Invitrogen). After 48 h, cells were harvested and fixed in 70% ethanol for 30 min. The samples were concentrated by removing the ethanol and treating with 10 μg/mL RNase (Roche). They were then stained with propidium iodine (Sigma) and analyzed for cell cycle distribution by DNA content analysis using flow cytometry.

Stable clone establishment

To prepare stable cell lines re-expressing SFRP2, we transfected KYSE30 cells with the pcDNA3.1/V5-HisA-SFRP2 expression vector encoding SFRP2 cDNA using Lipofectamine2000 (Invitrogen), according to the manufacturer's instructions. Cells transfected with pcDNA3.1 V5-HisA empty vector were used as controls. Transfected cells were selected by culturing with G418 (Merck, Darmstadt, Germany) at 300 μg/mL for 1 mo. Single colonies of stable transfectants were isolated and expanded for further analysis.

Detection of apoptosis by annexin V/FITC

KYSE30 cells stably transfected with pcDNA3.1/V5-HisA-SFRP2-expression vector or pcDNA3.1 V5-HisA empty vector were harvested, washed twice in phosphate buffered saline (PBS) (4 ℃), and re-suspended in 250 μL binding buffer. One hundred microliters of cells from each sample were aliquoted into FACS tubes and stained

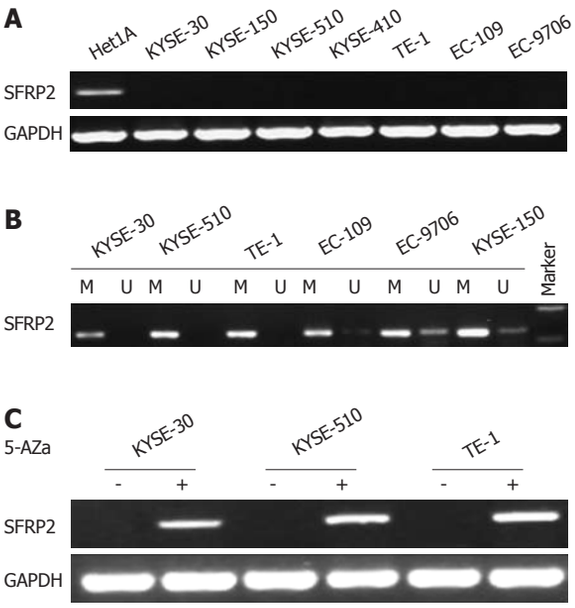

Figure 1 Expression and promoter methylation of secreted frizzled-related protein 2 in esophageal squamous cell carcinoma cell lines. A: Secreted frizzled-related protein 2 (SFRP2) mRNA expression as determined by reverse-transcription polymerase chain reaction (RT-PCR); B: The methylation status of the SFRP2 promoter as determined by methylation specific PCR; C: mRNA expression of SFRP2 (determined by RT-PCR) restored after treatment with the demethylation agent 5-Aza-dC in three esophageal squamous cell carcinoma cell lines. M: Methylated; U: Unmethylated.

with 5 μL annexin V/FITC and 10 μL of 20 μg/mL propidium iodine (Sigma). After gentle mixing, cells were incubated for 15 min at room temperature in the dark. Four hundred microliters of binding buffer was added immediately prior to analysis by flow cytometry.

Cell proliferation assay

Stable transfected KYSE30 cells with or without SFRP2 expression vector and parental cells were selected for measurement of cell proliferation. A quantity of 5×10^3 cells were reseeded in 96-well plates. After incubation, the medium was removed and 20 μL 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyltetrazolium bromide (5 mg/mL, Gibco) was added to each well. DMSO was added and the optical density of each well was read at 490 nm. Each experiment was performed in triplicate.

Colony formation assay

A quantity of 2×10^3 KYSE30 cells transfected with either SFRP2 expression vector or empty vector were cultured in 60-mm plates with G418 (Merck, Darmstadt, Germany) at 0.3 mg/mL for 4 wk in regular culture medium. Colonies with cell numbers of more than 50 cells per colony were counted. Colonies were fixed with methanol for 15 min and stained with Giemsa. All the experiments were performed in triplicate wells in three independent experiments.

In vivo tumorigenicity

Stable KYSE30 cells transfected with either the SFRP2 expression vector or empty vector cells and parental cells (1.5×10^6 cells in 0.2 mL PBS) were inoculated subcutaneously into the dorsal flank of 5-wk-old male Balb/c nude mice. Each group was comprised of 5 mice, and all were maintained under sterile conditions. Tumor formation was observed 2 wk later. Tumor diameter was measured every 3 d for 5 wk, and tumor volume (mm^3) estimated by measuring the longest and shortest diameter of the tumor and calculating the volume as follows: volume = $(\text{shortest diameter})^2 \times (\text{longest diameter}) \times 0.5$. After 5 wk of observation, the mice were sacrificed. Tumors were excised, measured, and weighed. Care of animals and all experimental procedures were approved by the Animal Ethics Committee of Beijing Friendship Hospital, and were carried out in accordance the "Guideline for the Welfare of Animals in Experimental Neoplasia"[22].

Statistical analysis

All statistical analyses were conducted using SPSS 11.5 software (SPSS Inc, Chicago, IL, United States). Data were expressed as the mean ± SD. P values < 0.05 were considered statistically significant. Statistical comparison between two groups was performed using the non-parametric Mann-Whitney U-test or Student's t-test. For comparison of more than three groups, we used one-way analysis of variance, followed by Tukey's multiple comparison.

RESULTS

Frequent transcriptional silencing of SFRP2 in ESCC cell lines

We first examined mRNA expression of SFRP2 by means of reverse-transcription-PCR in seven ESCC cell lines (TE-1, KYSE-30, KYSE-150, KYSE-410, KYSE-510, EC-109 and EC-9706), and one immortalized normal esophageal epithelial cell line (Het-1A). RT-PCR showed that the SFRP2 transcript was silenced in all seven ESCC cell lines, but not in the immortalized normal esophageal epithelial cell line (Het-1A) (Figure 1A). We next examined the role of promoter methylation in the silencing of SFRP2. By methylation-specific PCR, complete methylation was detected in three cell lines with silenced SFRP2 expression, and extensive methylation was observed in the other four silenced cell lines (Figure 1B). We analyzed SFRP2 methylation in more detail using high-resolution bisulfite genomic sequencing analysis. Extensive methylation was detected in three ESCC cell lines (KYSE-30, KYSE-510 and TE-1) with silenced SFRP2 expression, whereas no methylated CpG site was detected in the immortalized normal esophageal epithelial cell lines (Het-1A) (Figure 2).

SFRP2 reactivation in ESCC cell lines treated with 5-Aaza-dC

Since we had established that the SFRP2 mRNA expression was related to its methylation status, we next investigated whether DNA demethylation could restore the expression of SFRP2 mRNA in the three ESCC cell lines lacking SFRP2 expression using 5-Aza-dC. 5-Aza-dC restored the

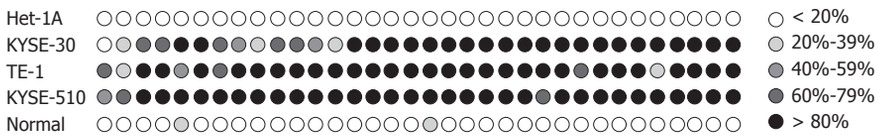

Figure 2 A representative picture of bisulfite genomic sequencing in the secreted frizzled-related protein 2 gene promoter. A-293bp region with 34 CpG site was analyzed. Each row of CpG site represents an individual allele of the secreted frizzled-related protein 2 promoter analyzed. Percentage methylation was determined as a percentage of methylated cytosine from 8 to 10 randomly sequenced colonies. Methylated and unmethylated CpG sites are indicated by black and white circles, respectively.

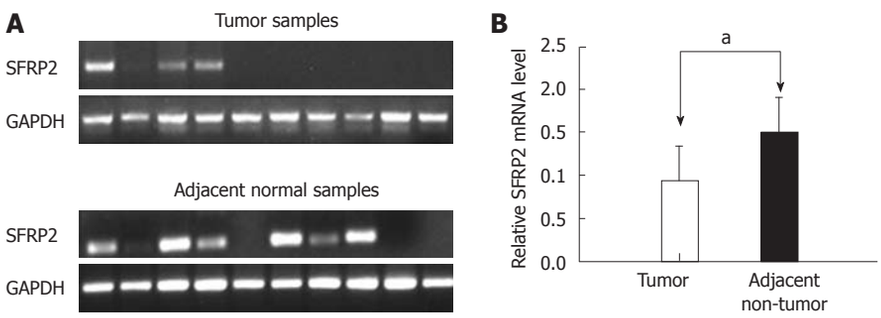

Figure 3 Expression of secreted frizzled-related protein 2 in esophageal squamous cell carcinoma and paired non-cancerous tissues. A: Reverse-transcription polymerase chain reaction (RT-PCR) analysis of secreted frizzled-related protein 2 (SFRP2) mRNA expression in 10 primary esophageal squamous cell carcinoma (ESCC) tissues and their adjacent non-cancerous tissues; B: The SFRP2 mRNA expression level normalized according to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level. Data are expressed as mean ± SD. ESCC tissues *vs* adjacent non-tumor tissues ($^aP < 0.01$).

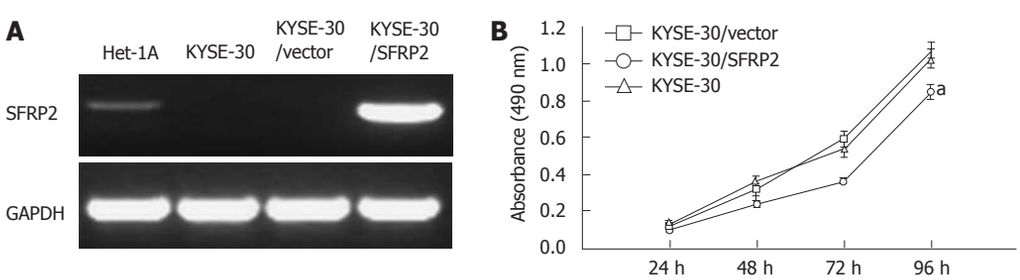

Figure 4 Effect of secreted frizzled-related protein 2 ectopic expression on cell proliferation. A: Strong secreted frizzled-related protein 2 (SFRP2) mRNA expression in KYSE30 cells transfected with pcDNA3.1/SFRP2, but not in KYSE30 and cells transfected with the empty vector; B: SFRP2-transfected KYSE30 cells growing significantly slower than control and parental cells ($^aP < 0.05$).

expression of SFRP2 in all of the cell lines (Figure 1C), further supporting the role of promoter methylation as a primary mechanism of SFRP2 inactivation.

Expression and methylation status of the SFRP2 in primary ESCC tumors

We determined the mRNA expression level of SFRP2 in 20 pairs of primary human ESCC tumors and adjacent non-cancerous tissues using RT-PCR. When compared with adjacent non-cancerous tissues, SFRP2 was found to be clearly down-regulated in primary ESCC tumor specimens (1.51 ± 0.399 *vs* 0.939 ± 0.398, $P < 0.001$) (Figure 3). SFRP2 promoter methylation was observed in 95% (19/20) of the ESCC tissues and 65% (13/20) of the paired normal esophageal epithelial tissues, as measured by methylation-specific PCR. The positive rate of methylation in ESCC tissue was significantly higher compared with that in the normal control ($P = 0.044$). Notably, using high-resolution bisulfite genomic sequencing analysis, no SFRP2 methylation was seen in normal esophageal epithelium specimens (Figure 2).

SFRP2 inhibits tumor cell proliferation and induces apoptosis

A cell proliferation assay was performed on stably transfected KYSE30 cells with or without the SFRP2 expression, and on parental cells. Re-expression of SFRP2 in the transfected KYSE30/SFRP2 cells was confirmed by reverse-transcription PCR (Figure 4A). Cells stably expressing SFRP2 grew significantly slower than the empty vector-transfected cells and parental cells ($P < 0.05$, Figure 4B). We carried out apoptosis and cell cycle analysis to investigate whether SFRP2 over-expression affects these parameters in ESCC cells. We found a significantly higher number of early apoptotic cells in SFRP2-transfected cells than in the control cells ($P = 0.025$, Figure 5). The mean cell number in the S and G2-M phases of the cell cycle was also significantly lower in SFRP2-

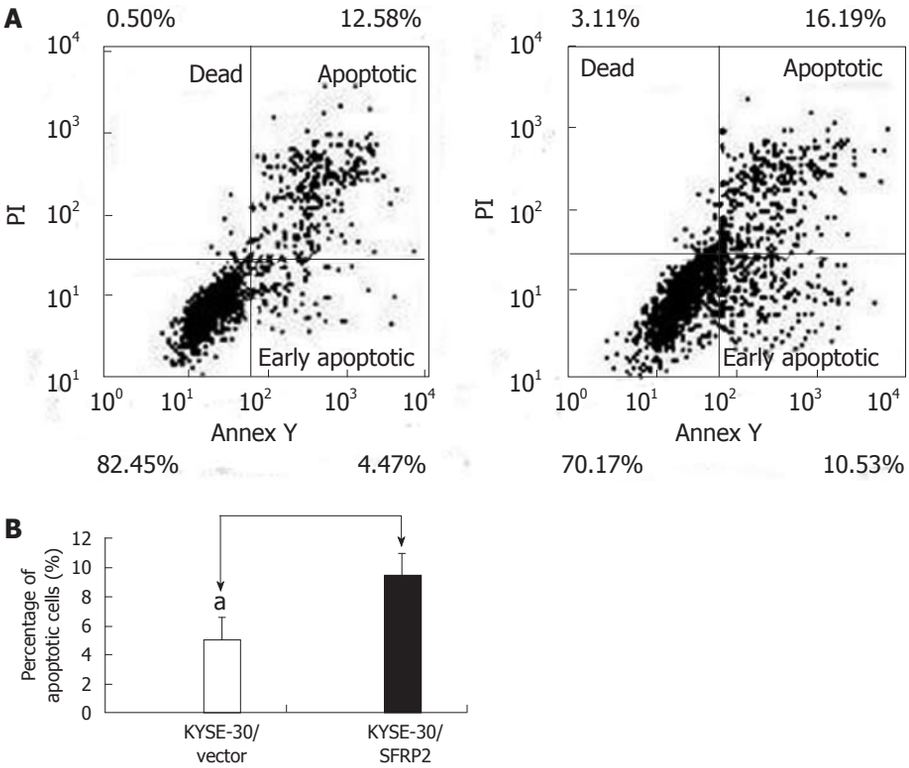

Figure 5 Ectopic expression of SFRP2 induces apoptosis in KYSE30 cells. A: The representative images of flow cytometry analysis; B: The data are the percentage of early apoptotic out of the total cell population of KYSE30 cells transfected with SFRP2 and cells with the empty vector.The early apoptotic cells in SFRP2-transfected cells were significantly higher than that in the control cells ($^aP < 0.05$).

Table 2 Effect of secreted frizzled-related protein 2 re-expression on the cell cycle distribution of KYSE30 cells (%)

Groups	G0/G1	S	G2/M
KYSE-30/vector	45.76 ± 3.74	48.64 ± 4.90	5.59 ± 5.18
KYSE-30/SFRP2	64.49 ± 6.11	30.52 ± 5.07	4.97 ± 6.60

transfected cells, suggesting SFRP2-induced G1 arrest in KYSE30 cells (Table 2).

SFRP2 suppresses colony formation of ESCC cell lines

Frequent silencing of SFRP2 by promoter methylation in ESCC cell lines, but not in the immortalized normal esophageal epithelial cell line, suggests a potential tumor-suppressor role of this gene. To test this speculation, we examined the effect of SFRP2 re-expression on growth characteristics of ESCC cells using colony formation assays. The colonies formed by SFRP2-transfected KYSE30 cells were significantly fewer in number (47.17% ± 15.61% *vs* 17% ± 3.6%, $P = 0.031$) and smaller in size than those in control cells (Figure 6), indicating that SFRP2 has a definite growth inhibitory effect.

SFRP2 inhibits tumor growth in nude mice

After studying the effect of SFRP2 on cell proliferation *in vitro*, the involvement of SFRP2 in carcinogenesis *in vivo* was investigated. SFRP2-transfected KYSE30 cells, control cells, and parental cells were injected into nude mice. The tumor growth curve of stably transfected KYSE30/SFRP2 expression vector and KYSE30/vector control in nude mice is shown in Figure 7. The tumor size was significantly lower in SFRP2 transfected nude mice than in the vector control mice (917.86 ± 249.35 mm^3 *vs* 337.23 ± 124.43 mm^3, $P < 0.05$); however, no difference was found between the weight and volume of tumors from parental cells and those from the control, suggesting that SFRP2 functions as a tumor suppressor in esophageal carcinogenesis.

DISCUSSION

The SFRPs are a group of negative modulators of the Wnt signaling pathway, of which five members (SFRP1-5) have been identified to date. Using microarray analysis, we previously confirmed that the expression of SFRP2 was 3.6-fold lower in ESCC tissues than in paired non-cancerous tissue samples, implying that down-regulation of SFRP2 may be involved in the carcinogenesis of ESCC. In this study, we extended our previous work on SFRP2 to ESCC and found that SFRP2 was silenced in seven ESCC cell lines, but not in an immortalized esophageal epithelial cell line. The silencing of expression was linked closely to promoter methylation, as confirmed by demethylation treatment and methylation analyses, suggesting that promoter methylation is the principal regulatory mechanism of SFRP2 inactivation in ESCC. There are unmethylated alleles in 4 ESCC cell lines with no expression of SFRP2 detected by reverse-transcription PCR, indicating the existence of other transcription regu-

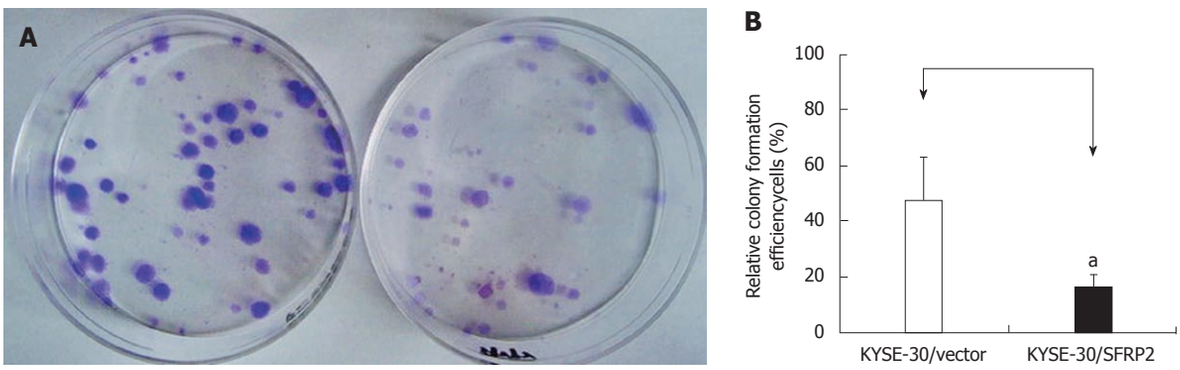

Figure 6 Colony formation assays. A: The representative dishes of transfection with pcDNA3.1V5HisA/secreted frizzled-related protein 2 (SFRP2) or empty vector (pcDNA3.1 V5HisA). Left: colonies formed by control cells. Right: colonies formed by cell strains with stably transfected SFRP2; B: Quantitative analyses of colony formation efficiency. Values are the mean ± SD of at least 3 independent experiments. The relative colony formation efficiency of SFRP2-transfected esophageal squamous cell carcinoma cells were lower than those of mock-transfected cells ($^aP < 0.05$).

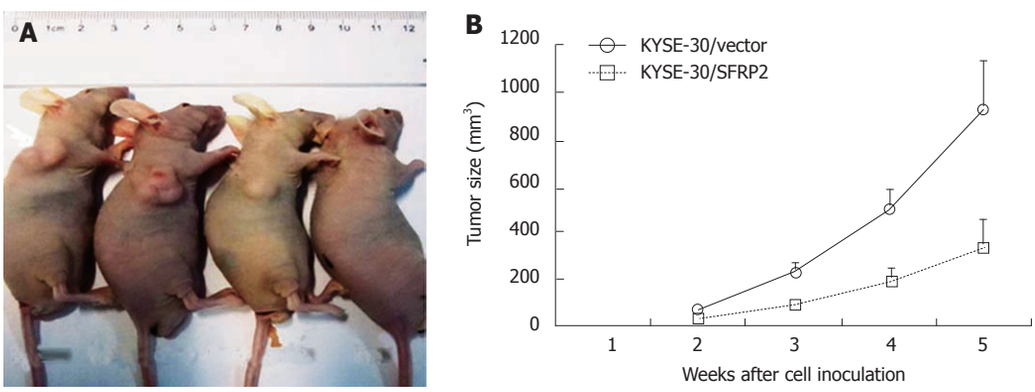

Figure 7 Secreted frizzled-related protein 2 inhibits growth of tumors derived from KYSE-30 *in vivo*. A: A representative picture of nude mice: at week 5 nude mice injected with KYSE-30, KYSE-30/vector, KYSE30/SFRP2 and phosphate buffered saline; B: The tumor growth curves of nude mice. The growth of tumors derived from the SFRP2-transfected esophageal squamous cell carcinoma cells cells were significantly slower than those from the mock-transfected cells ($^aP < 0.05$).

latory mechanisms that repress SFRP2 expression, such as histone remodeling or transcriptional repressors.

Similar results were found in the experiments on primary ESCC tissues and adjacent non-tumor tissues. SFRP2 was clearly down-regulated in primary ESCC tumor tissues when compared to the paired non-cancerous tissues. MSP analysis of the SFRP2 promoter revealed a higher prevalence of CpG methylation in ESCC tissues than in adjacent non-tumorous tissues, suggesting that aberrant methylation of the SFRP2 promoter region is not a cell line-specific event and is frequent in ESCC carcinogenesis.

Functional inactivation of SFRP2 by promoter methylation, which resulted in tumor cell proliferation and invasion, has been confirmed in many human cancers[23-27], suggesting that SFRP2 functions as a tumor suppressor. In contrast to these results, others have found that SFRP2 reduced apoptosis and promotes tumor progression[19,21]. Yamamura *et al*[20] found that ectopic expression of SFRP2 in renal cancer cells reduced UV-induced apoptosis and increased the G2 phase of the cell cycle, significantly promoting the growth of xenografts in nude mice. However, there has been no detailed report thus far describing the functional significance of SFRP2 in ESCC. We therefore analyzed the functional role of SFRP2 in esophageal carcinogenesis by both *in vitro* and *in vivo* assays. Ectopic expression of SFRP2 in KYSE30 cells with no SFRP2 expression induced low cloning efficacy in colony formation assay, along with reduced tumor size in nude mice. FACScan analysis of SFRP2-re-expressed KYSE30 cells revealed a significant decrease in cell proliferation, and an increase in apoptotic cells. Taken together, these results confirmed the role of SFRP2 as a functional TSG through suppression of cell proliferation and induction of cell apoptosis in ESCC. Previous studies of other tumors, such as colorectal and gastric cancer, showed that SFRP2 functioned as tumor suppressor through attenuating the Wnt signaling pathway[10-12]. When it came to ESCC in the present study, although we confirmed that ectopic expression of SFRP2 significantly reduced the expression lever of c-myc and cyclinD1, which were downstream genes of the Wnt pathway (data not shown), further experiments such as immunofluorescence and luciferase reporter assay are necessary to reach a similar conclusion.

SFRP2 methylation was proven to be a highly promising predictive biomarker in many human cancers. Cheng *et al*[17] reported that methylated SFRP2 was detected in 66.7% of serum samples from cancer patients, but not in normal controls and the frequency of SFRP2 hypermethylation decreased from 73.3% in gastric cancer to 37.5%

in intestinal metaplasia and 20% in adjacent non-cancer tissues, indicating that SFRP2methylation may have the potential for identifying individuals at risk of further histological progression. Muller *et al*[28] proposed SFRP2 hypermethylation as a sensitive marker able to detect 77%-90% of colorectal cancers (CRC); Huang *et al*[18] reported that methylated SFRP2 occurred in 94.2%, 52.4%, 37.5% and 16.7% of patients with CRC, adenoma, hyperplastic polyp, and ulcerative colitis, respectively. However, the clinical impact of SFRP2 hypermethylation in ESCC is unknown. Further studies of clinical correlation of SFRP2 methylation are required to answer this question.

In summary, we have presented the first convincing evidence that the epigenetic silencing of SFRP2 is a frequent event in human ESCC, and that SFRP2 is a potent TSG with key roles in suppressing cell proliferation and inducing apoptosis in the development of esophageal cancer. Further studies should be conducted to assess whether SFRP2 methylation might serve as a useful bio-marker for early detection and a potential target for treatment of esophageal carcinoma through reversal of SFRP2 inactivation by demethylating agents.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is one of the most frequent malignant neoplasms in China. Although the exact mechanisms remain unclear, several studies have indicated that functional inactivation of tumor suppressor genes (TSG) through promoter methylation were involved in the pathogenesis of ESCC. Secreted frizzled-related protein 2 (SFRP2) promoter methylation has been documented in several human malignancies. We therefore analyzed the methylation and expression status, as well as the function of this gene in ESCC.

Research frontiers

Aberrant promoter methylation of *SFRP2* gene has been documented in several human malignancies, such as colorectal cancer, gastric cancer, and breast cancer. This study investigated the role of SFRP2 in ESCC and showed for the first time that the hypermethylation of *SFRP2* gene promoter was the predominant mechanism for *SFRP2* gene silence in esophageal cancer.

Innovations and breakthroughs

The authors present the first convincing evidence that the epigenetic inactivation of SFRP2 is a frequent event in human ESCC, and that SFRP2 is a potent TSG with key roles in suppressing cell proliferation and inducing apoptosis in the development of ESCC.

Applications

SFRP2 methylation might serve as a useful bio-marker for early detection and a potential target for treatment of esophageal carcinoma.

Terminology

DNA methylation is an epigenetic modification that is important for normal development in higher organisms. Aberrant changes in methylation patterns play an important role in carcinogenesis, cancer progression and chemosensitivity.

Peer review

This is a study looking for the significance of epigenetic inactivation of SFRP2 in the development of ESCC. The data suggest that SFRP2 methylation might serve as a potential target for treatment of ESCC in the future. Overall ,this study is well designed and the data are convicing.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i6.541

World J Gastroenterol 2012 February 14; 18(6): 541-545
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Response evaluation of chemotherapy in metastatic colorectal cancer by contrast enhanced ultrasound

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Received: August 10, 2010 Revised: October 26, 2010
Accepted: November 9, 2011
Published online: February 14, 2012

Abstract

AIM: To evaluate whether contrast enhanced ultrasound (CEUS) might also be used for response prediction and early response evaluation in patients receiving bevacizumab based chemotherapy for metastasized colorectal cancer.

METHODS: Thirty consecutive patients with non primary resectable liver metastases from colorectal cancer underwent CEUS before treatment (CEUS date 1) and before the second (CEUS date 2) and fourth (CEUS date 3) cycle of bevacizumab based chemotherapy. Three parameters [PEAK, Time to peak (TTP) and RISE RATE]were correlated with radiological response.

RESULTS: For neoadjuvant purpose a reduction of tumour mass was required to assume clinical response. Based on these response criteria there was a significant ($P < 0.001$) correlation in TTP between metastases of responders (9.08 s) and non-responders (14.76 s) archived on CEUS date 1. By calculating a standardized quotient (metastases divided by normal liver tissue) we were able to define a cut off, predicting response with a sensitivity of 92.3 % and a specificity of 100 %. To reflect a palliative intention only those patients with progressive disease were classified as non-responders. In this stetting TTP was also significantly ($P < 0.01$) different between responders and non-responders. In contrast, Peak and Rise rate did not show any significant difference between responder and non-responder.

CONCLUSION: CEUS might serve as a surrogate marker to predict treatment response in patients with metastasized colorectal cancer who receive antiangiogenic therapy.

Key words: Colorectal cancer; Liver metastases; Response prediction to chemotherapy; Contrast-enhanced ultrasound; Bevacizumab

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INTRODUCTION

The liver is the most frequent site of colorectal cancer (CRC) metastases, with 15% to 25% of patients having liver metastases at diagnosis and further 30% developing liver metastases at a later point in the disease course[1-3]. While for decades prognosis for these patients was poor, introduction of multimodal management approaches such as highly active chemobiological therapy and innovative surgical techniques, have significantly improved both disease free and overall survival. Those patients who can have liver metastases resected even display 5-year survival rates of up to 40%, with 20% alive after 10 years[4]. Unfortunately, most patients initially present with unresectable disease. However, in some of these cases neoadjuvant treatment can reduce tumour load and allow secondary resections. Thus, the aim of systemic chemotherapy in patients with non primary respectable liver metastases has undergone a transition from a pure palliative to a neoadjuvant and thereby curative concept[5].

To improve anti-tumour activity, in clinical routine the vascular endothelial growth factor (VEGF)-antibody bevacizumab is often added to treatment[6-8]. While on the one hand such combination chemotherapies are associated with improved response rates, they are on the other hand related to high toxicity and costs, and it remains an unsolved problem to identify those patients that will benefit from therapy. Fluorodeoxyglucose positron emission tomography (FDG PET) demonstrated efficacy in early treatment monitoring of a variety of tumour diseases, however data supporting its use in patients with advanced colorectal cancer are conflicting[9,10]. A novel technique to improve response evaluation to anti-angiogenic agents represents contrast-enhanced ultrasound[11] with the opportunity to detect changes of tumour perfusion at early time-points after administration of chemotherapy.

In the present study we explore the use of CEUS for response prediction and early response evaluation in patients with liver metastases from CRC, which were treated with bevacizumab containing chemotherapy.

MATERIALS AND METHODS

Patients

Between October 2007 and October 2009, 30 consecutive patients with histological confirmed colorectal carcinoma and non primary resectable liver metastasis, according to the decision of our interdisciplinary tumour board, were enrolled. Five patients had metastatic rectal cancer and 25 patients had colonic cancer. The histological grading was in 13 cases G2 and in 17 cases G3. The number of individual liver metastases ranged from 4 to 25.

All eligible patients were > 18 years old and had no prior history of receiving chemo- or radiotherapy or major surgery within 28 d before initiation of study treatment. The protocol was approved by the institutional review board and carried out in accordance with the Declaration of Helsinki and local ethical and legal requirements.

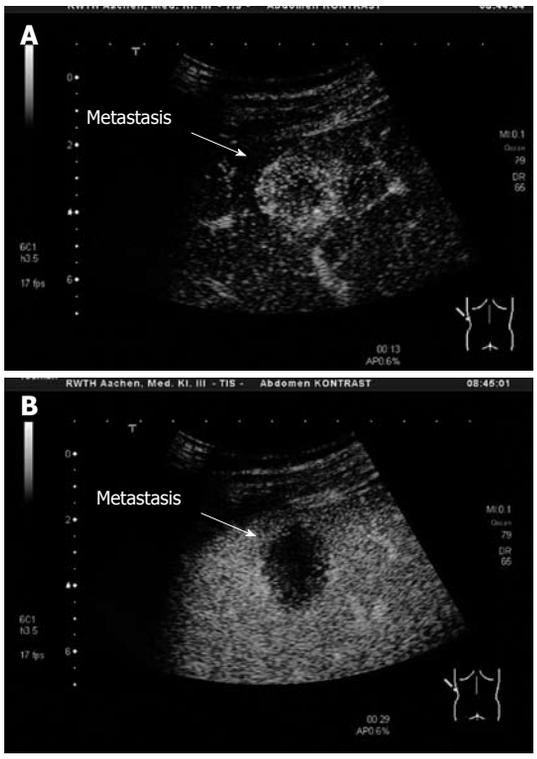

Figure 1 Contrast enhanced ultrasound. A: A representative liver metastasis after 13 s of contrast agent injection with early contrast enhancement; B: A representative liver metastasis after 29 s of contrast agent injection with lost of contrast enhancement.

Treatment

Chemotherapy consisted of Leucovorin (LV) 400 mg/m^2 per day as a 2 h infusion followed by bolus 5-Fluorouracil (5-FU) 400 mg/m^2 per day and a 46-h infusion of 5-FU 2400 mg/m^2 per day (simplified FOLFIRI). Bevacizumab was administered every two weeks at a dose of 5 mg per kg.

Contrast-enhanced ultrasound

Within 1 d before the first (day 1), second (day 15) and forth (day 43) application of chemotherapy a contrast enhanced ultrasound (CEUS) of one liver metastasis was performed (CEUS - date 1, 2 and 3). In case of multiple liver metastases, the metastasis which could be best positioned in the CEUS was selected. Ultrasound examinations were performed using the Aplio system (Toshiba Medical Systems Europe, Neuss, Germany). All patients were examined with a convex 3.5 MHz transducer as baseline US. The CEUS was performed using the contrast enhancer SonoVue® and predefined settings using the 3.5 MHz transducer. All patients were examined in wideband harmonic mode (pulse inversion) at low energy (low mechanical index) optimizing real-time detection of harmonic contrast response. The agent was injected as bolus in units of 2.4 mL through a peripheral venous catheter over 2 s, followed by bolus injection of 10 mL of 0.9% NaCl solution. Video documentation of all examination steps was obtained from the beginning of injection for a period of at least 2 min, allowing recording all steps of contrast enhancement characteristics (Figure 1).

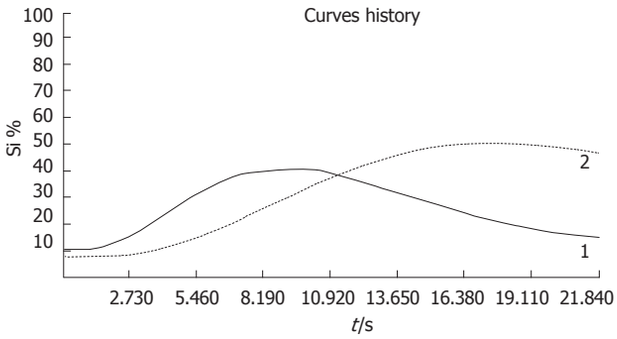

Figure 2 Curves of contrast behavior in a liver metastasis (solid line, 1) and normal liver tissue (dotted line, 2) over the time (s = seconds) and percent of contrast enhancement (Si %).

For quantitatively measuring vascularity in the metastasis and normal liver tissue by contrast dye characteristics we used the Bracco QONTRAST software (Version 4.00). This software is a specific sonographic quantification software, based on pixel by pixel signal intensity over time to obtain contrast-enhanced sonographic perfusion maps for each metastasis. In all cases, regions of interest (ROIs) were chosen over the whole area of the metastasis. Additionally we selected ROIs at least two centimetres lateral (same deepness) of the metastasis in a region with normal liver tissue. The first contrast enhancement seen in ROI determines the beginning of the measurement (Figure 2).

Three parameters were calculated including the PEAK (%; maximum peak of contrast intensity), the Time to peak (TTP) (s, time to reach the maximum peak of contrast intensity) and the RISE RATE (s-1), which is equal to contrast enhancement. The ultrasound exam was analyzed by two independent investigators blinded to the cases.

Response evaluation

All patients received before and after 3 mo of therapy a CT scan for response evaluation. Tumour response was defined by changes in diameter of target lesions on the basis of Multislice-CT results at baseline and after the first 3 mo of FOLFIRI-Avastin administration, according to published recommendations [Response Evaluation Criteria In Solid Tumors (RECIST) 1.1][12].

Statistical analysis

The multivariate analysis of variance model (MANOVA) was applied to test the within subject factors time (CEUS date 1, 2 and 3) and tissue (metastasis *vs* normal). Pair wise comparisons were performed with the paired *t*-test. The MANOVA was applied to test the between subject factor response (responder *vs* non-responder). For each separate date the unpaired *t*-test was used to compare the two groups. ROC curves were used to analyse the diagnostic power of the parameters. Cut points were chosen as to maximize the Youden index. Patients were classified as responder if the parameter is smaller or equal to the cut point.

RESULTS

Patients and response to chemotherapy

A total of 30 patients (8 women and 22 men) with a mean age of 62 (range: 50-78) were enrolled and received FOLFIRI supplemented by bevacizumab for metastasized colorectal cancer. All patients underwent radiological evaluation of tumour-load at baseline and after a period of 3 mo of treatment. According to RECIST criteria 1 patient showed a complete response (CR), 12 patients showed a partial response (PR), 8 patients had a stable disease (SD) and 9 patients had a progressive disease (PD). 4 of the 30 patients underwent resection of liver metastases within 4 mo after initiation of chemotherapy.

To reflect the clinical reality of patients with metastasized colorectal cancer, two distinct definitions of response were used: in a first scenario only those patients with PR and CR in radiological evaluation were classified as responders, while in a second scenario also patients with radiological SD were included in the group of responders.

Contrast-enhanced ultrasound

At baseline, all hepatic tumour sites were assessed by classical sonography. One metastasis was chosen as an index metastasis for further examination bei CEUS. The diameters of these selected lesions ranged from 1.9 cm to 15 cm (mean 2.7 cm). 26 lesions were classified as predominantly hypoechoic and 4 as hyperechoic according to B-mode ultrasound. In these lesions, all relevant quantitative parameters (TTP, PEAK and RISE RATE), were analyzed at baseline and during the course of treatment (CEUS-date 1, 2 and 3) by CEUS.

By applying the more strict criteria of the first scenario (neoadjuvant purpose), reflecting the need of tumour reduction in patients that could benefit from neoadjuvant therapy, we detected a significant ($P < 0.001$) difference of TTP in metastases between responders (9.08 s) and non-responders (14.76 s) already at baseline (CEUS date 1) shown in Table 1 and illustrated in Figure 3A. Furthermore in the group of the responders, a strong and continuous increase in TTP was observed during therapy and this reflects the effect of bevacizumab on tumour vascularisation. Strikingly no comparable therapy related increase in TTP was detectable within the group of non-responders. While on CEUS date 2 differences in TTP between responders and non-responders were still detectable, after 4 cycles (8 wk) of therapy identical TTP were detected in both groups. In contrast to these observations in metastatic tissue, no differences in TTP were found in normal liver tissue. To further standardize our data, a standard TTP-quotient was calculated by dividing the TTP measured in liver metastasis by TTP in the corresponding normal liver. Strikingly standardization of the described data did not change the described observations.

Chemobiological therapies are related to high toxicity. Response prediction would therefore allow restricting treatment to patients that will benefit from therapy. We therefore attempted to calculate a cut of point which

Table 1 Time to peak and to peak quotient in liver metastasis

	Date 1	Date 2	Date 3
Time to peak			
Responder (CR, PR)			
mean ± SD	9.1 ± 2.9	12.1 ± 2.3	14.6 ± 3.9
95% CI	7.3-10.8	10.7-13.6	12.2-17.0
Non-responder (SD[1],PD)			
mean ± SD	14.8 ± 3.7	16.7 ± 2.8	16.4 ± 2.6
95% CI	12.8-16.7	15.2-18.2	15.0-17.7
P value compared responder with non-responder	< 0.001	< 0.001	NS
Time to peak quotient			
Responder (CR, PR)			
mean ± SD	57.5 ± 11.8	80.5 ± 5.4	93.7 ± 9.2
95% CI	50.3-64.6	77.2-83.7	88.2-99.2
Non-responder (SD[1], PD)			
mean ± SD	98.7 ± 12.6	100.6 ± 13.3	96.9 ± 9.7
95% CI	92.2-105.2	93.8-107.5	91.9-101.9
P value compared responder with non-responder	< 0.001	< 0.001	NS

NS: Not significant; CR: Complete response; PR: Partial response; SD[1]: Stable disease; PD: Progressive disease; CI: Confidence interval.

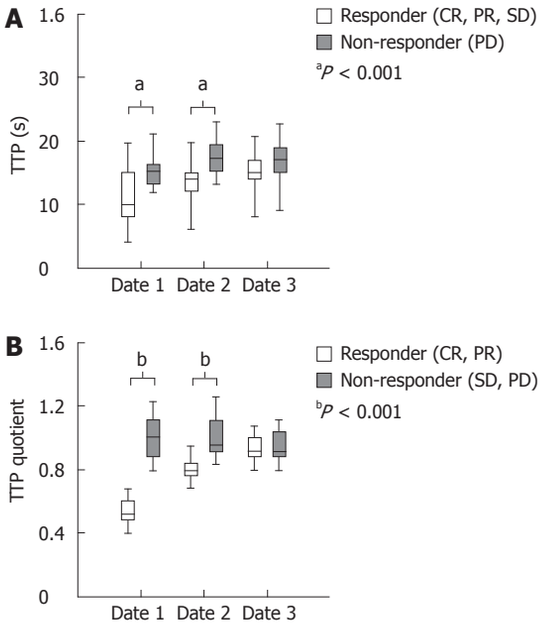

Figure 3 Time to peak parameters. A: Time to peak (TTP) values measured in the metastasis between responders and non-responders on contrast enhanced ultrasound (CEUS) date 1, date 2 and date 3 (responders with complete response (CR), partial response (PR) and stable disease (SD) (n = 21) *vs* non-responder with progressive disease (PD) (n = 9); B:The TTP quotient between responders and non-responders on CEUS date 1, date 2 and date 3 (CR and PR were classified as responders (n = 13) and patients with SD and PD as non-responders (n = 17).

predicted response with high specificity and sensitivity. According to our data, a TTP-quotient < 0.7 predicted a decrease of tumour load according to RECIST with a sensitivity of 92.3% and a specificity of 100% (Table 1 and Figure 3B).

In a second scenario we used less strict criteria for response definition, as patients with stable disease were also included in the group of responders, reflecting the clinical reality of patients in a palliative setting. Interestingly, also based on this response definition TTP and TTP-quotient were significantly lower in the group of the responders compared to non responders (Figure 3A). Here, a TTP quotient of 0.8 predicted response with a sensitivity of 61.9% and a specificity of 100%.

In contrast, the PEAK and RISE RATE parameter did not show any significant difference between responder and non-responder independent of response definition, use of the quotient or date of CEUS. In addition, there was no significant correlation between tumour response and differentiation of the tumour or the number, the location or the size of liver metastases (data not shown).

DISCUSSION

In the last years intensive efforts were conducted to identify surrogate markers that predict response to antiangiogenic combination chemotherapies. Previously a correlation between early metabolic response according to PET and patients outcome was demonstrated in patients receiving bevacizumab. However comparable data regarding colorectal cancer are insufficient. Indeed just recently two studies investigating the role of FDG-PET for treatment monitoring in patients with metastasized colorectal cancer revealed conflicting results[9,10]. Furthermore, despite these advances in monitoring of treatment, to our knowledge no data describing surrogate markers for prediction of response before starting the treatment are available.

In the present study we demonstrate that CEUS might evolve as an innovative tool in prediction of response and response evaluation in patients with metastasized colorectal cancer receiving bevacizumab based therapy. We clearly demonstrate that baseline TTP and TTP quotient are significantly lower in the group of the responders compared to the non-responders, meaning that low baseline TTP significantly correlates with tumor response according to RECIST. Furthermore, correlating to the antiangiogenic effect of bevacizumab we observed a strong increase in TTP and TTP quotient during chemotherapy, which was restricted to the group of the responders. In line with these data Varallyay *et al*[13] recently demonstrated a decrease in tumor perfusion after bevacizumab based chemotherapy by using dynamic MRI.

Chemobiological combination chemotherapies are associated with considerable toxicity and prediction of response could help to prevent non-responders from undesiderable side-effects. Based on the described perfusion analysis we established a cut off for TTP which predicted response with a specificity and sensitivity of up to 90%. To our knowledge we here for the fist time describe a parameter which reliably predicts tumor response even before starting therapy.

Despite these encouraging results, the present study has some limitations. The number of patients included is limited and at present the follow up is only 3 mo, so that

a conclusion on the use of CEUS for predicting survival is not possible. Additionally, we only included one index lesion that could be best positioned in the CEUS and thus it would be of interest to include different liver metastasis of one patient to further assess the power of this method.

In summary, our results indicate that CEUS might serve as a useful, noninvasive surrogate marker of early response in patients with liver metastases of a colorectal cancer receiving bevacizumab. However, larger trials with a longer follow-up are needed to clarify the clinical use of CEUS in this field.

COMMENTS

Background

In the last few years contrast enhanced ultrasound (CEUS) was established for differentiation between benign and malignant liver lesions. CEUS might also be used for response prediction and early response evaluation in patients receiving chemotherapy for metastasized colorectal cancer.

Research frontiers

In the present study we explore the use of CEUS for response prediction and early response evaluation in patients with liver metastases from CRC, which were treated with bevacizumab containing chemotherapy.

Innovations and breakthroughs

The results indicate that CEUS might serve as a useful, noninvasive surrogate marker of early response in patients with liver metastases of a colorectal cancer receiving bevacizumab.

Applications

CEUS for response evaluation may represent a future diagnostic pathway in the management of patients with liver metastases of a colorectal cancer.

Peer review

This is an interesting pilot study investigating the relevance of CEUS in metastatic colorectal cancer. For the first time a parameter, which reliably predicts tumor response even before starting therapy, was described.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i6.546

World J Gastroenterol 2012 February 14; 18(6): 546-550
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Ambispective comparative study of two surgical strategies for liver hydatidosis

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Received: March 23, 2011 Revised: November 12, 2011
Accepted: November 19, 2011
Published online: February 14, 2012

Abstract

AIM: To investigate the morbidity, mortality, recurrence and technical aspects of two distinct surgical strategies that were implemented in successive periods.

METHODS: Ninty-two patients with 113 cysts underwent surgical procedures. The study was divided into 2 periods. Data from first period (P1) were compiled retrospectively. The surgical strategy was conservative surgery. The second period (P2) included a prospective study conducted according to a protocol following the criterion that radical procedures should be performed whenever it is technically feasible.

RESULTS: Patients of both periods showed no statistically significant differences in age, gender, cyst location or mortality. Among the P2 group, patients exhibited more preoperative jaundice, and cyst size was smaller ($P < 0.05$). Changes in surgical strategy increased the rate of radical surgery, decreases morbidity and in-hospital stay ($P < 0.001$). A negative result in P2 was the death of two old patients (4.8%) who had undergone conservative treatments. The rate of radical surgery in P2 was around 75%.

CONCLUSION: Radical surgery should be the technique of choice whenever it is feasible, because it diminishes morbidity and in-hospital stay. Conservative surgery must be employed only in selected cases.

Key words: Hydatid disease; Surgery; Morbidity; Liver

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Ramia JM, Ruiz-Gomez F, De la Plaza R, Veguillas P, Quiñones J, García-Parreño J. Ambispective comparative study of two surgical strategies for liver hydatidosis. *World J Gastroenterol* 2012; 18(6): 546-550 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i6/546.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i6.546

INTRODUCTION

The primary therapeutic objective in hydatid disease of the liver (HDL) is to completely eliminate the parasite and prevent recurrences with minimal morbidity and mortality[1-6]. Although several treatment options exist, the mainstay of therapy is surgery[1,2,4,6-14]. At present, no scientific evidence exists regarding which of two possible technical options, radical surgery (RS) or conservative surgery (CS), is most appropriate[4,7,11]. The main difference between the two surgical options is that the parasitic cyst is completely excised in RS, whereas only a variable percentage of the cyst is excised in CS, which makes complementary therapeutic measures for the residual cavity necessary[3,9,10]. We made an ambispective (retrospective and prospective) study to compare two consecutive

periods in our center in which the preferential surgical strategy for HDL changed from conservative surgery to radical surgery.

MATERIALS AND METHODS

The inclusion criterion was: patients with HDL who underwent scheduled surgery in our center in the study period (January 2001 to November 2010). A total of 92 patients with 113 cysts made up the study group. The exclusion criterion was emergency surgery for HDL.

RS was defined as complete open or closed cystectomy and/or hepatectomy. All other interventions (subtotal cystectomy, Lagrot surgery, marsupialization, and others) were categorized as CS[2,7,13,15]. Blood tests, liver tests, indirect anti-Echinococcus antibody hemagglutination, abdominal CT and ultrasonography were done in all patients. All patients had CE1, CE2 or CE3 type cysts (World Health Organization classification).

The study had two periods. The data from the first period (January 2001 to April 2007) (P1) were compiled retrospectively. The preferred surgical strategy was conservative surgery with insertion of a Kehr tube if preoperative jaundice, intracystic bile, or bile duct dilation was present. The period from May 2007 to January 2010 (P2) was a prospective study conducted according to protocol guided by the criterion the RS should be performed whenever it was technically feasible. The decision to perform total cystectomy or hepatectomy depended exclusively on cyst location and the relation between the cyst and vascular and biliary structures. A Kehr tube was not inserted unless required to resolve situations that arose intraoperatively. Systematic preoperative endoscopic retrograde cholangiopancreatography (ERCP) was performed if a frank intrabiliary rupture was present. Albendazole was administered only if conservative surgery is going to be performed for one month preoperatively and 3 mo postoperatively.

Morbidity and mortality were assessed according to the Dindo-Clavien classification[15,16]. Data were analyzed with the SPSS 16.0 program for Mac. Approval of ethics committee was obtained for P2.

RESULTS

General findings

In the 2001-2007 period (P1), 50 patients with 60 cysts were treated (Table 1). The patients were 56% men (28/50) and the median age of patients was 53 years (range: 11-77). Previously operated recurrent cysts were present in 22% (11/50) of patients. The usual clinical manifestation was abdominal pain (60%) (30/50); only 10% (5/50) of patients debuted with obstructive jaundice. The median number of cyst was one. The cyst locations were: 18 in left liver, 23 in right liver, and 9 bilobar. Median cyst size was 8.5 cm (2-20 cm) and 48.3% of cysts were larger than 10 cm. Only 2 patients (4%) had preoperative ERCP.

Table 1 Comparison between radical and conservative surgery groups

	P1	P2	*P* value
Patients	50	42	NS
Cysts	60	53	NS
Age (yr)	53 (11-77)	52 (23-83)	NS
Men (%)	28/50 (56)	25/42 (59.6)	NS
Recurrent cysts (%)	11/50 (22)	7/42 (16.7)	NS
Primary symptom (%)	Abdominalpain (60)	Abdominalpain (69)	NS
Complicated cysts	5/50 (10)	13/42 (31)	< 0.01
Preoperative jaundice	5/50 (10)	9/42 (23.7)	< 0.01
Size (cm)	8.5 44%>10 cm	6.5 21.1%>10 cm	< 0.05
Location	18L/23R/9 bilobar	22L/15R/4 bilobar	NS
Radical surgery cyst (%)	4/60 (6.7)	39/53 (73.6)	< 0.001
Radical surgery patients (%)	3/50 (6)	31/42 (73.8)	< 0.001
Kehr tube insertion (%)	36/50 (72)	2/42 (4.8)	< 0.001
Morbidity (%)	21/50 (42)	7/42 (16.7)	< 0.001
Biliary fistula (%)	17/50 (34)	5/42 (12)	< 0.001
RS	0/31 (0)	2/31 (7.7)	
CS	4/11 (35)	3/11 (27.3)	
Mortality (%)	0/50 (0)	2/42 (4.8)	NS
RS	0/31 (0)	0/31 (0)	
CS	0/11 (0)	2/11 (18)	
Mean hospital stay	23 (6-71)	7 (4-50)	< 0.001
Recurrence	4	0	NS

L: Left; R: Right; NS: No stastistical significance.

In the 2007-2010 period (P2), 42 patients with 53 cysts were treated. Seven patients (16.7%) were HDL cyst recurrence. The 59.6 % of patients (25/42) were men. The median age was 52 years (range: 23-83 years). Abdominal pain was the primary symptom in 29 patients (69%). The debut was as complicated HDL in 31% (13/42) of patients (8 cases of obstructive jaundice due to frank intrabiliary rupture, 2 cases of septic shock, 1 cutaneous hydatid fistula, 1 bilio-bronchial fistula, and 1 upper gastrointestinal hemorrhage due to esophageal varices originated by portal hypertension). The median cyst size was 6.5 cm (range: 3-20 cm) and 21.1% of cysts were more than 10 cm in diameter. The cyst locations were: 22 in left liver, 15 in right liver, 4 bilobar cysts, 2 in spleen, and 2 subcutaneous cysts. Preoperative ERCP was performed in 9 patients who debuted with obstructive jaundice (8 patients with obstructive jaundice and one patient with a bilio-bronchial fistula). Preoperative percutaneous drainage was done in the two patients with septic shock and were operated on later.

Technical aspects

In P1, RS was performed in 3 patients (6%) and 4 cysts (6.6%) and consisted of 4 total cystectomies. In 47 patients (94%) with 56 cysts (93.4%), CS was performed, consisting of 47 partial cystectomies, which were accompanied by cyst marsupialization in 10 cases. A Kehr tube was installed in 36 patients (72%). Some type of morbidity occurred in 21 patients (42%). Postoperative biliary fistulas developed in 17 cases, that occurred in patients

that were treated by CS, as well as one stenosis of the intrahepatic biliary tract, one gas embolism, and two cases of bleeding of the residual cystic cavity, one of which required a new laparotomy (19 Clavien Ⅲa, 2 Clavien Ⅳb). The mortality was 0%. The median hospital stay was 23 d (6-71 d). Four recurrences (6.7%) were detected during follow-up. The mean follow-up was 74 mo (range: 36-108 mo).

In P2, RS was performed in 31 patients (73.8%) and 39 cysts (73.6%). The operations performed were left lateral sectionectomy (11), left hepatectomy (5), right hepatectomy (2), total cystectomy (15), two of them by laparoscopic approach, and splenectomy (2). CS was performed in 11 patients with 14 cysts; cystectomy was almost complete in 8 of these patients. The Lagrot procedure with omentoplasty was performed in 2 patients and one marsupialization. The most frequent reason for not performing RS was the presence of more than 5 cm of contact surface with the inferior vena cava (8/11). Two Kehr tubes (5%) were inserted. Seven patients experienced morbidity (17%); 5 patients had a Clavien-Dindo Ⅲa morbidity consisting in postoperative biliary fistulas that were resolved by postoperative ERCP. The patients presenting biliary fistula three have been treated by CS and 2 by RS. The two patients treated by RS had hepatic hilar damage, which was managed by hepatectomy and reconstruction of the biliary tree. Two patients with Clavien-Dindo V pathology, an 81-year-old patient and an 83-year-old patient with complicated cysts, were treated by means of CS (bilio-bronchial fistula and septic shock due to an infected cyst). These patients developed numerous complications and died (mortality: 2/42, 4.8%). The median hospital stay was 7 d (range: 4-50 d; in the follow-up conducted (range: 1-36 mo), no recurrences were observed.

P1-P2 comparison

The patients of the two periods showed no statistically significant differences in age, gender, primary symptom, cyst location, previous surgery, mortality, or recurrence (Table 1). Among the P2 patients, patients had more preoperative jaundice, more preoperative ERCPs were performed, and cyst size was smaller ($P < 0.05$). In addition, the change in surgical strategy substantially increased the rate of radical surgery and diminished the number of Kehr tubes installed, morbidity, particularly biliary fistula, and the mean stay ($P < 0.001$). In P2, the death of two patients occurred (5%) who had been treated by means of CS.

DISCUSSION

HDL is still an endemic disease in certain areas of the planet[2-7,10,11,13,14]. No consensus exists in the international literature regarding the optimal treatment for HDL[10,13,17-19]. Possible therapeutic options are observation, anthelmintics, percutaneous aspiration, or surgery (conservative or radical). Surgery is considered the therapy of choice[2,3,8-11,17-19]. There are many studies for and against both surgical options[8,19]. Current evidence on which type of surgery is optimal for HDL is supported only by evidence level Ⅳ grade C[7,8,13].

The two technical options, CS and RS, have their respective advantages and disadvantages. Only a randomized study with a large number of patients can provide a methodologically valid response. The most important problem is the bias inherent to the fact that CS is universally applicable, whereas RS sometimes is not feasible.

The characteristics of the cyst (number, size and location), presence of complications related to the HDL, the patient's age and comorbidities, presence of new or recurrent disease, and the surgeon's experience in hepatic surgery condition decision-making with regard to selection of the technique[2-4,9-11,17,19]. CS offers acceptable results, is easily performed at any center and on any cyst by surgeons with little experience in hepatic surgery, and has a low mortality rate and an appreciable morbidity rate, particularly biliary morbidity and recurrence[1,4,10,12,17,19]. Since laparoscopy came into use for the treatment of HDL, CS has become popular again as the preferred method because laparoscopic RS is technically more challenging[11,14,19].

RS is safe and efficient, and produces less morbidity, especially in terms of postoperative biliary fistula and cavity infection. RS eliminates the possibility of untreated satellite lesions in CS and achieves a shorter hospital stay with fewer recurrences[1,4,7,8,10,11,14,17]. RS is generally criticized as associated with high morbidity and mortality that are considered disproportionate in the case of a benign pathology, although the benignity of HDL is questionable[1,8,10,14,17,19]. The only prospective comparative study of RS and CS concluded that RS is associated with less morbidity and mortality, fewer recurrences, and a shorter hospital stay[20]. The election of total cystectomy or anatomic resection depends on cyst location and the anatomic relations of the cyst(s)[10]. RS, despite the advantages mentioned, has not been generally accepted as the treatment of choice.

RS is not feasible in certain cases of extensive contact (> 5 cm) with the inferior vena cava[9]. In our series, this was the main contraindication for RS. A small number of combined resections of the inferior vena cava and hydatid cyst have been reported[21], but we believe that this procedure should be performed only in cases of serious complications (Budd-Chiari syndrome, hemorrhage or other) that justify exeresis.

Major hepatectomy, which was once unthinkable, has been demonstrated to achieve excellent results with very low morbidity, mortality and recurrence rates[17]. The 20% rate of major hepatectomy in our series was high compared to 3.3%-10% in other series[14]. Major hepatectomy was performed for biliary rupture at the hilar plate or the presence of several cysts that affected the entire hepatic lobe. The hepatic resection most frequently performed was left lateral sectionectomy for cysts that affected segments Ⅱ-Ⅲ[14].

Published series on surgery for HDL usually involve cases of CS or a combination of patients treated by CS and/or RS[3,4,6,9,10,17,19]. Few publications exist on patients treated exclusively by RS (22). The percentage of RS in mixed series varies widely and ranges from 15% to 80%[1-14,17-21], but a few series have reported as many as 75% of patients treated with RS, including our series[11,17,22]. The use of an ultrasonic bisturi, a more refined hepatic surgery technique, to treat patients with HDL in hepato-bilio-pancreatic units that have experience in treating this condition, and the surgical team's awareness of the advantages of RS, are the key to attaining this RS rate, with CS being restricted to very specific cases[23].

The literature overall morbidity of RS is 3.2% to 32% lower than the morbidity of CS (15.7%-54%)[2-6,9-11,13,17,19,22]. In the second period, in which the RS rate was high, there was a drastic decrease in morbidity.

The literature rate of postoperative biliary fistula in RS was 0% to 7.7%, compared to 11.3%-25.6% for CS[2,8,11,14,18,19]. Traditionally, when CS is performed, if bile is present in the cyst, the bile ducts are dilated, or preoperative cholangitis is observed, the bile ducts found inside the cystic cavity are sutured and a Kehr tube is inserted in the choledochus or a biliary derivation is performed[1,3,6,13,19]. The rate of Kehr tube use in series of patients treated by CS is 30% to 50%[4].

Performance of preoperative ERCP in patients who debut with obstructive jaundice due to a frank intrabiliary rupture, established by protocol in our unit, facilitates optimal bile duct cleaning and helps to eliminate the need for bile duct opening[2,3]. The combination of preoperative ERCP and RS drastically reduces the need for a Kehr tube, thus eliminating the morbidity associated with this procedure and shortening the mean hospital stay[3,6,14]. In series in which RS is the most frequently used technique, the Kehr tube is used in only 4% compared to 4.8% in our series. Patients with frank biliary rupture into the cyst are at the highest risk of postoperative biliary fistula, so we consider them to be a sub-group of patients who may benefit especially from RS.

Infection of the residual cystic cavity is an exclusive complication of CS[1,2]. The frequency of abscess formation in the residual cavity ranges from 5.5% to 37%[1,8,10,12,19]. Several techniques exist to diminish this complication, including omentoplasty, introflection, capitonnage, external drainage, *etc.*[4]. In P2, a patient who debuted with cyst infection and septic shock in which CS with omentoplasty was practiced presented infection of the cystic cavity. Omentoplasty seems to be accompanied by less morbidity than other techniques[4,6,7,12]. In patients in whom RS is performed, an intra-abdominal abscess can develop in the dead space remaining after the hepatic parenchyma is resected, although the rate of this complication is lower (3%) than the rate of intracystic cavity infection[2,8] and we did not observe it in our series. An added problem of CS is the difficulty of conducting postoperative follow-up using imaging techniques, since it is complicated to assess residual cavities and differentiate between a residual cavity with disease recurrence and one without recurrence[24].

The recurrence rate after surgery for HDL is 0% to 25%. The recurrence rate differs between RS (0%-6.4%) and CS (6%-25%)[2,4,6,8-11,14,17,18]. However, although recurrence after RS is much less frequent than after CS, RS is not suitable for all patients[18]. Cases of HDL recurrence should be treated by RS if this is technically feasible[2,9]. Our recurrence rate was 6% in the first period, when patients were treated preferentially by CS. The follow-up of patients in the second period is too short to draw conclusions yet.

Mortality due to surgery for HDL is low and differs between patients treated by CS (0%-2.1%) and by RS, which has a slightly higher mortality (0%-2.9%)[2-4,6,9,10,13,14,17-19,22]. Higher mortality rates (4.5%) have been reported in series limited to cysts that communicate with the biliary tract, all treated by CS[2,25]. In our series, the two deaths that occurred in the second period were patients over 80 who had complicated cysts (septic shock due to an infected cyst and bilio-bronchial fistula) treated by means of CS. No deaths occurred in the patients of the RS group.

Despite the methodologic limitations of an ambispective study, we believe that radical surgery should be performed in HDL because it reduces morbidity, especially biliary complications, the duration of the hospital stay and, according to published series, has a lower recurrence rate. Conservative surgery is useful in certain extreme cases in which the risk is extremely high[8,11].

COMMENTS

Background

No evidence medicine data about best surgical option for liver hydatidosis exist. We have performed an ambispective study divided in 2 periods, first period we performed as first option conservative surgery, in second radical surgery. We compare morbidity, relapse and feasibility of radical surgery.

Research frontiers

The research hotspot is how to completely eliminate hydatid disease of the liver and prevent recurrences with minimal morbidity and mortality. But, which of two possible technical options, radical surgery or conservative surgery, is most appropriate? In this study, the authors attempt to answer this question.

Innovations and breakthroughs

Very few ambipective of liver hydatidosis exist. Randomized trial comparing radical and conservative surgery are scarce.

Applications

Radical surgery is the best surgical therapy but not always is feasible.

Peer review

Morbidity, relapse and hospital stay is reduced with radical surgery but these techniques are not always feasible. Preoperative endoscopic retrograde cholangiopancreatography in jaundiced patients is recommended.

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S- Editor Gou SX **L- Editor** O'Neill M **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i6.551

World J Gastroenterol 2012 February 14; 18(6): 551-556
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Tracheobronchial Polyflex stents for the management of benign refractory hypopharyngeal strictures

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Received: December 18, 2010 Revised: February 1, 2011
Accepted: February 8, 2011
Published online: February 14, 2012

Abstract

AIM: To describe a modified technique for placement of a tracheobronchial self-expanding plastic stent (SEPS) in patients with benign refractory hypopharyngeal strictures in order to improve dysphagia and allow stricture remodeling.

METHODS: A case series of four consecutive patients with complex hypopharyngeal strictures after combined therapy for laryngeal cancer, previously submitted to multiple sessions of dilation without lasting improvement, is presented. All patients underwent placement of a small diameter and unflared tracheobronchial SEPS. Main outcome measurements were improvement of dysphagia and avoiding of repeated dilation.

RESULTS: The modified introducer system allowed an easy and technically successful deployment of the tracheobronchial Polyflex stent through the stricture. All four patients developed complications related to stent placement. Two patients had stent migration (one proximal and one distal), two patients developed pharyngocutaneous fistulas and all patients with stents in situ for more than 8 wk had hyperplastic tissue growth at the upper end of the stent. Stricture recurrence was observed at 4 wk follow-up after stent removal in all patients

CONCLUSION: Although technically feasible, placement of a tracheobronchial SEPS is associated with a high risk of complications. Small diameter stents must be kept in place for longer than 3 mo to allow adequate time for stricture remodeling.

Key words: Larynx neoplasms; Hypopharynx; Strictures; Dilation; Stents

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Silva RA, Mesquita N, Nunes PP, Cardoso E, Pinto RM, Dias LM. Tracheobronchial Polyflex stents for the management of benign refractory hypopharyngeal strictures. *World J Gastroenterol* 2012; 18(6): 551-556 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i6/551.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i6.551

INTRODUCTION

Total laryngectomy or pharyngolaryngectomy combined with postoperative radiation therapy is the standard treatment for advanced laryngeal cancer. After therapy, the majority of patients will develop some degree of swallowing dysfunction because of the altered swallowing dynamics of the neopharynx[1]. Differential diagnosis between functional dysphagia, benign strictures and newly developing tumors is essential because of the different therapeutic approach and prognosis. Moreover, late onset dysphagia, usually due to hypopharyngeal or upper esophageal strictures, may occur in up to 58% of patients and can become a devastating complication compromising nutritional status and deteriorating quality of life[2]. These strictures are typically complex with an angulated, irregular and severely narrowed lumen that results from mucosal scarring (healing by secondary intention) after radiation induced mucositis and ischemia[3]. Although they can usually be dilated with bougies, most are difficult to treat and cannot be dilated to an adequate diameter for relief of dysphagia despite repeated sessions or tend to require multiple dilations due to recurrence of dysphagia. Furthermore, deployment of a self-expanding metal or plastic stent is not easy because they are placed very high in the pharynx with the upper flange of the stent at less than 12 cm from the dental arcade. Thereby, nutrition through feeding tubes, nasogastric or percutaneous gastrostomy, is often required.

We developed a modified introducer system for placement of a tracheobronchial self-expanding plastic stent (SEPS) (Polyflex™, Willy Rüsch GmbH/Boston Scientific Corporation, Natick, MA, United States) in patients with refractory and complex hypopharyngeal strictures after surgery and radiotherapy. The purpose was to alleviate dysphagia as well as to allow for hypopharynx conditioning, resulting in a less stenotic lumen. This report describes the technical aspects of the modified introducer system and evaluates the safety and efficacy of this stent in this group of patients.

MATERIALS AND METHODS

Four patients with refractory hypopharyngeal strictures after total laryngectomy followed by radiation therapy for laryngeal cancer were included. Before stent placement, all patients had been submitted to several sessions of dilations with Savary-Gilliard polyvinyl bougies over a spring-tip stainless steel guidewire (Cook Medical) or a 0.038 in × 260 cm Jagwire (Boston Scientific) under fluoroscopic guidance. Because of the rigidity of the stricture, it was not possible to introduce dilators larger than 12.8-15 mm even with sessions performed every 2 to 3 wk. Successive recurrence of the stricture was observed in all patients, with consequent dysphagia to semisolid foods. Each patient signed an informed consent form before the endoscopic procedure.

The tracheobronchial Polyflex™ stent used in this study is a SEPS made of silicone with a polyester mesh.

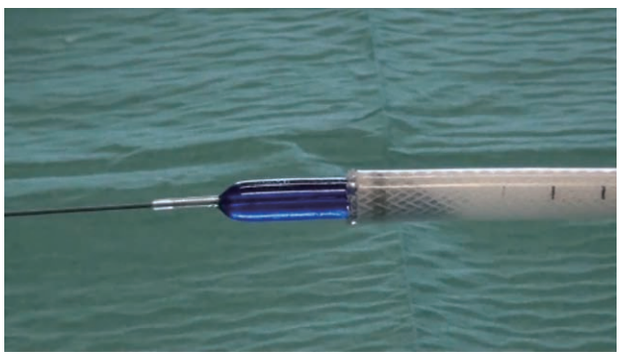

Figure 1 Tracheobronquial Polyflex stent with an esophageal wire-guided balloon inserted through the delivery system with the tip protruding from the introducer by 2 to 3 cm.

While this mesh structure on the outer surface reduces dislocation risk, the silicone coating throughout the stent prevents ingrowth of granulation tissue. The latter, along with the reduction of its cross-section when stretched lengthwise, would have the advantage of allowing easy removal or change of the stent without the usual difficulty associated with self-expanding metal stents. The stent is impregnated with markers at both ends to assist fluoroscopic visualization and endoscopic placement. Since strictures were very narrow and non-compliant and stents would be positioned very high in the hypopharynx, we used a tracheobronchial stent because this was the only available stent of small diameter and without flared ends. However, the Polyflex™ airway stent delivery system, unlike the esophageal one, is somewhat inflexible and stiff and has a blunt edge, making it difficult to transverse the angulation of the oropharynx and to position it through the area of stenosis. To overcome this difficulty we modified the introducer system, cutting both the basket of the stent loader and the opposite end and making the system hollow. An 8-10 mm controlled radial expansion (CRE) balloon dilation catheter (Boston Scientific) without the guidewire was then inserted through the delivery system with the tip protruding from the introducer by 2 to 3 cm and then inflated, resulting in a tapered edge with only a small indentation between the balloon and the delivery tube (Figure 1). The system was then threaded over a 0.035 in × 450 cm Jagwire (Boston Scientific) previously placed under fluoroscopy through the stricture into the esophagus, allowing easy and atraumatic passage and positioning of the stent delivery system (Figure 2). Subsequent stent deployment was performed under simultaneous fluoroscopic guidance and direct vision, with an ultra-thin videoendoscope passed alongside the introducer shaft.

RESULTS

Patient 1

A 71-year-old man with T3N0M0 laryngeal cancer underwent total laryngectomy followed by chemoradiation therapy 2 years previously. Three months after completing therapy, the patient developed dysphagia with solids

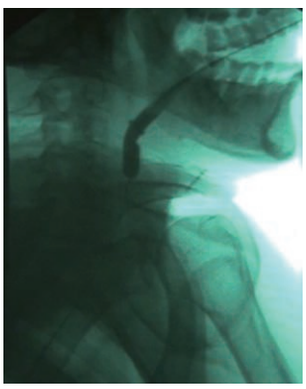

Figure 2 Passage of the stent delivery system through the stricture under fluoroscopic guidance.

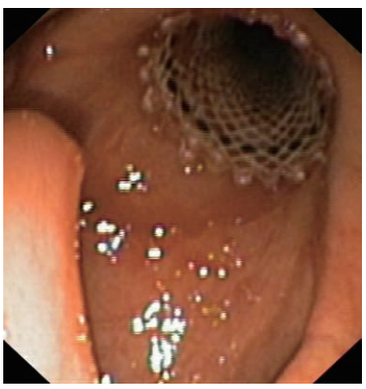

Figure 3 Endoscopic view of the uvula and the stent deployed in the hypopharynx.

which progressively worsened. Upper gastrointestinal endoscopy revealed a 4 cm regular stricture with a 4 mm lumen at 12 cm from the incisors. A percutaneous endoscopic gastrostomy tube was placed. The patient underwent 8 sessions of dilation for 6 mo with little improvement. At the time of tracheobronchial Polyflex™ stent placement, lumen dilation was performed from 7 to 11 mm, and a 14 mm × 6 cm stent was deployed (Figure 3). There was improvement in the dysphagia score and the patient was able to take solid meals until 2 mo later when he complained of recurrent symptoms. Endoscopy showed 1 cm distal stent migration with tissue overgrowth at the proximal end. Repositioning of the stent into the stricture was performed using an alligator jaw grasping forceps, with resolution of symptoms. However, 6 wk later the patient was readmitted with dysphagia for liquids due to complete obstruction of the upper end of the stent with granulomatous tissue. The stent was removed and no other treatment was performed since the gastroscope could easily be introduced into the stomach. Nevertheless, dysphagia relapsed after 1 mo, with recurrence of the stricture, requiring multiple sessions of dilation during the 5 mo follow-up.

Patient 2

A 54-year-old man with T4N1M0 laryngeal cancer underwent total laryngectomy followed by radiation therapy 7 years previously. Progressive dysphagia occurred 4 years later. Endoscopy revealed a 3 cm regular stricture with a 3 mm lumen at 10 cm from the dental arcade. The patient underwent 3 endoscopic dilations over 2 mo to 12.8 mm, with symptom relief. However, dysphagia relapsed 2 years later and endoscopic bougienage was restarted with dilations performed every 3-4 wk for 10 mo. At this time, it was still not possible to introduce a Savary-Gilliard dilator larger than 12.8 mm, and a 14 mm × 4 cm tracheobronchial Polyflex™ stent was successfully placed across the stricture. Two days after the procedure and subsequent to a coughing attack, the patient developed sudden total dysphagia. Endoscopic examination showed proximal stent migration with impaction in the oropharynx. The stent was therefore removed

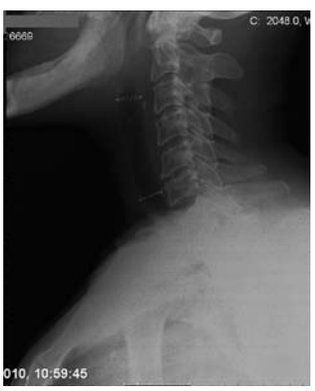

Figure 4 Cervical X-ray to assess adequate positioning and expansion of the stent.

and replaced by a same diameter but 2 cm longer Polyflex™ stent (Figure 4). The patient was able to tolerate a solid diet until 3 mo later when he complained of only being able to swallow liquids. Hyperplastic tissue growth with stricture formation at the upper end of the stent was observed. After dilation with a 13.5 mm CRE balloon catheter, we used a two-channel therapeutic scope and two alligator jaw forceps grasping at opposite sites to remove the plastic stent. At 4 wk post-procedure the patient had relapse of the stricture and additional dilations were required during the 3 mo follow-up to maintain lumen patency and oral feeding.

Patient 3

A 44-year-old man with T3N2M0 laryngeal cancer who had been treated with total laryngectomy followed by chemoradiation therapy developed progressive dysphagia 2 years after completion of the treatment. Endoscopy revealed a 2 cm long stricture with a 3 mm lumen at 13 cm from the incisors, and dilations were carried out monthly (7 sessions). Despite the stricture allowed passage of a 15 mm dilator, there was only brief relief of the dysphagia due to the inability to maintain lumen diameter. In view of the recurrence of the stricture, a 16 mm × 4 cm tracheobronchial Polyflex™ stent was deployed. There was improvement in the dysphagia score and the patient

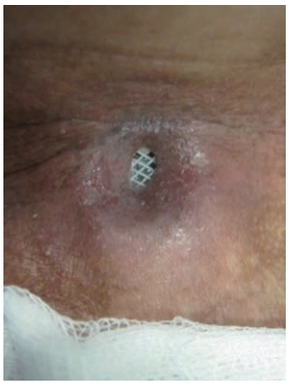

Figure 5 View of the lower end of the stent through the pharyngocutaneous fistula.

was able to take solid meals. In the meantime, cervicothoracic computed tomography demonstrated a large pulmonary mass suspected to be a second primary tumor, and chemotherapy was started. Two months after stent placement, though the patient had no dysphagia, he was readmitted with a pharyngocutaneous fistula with purulent drainage at the level of the lower end of the stent (Figure 5). Intravenous antibiotic therapy was started and the stent removed and replaced 3 d later with a same diameter but 2 cm longer Polyflex™ stent, so that it could occlude the fistula orifice. Although cicatrization was observed within 2 wk, reopening after each course of chemotherapy was necessary. Two months later the patient noted recurrence of dysphagia complicated by the constant leakage of saliva, ingested food and liquids through the fistula. After removal of the stent, a percutaneous endoscopic gastrostomy tube was placed for nutritional support. Even though the patient was given nothing *per os* for 2 wk, this was not successful in closing the fistula and ultimately a partially covered self-expanding metal stent was used to seal the leakage.

Patient 4

A 56-year-old man with a T3N3M0 carcinoma of the supraglottic larynx was treated by neoadjuvant chemotherapy followed by total laryngectomy and radiation therapy. Four months later the patient was referred to our unit with food impaction. Upper gastrointestinal endoscopy showed a tight 3 cm long stricture at 13 cm from the incisors. Four sessions of dilation at 3 wk intervals offered only a slight improvement and so a decision was made to introduce a 14 mm × 6 cm tracheobronchial Polyflex™ stent. However, 24 h after the procedure and despite a normal water-soluble contrast swallow, the patient complained of total dysphagia with pharyngonasal regurgitation. The stent was therefore removed and the patient discharged. One week later, the patient was readmitted with fever, productive cough and a pharyngocutaneous fistula with purulent drainage. The patient was treated with intravenous antibiotics and percutaneous gastrostomy tube placement, with resolution of the respiratory symptoms and spontaneous closure of the fistula track 6 wk later.

DISCUSSION

Hypopharyngeal stricture is a serious complication that may occur after total laryngectomy and radiotherapy. Contributing factors include continued alcohol and tobacco abuse, the dose-volume relationship of the radiotherapy, and normal tissue damage from the tumor and the treatment[4]. Endoscopic dilation is the mainstay of treatment for hypopharyngeal strictures. However, these strictures are usually complex, and even complete lumen obliteration requiring a transgastric endoscopic retrograde approach has been previously reported[5]. When strictures fail to respond to endoscopic dilations, they are defined as refractory. In addition to providing significant physical discomfort, ongoing dilation therapy in these patients carries a risk of perforation and mortality as high as 5% has been reported[6]. Although the use of intralesional steroid injection and electrocautery incisions have been considered for peptic and anastomotic strictures, respectively, no significant benefit has been established for this indication[7,8].

The use of self-expanding stents is another possibility to achieve tissue remodeling needed to promote longer lasting stricture resolution. The rationale is to offer a continuous and stable dilation that might prevent the scarring process and avoid the adhesion of damaged areas. Self-expanding metal stents are currently considered the most effective and widely used method of palliation of malignant dysphagia. However, several limitations preclude their routine use in the management of benign esophageal strictures. Actually, these stents can become embedded into the granulation tissue ingrowth through the mesh of the uncovered part of the stent, resulting in recurrent obstruction and difficult removal. To overcome these disadvantages, a SEPS Polyflex™, designed to induce less tissue ingrowth and to be easily removed, was developed by Boston Scientific Corporation and has been commercially available since 2003. This stent is made of a polyester mesh completely covered by a silicone layer with a smooth inner surface and a structured outer surface. Initial studies on the use of Polyflex™ stents in benign refractory strictures were favorable, with improvement of symptoms and few complications. Repici *et al*[9] reported 15 patients with caustic, post-radiation, anastomotic and peptic strictures. Stent placement was successful in all patients and long-term resolution of dysphagia after 23 mo follow-up was achieved in 80% of patients. Evrard *et al*[10] reported 21 patients, of whom 17 had hyperplastic (after metallic stent placement), caustic, peptic, anastomotic and post-radiation strictures, with 81% of patients remaining dysphagia-free during a mean follow-up of 21 mo. However, less encouraging results have since been reported, with a lower success rate and a high incidence of complications. Overall, for benign esophageal strictures, the success rates of SEPSs in the published literature range from 17% to 95%, with 6% being major complications[11].

In the current literature, there are only two small series evaluating the efficacy and safety of stents in the treat-

ment of benign refractory hypopharyngeal strictures. Conio *et al*[12] developed a modified fully covered self-expanding Niti-S stent with a body diameter of 10 mm, 12 mm or 14 mm and a flared 2 mm wider upper end. Although it effectively improved dysphagia in all patients, six of the seven patients developed complications requiring stent exchange 3 mo after previous stent placement. Due to the stricture recurrence observed after stent removal, prolonged stent placement with periodic stent exchange was necessary. Somani *et al*[13] described four patients who underwent temporary esophageal Polyflex™ stenting for 3 mo. Although three of the four patients remained symptom-free after a median follow-up of 14 mo, they were able to pre-dilate the strictures up to 15 mm to allow the passage of the stiff 13 mm introducer system and the deployment of a 20 mm body with 23 mm flare plastic stent. While the use of such a large stent can explain the good results, that would not be possible in the tight and rigid strictures observed either in the first study or in our group of patients.

In the present study, the modified introducer system for placement of tracheobronchial Polyflex™ stents allowed an easy and technically successful deployment of the stent through the stricture. The use of small diameter and unflared upper end stents can explain the absence of significant pain or a foreign body sensation in the throat. Nevertheless, all patients developed complications as stent migration, a pharyngocutaneous fistula, and hyperplastic tissue growth with stent obstruction. Actually, although migration is likely to occur with this type of stent, they may be easily recovered or repositioned with an alligator jaw forceps. Moreover, the features of these stents reduce the risk of intestinal complications that may occur after migration of larger or flared stents. Fistula formation can also occur spontaneously after chemoradiation for head and neck cancer, or be precipitated by concurrent chemotherapy or infection. However, there is little doubt that in these patients the high radial expansive force and relative inflexibility of the Polyflex™ stent could have played an important role in the development of the pharyngocutaneous fistulas. Hyperplastic tissue growth with fibrous stricture formation at the upper end of the stent was observed in all patients who had their stents *in situ* for more than 8 wk, requiring stent removal. This has been previously reported to occur with the Polyflex™ stent in only a small number of patients in the literature and may be related to the stimulation of the underlying pathologic process by the upper edge of the stent, with ongoing obliterative endarteritis and ischemia after radiation injury[14]. Finally, stricture recurrence with dysphagia was observed in all patients at 4 wk follow-up after stent removal indicating that, in strictures with this etiology, small diameter stents must be kept in place for longer than 3 mo to allow adequate time for stricture remodeling.

In conclusion, these results suggest that Polyflex™ stents have limited efficacy in benign hypopharyngeal stricture resolution. New smaller diameter stents are needed to manage these narrow and non-compliant refractory strictures and to provide an alternative approach to continued serial dilation and feeding tube nutrition.

COMMENTS

Background

The management of patients who have benign refractory hypopharyngeal strictures after surgery combined with chemoradiation therapy is often unsatisfactory and disappointing. Repeated dilation therapy or long-term feeding through a nasogastric tube or percutaneous gastrostomy is frequently required.

Research frontiers

The authors aimed to evaluate the efficacy and safety of a tracheobronchial self-expanding plastic stent (SEPS) (Polyflex™) placed with modified technique in the treatment of refractory hypopharyngeal strictures after combined therapy for laryngeal cancer.

Innovations and breakthroughs

This case series has shown the technical feasibility of tracheobronchial Polyflex stent insertion for hypopharyngeal strictures. The modification of the stiff delivery system, with insertion of a balloon dilation catheter through the introducer resulting in a wire-guided tapered edge, allowed an easy and atraumatic transverse of the oropharynx and stricture. However, the results suggest that these stents have limited efficacy in stricture remodeling and have a high incidence of side effects.

Applications

Placement of SEPSs is associated with early hyperplastic tissue growth requiring short term stent replacement. New smaller diameter stents are needed to manage these narrow and refractory hypopharyngeal strictures.

Peer review

Although the use of temporary SEPS is a well recognized option for treatment of refractory stenosis of gastrointestinal tract, both benign or malignant, this report is interesting because it describes a personal modification of the introducer that should be effectively used for some more complex strictures.

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S- Editor Tian L **L- Editor** Cant MR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i6.557

World J Gastroenterol 2012 February 14; 18(6): 557-562
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Leptin and peroxisome proliferator-activated receptor γ expression in colorectal adenoma

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Supported by Grant from Inje University College of Medicine (2010)
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Received: April 28, 2011 Revised: September 7, 2011
Accepted: September 14, 2011
Published online: February 14, 2012

Abstract

AIM: To investigate the expressions of leptin and peroxisome proliferator-activated receptor γ (PPARG) in relation to body mass index (BMI).

METHODS: We evaluated leptin and PPARG expression in 30 adenomas over 1 cm in size by immunohistochemical staining. In addition, clinicopathologic features including BMI were assessed.

RESULTS: PPARG and leptin expression showed a strong positive correlation (P = 0.035). The average BMI of the leptin-positive group was higher than that of the leptin-negative group (25.4 ± 3.4 kg/m^2 *vs* 22.6 ± 2.4 kg/m^2, P = 0.018), and leptin expression was significantly correlated with high BMI (P = 0.024). Leptin expression was more frequently observed in intermediate/high grade dysplasia than in low grade dysplasia (P = 0.030). However, PPARG expression was not correlated with BMI and grade of dysplasia.

CONCLUSION: BMI has influenced on the leptin expression of colorectal adenoma. The exact mechanism underlies the strong correlation between leptin and PPARG expression needs further study.

Key words: Leptin; Peroxisome proliferator-activated receptor γ; Obesity; Body mass index; Colorectal adenoma

Peer reviewer: Benjamin Perakath, Professor, Department of Surgery Unit 5, Christian Medical College, Vellore 632004, Tamil Nadu, India

Kim HH, Kim YS, Kang YK, Moon JS. Leptin and peroxisome proliferator-activated receptor γ expression in colorectal adenoma. *World J Gastroenterol* 2012; 18(6): 557-562 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i6/557.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i6.557

INTRODUCTION

Colorectal cancer is a major cause of cancer-related mortality and morbidity[1]. Many Asian countries, including South Korea, China, Japan, and Singapore, have experienced an increase of 2 to 4 times in the incidence of colorectal cancer during the past few decades[2]. Obesity is a risk factor of colorectal cancer and colorectal adenoma, and is an independent poor prognostic variable in colorectal cancer survivors[3-5]. Although mechanism by which obesity increases the risk of colorectal cancer is not clearly understood, obesity-induced changes in hormonal metabolism are known to distort the normal balance between cell proliferation, differentiation, and apop-

tosis[3]. Circulating levels of insulin, insulin-like growth factor, and leptin are increased in obesity and may mediate the increased risk of colorectal cancer[6,7]. Leptin plays a central role in mammalian feeding behavior and energy expenditure[8]. In addition to its neurohormonal action in the brain, several *in vitro* studies have shown that leptin can act as a mitogenic, antiapoptotic, and tumorigenic factor for different cancer cell lines[9,10]. Moreover, leptin is overexpressed in human colorectal cancer[11], and its expression gradually increases during the transition from normal adenoma to adenocarcinoma, suggesting that it is associated with colorectal carcinogenesis[12]. Interestingly, however, high leptin expression in colorectal cancer is an indicator of favorable tumor features and better survival in colorectal cancer patients[12]. One interesting fact revealed in our previous study was that there was a linear trend between leptin expression of colorectal adenomas and patients' body mass index (BMI)[13]. This suggests that obesity postulated from high BMI can affect tumorigenesis through local leptin activity.

Peroxisome proliferator-activated receptor γ (PPARG) is a member of the nuclear hormone receptor PPAR superfamily[14]. PPARG plays an important role in adipose cell differentiation, modulation of metabolism, modulation of the inflammatory response, and cellular apoptosis[15,16]. Unlike leptin, PPARG is involved in cell cycle regulation and cellular differentiation in colonic epithelium, supporting its antineoplastic effect[15,17,18]. Therefore, some researchers have tried to use PPARG agonists as chemotherapeutic agents for malignancy[19]. Moreover, the overexpression of PPARG in colorectal cancer is independently correlated with longer survival of patients, so PPARG expression appears to mark an indolent subset of colorectal cancers as dose leptin expression[20].

Despite the antagonistic effects on the development of neoplasm, the expressions of PPARG and leptin are related with favorable prognosis respectively[12,20]. This phenomenon led us consider the possible interaction of PPARG and leptin in the development of colon cancer and adenoma. Colorectal adenoma is the well known premalignant lesion of colorectal cancer, and remove of it is accepted as a most effective prevention of colorectal cancer. However, there are only few studies regarding leptin and PPARG expression in colorectal adenoma[12]. Moreover, there has been no report about the correlation between leptin expression and PPARG expression. Therefore, we aimed to investigate leptin and PPARG expression in colorectal adenomas in relation to BMI. We also investigated whether the detailed characteristics of adenomas such as dysplasia grade, number, and location, are correlated with leptin or PPARG expression. Finally, we aimed to establish whether the expression of leptin is correlated with that of PPARG in colorectal adenomas.

MATERIALS AND METHODS

Patients and specimens

Thirty patients with colorectal adenomas of over 1 cm in size were enrolled in this study. All patients, less than 55 years old, were diagnosed and treated by polypectomy or endoscopic mucosal resection at the Seoul Paik Hospital (Seoul, South Korea) between August 2007 and August 2008. We excluded adenomas, which had carcinomatous components. All tissue samples were formalin fixed and paraffin embedded. Hematoxylin and eosin (HE) slides, pathologic reports, and medical records were retrospectively reviewed to confirm the diagnosis and clinicopathologic parameters including age, gender, BMI, adenoma location, adenoma size, pathologic type, and dysplasia grade. We followed the guidelines for human studies and animal welfare regulations. Subjects had given their informed consent, and the study protocol had been approved by the institutional review board on human research.

Tissue microarray construction

The most representative area of each adenoma was carefully selected and marked on a HE stained slide. Core tissue biopsies (2 mm in diameter) were taken from the corresponding donor blocks and arranged in a new recipient paraffin block (tissue array block) using a trephine apparatus (SuperBioChips Laboratories, Seoul, South Korea). Duplicate tissue cores were taken from each donor block to minimize the limitations of identifying a representative area of the tumor.

Immunohistochemical staining

Serial 4-μm thick sections of tissue array blocks were examined immunohistochemically. Sections were deparaffinized, and antigen retrieval was performed in 10 mmol/L sodium citrate buffer (pH 6.0) for 15 min at 95 ℃ using a microwave oven. Endogenous peroxidase was blocked for 10 min with 30 mL/L H_2O_2 and slides were labeled with a mouse monoclonal antibody to PPARG (E-8, 1/50 dilutions; Santa Cruz Biotechnology, Santa Cruz, CA, United States) or a rabbit polyclonal antibody to leptin (A-20, 1/100 dilution; Santa Cruz Biotechnology) for 1 h. After washing with phosphate-buffered saline, a chromogen reaction was carried out using an Ultravision LP kit (Labvision, Fremont, CA, United States). Briefly, sections were incubated with primary antibody enhancer for 20 min and horseradish peroxidase for 30 min. 3, 3'-diaminobenzidine tetrahydrochloride was used as a chromogen and Mayer's hematoxylin counterstain was applied. Negative controls without primary antibody were run simultaneously. The expressions of both proteins were evaluated based on the intensity and extent of the staining. Staining intensity was scored as 0 (negative), 1 (weak), or 2 (strong). Extent of staining was scored as 0 (0%), 1 (1%-20%), 2 (20%-50%), or 3 (50%-100%). PPARG positivity was defined as the presence of weak nuclear staining in at least 20% of adenoma cells, or the presence of strong nuclear staining (Figure 1A and B). Leptin positivity was defined as the presence of weak cytoplasmic staining in at least 20% of adenoma cells, or the presence of strong cytoplasmic staining (Figure 1C and D).

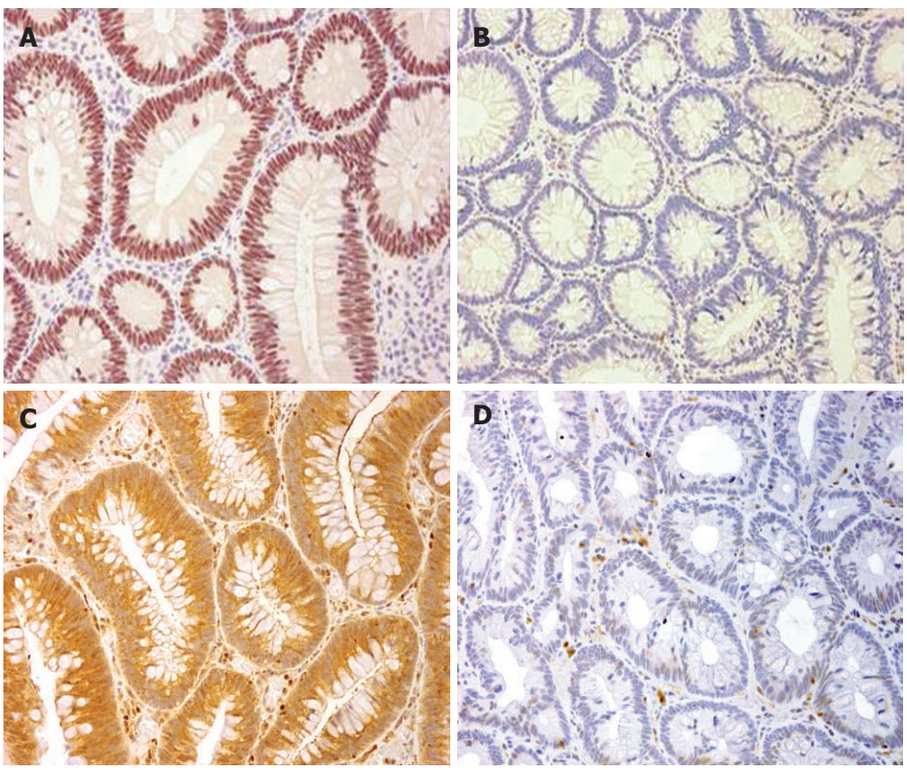

Figure 1 Immunohistochemical detection of peroxisome-proliferator-activated receptor γ and leptin expression in colorectal adenomas. A and B: Strong nuclear expression of peroxisome-proliferator-activated receptor γ (PPARG) (A) and negative expression (B) in representative colorectal adenomas; C and D: Strong cytoplasmic expression of leptin (C) and negative expression (D) in representative colorectal adenomas. Original magnification:×200 (A-D).

Table 1 Baseline characteristics of subjects

Baseline characteristics	Value
Total patients	30
Sex (male) *n* (%)	23 (77)
Age, year	44.6 ± 6.5
BMI (kg/m^2)	24.2 ± 3.83
Polyp size (cm)	1.2 ± 0.3
Polyp number	2.2 ± 1.4
Location[1] *n* (%)	
Left colon	17 (56.7)
Right colon	13 (43.3)
Dysplasia grade *n* (%)	
Low	13 (43.3)
Intermediate/high	17 (56.7)
Pathology *n* (%)	
Tubular	25 (83.0)
Villotubular	5 (17.0)

[1]Left colon consists of transverse, descending and sigmoid colon and rectum, and right colon is composed of cecum and ascending colon. BMI: Body mass index.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software (version 12.0, SPSS, Chicago, IL, United States). The relationship between PPARG and leptin was evaluated using the Fisher's exact test. The relationships of PPARG or leptin with clinicopathologic features were investigated by the Fisher's exact test and *t* test. Two tailed *P* values less than 0.05 were regarded as statistically significant.

RESULTS

The study sample included 23 male and 7 female patients. Patient age ranged from 29 to 53 years (44.6 ± 6.5 years). Tumors consisted of 25 tubular adenomas and 5 villotubular adenomas. Seventeen adenomas located in the left colon: transverse, descending and sigmoid colon and rectum, and 13 adenomas located in the right colon: cecum and ascending colon. The sizes of adenomas ranged from 1 to 2.5 cm (1.2 ± 0.3 cm). The number of polyps ranged from 1 to 5 (2.2 ± 1.4). Thirteen adenomas showed low grade dysplasia. Seventeen adenomas had intermediate grade dysplasia or high grade dysplasia. There were no polyps containing carcinoma-*in-situ*. These results were described in Table 1.

In colorectal adenomas, 22 of 30 cases (73.3%) showed PPARG expression, and 17 cases (56.7%) showed leptin expression (Table 2; representative photomicrographs in Figure 1). PPARG and leptin expression showed significant correlation each other ($P = 0.035$).

The BMI of the leptin positive group (25.4 ± 3.4 kg/m^2) was significantly higher than that of the leptin negative group (22.6 ± 2.4 kg/m^2, $P = 0.018$, Figure 2A), and leptin expression was significantly correlated with high BMI (≥ 25 kg/m^2, $P = 0.024$, Table 2). In contrast, PPARG expression was not correlated with BMI ($P = 0.162$, Figure 2B).

Leptin expression was observed more frequently in the intermediate/high grade dysplasia group than in the low ($P = 0.030$, Table 2). However, PPARG expression was not

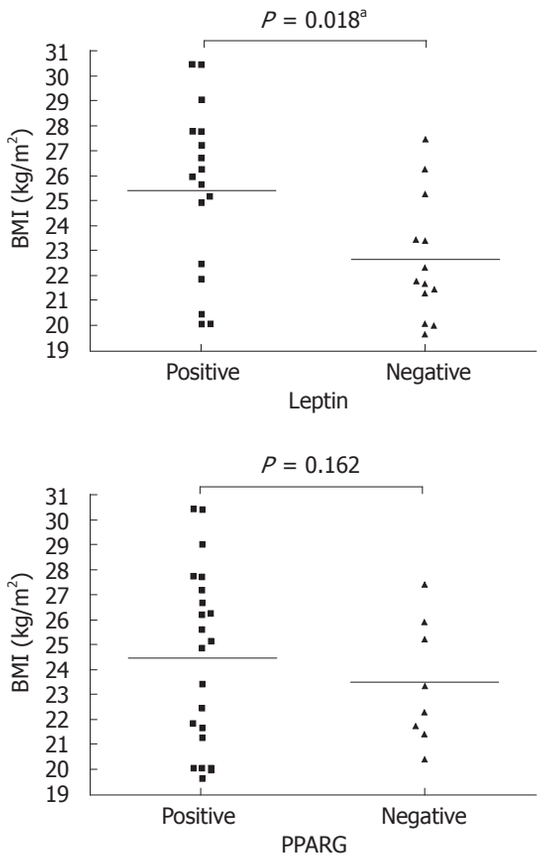

Figure 2 Leptin and peroxisome-proliferator-activated receptor γ expression in colorectal adenomas and body mass index. A: The body mass index (BMI) of leptin positive group (25.4 ± 3.4 kg/m^2) was significantly higher than the BMI of leptin negative group (22.6 ± 2.4 kg/m^2, P = 0.018); B: There is no significant difference between the BMI of peroxisome-proliferator-activated receptor γ (PPARG) positive group (24.47 ± 0.75 kg/m^2) and PPARG negative group (23.52 ± 0.87 kg/m^2, P = 0.162).

correlated with dysplasia grade (P = 0.295, Table 2). Neither leptin nor PPARG expression were correlated with the location or pathologic types of adenomas (Table 2).

Table 2 Leptin expression in colorectal adenomas and clinicopathologic characteristics of patients *n* (%)

Clinicopathologic features	Leptin expression			PPARG expression		
	Positive	Negative	*P* value	Positive	Negative	*P* value
	17 (56.7)	13 (43.3)		22 (73.3)	8 (26.7)	
BMI						
⩾ 25 kg/m^2	11 (36.7)	3 (5.0)	0.024	11 (36.7)	3 (9.9)	0.689
< 25 kg/m^2	6 (20.0)	10 (33.3)		11 (36.7)	5 (16.7)	
Dysplasia grade						
Low	5 (16.7)	9 (30.0)	0.03	9 (29.7)	5 (16.7)	0.295
Intermediate/high	12 (40.0)	4 (13.3)		13 (43.7)	3 (9.9)	
Location[1]						
Left colon	11 (36.7)	6 (20.0)	0.794	8 (26.7)	3 (9.9)	0.954
Right colon	6 (20.0)	4 (13.3)		14 (46.7)	5 (16.7)	
Histological type						
Tubular	14 (46.7)	11 (36.7)	0.869	12 (40.0)	13 (43.4)	0.743
Villotubular	3 (10.0)	2 (6.6)		2 (6.7)	3 (9.9)	

[1]Left colon consists of transverse, descending and sigmoid colon and rectum, and right colon is composed of cecum and ascending colon. BMI: Body mass index; PPARG: Peroxisome proliferator-activated receptor γ.

DISCUSSION

Our study revealed a strong correlation between leptin expression in colorectal adenomas and high BMI. Although BMI is just one measure of obesity, the strong correlation between high BMI and leptin expression nonetheless suggests that obesity may influence leptin expression in adenomas. In our previous study, we found a trend between leptin expression and BMI[13]. However, when we enrolled colorectal adenoma lager than 1 cm size in this study, we could observe the strong correlation between high BMI and leptin expression in colorectal adenomas.

Several studies of leptin expression in cancers have suggested that local rather than endocrine leptin might play a significant role in breast tumorigenesis[21]. More specifically, both leptin and leptin receptor were found in breast tumors, indicating that leptin could influence cancer cells through autocrine or paracrine mechanisms[21-23]. However, given the induction of leptin overexpression by obesity-related stimuli, it is reasonable to also consider systemic effects on leptin expression[23].

Several studies have suggested that leptin expression is related to local hypoxia. Indeed, in several cellular systems, including breast cancer cells, leptin mRNA expression is induced by hypoxia, and the leptin gene promoter is regulated by hypoxia-inducible factor-1α[23-25]. However, we would argue that high BMI is a more likely potential marker for leptin expression than hypoxia in colorectal adenomas, for the following 2 reasons. First, previous studies regarding leptin expression and hypoxia dealt with cancers rather than adenomas. It is reasonable to accept that leptin is induced under the effect of hypoxia in cancers due to their large mass, but colorectal adenomas are much smaller than typical cancers. Furthermore, PPARG expression was strongly correlated with leptin expression in our study. PPARG levels are known to be reduced by hypoxia[26], so the strong correlation between leptin and PPARG further suggests that leptin expression is probably not caused by hypoxia in colorectal adenomas.

When we divided adenomas into low and intermediate/high dysplasia grade groups, leptin was expressed more frequently in the intermediate/high grade dysplasia group. Interestingly, the ratio of overexpressed leptin was gradually increased from normal mucosa, to adenoma, to carcinoma[11,12]. Therefore, increased leptin expression ratio from low grade to intermediate/high grade dysplasia appears to follow the pattern of increased leptin expression from normal mucosa to adenoma, to carcinoma by stages. Moreover, this may connote that as dysplasia progresses, autocrine or paracrine mechanisms of leptin may thus be progressively enhanced in colorectal adenomas.

In our study, PPARG expression was positively correlated with leptin expression in colorectal adenomas, but was not correlated with high BMI or adenoma dysplasia grade. PPARG and leptin functionally intersect *via* the Ja-

nus-activated kinase/signal transducers and activators of transcription (JAK/STAT) pathway. Leptin signaling is transmitted mainly by the JAK/STAT pathway[27] and terminated by the induction of suppressor of cytokine signaling-3[28]. In contrast to leptin, PPARG agonists inhibit cytokine-induced activation of the JAK-STAT pathway[29] and STAT-3[30]. These findings show that leptin and PPARG clearly affect the JAK/STAT pathway in different ways and possibly interact each other by unrevealed feedback mechanisms[31]. Regarding 2 molecules, several recent studies revealed that leptin might exert an inhibitory effect on PPARG protein expression. Zhou *et al*[32] suggested leptin induced extracellular signal-regulated kinases (ERK) 1/2 activation, which cause the subsequent decline in PPARG expression in hepatic stellate cells. In addition, leptin could inhibit the PPARG expression in TallyHo/Jng mouse[33]. On the other hands, PPARG ligands has the inhibitory effect on leptin induced hepatic stellate cells proliferation through the reversion of ERK 1/2 activation[33,34]. However, molecules may interact *via* different mechanism or interact with each other differently depend on cells or organs. Therefore, to clarify the exact mechanism between leptin and PPARG in colorectal adenomas, we think that additional in vitro studies are needed.

There were several limitations in our study. First of all, enrolled numbers in this study was relatively small. We wanted exclude the age factor which strongly influence on development of colorectal adenoma and carcinoma. Indeed, the prevalence of colorectal adenoma in Korea revealed that most colorectal adenomas were detected in aged group[35]. To determine the pure effect of obesity on leptin and PPARG expression in colorectal adenomas, we enrolled the patients who were under age of 55. However, information from 30 cases was enough to generate appropriate statistical values. Second, limitation was that we were able to investigate the only one indicator of obesity, BMI. Other variables accessing obesity such as wait circumference and waist-hip ratio were not investigated due to lack of information. Third, we did not investigate a control group. If we had evaluated leptin and PPARG expression in hyperplastic polyps or normal mucosa, we would establish specific criteria for the overexpression of 2 molecules in the present study. Fourth, there was no in vitro study about the interaction of PPARG and leptin. These two molecules may affect the JAK/STAT or ERK 1/2 pathways in different ways and possibly interact each other by unrevealed mechanisms. In this study, however, we did not evaluate the mechanism regarding the relationship of leptin and PPARG expression in the development of colorectal adenoma. Our study suggested the correlation between PPRAG and leptin expression but could not explain the causal relationship. For understanding the causal relationship, in vitro study should be performed in the near future.

In summary, our study is the first to evaluate simultaneous leptin and PPARG expression in colorectal adenomas in relation to BMI and characteristics of adenomas. Based on the strong correlation between leptin expression and high BMI, we postulated that energy accumulation might induce overproduction of leptin in colorectal adenomas. The known positive correlation between leptin expression and dysplasia grade further strengthens the possibility that leptin might contribute to the sequential progression of carcinogenesis from low grade dysplasia to colorectal carcinoma and through high grade dysplasia[11,12]. In contrast, PPARG expression showed no correlation with high BMI or dysplasia grade in colorectal adenomas. The correlation between leptin and PPARG expression suggests that a possible interaction between these 2 molecules during the development of adenomas, likely mediated by the JAK/STAT or ERK 1/2 pathway.

COMMENTS

Background

Colorectal cancer is a major cause of cancer-related mortality and morbidity. Leptin regarded to be associated with colorectal carcinogenesis. Unlike leptin, peroxisome-proliferator-activated receptor γ (PPARG) is involved in antineoplastic effect. Despite the antagonistic effects on the development of neoplasm, the expressions of PPARG and leptin are related with favorable prognosis respectively. This phenomenon suggests that the possible interaction of PPARG and leptin in the development of colon cancer and adenoma.

Research frontiers

Mechanism of leptin expression was one imperative issue in the research field related to the article. Obesity, expressed as body mass index (BMI), can be related with leptin expression in colorectal adenomas. Second issue was the relationship between PPARG expression and leptin expression in colorectal adenomas because these two molecules behave antagonistically.

Innovations and breakthroughs

The study is the first to evaluate simultaneous leptin and PPARG expression in colorectal adenomas in relation to BMI and characteristics of adenomas. Based on the strong correlation between leptin expression and high BMI, we postulated that energy accumulation might induce overproduction of leptin in colorectal adenomas. The correlation between leptin and PPARG expression suggests a possible interaction between these 2 molecules during the development of adenomas, likely mediated by the janus-activated kinase / signal transducers and activators of transcription or extracellular signal-regulated kinase 1/2 pathway.

Applications

Present study showed the first step to widen our understating about the interaction of PPARG and leptin in the development of colorectal adenomas. New findings about this molecular interaction can be applicable in the prevention of colorectal adenomas, premalignant lesion of colorectal cancer, and can provide pivotal hint for chemotherapy for colorectal cancer.

Peer review

The paper demonstrated interesting findings. Strong correlation between leptin and PPARG expression in colorectal adenomas, correlation between leptin expression and high BMI, and Leptin expression was more frequently observed in intermediate/high grade dysplasia than in low grade dysplasia, while PPARG expression was not correlated with BMI or grade of dysplasia.

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S- Editor Gou SX **L- Editor** O'Neill M **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i6.563

World J Gastroenterol 2012 February 14; 18(6): 563-569
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

ABO blood type, diabetes and risk of gastrointestinal cancer in northern China

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Supported by The National Basic Research Program, 973 Program No. 2010CB912802 and No. 2006CB910700; the China National Science Foundation, No. 81071953, No. 30890033 and No. 30620120433; the Chinese Academy of Sciences No. KSCX1-YW-R-40; and the 44 Postdoctoral Fund of China, No. 20080441314
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Received: June 18, 2010 Revised: March 14, 2011
Accepted: March 21, 2011
Published online: February 14, 2012

Abstract

AIM: To explore the potential risk factors related to gastrointestinal cancer in northern China.

METHODS: A total of 3314 cases of gastrointestinal cancer (esophageal, gastric, pancreatic and biliary) and 2223 controls (including healthy individuals, glioma and thyroid cancer) were analyzed by case-control study. Multivariable logistic regression analysis was applied to evaluate the association between different cancers and hepatitis B surface antigen, sex, age, blood type, diabetes, or family history of cancer.

RESULTS: Type 2 diabetes was significantly associated with gastric, biliary and pancreatic cancer with an OR of 2.0-3.0. Blood type B was significantly associated with esophageal cancer [odd ratio (OR) = 1.53, 95% confidence interval (CI) = 1.10-2.14] and biliary cancer (OR = 1.49, 95% CI = 1.09-2.05). The prevalence of type 2 diabetes was significantly higher in gastric, biliary and pancreatic cancers compared with other groups, with ORs ranging between 2.0 and 3.0. Family history of cancer was strongly associated with gastrointestinal compared with other cancers.

CONCLUSION: Blood type B individuals are susceptible to esophageal and biliary cancer. Type 2 diabetes is significantly associated with gastric, biliary and especially pancreatic cancer.

Key words: Blood type; Type 2 diabetes; Gastric cancer; Esophageal cancer; Pancreatic cancer

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Gong Y, Yang YS, Zhang XM, Su M, Wang J, Han JD, Guo MZ. ABO blood type, diabetes and risk of gastrointestinal cancer in northern China. *World J Gastroenterol* 2012; 18(6): 563-569 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i6/563.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i6.563

INTRODUCTION

The incidence and mortality of gastrointestinal cancers (e.g., esophagus, stomach, pancreas, and biliary tract) are higher in China compared with Western countries, but the reason remains unclear.

In contrast to most Western countries, esophageal squamous cell cancer is common in many areas of China[1].

Gastric cancer ranks as the third most common cancer in China for both men and women, with 5-year survival rate < 20%[2]. Pancreatic cancer is the sixth leading cause of death among malignant diseases in China, with an overall cumulative 5-year survival rate of only 1%-3%[3].

A recent seroepidemiological study conducted in mainland China showed that hepatitis B surface antigen (HBsAg) prevalence was 7.2% in the general population and 1.1% in children under 5 years of age[4]. Hepatitis B virus (HBV) is considered to be hepatotropic and is significantly associated with end-stage chronic liver diseases, including hepatocellular carcinoma (HCC) and cholangiocarcinoma[5]. HBV may travel through the bloodstream and deposit in other organs[6]. The possible relationship between HBV and pancreatic or bile duct cancer has been reported[7,8], but the inverse relationship has been reported by others[9].

The most common type of diabetes mellitus, type 2, seems to be associated with cancers of the biliary tract and pancreas. It is significant that the greatest risk of cancer in diabetic patients is to the organs in which concentrations of endogenous insulin reach particularly high levels (i.e., liver and pancreas)[10]. Meta-analyses have indicated that diabetes mellitus is associated with a 1.7-fold increased risk of pancreatic cancer and a 2.5-fold increased risk of HCC[11].

Studies conducted several decades ago have suggested a link between inherited human blood group antigens and the risk of various malignancies[12]. Human blood antigens are glycoproteins expressed on the surface of red blood cells and a few other cell types, including cells from the gastrointestinal tract. The sugar residues of these glycoproteins are attached to a protein backbone, the H antigen, by a glycosyltransferase that is encoded by the *ABO* gene[13]. Alterations of surface glycoconjugates may lead to modifications and could be related to tumor development and spread.

Recently, Wolpin *et al*[14] have found that ABO blood type is significantly associated with the risk of pancreatic cancer. Hassan *et al*[7] have reported a higher risk of pancreatic cancer in HBV carriers.

Although it seems reasonable that ABO blood type, HBsAg, and type 2 diabetes may have a close relationship with gastrointestinal tract tumors in Western countries, to the best of our knowledge, no previous studies have been conducted to investigate the possible association between these factors and the risk of gastrointestinal cancers in China. Therefore, we embarked on the present large case-control study to evaluate whether ABO blood type, HBsAg, sex, age, type 2 diabetes, and family history of cancer are associated with gastrointestinal cancer in northern China.

MATERIALS AND METHODS

Study population

This study is a retrospective hospital-based, case-control investigation conducted in Beijing, China, at the Chinese PLA General Hospital. A total of 3314 cases and 2223 controls were recruited from January 2004 to November 2008. Cases were patients with newly diagnosed gastrointestinal tract tumors, who were evaluated and treated at the Chinese PLA General Hospital. The population mainly came from northern China and the majority was Han people. The inclusion criteria for cases were as follows: pathologically confirmed diagnosis of gastrointestinal tract cancer; laboratory data available for ABO blood type, HBsAg, and diabetes screening; and detailed record of disease course and history. The exclusion criteria were the presence of other types of digestive disease (such as neuroendocrine tumors, adenomas, cysts or unknown primary tumors) and the absence of laboratory data on blood types, HBsAg and plasma glucose.

Controls were healthy cohorts (undergoing physical examination in Chinese PLA Hospital, mainly from northern China) or patients with other cancers (such as glioma and thyroid cancer). The inclusion criteria for controls were the same as those for cases, except for the cancer diagnosis. The cases and controls were selected at the same period, with integrated laboratory data on blood types, HBsAg and plasma glucose to reduce the bias inherent in retrospective studies. The research proposal was approved by the hospital institutional review board and ethics committee.

Sampling and subject recruitment

The fasting blood samples were collected from patients and controls. Plasma samples were separated and tested for the presence of HBsAg using a third-generation ELISA or chemiluminescence assay, venous glucose by enzyme methods, and blood type by immune assay. The laboratory researcher running these assays was blinded to the disease status (cases or controls) of the subjects' blood samples. Patients whose fasting glucose was > 7.8 mmol/L at least twice were diagnosed as having diabetes. The blood type was ABO. Family history of cancer was defined as any of the first-degree relatives (parents, brother or sister, children) with a tumor history.

Sample size and statistical methods

Cases were comprised of a total of 855 esophageal, 824 gastric, 809 pancreatic and 826 biliary cancer patients. Controls included 674 glioma and 798 thyroid cancer patients, as well as 751 healthy controls. Stata software version 10 (http://www.stata.com/stata10/) was used for data management and statistical analysis. We compared the proportions of potential risk factors among cases and controls. Student's *t* test was used to compare mean age between cases and controls. The χ^2 test was used to compare proportions. We performed multivariable unconditional logistic regression analyses using all variables significant at $P < 0.05$ in the single factor analyses. For each factor, we calculated the adjusted odds ratio and 95% CI using maximum likelihood estimation.

RESULTS

Association of sex, age, family history of cancer, HBsAg and diabetes with gastrointestinal cancer

To determine whether sex, age, family history of cancer,

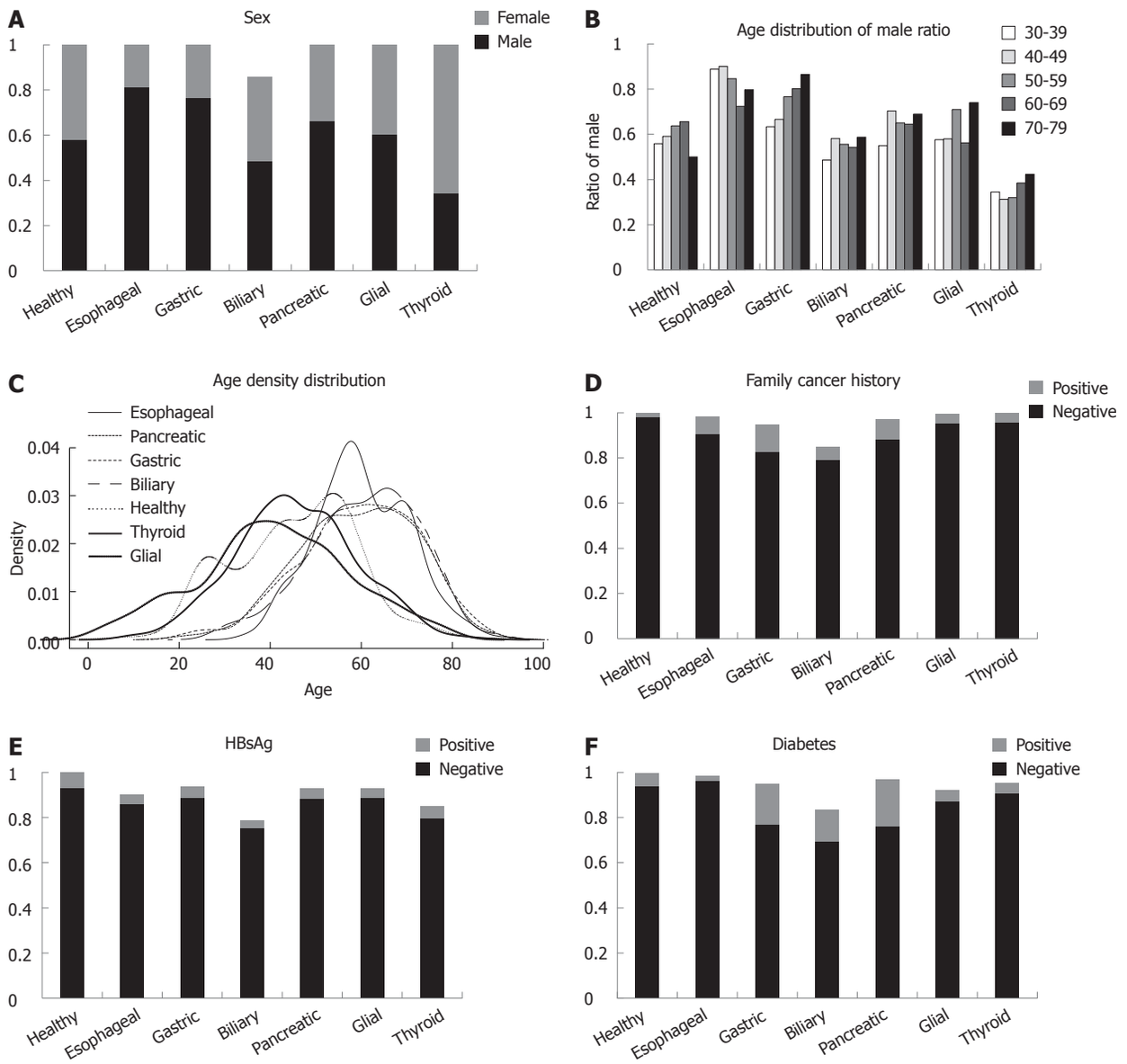

Figure 1 Distributions of clinical traits among different patient groups. A: Sex distribution in different patient groups. Blank areas in this figure represent missing values. Y axis represents sex composition. Grey color represents female individuals, black color represents male individuals; B: Proportion of male patients in different age groups with various cancers, and in healthy controls. Different colors represent different age groups. Y axis represents male ratio; C: Age distribution of different patient groups; D: Frequency of family cancer history (positive or negative) among different population groups; E: Proportion of population with HBsAg among different population groups. Grey color represents HBsAg-positive groups. Black color represents HBsAg-negative groups; F: Proportion of patients with diabetes among different population groups. Grey color represents diabetes-positive groups. Black color represents diabetes-negative groups.

Table 1 Number of cancer patients and healthy individuals in this study

Population	Number
Healthy controls	751
Esophageal cancer	855
Gastric cancer	824
Biliary cancer	826
Pancreatic cancer	809
Glioma	674
Thyroid cancer	798

HBsAg and diabetes were associated with gastrointestinal cancer, we evaluated the distribution of these clinical traits among the healthy controls, and patients with digestive system and other system cancers (Figure 1A), and calculated the OR to measure the association of each trait with gastrointestinal or other cancers, compared with healthy controls (Table 1), using multivariable logistic regression (Table 2).

Sex: The esophageal and gastric cancer groups were dominated by male patients, whereas female patients comprised the majority in the thyroid cancer group (Figure 1). Therefore, thyroid cancer showed a protective effect of male sex (OR = 0.37, 95% CI = 0.30-0.47, Table 2), whereas all other cancers were positively associated with male sex compared with healthy controls (Table 2).

Table 2 Associations between different risk factors and cancer types

Cancer type	Sex		Age		Family history		HBsAg		Diabetes	
	OR[1]	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Esophageal	3.22	[2.42–4.28]	1.12	[1.10–1.13]	6.20	[3.03–12.68]	0.66	[0.39–1.11]	0.29	[0.15–0.54]
Gastric	1.80	[1.38–2.35]	1.08	[1.07–1.09]	9.61	[5.07–18.22]	0.72	[0.43–1.19]	2.07	[1.40–3.08]
Biliary	0.86	[0.67–1.10]	1.10	[1.09–1.11]	5.27	[2.58–10.75]	0.94	[0.56–1.58]	2.13	[1.43–3.17]
Pancreatic	1.21	[0.94–1.55]	1.08	[1.07–1.09]	6.18	[3.22–11.86]	0.72	[0.43–1.19]	2.87	[1.97–4.19]
Glioma	1.17	[0.93–1.47]	0.98	[0.97–0.99]	2.83	[1.42–5.64]	0.68	[0.42–1.09]	1.06	[0.65–1.72]
Thyroid	0.37	[0.30–0.47]	1.00	[1.00–1.01]	2.46	[1.24–4.88]	0.95	[0.61–1.47]	0.89	[0.54–1.45]

[1]OR > 1 means males are more susceptible than females to cancers. Odd ratios (ORs) and respective 95% confidence intervals (CIs) were calculated using multivariable logistic regression. For each cancer, regression analysis was performed *vs* healthy controls. Factors including HBsAg and diabetes, as well as sex, age and family history of cancer were considered.

Table 3 Association between blood types and cancer types

Cancer type	Control	Type A		Type B		Type AB		Type O	
		OR[1]	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Esophageal	Type O	0.99	[0.70–1.40]	1.53	[1.10–2.14]	1.25	[0.76–2.08]	–	–
	All others	0.80	[0.60–1.05]	1.49	[1.12–1.97]	1.11	[0.71–1.74]	0.80	[0.60–1.07]
Gastric	Type O	0.99	[0.71–1.37]	1.03	[0.74–1.46]	1.28	[0.78–2.10]	–	–
	All others	0.93	[0.71–1.23]	1.03	[0.78–1.37]	1.24	[0.79–1.93]	0.96	[0.73–1.26]
Biliary	Type O	0.78	[0.56–1.08]	1.49	[1.09–2.05]	0.95	[0.57–1.57]	–	–
	All others	0.65	[0.49–0.86]	1.66	[1.27–2.17]	0.91	[0.58–1.43]	0.92	[0.70–1.21]
Pancreatic	Type O	1.05	[0.76–1.44]	1.26	[0.91–1.75]	1.44	[0.89–2.31]	–	–
	All others	0.88	[0.67–1.15]	1.20	[0.92–1.57]	1.28	[0.84–1.95]	0.86	[0.65–1.12]
Glioma	Type O	0.83	[0.62–1.12]	1.15	[0.86–1.54]	1.22	[0.81–1.84]	–	–
	All others	0.77	[0.60–0.98]	1.21	[0.95–1.56]	1.21	[0.84–1.76]	0.99	[0.78–1.26]
Thyroid	Type O	0.74	[0.56–0.99]	1.16	[0.87–1.55]	1.01	[0.66–1.54]	–	–
	All others	0.70	[0.54–0.89]	1.30	[1.02–1.66]	1.07	[0.73–1.57]	1.07	[0.84–1.35]

[1]Only Odd ratios (ORs) for blood type are shown here, ORs for other factors are similar to Table 2. Here HBsAg, diabetes, sex, age and family history of cancer were excluded. ORs and respective 95% confidence intervals were calculated using logistic regression. For each cancer, every blood type group was compared with type O group and all the other groups separately.

The age peak of cancer occurrence was different among various cancers, therefore, we calculated sex ratios within different age groups to ensure the distribution was not skewed by any particular age group. Sex ratios were largely consistent among different age groups (Figure 1B). Thus, the association of sex with thyroid, esophageal and gastric cancers seems to be independent of age.

Age: The age distribution of patients with gastrointestinal cancers (esophageal, gastric, biliary and pancreatic) was similar, whereas that of healthy controls and patients with other cancers (glial and thyroid) had relatively younger age profiles (Figure 1C). These different age distributions suggested that older individuals were more susceptible to gastrointestinal cancer. Only glioma had a weak negative association with increasing age compared with healthy controls (OR = 0.98, 95% CI = 0.97-0.99). All the other cancers analyzed here showed weak positive association with age (Table 2).

Family history of cancer: The prevalence of a positive family history of cancer seemed to be much higher in the cases compared with controls (Figure 1D). Association analysis also revealed stronger associations of family cancer history with gastrointestinal cancer compared with other cancers (Table 2), although the two other cancers (glioma and thyroid) were also significantly associated with cancer family history when compared with healthy controls (OR = 2.83, 95% CI = 1.42-5.64; OR = 2.46, 95% CI = 1.24-4.88, respectively).

HBsAg: There were no associations found between HBsAg and any of the different patient groups (Figure 1E and Table 2).

Diabetes: Type 1 diabetes mellitus is relatively rare in most populations and cancer risk investigations have been scarce. Our study indicates that the prevalence of type 2 diabetes was significantly higher in gastric, biliary and pancreatic cancers compared with other groups (Figure 1F), with ORs ranging between 2.0 and 3.0 (Table 2). However, esophageal cancer was strongly inversely associated with type 2 diabetes compared with healthy controls (OR = 0.29, 95% CI = 0.15-0.54).

Association of ABO blood types with gastrointestinal cancer

The risk of thyroid cancer was lower in blood type A than in type O (OR = 0.74, 95% CI = 0.56-0.99). The risk of esophageal cancer (OR = 1.53, 95% CI = 1.10-2.14) and

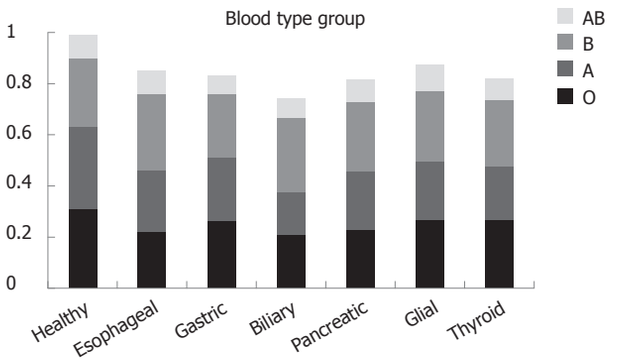

Figure 2 Fraction of each blood type with different population groups. Different colors represent different blood types (AB, A, B, or O), Blank areas in this figure represent missing values.

biliary cancer (OR = 1.49, 95% CI = 1.09-2.05) (Table 3) was significantly associated with blood type B. These were confirmed by comparison of each blood type with a combination of all the other blood types (Table 3, Figure 2). In addition, the risk of thyroid cancer in blood type B was significantly higher when compared with the combination of all the other blood types (OR = 1.30, 95% CI = 1.02-1.66). The risk of biliary cancer and glioma was significantly lower in blood type A when compared with combination of all the other blood types (OR = 0.65, 95% CI = 0.49-0.86; OR = 0.77, 95% CI = 0.60-0.98).

No significant associations of cancer risk were found with blood type AB. This may have been caused by the small number of patients with this blood type in this study.

DISCUSSION

This was a retrospective study on the risk factors (including ABO blood type, HBsAg, diabetes, sex, age and family history of cancer) associated with digestive system cancer in northern China. To reduce the bias, we tried to enlarge our samples, and the cases and controls were selected using the same criteria and in the same period. The association of type 2 diabetes with gastric, biliary and pancreatic cancers was significant, and blood type B was significantly associated with esophageal and biliary cancers.

The ABO blood groups are defined by carbohydrate moieties displayed on the surface of red blood cells and attached to a protein backbone, known as the H antigen. Three variant alleles (A, B and O) of a single gene on chromosomes 9q34, the *A, B* or *O* gene, determine a person's blood type by encoding three glycosyltransferases with different substrate specificities. In addition to their expression on the surface of red blood cells, the A, B and O antigens are highly expressed on the surface of epithelial cells of the gastrointestinal, bronchopulmonary, and urogenital tracts.

One hospital-based case-control study has shown some evidence of a positive association between blood type A and risk of pancreatic cancer[15]. Another study has demonstrated an increased prevalence of pancreatic cancer among patients with blood group B and a decreased prevalence in patients with blood group O[16]. Wolpin *et al*[14] have found that ABO blood type is significantly associated with the risk of pancreatic cancer. Compared with blood group O, patients with groups A, AB and B were more likely to develop pancreatic cancer (adjusted hazard ratios for incidence of pancreatic cancer were 1.32, 95% CI = 1.02-1.72; 1.51, 95% CI = 1.02-2.23; and 1.72, 95% CI = 1.25-2.38, respectively). However, in our study, no significant associations between A, B and O blood types and pancreatic cancer were found. Environmental and dietary factors may play an important role in pancreatic cancer. However, a lower risk of biliary cancer associated with blood type A was found in our study, whereas patients with blood type B were more susceptible to esophageal and biliary cancers.

The functional significance of ABO blood group distribution might be associated with biological characteristics such as differentiation, mean size of the tumor, venous invasion, and TNM stages of esophageal squamous cell cancer[17]. A previous study from China has shown that blood group B is associated with the incidence of upper esophageal squamous cell cancer in men[18]. Our results are consistent with these findings.

Previous studies have reported an association between hepatitis B and pancreatic and biliary cancers. Hassan *et al*[7] have reported a higher risk of pancreatic cancer in HBV carriers. Meanwhile, a case-control study in China has reported that HBV infection and hepatolithiasis are risk factors in the development of cholangiocarcinoma[8]. In Shanghai, chronic HBV infection was associated with a 2.4-fold increased risk of extrahepatic bile duct cancer[19]. However, no association was found between HBsAg and pancreatic or biliary cancer in our study. However, there were some limitations to this study for evaluation of HBV infection. We only acquired HBsAg data, and not data from occult HBV infection. Occult HBV infection has been described in patients who are negative for HBsAg, and who have been previously exposed to HBV and have recovered from acute or chronic infection[20,21]. Currently, it is generally accepted that occult HBV infection among patients without any serological evidence for infection is a risk factor for HCC development[22-24]. For biliary cancer, we did not separate our cases by extrahepatic and intrahepatic origin although most cases were extrahepatic in origin.

Diabetes has been found in previous studies to be associated with a number of cancers. Recent studies among Japanese men and women have shown that a past/present history of diabetes is associated with cancer risk for all sites in both sexes (OR = 1.44, 95% CI = 1.28-1.62; OR = 1.39, 95% CI = 1.19-1.62, respectively). A significantly increased risk was found for cancers of the pharynx, esophagus, colorectum, liver, pancreas, and lung among men, and the stomach, liver, lung and uterine cervix among women[25,26]. Diabetes mellitus and hyperglycemia have also been found to increase the risk of gastric cancer associated with *Helicobacter pylori* infection[27]. The relationship between diabetes mellitus and esophageal

cancer has also been investigated in a Danish study that has shown a 30% increase in risk for esophageal cancer among men with diabetes. No increased risk was seen in women[28].

Diabetes often precedes pancreatic cancer and is thus regarded as a potential risk factor for malignancy. Conversely, pancreatic cancer may secrete diabetogenic factors. Given these findings, there is increasing interest in whether close monitoring of the glycemic profile may help with early detection of pancreatic cancer[29]. The success of a strategy using new-onset hyperglycemia and diabetes as a screening tool to identify people with a high likelihood of asymptomatic pancreatic cancer will depend largely on our ability to differentiate pancreatic-cancer-associated diabetes from the more common type 2 diabetes, by use of a serological biomarker[30].

The risk from diabetes varies according to tumor site, and in our study, diabetes had the strongest association with pancreatic cancer, a moderately increased risk for gastric and biliary cancers, and a decreased risk for esophageal cancer. Given the food intake difficulties of patients with advanced esophageal cancer, the inverse association of diabetes with esophageal cancer could be an effect of diet restriction. Substantial public investment in preventing diabetes mellitus is important to have a major impact on its adverse health effects including cancer.

In conclusion, we found that blood type A was associated with a lower risk of biliary cancer, glioma and thyroid cancer, while those with blood type B were more susceptible to esophageal, biliary and thyroid cancers. Patients with type 2 diabetes were at high risk of pancreatic cancer, moderate risk for gastric and biliary cancers, and decreased risk for esophageal cancer. We did not find any association between HBsAg and the four gastrointestinal cancers in the Chinese population that we studied here.

COMMENTS

Background

The incidence and mortality of gastrointestinal cancer (such as cancer of the esophagus, stomach, pancreas, and biliary tract) are higher in China compared with Western countries, but the reason remains unclear.

Research frontiers

Although it seems reasonable that ABO blood type, hepatitis B surface antigen (HBsAg), and type 2 diabetes have a close relationship with gastrointestinal tract tumors in Western countries, to the best of our knowledge, no previous studies have been conducted to investigate the possible association between these factors and the risk of gastrointestinal cancer in China.

Innovations and breakthroughs

This was a retrospective study on the risk factors (including ABO blood type, HBsAg, diabetes, sex, age and family history of cancer) associated with digestive system cancer in northern China. The association of type 2 diabetes with gastric, biliary and pancreatic cancer was significant, and blood type B was significantly associated with esophageal and biliary cancers.

Applications

It is important for early cancer detection and prevention that type 2 diabetes is related to gastric, biliary, and particularly pancreatic cancers.

Peer review

This study describes a very large case-control study of risk factors (age, sex, ABO blood type, diabetes, hepatitis B virus, and family cancer history) for gastrointestinal cancers in northern China. ABO blood group and type 2 diabetes are correlated with gastrointestinal cancer in northern China, and the family history findings are important. The study design is good and the methods are standard and reliable.

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S- Editor Sun H **L- Editor** Kerr C **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i6.570

World J Gastroenterol 2012 February 14; 18(6): 570-575
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Identification of differential gene expressions in colorectal cancer and polyp by cDNA microarray

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Supported by Grant from Medical Technology Innovation Project of Nanjing Military, No. 09MA066
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Received: May 31, 2011 Revised: July 17, 2011
Accepted: August 15, 2011
Published online: February 14, 2012

Abstract

AIM: To screen the differential expressed genes in colorectal cancer and polyp tissue samples.

METHODS: Tissue specimens containing 16 cases of colorectal adenocarcinoma and colorectal polyp *vs* normal mucosae were collected and subjected to cDNA microarray and bioinformatical analyses. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to confirm some of the cDNA microarray data.

RESULTS: The experimental data showed that eight genes were differentially expressed, most of which were upregulated in adenomatous polyp lesions. Forty-six genes expressions were altered in colorectal cancers, of which 29 were upregulated and 17 downregulated, as compared to the normal mucosae. In addition, 18 genes were similarly altered in both adenomatous polyps and colorectal cancer. qRT-PCR analyses confirmed the cDNA microarray data for four of those 18 genes: *MTA1*, *PDCD4*, *TSC1* and *PDGFRA*.

CONCLUSION: These differentially expressed genes likely represent biomarkers for early detection of colorectal cancer and may be potential therapeutic targets after confirmed by further studies.

Key words: Colorectal polyp; Colorectal cancer; cDNA microarray; Quantitative reverse transcription-polymerase chain reaction

Peer reviewers: Jean Wang, Professor, Washington University, 660 S. Euclid Ave, Campus Box 8124, St. Louis, United States; Zoran Krivokapic, Professor, MD, FRCS, Institute for Digestive Disease, First Surgical Clinic, Clinical Center of Serbia, 6, Dr Koste Todorovica, Belgrade, 11 000, Serbia

Dai YC, Zhu XS, Nan QZ, Chen ZX, Xie JP, Fu YK, Lin YY, Lian QN, Sang QF, Zhan XJ. Identification of differential gene expressions in colorectal cancer and polyp by cDNA microarray. *World J Gastroenterol* 2012; 18(6): 570-575 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i6/570.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i6.570

INTRODUCTION

Colorectal polyp (CRP) is considered as a premalignant lesion for development of colorectal cancer (CRC)[1]. Although the mechanism underlying colorectal cancer de-

velopment remains to be defined, a series of genetic and epigenetic events are thought to play important roles in colorectal carcinogenesis, including oncogene activation and tumor suppressor gene inactivation[2,3].

By attaining a detailed understanding of the altered gene expression profile of colorectal cancer novel strategies may be developed for earlier detection and more effective prevention and treatment, thereby reducing colorectal cancer incidence and increasing survival rates.

In this study, we performed a cDNA microarray analysis to profile differential gene expressions in tissue specimens of polyps and colorectal carcinoma and compared the expression profiles to that in corresponding normal tissues. We chose the genes with marked differential expressions for verification by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). These data provide insightful information into the genetic mechanisms of colorectal cancer and identify genes that may be useful as biomarkers for early disease detection.

Table 1 Clinical characteristics of patients with colorectal cancer or polyps

Case	Sex	Age	Size in cm/*n*		Differentiation	Depth	Dukes
			CRC	CRP	CRC	Polyps	
1	F	41	1.7/2	0.6/2	High	S	B1
2	M	37	0.9/1	1.2/3	Poor	Ss	C1
3	M	68	2.9/2	0.7/1	Poor	Sm	D1
4	F	27	2.8/1	1.4/1	Poor	Ss	C2
5	M	59	1.8/1	0.5/3	Moderate	S	C2
6	F	52	2.9/1	1.9/4	Poor	Ss	B2
7	M	71	3.3/2	0.4/1	High	Sm	B1
8	M	46	2.7/1	3.1/1	Moderate	Ss	C1
9	M	28	0.9/1	2.8/2	Poor	Sm	B1
10	F	36	2.6/1	0.4/1	Poor	Ss	C2
11	F	30	2.4/1	2.8/2	Moderate	S	C1
12	M	46	3.1/1	1.3/2	Poor	Sm	B1
13	M	62	1.5/1	2.0/3	Poor	Ss	C2
14	F	24	0.8/1	2.4/2	Moderate	Sm	C2
15	M	70	2.5/1	4.3/1	Poor	S	B2
16	M	43	3.2/2	3.7/1	High	Ss	B2

S: Serosa; Ss: Subserosa; Sm: Submucosa.

MATERIALS AND METHODS

Ethics

This study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association and approved by the Medical Ethics Committee of Fujian Province, China. All patients read and signed an informed consent form prior to surgery and sample collection.

Patient tissue

A total of 16 patients with colorectal adenocarcinoma and adenomatous polyp lesions were collected from The 174th Hospital of the Chinese PLA between May 2006 and December 2010. Diagnosis of these patients was confirmed by surgical pathology. None of the patients received any pre-surgical chemo- or radiation-therapy. All tissue specimens were immediately taken from the operation room upon excision from the patient, snap frozen in liquid nitrogen, and stored at -80 ℃ until use. The tissue specimens from these patients were divided into two groups: adenomatous polyp lesions *vs* proximal non-cancerous colorectal mucosae (Group A) or colorectal cancer *vs* proximal non-cancerous colorectal mucosae (Group B). The clinicopathological characteristics of these patients are summarized Table 1.

RNA isolation and cDNA microarray analysis

Total cellular RNA was extracted from the tissue samples by using the Trizol reagent (Sigma-Aldrich Inc., Germany)[2,4]. mRNA isolation was then carried out with Qiagen Oligotex beads, (Valencia, CA, United States) according to the manufacturer's instructions. The final concentration of mRNA was measured by spectrophotometer.

Next, the mRNAs from colorectal cancer or adenomatous polyp lesions were reverse transcribed into cDNA by means of Cy5-dUTP labeling, while the mRNAs from the normal mucosae were processed with Cy3-dUTP labeling by following the manufacturer's protocols (NEN Company, Boston, MA, United States). The labeled probes were then hybridized to the cDNA microarray (Chipscreen, Shenzhen, China), which contained 8064 human genes.

Microarray scanning and data analysis

Hybridized cDNA microarrays were scanned using a Gene PIX 4000 microarray fluorescence scanner (Axon Instruments, Foster City, CA, United States). Accompanying bioinformatical software was used convert the output images to data form and perform analysis. Ratios of Cy5: Cy3 were normalized to the median ratio value of all the microarray spots detected. Spots with intensities in both channels that were 0.5 to 2.0-fold higher than the local background were excluded from further analysis. SPSS v13.0 statistical software (Chicago, IL, United States) was used to carry out Student's *t*-test statistical analysis to determine significant intergroup differences of gene expression. *P*-values < 0.05 were considered statistically significant.

Quantitative reverse transcription-polymerase chain reaction

Differentially expressed genes between the adenomatous polyp lesions and colorectal cancer samples detected by the cDNA microarray, as compared to non-cancerous tissues, were verified by using qRT-PCR. The primers for these mRNAs used for qRT-PCR is listed in Table 2.

RESULTS

Qualification of the isolated total RNA and mRNA from the tissue samples

Quality of the isolated total RNA and mRNA from the tissue samples was found to have high correlation coefficients (Figure 1). The fluorescence signal was consistent

Table 2 Primers used for quantitative reverse transcription polymerase chain reaction analysis

Gene tag	Sequences	Tm (℃)	Cycle	Product (bp)
MTA1	5′-AGCCGTGCTTCGGTATCTT-3′ 5′-CCCGTTGTGCTGCTCGTA-3′	57	30	580
PDCD4	5′-GCTGAATTCGGATGGATG-TAGAAAATGAGCAGA-3′ 5′-CTGCTCGAGTCAG-TAGCTCTCTGGTTTAAGA-3′	54	27	470
TSC1	5′-ATCGCCTTTATGGAATGT-3′ 5′-GCTTGTGGTGGTTCAGTT-3′	49	29	510
PDGFRA	5′-ACCATAAGGCTCTTACTCT-3′ 5′-TTCTGGCACTTACCTACA-3′	45	31	490

with the expectations and standards (Figure 2); for example, the good 28S to 18S RNA subunit ratio in these samples indicated that there was no significant degradation (data not shown).

Identification of differential gene expression profile between colorectal cancer and adenomatous polyp lesions

cDNA microarray analysis of these tissue mRNAs revealed that eight genes were differentially expressed between adenomatous polyp lesions and the normal mucosae, and most of these were upregulated in the polyps (Table 3, $P < 0.05$). Meanwhile, 46 genes were differentially expressed between colorectal cancer and normal tissues, of which 29 were up-regulated in the cancer samples (Table 3, $P < 0.05$). A total of 18 genes were found to be similarly altered in both adenomatous polyp lesions and colorectal cancer samples (Table 4). qRT-PCR confirmed the observed patter for four of those 18 genes: *MTA1*, *PDCD4*, *TSC1* and *PDGFRA* (Table 5).

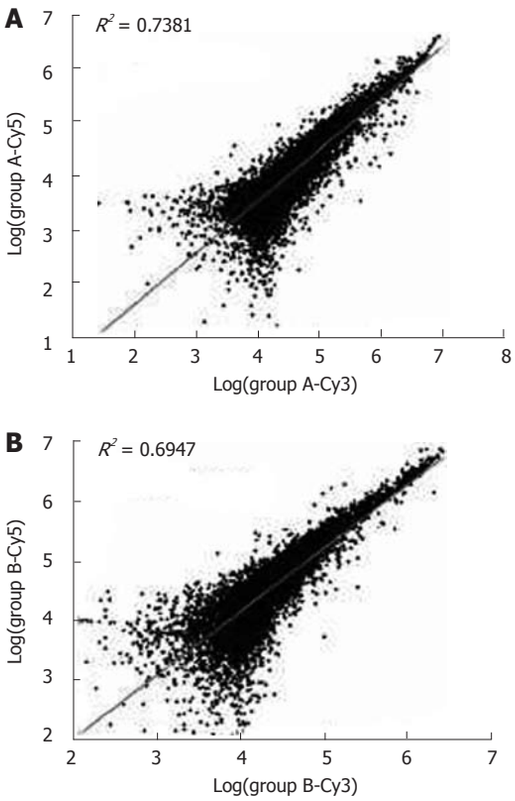

Figure 1 Correlation of CY5/CY3 hybridization signal intensity of all gene spots in Group A (polyps *vs* normal) and B (colorectal cancer *vs* normal) (*R* = 0.8591 and 0.8335, respectively). For visualization of these gene distributions, dispersion plots containing the log2 (normal tissues) and log2 (polyps or colorectal cancer) values were constructed.

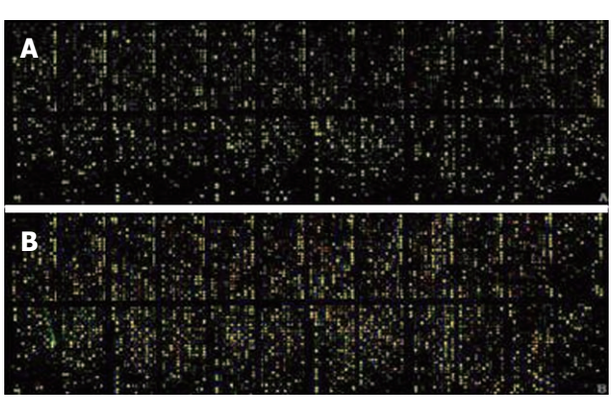

Figure 2 Microarray scanned data from Group A and B.

DISCUSSION

Colorectal carcinoma is a major cause of cancer-related deaths in China[5,6]. Unfortunately, little is known about the gene expression profiles between colorectal cancer and adenomatous polyp lesions. Genes that are differentially expressed in adenomatous polyp lesions may represent useful biomarkers for risk of colorectal cancer development, while altered genes in cancerous tissues may be used as therapeutic targets for colorectal cancer treatment[7].

Adenomatous polyp is the premalignant lesion of colorectal cancer[8-12]. Therefore, we conducted the current study to profile the differential gene expressions between normal mucosae and adenomatous polyp lesions, between normal mucosae and colorectal cancer, and between adenomatous polyp lesions and colorectal cancer. We found that eight genes were differentially expressed in adenomatous polyp lesions, as compared to normal mucosae. In addition, 46 genes were differentially expressed in colorectal cancer, as compared to the normal mucosae; twenty-nine of which were up-regulated. A total of 18 genes were significantly upregulated in both colorectal cancer and adenomatous polyp lesions, further indicating that adenomatous polyp is a precancerous lesion. However, some genes were downregulated in adenomatous polyp lesions (such as *MGST1* and *PDGFRA*) or upregulated in colorectal cancers only (*PLAGL1*). These genes encode proteins that are known to be involved in cell growth, apoptosis, and metastasis and are likely to contribute to colorectal carcinogenesis, as purported by previous studies[13,14].

The *MGST1* gene is located in 12p13.1-13.2 and was previously considered to be a "housekeeping" gene[15].

Table 3 Identification of differential gene expression profile between colorectal cancer and adenomatous polyp lesions

Accession	Gene function	Gene tag	Ratio
AY421086	Programmed cell death 4	*PDCD4*	6.41 ± 0.10
AA630800	Interferon, γ-inducible protein 30	*IFI30*	1.31 ± 0.18
AA400973	Lipocalin 2 (oncogene 24p3)	*LCN2*	3.16 ± 0.22
AI817942	Zeta-chain associated protein kinase (70 kD)	*ZAP70*	3.29 ± 0.31
AA447515	Mad4 homolog	*MAD4*	2.35 ± 0.20
W47350	Retinoic acid receptor responder 3	*RARRES3*	0.18 ± 0.13
AA436401	TU3A protein	*TU3A*	5.40 ± 0.27
AI650283	Serum/glucocorticoid regulated kinase 2	*SGK2*	1.29 ± 0.13
NM003542	H4 histone family, member G	*H4FG*	7.04 ± 0.17
NM205510	Fibroblast growth factor receptor 1	*FGFR1*	2.94 ± 0.21
NM204434	Cyclin-dependent kinase inhibitor 2A	*CDKN2A*	3.16 ± 0.28
NM005438	FOS-like antigen-1	*FOSL1*	1.93 ± 0.25
NM005439	Myeloid leukemia factor 2	*MLF2*	2.17 ± 0.24
BC08072	v-raf murine sarcoma 3611 viral oncogene homolog 1	*ARAF1*	0.98 ± 0.15
NM020531	Chromosome 20open reading frame 3	*C20ORF3*	2.23 ± 0.21
NM033158	Hyaluronoglucosaminidase 2	*HYAL2*	4.16 ± 0.28
NM001950	E2F transcription factor 4,p107/p130-binding	*E2F4*	5.26 ± 0.28
BC059522	Ribosomal protein S30	*FAU*	2.58 ± 0.13
NM008583	Multiple endocrine neoplasia I	*MEN1*	3.89 ± 0.11
NM011492	Serine/threonine kinase 11	*STK11*	5.12 ± 0.27
NM133862	Fibrinogen, gamma polypeptide	*FGG*	3.0 4 ± 0.28
BC162533	GRO2 oncogene	*GRO2*	1.39 ± 0.30
NM000612	Insulin-like growth factor 2 receptor	*IGF2*	5.06 ± 0.29
NM005343	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	*HRAS*	4.74 ± 0.27
NM002634	Prohibitin	*PHB*	2.98 ± 0.15
BC046375	p53-induced protein	*PIG11*	6.67 ± 0.29
NM004448	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2	*ERBB2*	3.15 ± 0.17
NM010658	v-maf musculoaponeurotic fibrosarcoma oncogene family	*MAFG*	5.61 ± 0.04
NM022012	Mitogen-activated protein 3 kinase 11	*MAP3K11*	3.32 ± 0.06
NM023983	Melanoma adhesion molecule	*MCAM*	7.38 ± 0.14
NM014567	Breast cancer anti-estrogen resistance 1	*BCAR1*	5.12 ± 0.23
NM000535	Postmeiotic segregation increased 2	*PMS2*	5.17 ± 0.25
NM183243	Inosine monophosphate dehydrogenase 1	*IMPDH1*	7.14 ± 0.10
NM005380	Neuroblastoma, suppression of tumorigenicity 1	*NBL1*	5.62 ± 0.16
NM002429	Matrix metalloproteinase 19	*MMP19*	3.90 ± 0.22
NM002466	v-myb avian myeloblastosis viral oncogene homolog-like 2	*MYBL2*	9.70 ± 0.21
NM022588	Metastasis associated 1	*MTA1*	10.41 ± 0.37
NM017045	Retinoblastoma 1	*RB1*	2.81 ± 0.14
NC006104	SET translocation	*SET*	2.69 ± 0.11
NM002439	Phosphatase and tensin homolog	*PTEN*	3.94 ± 0.21
NM053455	Fibrinogen-like 2 GTPase activating	*FGL2*	3.40 ± 0.27
NM005638	ADP-ribosylation factor protein 1	*ARFGAP*	5.14 ± 0.25
NM032415	Mucosa associated lymphoid tissue lymphoma translocation gene 1	*MALT1*	4.84 ± 0.21
NM006283	Transforming acidic coiled-coil containing protein 1	*TACC1*	3.41 ± 0.13
NM00288	v-ral simian leukemia viral oncogene homolog B	*RALB*	4.12 ± 0.18
NM003766	Myosin-like BCL2-interacting protein	*BECN1*	5.05 ± 0.14
NG027821	TRK-fused gene	*TFG*	4.33 ± 0.28
NM005805	Cadherin 1,type 1,E-cadherin (epithelial)	*CDH1*	4.11 ± 0.26
NM001982	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 3	*ERBB3*	5.82 ± 0.15
NM133250	MutS (*Escherichia coli*) homolog 2	*MSH2*	2.13 ± 0.21
AY805747	Ras homolog gene family, member E	*ARHE*	3.61 ± 0.22
NM002884	RAP1A, member of RAS oncogene family	*RAP1A*	5.18 ± 0.27
NM000368	Tuberous sclerosis 1	*TSC1*	6.02 ± 0.14
L36953	Mothers against decapentaplegic homolog 4	*MADH4*	4.93 ± 0.26

However, it has been frequently observed as upregulated in various cancers. A recent *in vitro* study has implicated the role of MGST1 in development of multiple drug resistance during breast cancer chemotherapy with several cytostatic drugs (such as cisplatin)[16]. Polymorphisms in MGST1 have also been associated with colorectal cancer risk in Chinese[17]. In this study, we found that MGST1 mRNA levels were upregulated in colorectal cancer, as compared to those detected in normal mucosae (2.90 ± 0.16). Intriguingly, MGST1 was down-regulated in adenomatous polyps, as compared to the normal mucosae (2.14 ± 0.23), but further study is necessary to fully understand the implications of this finding.

PDGFRA gene mutation is commonly observed in tissues of gastrointestinal stromal tumors[18]. Mutated PDGFRA proteins demonstrate constitutively elevated tyrosine

Table 4 Differentially expressed genes between polyps and colorectal cancer

Accession	Gene function	Gene tag	Ratio	
			A	B
AA191692	Stratifin	*SFN*	2.36 ± 0.25	3.86 ± 0.11[a]
H15456	Calpain 1, (mu/I) large subunit	*CAPN1*	8.17 ± 0.20	10.25 ± 0.24[a]
AA043501	v-maf musculoaponeurotic fibrosarcoma (avian) oncogene homolog	*MAF*	1.36 ± 0.08	4.85 ± 0.27[a]
AA495936	Microsomal glutathione S-transferase 1	*MGST1*	-2.14 ± 0.23	2.90 ± 0.16[a]
H23235	Platelet-derived growth factor receptor	*PDGFRA*	-0.15 ± 0.31	4.81 ± 0.14[a]
AA430032	Pituitary tumor-transforming 1	*PTTG*	4.91 ± 0.23	11.46 ± 0.18[a]
N94468	Jun B proto-oncogene	*JUNB*	1.27 ± 0.20	11.09 ± 0.14[a]
T61948	FBJ murine osteosarcoma viral oncogene B homolog	*FOSB*	1.37 ± 0.31	10.21 ± 0.20[a]
AA460168	Growth arrest and DNA damage inducible 34	*GADD34*	1.07 ± 0.15	4.15 ± 0.20[a]
L36870	MAP kinase kinase 4	*MKK4*	4.01 ± 0.23	6.93 ± 0.24[a]
AA426216	Malignant cell expression-enhanced gene	*LENG4*	7.03 ± 0.16	8.81 ± 0.23[a]
AA486219	SRp25 nuclear protein	*LOC51329*	2.09 ± 0.12	6.18 ± 0.23[a]
AA457705	Immediate early response 3	*IER3*	1.94 ± 0.25	9.17 ± 0.20[a]
AA485377	v-fos FBJ murine osteosarcoma viral oncogene homolog	*FOS*	4.03 ± 0.27	7.21 ± 0.05[a]
AA463204	Pleiomorphic adenoma gene-like 1	*PLAGL1*	7.11 ± 0.24	-4.16 ± 0.06[a]
AA434373	E74-like factor 3 (epithelial-specific)	*ELF3*	1.09 ± 0.29	7.03 ± 0.15[a]
AI677994	Fms-associated tyrosine kinase 3 ligand	*FLT3LG*	1.94 ± 0.32	4.35 ± 0.02[a]
AA464600	v-myc avian myelocytomatosis viral oncogene homolog	*MYC*	3.27 ± 0.17	8.01 ± 0.13[a]

[a]$P < 0.05$. "-" indicates down-regulation. A: Polyps *vs* normal mucosae; B: Colorectal cancer *vs* normal mucosae.

Table 5 cDNA microarray data confirmed by quantitative reverse transcription-polymerase chain reaction

Gene function	Gene tag	Ratio	
		cDNA microarray	qRT-PCR
Metastasis associated 1	*MTA1*	8.01 ± 0.47	6.72 ± 0.20[a]
Programmed cell death 4	*PDCD4*	6.41 ± 0.10	5.35 ± 0.01[a]
Tuberous sclerosis 1	*TSC1*	6.02 ± 0.14	4.83 ± 0.26[a]
Platelet-derived growth factor receptor	*PDGFRA*	-0.15 ± 0.31	0.03 ± 0.07[a]

[a]$P < 0.05$. "-" indicates down-regulation.

kinase activity and possess transforming ability, which can be reversed through PDGFR blockade[19]. Thus, mutants of PDGFRA protein behave as oncogenes, as has been demonstrated in glioma samples[20]. Here, we observed high expression of PDGFRA in colorectal cancers, as compared to that in normal tissues (4.81 ± 0.14). This observation suggests that PDGFRA may contribute to cancer development or maintenance of the tumor phenotype, possibly by supporting properties of tumor cell growth and invasiveness. However, to the reason why PDGFRA was down-regulated in adenomatous polyps remains unclear.

Finally, *PLAGL1*, a tumor suppressor gene, is localized on the chromosome 6q24-25 and is the target of several types of chromosomal rearrangement, including one identified in pleomorphic adenomas and malignant tumors. PLAGL1 is ubiquitously expressed in many human tissues where it regulates normal physiological functions; however, it has also been demonstrated to functionally contribute to complex pathologies such as cancer[21-23]. Our current study showed that PLAGL1 mRNA was down-regulated in colorectal cancer, as compared to adenomatous polyps, suggesting that PLAGL1 protein may also play a role in suppressing colorectal cancer development.

The functional roles for each of these genes in colorectal tumorigenesis remain to be verified. Nonetheless, our data provide insightful information into their potential roles in this complex and diverse disease[24]. Future studies will aim to verify these differentially expressed genes as biomarkers for early detection and/or therapeutic targets for treatment of colorectal cancers.

COMMENTS

Background

The mechanism of colorectal carcinogenesis remains to be defined and this study aims to obtain the gene expression profiles between colorectal cancer and adenomatous polyp lesions.

Research frontiers

Many researchs on genetic and epigenetic events which are thought to play important roles in colorectal carcinogenesis, such as oncogene activation and tumor suppressor gene inactivation.

Innovations and breakthroughs

The study firstly screened the differential expressed genes in colorectal adenocarcinoma and colorectal polyp *vs* normal mucosae.

Applications

These differentially expressed genes maybe as biomarkers for early detection and/or therapeutic targets for treatment of colorectal cancers.

Peer review

Study was well designed and performed methodologically.

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S- Editor Lv S **L- Editor** Webster JR **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i6.576

World J Gastroenterol 2012 February 14; 18(6): 576-582
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Study on *RIZ1* gene promoter methylation status in human esophageal squamous cell carcinoma

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Supported by Grant-in-aid from Specialized Research Fund for the Doctoral Program of Higher Education, No. 20091202110009; and Grant-in-aid from Natural Science Foundation of Tianjin, No. 10JCYBJC11300
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Received: April 27, 2011 Revised: July 9, 2011
Accepted: November 9, 2011
Published online: February 14, 2012

Abstract

AIM: To investigate the promoter region methylation status of retinoblastoma protein-interacting zinc finger gene 1 (RIZ1) in the human esophageal squamous cell carcinoma (ESCC) cell lines and tissues and verify the relationship between methylation of RIZ1 and oncogenesis, tumor progression and metastasis etc of ESCC.

METHODS: Methylation-specific polymerase chain reaction (MSP) was used to investigate the promoter region methylation status of RIZ1 in 6 ESCC cell lines. One cell line where RIZ1 promoter region methylation was detected was selected for the next study, where the cell line was treated with 5-aza-CdR. Real-time polymerase chain reaction was used to investigate its influence on the transcription of RIZ1. Experiments using frozen pathological specimens from 47 ESCC patients were performed using the same MSP methodology.

RESULTS: Promoter methylation of *RIZ1* gene was detected in TE13, CaEs17 and EC109 cell lines and the cell line TE13 was chosen for further study. The expression of RIZ1 mRNA in TE-13 was up-regulated after treatment with 5-aza-CdR. The rate of methylation in carcinomas tissues was significantly higher than those in matched neighboring normal and distal ending normal tissue, and the deviation of data was statistically significant (χ^2 = 24.136, P < 0.01). Analysis of the gender, age familial history, tumour deviation, tumour saturation, lymph gland displacement and clinical staging of 47 samples from ESCC patients showed that the fluctuation of data was not statistically significant.

CONCLUSION: Promoter methylation may play an important role in the epigenetic silencing of *RIZ1* gene expression in human ESCC. RIZ1 is considered to be a potential tumor suppressor gene and may be a biological parameter for testing early stage human ESCC.

Key words: Retinoblastoma protein-interacting zinc finger gene 1; Tumor suppressor genes; Esophageal squamous cell carcinoma; Promoter methylation; Methylation-specific polymerase chain reaction

Peer reviewers: Roderick M Quiros, MD, FACS, Surgical Oncologist, Cancer Care Associates, 801 Ostrum Street, Bethlehem, PA 18015, United States; Haruhiko Sugimura, MD, PhD, Professor, Department of Pathology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu, 431-3192, Japan

Dong SW, Zhang P, Liu YM, Cui YT, Wang S, Liang SJ, He Z, Sun P, Wang YG. Study on *RIZ1* gene promoter methylation status in human esophageal squamous cell carcinoma. *World J Gastroenterol* 2012; 18(6): 576-582 Available from: URL: http://

www.wjgnet.com/1007-9327/full/v18/i6/576.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i6.576

INTRODUCTION

Esophageal cancer is one of the most aggressive malignancies with poor prognosis in the world. Esophageal squamous cell carcinoma (ESCC) is a major histological form of the disease, especially in the Northern part of China[1], which is different from Europe and America. Like other types of solid tumors, the development of ESCC is due to the accumulation of the abnormal expression of oncogenes and tumor suppressor genes (*TSG*). Several genetic alterations have been associated with the development of ESCC including p53 and p16 mutations, amplification of cyclin D, c-myc, and EGFR, and allelic loss on chromosomes[2-5]. In mammalian development, DNA methylation has an essential regulatory function which suppresses gene activity by changing chromatin structure[6,7]. It has become apparent that aberrant DNA methylation of promoter region CpG islands may serve as an alternate mechanism to genetic defects in the inactivation of TSG in human malignancies[8,9].

In recent years, researchers found that the retinoblastoma protein-interacting zinc finger gene (*RIZ*) maps to the distal short arm of human chromosome 1 (1p36), a region thought to harbor TSG for a variety of human cancers. The *RIZ* gene normally produces two protein products of different length, RIZ1 and RIZ2. RIZ1 contains the positive regulatory (positive regulatory domain I binding factor 1 and RIZ) domain, but RIZ2 lacks this domain[10]. In many human cancers, RIZ1 is considered a TSG because RIZ1 can induce G2-M arrest and apoptosis. Moreover, a knockout study showed that RIZ1 is a tumor susceptibility gene in mice[11]. The expression of RIZ1 is frequently silenced in many human malignant tumours, including carcinomas of the breast, prostate, and thyroid gland[12-14]. Recently, methylation of RIZ1 promoter CpG islands has been proposed as a common mechanism in inactivating RIZ1. Increasing clinical evidence reveals a positive correlation of reduced RIZ1 expression with increased risk for metastasis, indicating that RIZ1 may be a potential new TSG[12-14]. Although RIZ1 is a putative tumor suppressor in several cancer types, for instance breast cancer[15], gastric cancer[16], lung cancer[17] and so on, the role of RIZ1 in human ESCC has not been reported. In this study, we analyzed methylation status of the RIZ1 promoter and its relationship with RIZ1 mRNA expression in human ESCC cell lines. In addition, the study examined the relationship between methylation of the *RIZ1* gene in the promoter region, oncogenesis, tumor progression, metastasis and hereditary factors etc of ESCC.

MATERIALS AND METHODS

Cell lines and tissues

The human ESCC cell lines KYSE150, KYSE510, TE13, EC9706, CaEs17 and EC109 were provided by the Institute of Cellula Nervosa in Tianjin Huanhu Hospital and were cultured in recommended media RPMI1640 (GIBCO, HEPES 4.76 g/$NaCO_3$ 2.0 g/RPMI-1640 10.4 g/ddH_2O 1000 mL) supplemented with 10% newborn bovine serum (GIBCO), 1 × L-glutamine and 1 × penicillin-streptomycin. Cells were maintained at 37 ℃ in a humidified environment with 5% CO_2.

Carcinoma, matched adjacent normal (> 2 cm from the tumor) and distal ending normal (> 5 cm from the tumor) tissues were obtained in our department during surgical excision from 47 patients with ESCC. All specimens were placed in liquid nitrogen immediately after resection and stored at -80 ℃ until RNA or genomic DNA (gDNA) extraction. No patient had received chemotherapy or radiation therapy prior to surgery. All patients were confirmed to have ESCC by pathologic test.

DNA extraction, purification and bisulfite modification

gDNA from cell lines or ESCC frozen tissues was extracted by using a Dneasy kit (Biomiga). All extracted genomic DNA was treated with sodium bisulfite (Sigma) as reported previously. Briefly, 2 μg gDNA was denatured by 5.5 μL of 3 mol fresh NaOH (final concentration 0.3 mol/L) for 10 min at 37 ℃. 30 μL of 10 mmol/L hydroquinone (Sigma) and 520 μL of 3 mol/L sodium bisulfite (pH 5.0) were added, away from light. The mixture was inverted, added to 200 μL liquid paraffin to prevent water evaporation and reagent oxidation, then incubated at 50 ℃ for 16 h. The modified DNA was purified using the Wizard DNA clean-up system (Promega). The purified DNA was treated again with NaOH and precipitated. DNA was resuspended in 20 μL of LoTE, 2 μL of which were subjected to polymerase chain reaction (PCR) amplification.

Methylation-specific polymerase chain reaction and sequencing

Methylation-specific primers were designed to cover 23 CpG dinucleotides numbered -124--103 (forward) and 32-52 (reverse). Similarly, unmethylation-specific primers were designed to cover 23 CpG dinucleotides numbered -123--103 (forward) and 32-52 (reverse). Primers specific for methylated DNA (forward 5'-GTGGTGGTTATTGGGCGACGGC-3'; reverse 5'-GCTATTTCGCCGACCCCGACG-3') and unmethylated DNA (forward 5'-TGGTGGTTATTGGGTGATGGT-3'; reverse 5'-ACTATTTCACCAACCCCAAGA-3') were added to the reaction and expected to generate 177-bp and 175-bp products, respectively. PCR conditions were 40 cycles of denaturation at 94 ℃ for 30 s, annealing at 68 ℃ for methylation-specific amplification or at 60 ℃ for unmethylation-specific amplification for 45 s and extension at 72 ℃ for 60 s, then sequencing of PCR products.

Reexpression of RIZ1 through 5-aza-CdR treatment

2×10^5 TE13 cancer cells were seeded into 6-well plates and treated with 10 μmol/L special DNA methyltransferase (DNMT) 5-aza-CdR (Sigma) for 3 d. The drug liquid was replaced every day, reagent was wiped out and

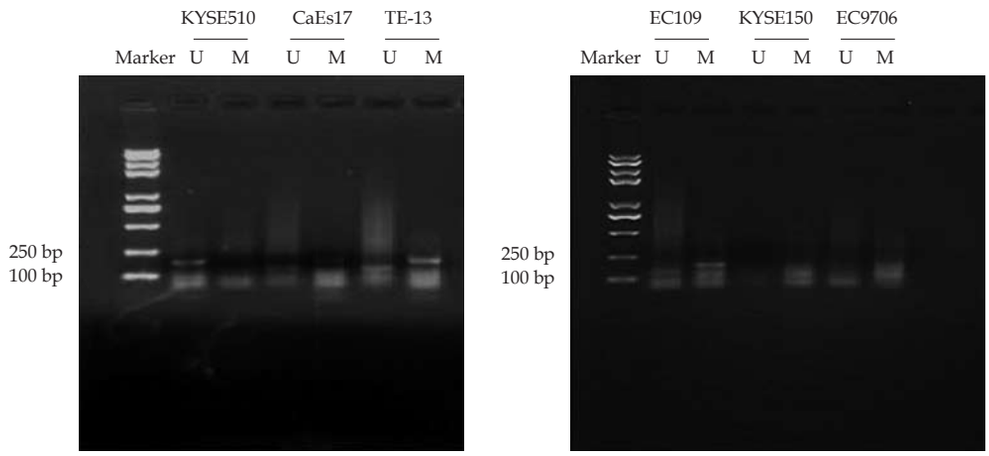

Figure 1 Methylation-specific polymerase chain reaction analyses of retinoblastoma protein-interacting zinc finger 1 promoter methylation status using genomic DNA extracted from 6 human esophageal squamous cell carcinoma cell lines, products of 177 bp and 175 bp were expected for methylated (M) and unmethylated (U) DNA. M: Methylation-specific amplification (177 bp); U: Unmethylation-specific amplification (175 bp).

incubation was continued routinely for 5 d. RNA was isolated, and real-time PCR was performed as described previously.

RNA extraction and reverse transcription reaction

Total cellular and tissue RNA was isolated by Trizol (Invitrogen) reagent according to the manufacturer's recommendations. Cellular RNA was isolated from 5×10^6 to 1×10^7 cells by 1 mL Trizol decomposition and tissues samples were ground into a fine powder using a mortar and pestle, and incubated in Trizol solution (100 g/L) for 15 min. Then 1/5 volume of chloroform was added. After vigorous agitation standing for 5 min, the inorganic phase was separated by centrifugation at 12000 *g* for 15 min at 4 ℃; RNA was then precipitated in the presence of equivolume isopropanol and centrifuged at 12000 *g* for 10 min at 4 ℃. RNA pellets were washed with 1 mL 75% ice-cold ethanol [diethypyrocarbonate (DEPC) treated] and centrifuged at 8000 *g* for 5 min at 4 ℃ then dissolved in DEPC-treated H_2O. Total RNA was quantified and concentration determined using ultraviolet (UV) spectrophotometry (Beckman Coulter) by absorbency at 260/280 nm and 1.2% denaturing agarose gel. For real-time polymerase chain reaction (real-time quantitative PCR) analysis, 2 μg RNA was reverse transcribed using reverse transcriptase M-MLV (Takara), Ribonuclease inhibitor (Takara) and dNTP mixture (Takara), according to the manufacturer's protocol; the cDNA templates were subjected to PCR amplification.

RT-PCR, sequencing and Real-time PCR

One μL cDNA from the TE13 cell line treated or not by 5-aza-CdR was used as the template to amplify specific fragments in 25 mL reaction mixture (10 × easy taq buffer 2.5 μL, 2 mol/L dNTP 2.5 μL, F primer 1 μL, R primer 1 μL, cDNA 1 μL, Easy taq 0.3 μL, ddH_2O 16.7 μL) under the following conditions: denaturation at 94 ℃ for 3 min, 35 cycles at 94 ℃ for 30 s, at 56 ℃ for 30 s, at 72 ℃ for 20 s, then extensions at 72 ℃ for 10 min. The primer (10 μmol/L) sets were: RIZ1, forward 5'-TCTGCTGTTGACAAGACCC-3', reverse 5'-GCATCAATGCACATCCATC-3'. The RIZ1 primer set yielded a band at 167 bp. 12 mL RT-PCR reaction product was analyzed by electrophoresis on a 12 g/L agarose gel. The electrophoresis images were scanned by UV spectrophotometer (Beckman Coulter). Sequencing of 0.75 μL of the resultant cDNA from TE13, which was mixed with 2 × SYBR Premix Ex Taq™ (Takara), was then performed. The primer (10 μmol/L) sets used were: RIZ1, forward 5'-TCTGCTGTTGACAAGACCC-3', reverse 5'-GCATCAATGCACATCCATC -3'; GAPDH, forward 5'-GAAGGTGAAGGTCGGAGTC-3', reverse 5'-GGGTGGAATCATATTGGAAC -3'. The amplifications were performed in LightCycler (Roche) real-time PCR system according to the manufacturer's protocol. Each sample was run in triplicate for each gene. An initial denaturation step at 94 ℃ for 5 min was followed by 45 cycles of denaturation at 95 ℃ for 5 s, annealing at 59 ℃ for 20 s, extension at 72 ℃ for 10 s, then the solubility temperature curve assay was performed.

Statistical analysis

t-test was used to compare the measurement data, for instance the RIZ1 mRNA expression levels with primary ESCC and the adjacent and distal ending normal tissues by Real-time PCR. The relative quantitative results were analyzed by comparison of $2^{-\text{average}\Delta\Delta CT} \times 100\%$. χ^2 test was also used to estimate the enumeration data, for example the results. P values < 0.05 were considered statistically significant.

RESULTS

Methylation status detecting of RIZ1 in human ESCC cell lines

MSP analyses of RIZ1 promoter methylation status using genomic DNA extracted from 6 human ESCC cell lines, products of 177 bp and 175 bp were expected for methylated (M) and unmethylated (U) DNA. Promoter methylation of *RIZ1* gene was detected in TE13, CaEs17, EC109

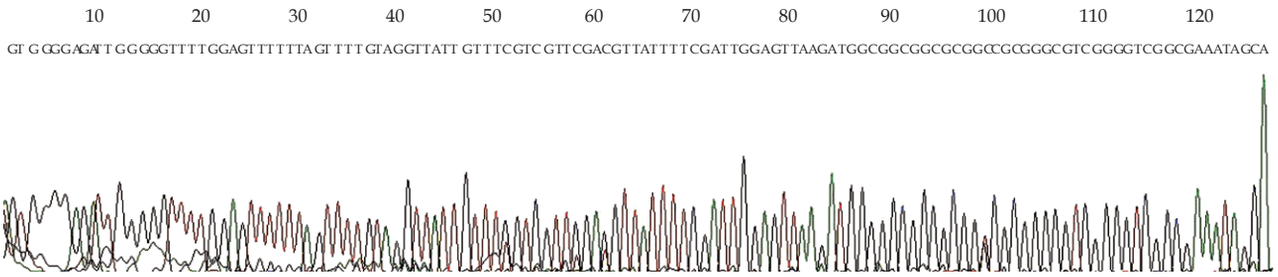

Figure 2 Sequencing analysis of methylated (M) polymerase chain reaction products of methylation-specific polymerase chain reaction from TE13

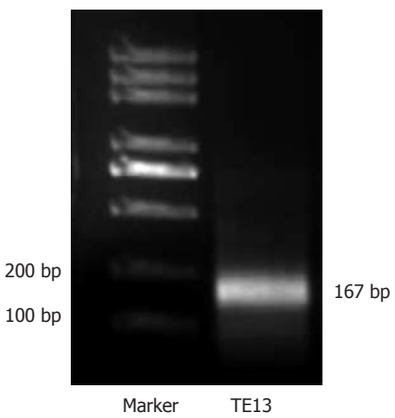

Figure 3 Reverse transcription-polymerase chain reaction for RIZ1 expression in TE13 treated or not by 5-aza-CdR.

but not KYSE510, KYSE150 and EC9706 (Figure 1).

All cytosines were changed to thymines, except cytosines in CpG dinucleotide in M-sequences obtained from amplification using M-primer (Figure 2).

RIZ1 mRNA expression of treated TE13 by DNMT compared with expression in untreated TE13

The expression of RIZ1 was higher after use of 5-aza-CdR than before its use ($P < 0.01$) (Figures 3-5).

Methylation of RIZ1 gene in promoter region of ESCC tissues

Among 47 nonselective ESCC patients and matched adjacent normal and distal ending normal esophageal tissue, 26, 3 and 0 cases, respectively, exhibited methylation in the CpG island of the RIZ1 promoter. The corresponding methylation ratios were 55.3%, 6.4% and 0.0%. The rate of methylation in carcinomas tissues was significantly higher than that in matched adjacent normal and distal ending normal tissues, and the deviation of data was statistically significant ($P < 0.01$). The difference in methylation rates between matched adjacent normal and distal ending normal tissues possesses no statistical significance ($P > 0.05$). In the 3 samples where methylation was positive in matched neighbouring normal tissues, methylation also existed in the corresponding carcinoma tissues. MSP electrophoresis of ESCC patients with RIZ1 methylation positive amplification in both carcinomas and matched normal tissues is illustrated in Figure 6A, while that with RIZ1 methylation positive amplification only in carcinomas tissues but not in matched normal tissues is shown in Figure 6B.

The relation between analysis of the methylation state of the CpG island in the *RIZ1* gene promoter by χ^2 verification and the clinical information of the 47 sufferers of the ESCC is illustrated in Table 1. It can be seen that there was no correlation between the methylation state of the CpG island in the *RIZ1* gene promoter of the 47 samples and the sufferers' gender, age, familial history, tumour deviation, tumour saturation, lymph gland displacement and clinical stages, respectively. The fluctuation of data was not statistically significant ($P > 0.05$ in all groups) (Table 1).

DISCUSSION

Among the numerous genes that are known to be silenced in human cancers, for example p53, Syk, APC, BRCA1, *etc.*[18-20], RIZ1 is one of the few with a proven role in causing cancer as demonstrated. Whereas previous studies demonstrate reduced *RIZ1* gene expression to be common in cancers, this study confirms that RIZ1 is commonly silenced by DNA methylation. The RIZ1 promoter has been demonstrated to have the characteristics of a CpG island, which suggests that RIZ1 is a target of inactivation by epigenetic mechanisms[21]. In prostate cancer, 42.6% of cancer cases were reported to have RIZ1 methylation, and was more frequent in patients with a high-grade malignancy[13]. In gastric adenocarcinoma, hypermethylation of RIZ1 was found in 69% of cancer tissues and in 21% of corresponding non-neoplastic mucosa[22]. In thyroid carcinoma Lal *et al*[14] reported that all of the 31 cancerous cases were methylated, and methylation was significantly frequent compared with normal thyroid tissues (33%). Du *et al*[21] reported that methylation of RIZ1 was detected in 44% (11/25) of breast cancer specimens and 62% (20/32) of liver cancer specimens. However, RIZ1 mutation has not been detected in these cancers[23]. Thus, DNA methylation may represent the preferred mechanism of RIZ1 inactivation in these cancers. Furthermore, because many types of human cancer cell lines exhibit reduced RIZ1 expression, we predict that *RIZ1* gene methylation will be commonly found in many types of human cancer tissue. In a previous study, we found that, compared with normal tissues, the expres-

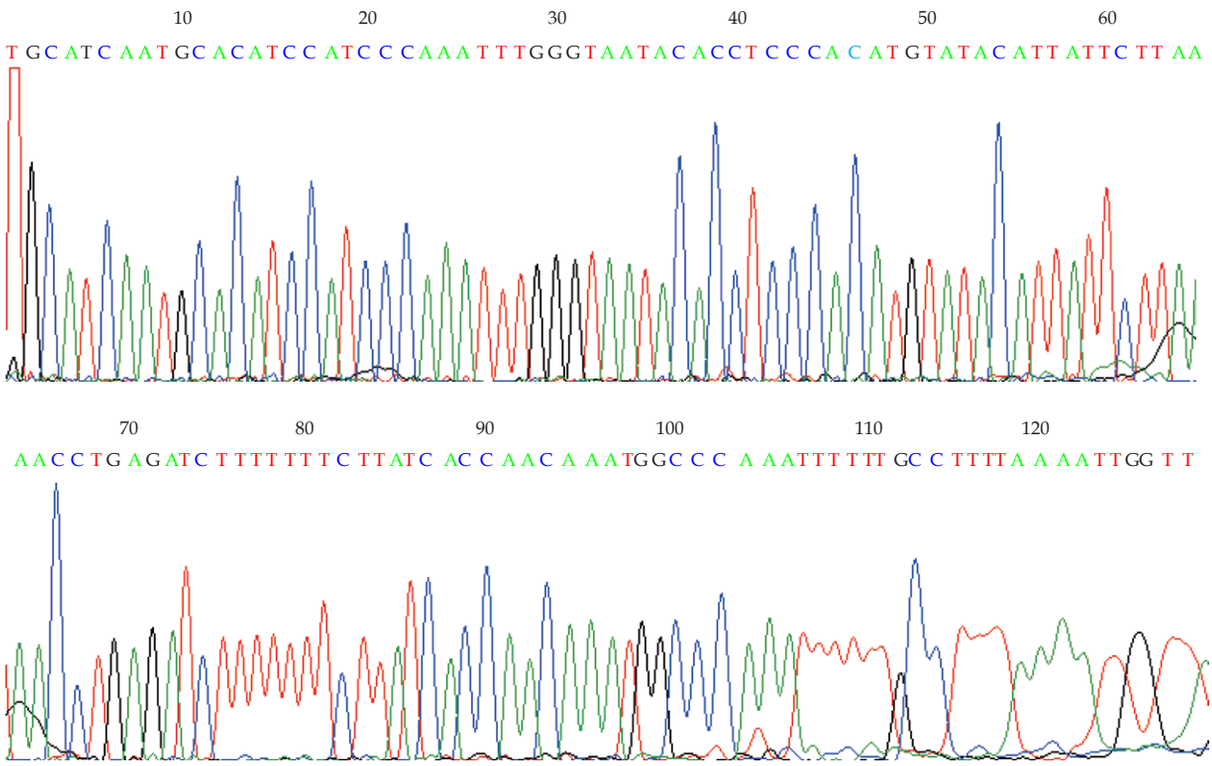

Figure 4 Sequencing analysis of reverse transcription-polymerase chain reaction production using retinoblastoma protein-interacting zinc finger gene 1 F-primer, which is the fragment aimed at.

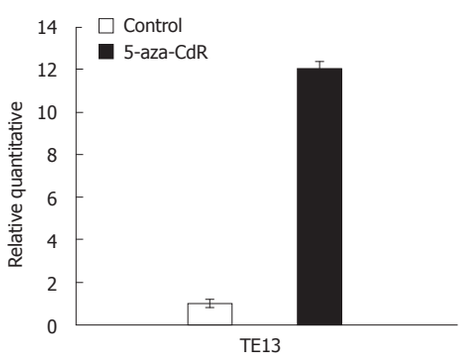

Figure 5 The expression of retinoblastoma protein-interacting zinc finger 1 when the drug 5-aza-CdR is used.

sion of RIZ1 mRNA was significantly lower in cancer tissues than in the adjacent non-cancerous tissues ($P < 0.01$). But in this study there was no significant difference between cancer tissues and the adjacent non-cancerous tissues ($P = 0.067$). No RIZ1 protein expression was observed in the cancer tissues, as it was 0% (0/12), while the expression level in the normal tissues was 66.67% (8/12). There was a statistically significant difference between RIZ1 mRNA and protein expression ($P < 0.05$) which indicates that RIZ1 expression may reduce the occurrence of ESCC. RIZ1 may be a candidate tumor suppressor in ESCC.

In this paper, we explored DNA methylation of RIZ1 in the promoter region among human ESCC cell lines, malignant human ESCC, its matched adjacent normal and distal ending normal tissues. MSP was used to detect

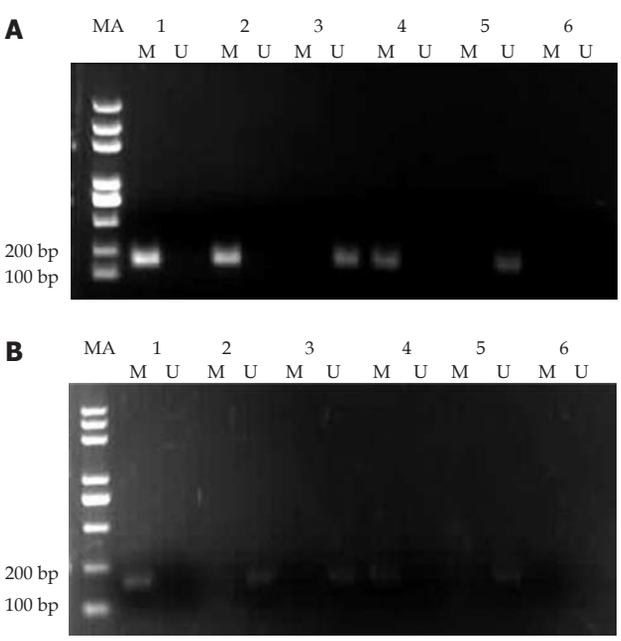

Figure 6 Methylation-specific polymerase chain reaction electrophoresis for cancerous, neighboring and distal ending normal tissues. A: The results of electrophoresis for the polymerase chain reaction (MSP) products of positive retinoblastoma protein-interacting zinc finger gene 1 (RIZ1) methylation amplification sufferers in both their cancerous and neighboring normal tissues; B: The results of electrophoresis for the MSP products of sufferers in whose cancerous tissue positive RIZ1 methylation amplification appears while positive unmethylation amplification is present in the corresponding neighboring normal tissues. MA: Marker; M: Methylation (177bp); U: Unmethylation (175bp);1: Carcinomas; 2: Matched neighboring normal esophageal tissues; 3: Distal ending normal esophageal tissues; 4: Postive control for methylation; 5: Postive control for unmethylation; 6: Negative control.

Table 1 Correlation between methylation of retinoblastoma protein-interacting zinc finger gene 1 gene promoter region and clinicopathologic factors in 47 esophageal squamous cell carcinoma patients' tissues

Factor	Methylation	Unmethylation	*n*	χ^2	*P* value
Gender				0.340	0.560
Male	23	15	38		
Female	7	2	9		
Age				0.029	0.865
> 50	14	20	34		
≤ 50	5	8	13		
Family history				0.151	0.698
Present	6	3	9		
Absent	20	18	38		
Differentiation				0.346	0.841
Well	6	9	15		
Moderately	6	13	19		
Poorly	4	9	13		
Depth of invasion				1.420	0.492
Mucosa and submuscosa	4	5	9		
Muscle	3	6	9		
Serosa	16	13	29		
Lymph node metastasis				3.489	0.062
Present	10	12	22		
Absent	5	20	25		
Stage				3.670	0.55
Ⅰ-Ⅱ	6	18	24		
Ⅲ-Ⅳ	12	11	23		

the promoter region methylation status of *RIZ1* gene in human ESCC cell lines including KYSE150, KYSE510, TE13, EC9706, CaEs17 and EC109. Promoter methylation of *RIZ1* gene was detected in TE13, CaEs17 and EC109. TE13 was chosen for further research and treated with 5-aza-CdR. Real-time PCR shows us that the expression of RIZ1 mRNA in TE-13 was up-regulated after treatment by the drug. The results of study using ESCC pathological frozen specimens illustrate that the R1Z1 gene promoter of the ESCC possesses a 55.3% methylation positive ratio (26/47). RIZ1 methylation positive amplification was seen in 26 sets of carcinoma tissue from 47 sufferers and unmethylation positive amplification was seen in 21. In matched neighboring normal esophageal tissues, the RIZ1 methylation positive amplification appeared in 3 cases, while unmethylation positive amplification was found in 44 cases. Furthermore, in distal ending normal esophageal tissues, the methylation positive amplification did not exist, i.e., all 47 studied cases possessed unmethylation positive amplification. Among those 3 sufferers with RIZ1 methylation positive amplification in matched neighbouring normal esophageal tissues, the corresponding carcinoma tissue also contained RIZ1 methylation positive amplification. In the 47 sufferers of ESCC, 3 possessed methylation in carcinoma tissue and matched neighbouring normal tissue but possessed unmethylation in distal ending normal tissue. Twenty three sufferers showed methylation in carcinoma tissues, but unmethylation in matched neighbouring normal tissue and distal ending normal tissue. Twenty one sufferers possessed unmethylation in carcinoma, matched neighbouring normal and distal ending normal tissues. The rate of methylation of RIZ1 promoter in ESCC was higher than that of adjacent normal tissues ($\chi^2 = 24.1$, $P < 0.01$).

DNA methylation in the promoter region may play an important role in the epigenetic silencing of *RIZ1* gene expression. However, the test to determine *RIZ1* gene promoter methylation shows there is no methylation found in 2 other human ESCC cell lines. This means that dimunition of RIZ1 expression is not always triggered by promoter methylation. One such potential mechanism is that RIZ1 silencing could be caused by a defect in a certain transcription factor that normally activates the RIZ1 promoter. Another potential mechanism is mutation in the RIZ1 promoter. However, given the prevalence of DNA methylation, these other mechanisms are not likely to be commonly involved, and further research is required to find out the true mechanism. Furthermore, we did not observe any significant correlation between RIZ1 methylation and tumour grade. This may be attributable to a relatively small sample size and the complexity of the unselected patient population. Additional detailed studies using a patient cohort should be done needed to examine the value of RIZ1 methylation as a diagnostic or prognostic marker. Another explanation is that gene methylation exists in the early stage of ESCC, but not in the middle and late stages. Hence, there is a statistically insignificant discrepancy during the development of tumour and the displacement of lymph gland. We guess that the methylation of the CpG island in RIZ1 may be an important molecular mechanism during the appearance and development of ESCC in the early stage, and may become a biological parameter for testing early stage of ESCC.

The vital importance of the epigenetic changes on the generation and development of tumours has been thoroughly realized by human beings. Nowadays, there exists an expert database of DNA methylation for researchers (www.methdb.de). The DNA methylation abnormality of malignancy characterizes significantly according to: specifics of the tumour, specifics and reversibility of genes and tissues, *etc*. Methylation testing techniques possess high sensibility and specificity. Thereinto, the MSP technique can be applied for testing some small quantity of tissues sections, phlegm and urine, *etc*., as well as for clinical follow-up through quantitative analysis of relevant genes. Therefore, further research on the mechanisms of the tumour suppressor gene *RIZ1* on esophageal cancer may show some new parameters of early diagnosis and prognosis evaluation for esophageal cancer. The DNMT, 5-aza-CdR, has been clinically applied for curing some solid tumors and some hematological diseases, such as myelodysplastic syndrome, acute myeloid leukemia, etc. A new therapy target for esophageal cancer may be found by strengthening the research on DNA methylation of the genes and the significance of application of 5-aza-CdR.

ACKNOWLEDGMENTS

We thank Dr. Si-Cheng Zhao for verbal changes in the material.

COMMENTS

Background

Recently, methylation of the *retinoblastoma protein-interacting zinc finger* gene (*RIZ1*) promoter CpG islands has been proposed as a common mechanism in inactivating RIZ1. Increasing clinical evidence reveals a positive correlation of reduced RIZ1 expression with increased risk for metastasis, indicating that RIZ1 may be a potential new tumour suppressor gene (*TSG*). However, although RIZ1 is a putative tumour suppressor in several cancer types, for instance breast cancer, gastric cancer, lung cancer, the role of RIZ1 in human esophageal squamous cell carcinoma (ESCC) has not been reported.

Research frontiers

In mammalian development, DNA methylation has an essential regulatory function of suppressing gene activity by changing chromatin structure. It has become apparent that aberrant DNA methylation of promoter region CpG islands may serve as an alternate mechanism to genetic defects in the inactivation of TSG in human malignancies.

Innovations and breakthroughs

China is a country with a high incidence of ESCC, and the pathological type is mainly squamous cell carcinoma, which is different from adenocarcinoma reported in other countries. The present study aimed to discover the effect and mechanism of the anti-cancer gene *RIZ1* on ESCC. The results illustrate that low expression of RIZ1 in ESCC is relevant to promoter methylation. Methylation takes place in the early stage of carcinogenesis, and it may become a molecular biological parameter for early diagnosis.

Applications

The methylation of RIZ1 is the major reason for the low expression in ESCC. This could take place in early stages of ESCC, and is expected to be a molecular biological parameter for early diagnosis.

Peer review

This paper reports RIZ1 promoter methylation in esophageal cancer in a usual way.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i6.583

World J Gastroenterol 2012 February 14; 18(6): 583-586
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Pulmonary embolism with acute pancreatitis: A case report and literature review

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Received: July 15,2011 Revised: September 12,2011
Accepted: October 27, 2011
Published online: February 14, 2012

Abstract

Acute pancreatitis is an inflammatory disease characterized by local tissue injury which can trigger a systemic inflammatory response. So vascular complications of pancreatitis are a major cause of morbidity and mortality. Pulmonary embolism in acute pancreatitis has been reported to be very rare. We reported a case of pulmonary embolism with acute pancreatitis. A 38-year-old woman broke out upper abdomen pain without definite inducement. She had no nausea and vomiting, fever, dyspnea, cough and expectoration, chest pain. The patient had been diagnosed with acute pancreatitis in local hospital. The patient was treated with antibiotics and proton pump inhibitors, and the abdomen pain was alleviated slightly. But the patient came forth cough and expectoration with a little blood, progressive dyspnea. A computed tomographic scan of the abdomen revealed pancreatitis. Subsequent computer tomography angiography of chest revealed pulmonary embolism (both down pulmonary arteries, left pulmonary artery and branch of right pulmonary artery). Dyspnea of the patient got well with thrombolytic treatment and anticoagulation therapy. Pulmonary embolism is a rare but potentially lethal complication of pancreatitis. Familiarity with this complication will aid in its early diagnosis, therapy and prevent pulmonary embolism, a rare but catastrophic phenomenon.

Key words: Pulmonary embolism; Pancreatitis

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Zhang Q, Zhang QX, Tan XP, Wang WZ, He CH, Xu L, Huang XX. Pulmonary embolism with acute pancreatitis: A case report and literature review. *World J Gastroenterol* 2012; 18(6): 583-586 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i6/583.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i6.583

INTRODUCTION

Pulmonary embolism (PE) is a blockage of the main artery of the lung or one of its branches by a substance that has travelled from elsewhere in the body through the bloodstream (embolism). Usually this is due to embolism of a thrombus (blood clot) from the deep veins in the legs, a process termed venous thromboembolism. A small proportion is due to the embolization of air, fat, talc in

drugs of intravenous drug abusers or amniotic fluid. Untreated, PE has a mortality rate of approximately 30%.

Vascular complications of pancreatitis are a major cause of morbidity and mortality and are related to haemorrhage resulting from arterial erosion or pseudoaneurysms, ischaemic complications (either "local" or related to remote vascular events) and venous or arterial complications - specifically splanchnic thrombosis and associated varices[1-3]. The frequency of pulmonary embolism in acute pancreatitis has been reported to be very rare. The thrombohemorrhagic complications in pancreatitis are playing a tremendous part in the development of its most severe forms and fatal outcome. So we described a case of pulmonary embolism with acute pancreatitis and reviewed the literature for the occurrence of this complication.

CASE REPORT

A 38-year-old woman broke out upper abdomen pain after engorgement. She had no nausea and vomiting, fever, dyspnea, cough and expectoration, chest pain. The patient had been diagnosed with acute pancreatitis in local hospital. The relevant laboratory findings on admission were: white blood cell (WBC) 12.71 × 10^9/L, red blood cell 3.65 × 10^{12}/L, hemoglobin 109 g/L, platelets 100 × 10^9/L, blood amylase 1130 U/L, alanine aminotransferase 261 U/L, total bilirubin 33.9 μmol/L, lactate dehydrogenase 614 U/L, urea nitrogen 10.26 mmol/L, serum creatinine 149.3 mmol/L, serum potassium 3.9 mmol/L, serum calcium 1.89 mmol/L, fasting blood glucose 12.1 mmol/L, c-reactive protein 162 mg/L. The patient was treated with antibiotics and proton pump inhibitors, and the abdomen pain was alleviated slightly. But the patient came forth cough and expectoration with a little blood, progressive dyspnea. So the patient was transferred to our institution. We found the following signs with physical examination: The temperature was 36 ℃, the breath rate was 30 breaths per minute, the heart rate was 120 beats per minute, the blood pressure was 90/60 mmHg. The patient had cyanopathy of lip, breathed rapidly. Coarse breath sounds were heard in whole lung, breath sounds were decreased in the bilateral low lung fields, moist rales were heard in bilateral lung. Cardiac examination revealed tachycardia of 120 beats per minute without pathological murmur. Abdomen was flat and soft. Tenderness was obvious in upper abdomen. There was no rebound tenderness on abdomen. Liver and spleen were untouched below the costal margin. Shifting dullness was negative. The remainder of the examination was unremarkable, with no clinical evidence of deep venous thrombosis.

Oxygen saturation of blood was 78% on room air, 82% on 4 L/min oxygen nasal cannula, and 89% with noninvasive ventilator. Laboratory evaluation showed a WBC count of 13.4 × 10^9/L, with otherwise normal complete blood count; normal basic metabolic panel results; and arterial blood gas measurements showed a pH of 7.30, PCO_2 43.6 mmHg, PO_2 68.4 mmHg, and bicarbonate 20.8 mEq/L on oxygen flow rate of 6 L/min face mask. Plasma dimerized plasmin fragment D (D-dimer) assay was > 500 μg/L. blood amylase was 850 U/L.

Electrocardiogram of the patient showed the sinus tachycardia and T-wave change. Doppler echocardiography revealed that right atrial was enlarged. There were no signs of celiac and pelvic vein thrombosis or proximal leg deep venous thrombosis with venous ultrasonography. A computed tomographic (CT) scan of the abdomen revealed pancreatitis (Figure 1). A CT scan of the chest revealed bilateral pleural effusion, lung infection and pulmonary hypertension (Figure 2). Subsequent computer tomography angiography of chest revealed pulmonary embolism (both down pulmonary arteries, left pulmonary artery and branch of right pulmonary artery) (Figure 3). Dyspnea of the patient got well with thrombolytic treatment and anticoagulation therapy.

DISCUSSION

Acute pancreatitis is an inflammatory disease characterized by local tissue injury which can trigger a systemic inflammatory response. There is increasing evidence that endothelial dysfunction is one of the critical pathophysiologic manifestations in patients with severe form of acute pancreatitis[1-4]. In keeping with this, we report the case of pulmonary embolism with acute pancreatitis. The case was reported for the reason: because pulmonary thrombus is a rare complication of pancreatitis.

Pulmonary embolism is a rare complication of pancreatitis, and there have been very few descriptions of it. Vascular thrombosis and hypercoagulable states complicating pancreatitis are thought to due to release of proteolytic enzymes from the pancreas and direct vasculitis. So some researchers consider the mechanism of formation of the pulmonary thrombus in the present case to be as follows: (1) a cyst communicating with the pancreatic duct penetrates into the vascular; (2) pancreatic juice enters the vascular and triggers the formation of a thrombus secondary to vasculitis; (3) hypercoagulability complicates pancreatitis and is thought due to a combination of hepatic dysfunction and hypertrypsinaemia (resulting in raised fibrinogen and Factor Ⅷ concentrations) and cachexia; (4) vascular changes, due to proteolytic damage or inflammation, may also play a significant part; and (5) the acute pancreatitis provokes deleterious effects in endothelium-dependent relaxing response for ACh in isolated mesenteric rings that were strongly associated with high plasma NO_x-levels as consequence of intense inflammatory responses. Furthermore, the subsensitivity of contractile response to phenylephrine in both mesenteric and pulmonary rings might be due to the complications of this pathological condition in the early stage of pancreatitis[5-7].

Pulmonary thromboembolism is a dreadful complication of vascular thrombosis in acute pancreatitis. Symptoms of pulmonary embolism include difficulty breathing, chest pain on inspiration, and palpitations.

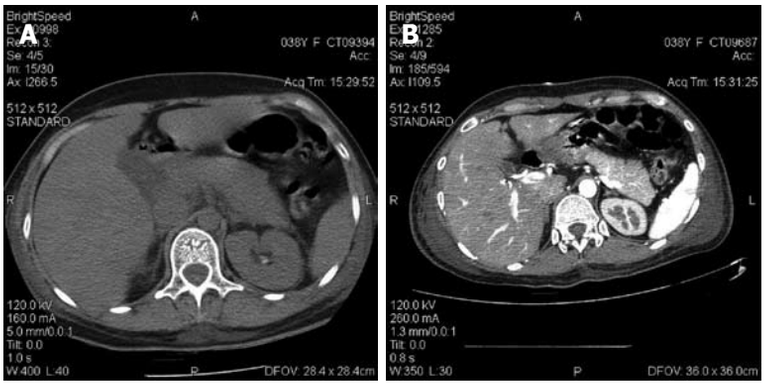

Figure 1 A computed tomographic scan of the abdomen revealed pancreatitis. A: Plain computed tomographic (CT) scan; B: Enhanced CT scan.

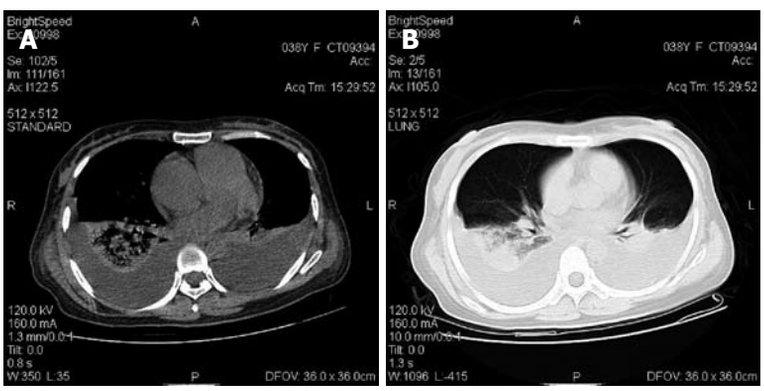

Figure 2 A computed tomographic scan of the chest revealed both sides pleural effusion, lung infection and pulmonary hypertension. A: Mediastinal window; B: Lung window.

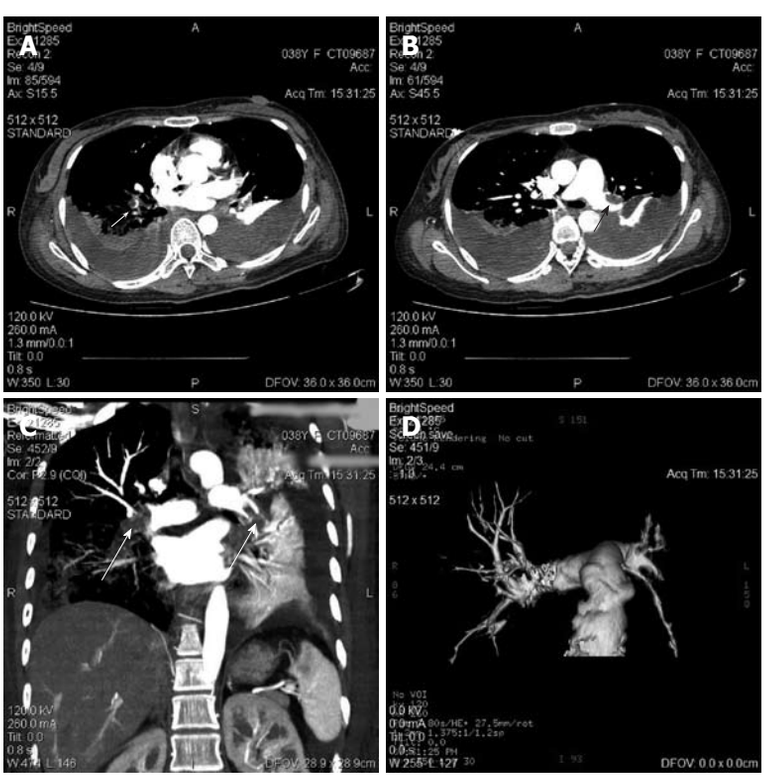

Figure 3 A Computer Tomography angiography of chest revealed pulmonary embolism (both down pulmonary arteries, left pulmonary artery and branch of right pulmonary artery). A: Embolism of rihgt down pulmonary artery (arrow); B:Embolism of left down pulmonary artery (arrow); C: Embolism of left pulmonary artery and branch of right pulmonary artery in coronal view of chest-3D slab image (arrows); D: 3D reconstruction of pulmonary arteries.

Clinical signs include low blood oxygen saturation and cyanosis, rapid breathing, and a rapid heart rate. Severe cases of PE can lead to collapse, abnormally low blood pressure, and sudden death. Diagnosis is based on these clinical findings in combination with laboratory tests (such as the D-dimer test) and imaging studies. So virtually all radiological modalities have been applied to the diagnosis of vascular thrombosis in acute pancreatitis. Contrast venography, ultrasonography, contrast-enhanced CT scan and magnetic resonance imaging (MRI) all have defined roles in the diagnosis of vascular thrombosis. Radionuclide 99mTc-venography and lung perfusion scintigraphy

are useful in diagnosing pulmonary thromboembolism secondary to vascular thrombosis in pancreatitis, showing abnormal large hot spots at the level of the pancreas and pulmonary embolism[2,8,9].

Early recognition and investigation of thromboembolism is imperative because accurate diagnosis and timely radiological interventional procedures can reduce mortality. Early treatment with intravenous heparin is effective. A vascular filter is sometimes used in the management of vascular thrombosis in acute pancreatitis to prevent pulmonary thromboembolism[10-13].

This case report describes a rare complication of acute pancreatitis. Familiarity with this complication will aid in its early diagnosis, therapy and prevent pulmonary embolism, a rare but catastrophic phenomenon.

In conclusions, pulmonary thrombosis is a rare but potentially lethal complication of pancreatitis. Where the patient developing acute shortness of breath, or leg edema, superficial thrombophlebitis or thromboembolic events arouse clinical suspicion of vascular thrombosis, evaluation by CT scanning, MRI, radionuclide 99mTc-venography and lung perfusion scintigraphy is required. Early recognition and investigation of thromboembolism is imperative because accurate diagnosis and timely radiological interventional procedures can reduce mortality. Early treatment with intravenous heparin or thrombolysis is effective. Vascular filter insertion may be a life-saving measure for such patients.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i6.587

World J Gastroenterol 2012 February 14; 18(6): 587-588
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Gastrointestinal stromal tumor and mitosis, pay attention

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Received: June 8,2011 Revised: September 9,2011
Accepted: October 14, 2011
Published online: February 14, 2012

Abstract

The difference between stages Ⅰ and Ⅲ of gastric gastrointestinal stromal tumor depends principally on the number of mitosis. According with TNM classification, the presence in the tumor of high mitotic rate determines the upgrading. Many studies exposed different count techniques in evaluating the number of mitosis. An international standardized method to assess mitotic rate is needed.

Key words: Gastrointestinal stromal tumor; Mitosis; Classification; Gastroesophageal; Enucleation

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Coccolini F, Catena F, Ansaloni L, Pinna AD. Gastrointestinal stromal tumor and mitosis, pay attention. *World J Gastroenterol* 2012; 18(6): 587-588 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i6/587.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i6.587

TO THE EDITOR

We read with great interest the comment to our article[1] by Peparini *et al*[2]. They posed the attention on the new TNM (Tumor, Node, Metastasis) classification[3] which included the gastrointestinal stromal tumors (GISTs). They particularly stressed the attention on the staging differences and the consequential therapeutic approach of the Ⅰ and Ⅲa stage of gastric GIST. The difference between these two stages depends principally on the number of mitosis. In fact, according with TNM classification, the presence in the tumor of high mitotic rate determines the upgrading, with a major risk and the proposed necessity of a more aggressive therapeutical behaviour. High mitotic rate for TNM classification is defined as more than 5 mitosis in 50 high-power fields (HPF) using the 40X magnification objective (total area 5 square mm in 50 fields)[3]. TNM classification states that stringent criteria have to be followed when defining mitosis: pyknotic or dyskaryotic nuclei must not be counted as mitoses[3].

On one hand we agree with the comment of Peparini *et al*[2] about the necessity to be carefull in deciding which surgical and target therapeutic strategy should be adopted, on the other hand however we consider mandatory to stress the possible bias in evaluating the number of mitosis.

Many studies in fact exposed different count techniques in evaluating the number of mitosis, which could lead to a different staging and consequentially to a different surgical and target therapeutic approach for the same lesion[4]. The number of different staging systems[4] and the uncertain behaviour of the GISTs, especially those of the upper gastrointestinal tract, which seem to be less aggressive than those of the lower tract, left uncertain the best treatment for the gasto-esophageal junction GISTs. In fact, it has been demonstrated as the gastric GISTs differentiated one from each other depending on the localization (gastoesophageal junction and body *vs* distal antrum)[5]. Moreover high intratumoral discrepancies in mitotic rate have been reported[6].

The anatomical site and the peculiar charachteristics of the tumor impose to be extremely careful to the risk-

benefit balace. Lastly, the urgent need for an international standardized method to assess mitotic rate which define the spectrum of mitotic figures, the total field area of 50 HPF and the best tumor area to be used for the count is emphasized from the present interesting correspondence.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2012 February 14; 18(6): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Online Submissions: http://www.wjgnet.com/1007-9327office
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www.wjgnet.com

World J Gastroenterol 2012 February 14; 18(6): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

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Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

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Amman, Jordan

May 7-10, 2012
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Chicago, IL 60601, United States

May 17-21, 2012
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Hollywood, FL 1300, United States

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San Diego, CA 92101, United States

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PancreasFest 2012
Pittsburgh, PA 15260, United States

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Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

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The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
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September 15-16, 2012
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Kiev, Ukraine

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Online Submissions: http://www.wjgnet.com/1007-9327office
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www.wjgnet.com

World J Gastroenterol 2012 February 14; 18(6): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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Name of journal

World Journal of Gastroenterology

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

Editorial office
World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
Ocean International Center,
No. 62 Dongsihuan Zhonglu,
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-59080039
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Indexed and abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifer, and Directory of Open Access Journals. ISI, Thomson Reuters, 2010 Impact Factor: 2.240 (35/71 Gastroenterology and Hepatology).

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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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World Journal of
Gastroenterology ®
Volume 18 Number 7
February 21, 2012

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ISSN 1007-9327
9 771007 932045 07>

ISSN 1007-9327 CN 14-1219/R Local Post Offices Code No. 82-261

World Journal of Gastroenterology
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Volume 18 Number 7 Feb 21 2012

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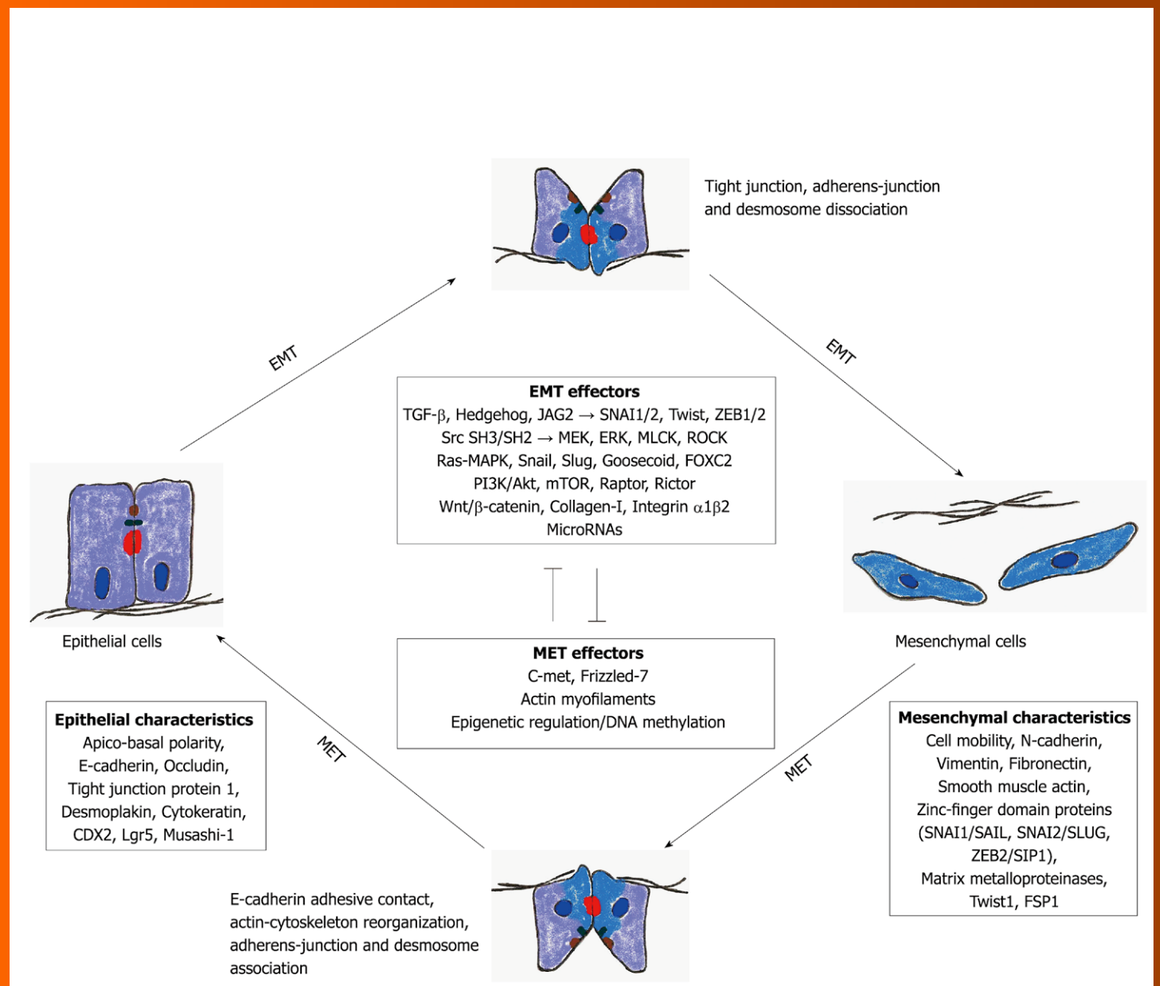

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World Journal of Gastroenterology
Volume 18 Number 7 February 21, 2012

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World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

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PRINT SUBSCRIPTION
RMB 300 Yuan for each issue, RMB 14400 Yuan for one year.

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

EDITORIAL

Herbal medicines for the management of irritable bowel syndrome: A comprehensive review

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Telephone: +98-21-66959104 Fax: +98-21-66959104
Received: June 7, 2011 Revised: August 21, 2011
Accepted: October 28, 2011
Published online: February 21, 2012

Abstract

Irritable bowel syndrome (IBS) is a functional gut disorder with high prevalence. Because of various factors involved in its pathophysiology and disappointing results from conventional IBS medications, the treatment of IBS is challenging and use of complementary and alternative medicines especially herbal therapies is increasing. In this paper, electronic databases including PubMed, Scopus, and Cochrane library were searched to obtain any *in vitro*, *in vivo* or human studies evaluating single or compound herbal preparations in the management of IBS. One *in vitro*, 3 *in vivo* and 23 human studies were included and systematically reviewed. The majority of studies are about essential oil of Menta piperita as a single preparation and STW 5 as a compound preparation. Some evaluated herbs such as Curcuma xanthorriza and Fumaria officinalis did not demonstrate any benefits in IBS. However, it seems there are many other herbal preparations such as those proposed in traditional medicine of different countries that could be studied and investigated for their efficacy in management of IBS.

Key words: Herbal medicines; Irritable bowel syndrome; Systematic review

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Rahimi R, Abdollahi M. Herbal medicines for the management of irritable bowel syndrome: A comprehensive review. *World J Gastroenterol* 2012; 18(7): 589-600 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/589.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.589

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gut disorder characterized by abdominal pain or discomfort, bloating, and bowel disturbances[1] with a higher prevalence ratio of women to men (ratio of 2:1)[2]. Studies from Asia suggest higher prevalence of IBS in more developed countries such as Singapore (8.6%) and Japan (9.8%) compared with India with the lowest prevalence (4.2%)[3]. The pathophysiology of IBS is most likely multifactorial involving visceral hypersensitivity, abnormal gut motility, intestinal microbiota, inflammation and immune disturbance, genetic factors, abnormal gas handling, psychosocial factors, intestinal infections, central nervous system, and serotonin[1,4,5]. Pharmacological treatment of IBS varies from antidepressants including tricyclic antidepressants[6] and selective serotonin reuptake inhibitors[7], to antispasmodics[8,9], 5-hydroxytryptamine-3 receptor (5-HT3) antagonists[10], 5-HT4 agonists[11], antibiotics[12], probiotics[13], and melatonin[14]. But involvement of numerous factors in pathophysiology and a very significant

placebo effect[15] cause therapy of this disease to be more complex. Due to disappointing results with conventional IBS treatments, complementary and alternative medicines are becoming attractive options for many patients[16].

In the present paper, management of IBS by herbal medicines and their modes of action have been evaluated in detail. For this purpose, electronic databases including PubMed, Scopus, and Cochrane library were searched to obtain studies giving any *in vitro*, *in vivo*, or human evidence of the efficacy of herbs in the treatment of IBS. Data were collected for the years 1966 to 2011 (up to February). The search terms were: "complementary and alternative medicine", "plant", or "herb" and "irritable bowel syndrome". Reference lists of the retrieved articles were also reviewed for additional applicable studies. The title and abstract of each article were examined to eliminate duplicates, reviews, studies examining functional bowel diseases other than IBS, and studies assessing complementary and alternative medicines other than herbs. Figure 1 shows a flow diagram of the study selection process.

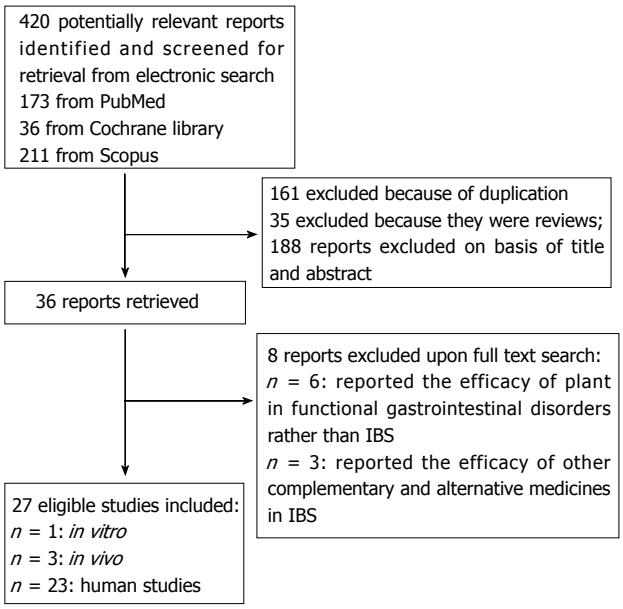

Figure 1 Flow diagram of the study selection process. IBS: Irritable bowel syndrome.

INVESTIGATED HERBAL PREPARATIONS IN IBS

Single preparations

Preparations containing only one herb and investigated in IBS are discussed below and also in Table 1.

Aloe vera

Fifty-eight patients with IBS were randomized to receive *Aloe vera* or matching placebo for a month but no significant difference was found between Aloe and placebo groups[17].

Curcuma species

Eight-week treatment of IBS patients with Curcuma longa extract tablet decreased IBS prevalence and abdominal pain/discomfort score significantly between baseline and after treatment. There were significant improvements in the IBS quality of life (QOL) scales. Approximately two thirds of all subjects reported an improvement in symptoms after treatment, and there was a favorable shift in self-reported bowel pattern[18]. In a randomized, double-blind, placebo-controlled trial, IBS patients were randomly assigned to receive Curcuma xanthorriza or placebo. IBS-related pain increased in the Curcuma group and decreased in the placebo group. IBS-related distension showed a greater reduction in the placebo group compared to the curcuma group. Additionally, the global assessment of changes in IBS symptoms and psychological stress due to IBS did not differ significantly among the two treatment groups. Thus, Curcuma xanthorriza did not show any therapeutic benefit over placebo in patients with IBS[19]. Thus, the species of Curcuma used is an important factor in determining its efficacy in IBS. The efficacy of Curcuma in IBS may be due to bactericidal[20], anti-inflammatory[21], and spasmolytic[22] activities.

Cynara scolymus

Cynara scolymus was demonstrated to have both preventive and curative roles in IBS. The leaf extract of Cynara scolymus was evaluated in healthy volunteers suffering concomitant dyspepsia and showed a 26.4% fall in IBS incidence after treatment. A significant shift in self-reported usual bowel pattern away from "alternating constipation/diarrhea" toward "normal" was observed. The nepean dyspepsia index (NDI) total symptom score significantly decreased by 41% after treatment. Similarly, there was 20% improvement in the NDI total QOL score in the subset after treatment[23]. When the leaf extract of Cynara scolymus was administered to patients with IBS for 6 wk, a significant reduction in the severity of symptoms was observed. Ninety-six percent of patients rated this extract better than or at least equal to previous therapies administered for their symptoms. Furthermore, the tolerability of Cynara scolymus extract was very good[24]. It was reported that Cynara scolymus affects intestinal microbiota[25] and has antispasmodic activity[26].

Fumaria officinalis

The efficacy of Fumaria officinalis, because of its antispasmodic activity, has been investigated in IBS patients. In the randomized, double-blind, placebo-controlled trial, IBS-related pain decreased more in the fumitory group compared to the placebo group. IBS-related distension decreased in the placebo group and increased in the fumitory group. Additionally, the global assessment of changes in IBS symptoms and psychological stress due to IBS did not differ significantly among the two treatment groups[19].

Table 1 Single herbs used for treatment of irritable bowel syndrome

Scientific name	Part	Type of study	Model	Concomitant drugs	Duration of study	Results	Ref.
Aloe vera	Gel	Placebo-controlled double-blind trial	IBS patients	-	1 mo	No difference between treatment and placebo groups in response to treatment at 1 mo diarrhea-predominant patients showed a trend towards a response to treatment at 1 mo	[17]
Curcuma longa	Rhizome	Partially blinded, randomized, two-dose, pilot study	IBS patients	-	8 wk	↓Abdominal pain/discomfort score Significant improvements in IBS QOL scales Approximately two thirds of all subjects reported an improvement in symptoms after treatment	[18]
Curcuma xanthorrhiza	Rhizome	Randomized, double-blind, placebo-controlled trial	IBS patients	-	18 wk	↑IBS-related pain ↓IBS-related distension but more decrease was seen in placebo The global assessment of changes in IBS symptoms and psychological stress due to IBS did not differ significantly among groups	[19]
Cynara scolymus	Aqueous-alcohol extract of leaf	Postmarketing surveillance study	IBS patients	-	6 wk	↓Severity of symptoms reported by both physicians and patients 96% of patients rated this drug as better than or at least equal to previous therapies Very good tolerability	[24]
	Aqueous extract of leaf	Dose-ranging, open, postal study	IBS patients	-	2 mo	↓IBS incidence by 26.4% A significant shift in self-reported usual bowel pattern away from "alternating constipation/diarrhea" toward "normal" ↓NDI total symptom score by 41% 20% improvement in the NDI total QOL score	[23]
Fumaria officinalis	Whole plant	Randomized, double-blind, placebo-controlled trial	IBS patients	-	18 wk	↓IBS-related pain ↑IBS-related distension The global assessment of changes in IBS symptoms and psychological stress due to IBS did not differ significantly among group	[19]
Hypericum perforatum	Aerial parts	Randomized, double-blind, placebo-controlled trial	IBS patients	-	12 wk	↓Overall BSS in both groups with the placebo arm having significantly lower scores at 12 wk compared with Hypericum group A similar proportion of subjects in each treatment group believed that the study drug they received decreased IBS life interferences	[28]
		Open-label, uncontrolled trial	IBS women	-	8 wk	↓autonomic nervous system to different stressor Improvement of Gastrointestinal symptoms of IBS	[29]
Iberis amara	Whole plant extract	Double-blind, randomized, placebo-controlled, multicentre trial	IBS patients	-	4 wk	Significant improvement in IBS symptom scale and abdominal pain scale in Iberis group compared with placebo	[44]
Maranta arundinacea	Root	Uncontrolled	Diarrhea predominant- IBS patients	-	1 mo	↓Diarrhoea ↓Abdominal pain	[30]
Menthe piperita	Essence	Prospective double blind placebo-controlled randomized trial	IBS patients	-	4 wk	75% of the patients in the treatment group showed a > 50% reduction of basal total IBS symptoms score compared with 38% in the placebo group ($P < 0.009$) a statistically significant reduction of the total IBS symptoms score in treatment group compared with T (0), while no change was found with the placebo	[34]
		Randomized double-blind placebo-controlled study	IBS patients	-	8 wk	The number of subjects free from abdominal pain or discomfort changed from 0 at wk 0 to 14 at wk 8 in the treatment group and from 0 to 6 in controls ($P < 0.001$). ↓Severity of abdominal pain significantly in the drug group as compared to controls Improvement in the QOL in the treatment group There was no significant adverse reaction	[33]
		Randomized, double-blind controlled trial	IBS patients	-	2 wk	76% of the patients receiving peppermint oil reported changes in the severity of symptom scale at the end of trial compared with 19% receiving placebo Improvements in the change of symptom scale in 71% of the patients receiving peppermint oil compared with 43% receiving placebo	[35]

			IBS patients	-	1 mo	No significant differences between groups in the Gastrointestinal Symptom Rating Scale No changes in symptoms such as abdominal rumbling, abdominal distention, belching, gas, and heartburn in treatment group compared with placebo Mean severity of pain symptoms in the treatment group was significantly lower than that in the placebo Significant reduction in the abdominal pain, abdominal distension, stool frequency, borborygmi, flatulence in the treatment group compared to placebo Symptom improvements after essence therapy were significantly better than after placebo No significant changes in liver function test results.	[32]
Paeonia lactiflora	Paeoniflorin; Active principle of root	*In vivo*	Neonatal maternal separation-induced visceral hyperalgesia in rats	-	Single dose	A dose-dependent analgesic effect Blockage of analgesic effect of Paeoniflorin by norbinaltorphimine, dl-α-methyltyrosine, and yohimbine. Analgesic effect may be mediated by kappa-opioid receptors and α(2)-adrenoceptors in the central nervous system	[39]
Plantago psyllium	Seed	Randomized placebo controlled trial	IBs patients	-	12 wk	Significantly greater proportion of responders in the psyllium group than in the placebo group ↓Symptom severity significantly in the psyllium group compared with the placebo No differences in QOL	[40]

IBS: Irritable bowel syndrome; QOL: Quality of life; NDI: Nepean dyspepsia index; BSS: Bowel symptom score.

Hypericum perforatum

Hypericum perforatum is a popular herbal medicine for the treatment of depression and it may be beneficial in the management of IBS by modulating psychological stress and serotonin[27]. The efficacy of Hypericum perforatum (St John's wort) was evaluated in IBS patients during a 12-wk randomized, double-blind, placebo-controlled trial. The overall bowel symptom score (BSS) from baseline was decreased both in Hypericum and placebo groups whereas the placebo arm showed significantly lower scores at the end of treatment. Individual BSS for diarrhea (D-BSS), constipation (C-BSS), pain or discomfort, and bloating, adequate relief (AR) of IBS of at least 50% during the last 4 wk of therapy and IBS quality-of-life score showed greater improvement in the placebo group when compared with the Hypericum group. Thus Hypericum perforatum showed lower efficacy for treatment of IBS than placebo[28]. Another study showed that Hypericum perforatum can improve the psychologic symptoms and the ANS reactivity to stress and relieve intestinal symptoms in women with IBS. In patients with IBS, intestinal symptoms of IBS were also relieved significantly[29].

Maranta arundinacea

The efficacy of powdered root of Maranta arundinacea (Arrowroot) was assessed in patients with diarrhea predominant-IBS. It reduced diarrhea with a long-term effect on constipation. It also reduced abdominal pain[30].

Mentha × piperita

The evidence for the efficacy of essential oil of Mentha × piperita (peppermint oil) in IBS seems to be more than other herbal preparations[9,31]. In a prospective, randomized, double-blind, placebo-controlled clinical study, the efficacy of an enteric-coated peppermint oil formulation was evaluated in outpatients with IBS. Seventy-nine percent of patients on Mentha capsule experienced an alleviation of the severity of abdominal pain, 83% had less abdominal distension, 83% showed a reduced stool frequency, 73% had fewer borborygmi, and 79% less flatulence. Corresponding figures for the placebo group were: 43% with reduced pain, 29% with reduced distension, 32% with reduced stool frequency, 31% with fewer borborygmi, and 22% with less flatulence. Symptom improvements after Mentha capsule were significantly better than after placebo. No significant side effect was seen in the Mentha group and peppermint oil was well tolerated[32]. In another randomized, double-blind, placebo-controlled study conducted on outpatients with IBS, the number of subjects free from abdominal pain or discomfort changed from 0 at week 0 to 14 at week 8 in the peppermint oil group and from 0 to 6 in the placebo group. The severity of abdominal pain was reduced significantly in the peppermint group as compared to the placebo group. Furthermore, peppermint oil capsule significantly improved the QOL. No significant adverse reaction was reported from peppermint oil capsule[33]. In another study, the effectiveness of an enteric-coated peppermint oil capsule was evaluated in patients with IBS in whom small intestinal bacterial overgrowth, lactose intolerance and celiac disease were excluded. The symptoms evaluated were: abdominal bloating, abdominal pain or discomfort, diarrhea, constipation, feeling of incomplete evacuation, pain at defecation, passage of gas or mucus and urgency at defecation. The number of patients who

showed reduction of the basal total IBS symptoms score in the peppermint oil group was greater than that in the placebo group[34]. In a randomized, double-blind controlled trial of children with IBS, 75% of those receiving peppermint oil showed a reduced severity of pain associated with IBS. At the end of the trial, the peppermint oil group reported a more significant improvement in the change of symptom scale than the placebo group. Symptoms such as changes in abdominal rumbling, abdominal distention, belching, gas, and heartburn exhibited no changes when peppermint oil was compared with placebo. The most predominant effect of peppermint oil was reduction in the severity of abdominal pain. No side effects were reported by either the investigator or patients during the 2-week study period[35]. Mentha was shown to have antimicrobial[36] and antispasmodic[37] activity and cause reduction in gastric motility[38].

Paeonia lactiflora

The root of Paeonia lactiflora is used in many herbal preparations for IBS. Paeoniflorin (PF) is one of the principle active ingredients of the root of Paeonia lactiflora. A dose-dependent analgesic effect was produced by both intraperitoneal and central administration of PF on visceral pain in rats with neonatal maternal separation. Further investigation showed that this effect may be mediated by kappa-opioid receptors and α (2)-adrenoceptors in the central nervous system[39].

Plantago psyllium

The efficacy of seed from Plantago psyllium in IBS was determined during a randomized controlled trial. The proportion of responders was significantly greater in the Plantago group than in the placebo group during the first month and the second month of treatment. After three months of treatment, symptom severity in the Plantago group was reduced by 90 points, compared with 49 points in the placebo group. No differences were found with respect to QOL[40].

COMPOUND PREPARATIONS

Preparations containing more than one herb are discussed below and summarized in Table 2.

Carmint

Carmint is an Iranian herbal medicine containing total extracts of Melissa officinalis, Mentha spicata, and Coriandrum sativum. Thirty-two IBS patients randomly received either carmint or placebo, plus loperamide or psyllium (based on their predominant bowel function), for 8 wk. The severity and frequency of abdominal pain/discomfort were significantly lower in the carmint group than the placebo group at the end of the treatment according to severity and frequency of bloating[41].

A Chinese herbal medicine

A randomized, double-blind, placebo-controlled trial was conducted to determine whether a Chinese herbal medicine (CHM) is of any benefit in the treatment of IBS. This formulation composed of 20 different herbs (Table 2). Compared with patients in the placebo group, patients in the CHM groups showed a significant improvement in bowel symptom and global improvement scores as rated by patients and by gastroenterologists. Patients reported that treatment significantly reduced the degree of interference with life caused by IBS symptoms[42].

Padma Lax

Padma Lax, a complex Tibetan herbal formula (Table 2), was evaluated for safety and effectiveness in treating constipation-predominant IBS in a 3-mo double-blind randomized pilot study. Significant improvement was demonstrated after 3 mo in the Padma Lax group compared to placebo in constipation, severity of abdominal pain, daily activities, incomplete evacuation, abdominal distension and flatus/flatulence. Significantly more Padma Lax patients than placebo patients rated the current treatment superior to previous therapies tried for IBS. Laboratory parameters displayed no clinically significant changes. The primary side effect of Padma Lax was loose stools which improved by lowering dose[43].

STW 5

STW 5 (Iberogast), a formula composed of hydroethanolic extracts of nine herbs, has been prepared for functional gastrointestinal disorders like IBS and its efficacy and mechanisms of action were investigated in several studies. It was significantly better than placebo in reducing the total abdominal pain and the IBS symptom scores. In a double-blind, placebo-controlled, multicentre trial, STW 5-Ⅱ, another formula composed of 6 of 9 herbal extracts used in STW 5, and a preparation containing only one of 9 herbal extracts (extract of Iberis amara) were compared with placebo. STW 5-Ⅱ like STW 5 showed more significant reduction in the total abdominal pain and the IBS symptom scores compared with placebo. There were no statistically significant differences between the mono-extract group and the placebo group[44]. Different mechanisms have been proposed for the efficacy of STW 5 in IBS. A study evaluating STW 5 effects on mucosal secretion in human intestinal mucosa/submucosa preparations and in the human epithelial cell line T84 suggested that this herbal preparation is a secretogogue in the human intestine by direct epithelial actions and through activation of enteric neurons. The prosecretory effect is due to increased epithelial chloride fluxes via cystic fibrosis transmembrane conductance regulator and calcium dependent chloride channels[45]. STW 5 was studied *in vitro* for binding affinities to serotonin (5-HT3 and 5-HT4) and muscarinic (M3) receptors of the intestine that play central roles in the etiology of IBS. STW 5 showed binding affinity to 5-HT4, M3 and to a lesser degree 5-HT3[46]. STW 5 has also controlled visceral hypersensitivity by reducing intestinal afferent sensitivity to mechanical and chemical stimuli in the upper gastrointestinal

Table 2 Combination herbal therapies used for irritable bowel syndrome

Name of preparation	Composition (part)	Type of study	Model	Concomitant drugs	Duration of study	Results	Ref.
Carmint	Mentha piperita (leaf) Melissa officinalis (leaf) Coriandrum sativum (fruit)	Double-blind, randomized, placebo-controlled, multicenter clinical trial	IBS patients	Loperamide or psyllium (based on their predominant bowel function)	8 wk	Severity and frequency of abdominal pain/discomfort were significantly lower in the Carmint group than the placebo group	[41]
CHM	Codonopsis pilosulae (root) Agastaches seu pogostemi (whole plant) Ledebouriella sesloidis (root) Coicis lachryma-jobi (seed) Bupleurum chinensis (whole plant) Artemisia capillaries (whole plant) Atractylodis macrocephalae (rhizome) Magnolia officinalis (bark) Citrus reticulata (pericarp) Zingiber officinale (rhizome) Fraxinus spp. (bark) Poria cocos (sclerotium) Angelica dahurica (root) Plantago spp. (seed) Phellodendron spp. (bark) Glycyrrhiza uralensis (root) Paeonia lactiflora (root) Saussurea lappa (root) Coptidis spp. (rhizome) Schisandra spp. (fruit)	Randomized, double-blind, placebo-controlled trial	IBS patients	-	16 wk	Significant improvement in bowel symptom scores as rated by patients and by gastroenterologists Significant global improvement as rated by patients and by gastroenterologists Patients reported that treatment significantly ↓ the degree of interference with life caused by IBS symptoms	[42]
C-IBS formula	Lactulose Ulmus fulva (bark) Glycyrrhiza glabra (root) Avena sativa (bran)	A two arm, open-label, uncontrolled pilot study	Constipation-predominant IBS	-	2 wk	A 20% increase in bowel movement frequency ↓ in straining, abdominal pain, bloating, and global IBS symptom severity improvements in stool consistency well-tolerated	[54]
DA-IBS formula	Vaccinium myrtillus (fruit) Ulmus fulva (bark) Cinnamomum zeylanicum (bark) Agrimonia eupatoria (aerial part)	A two arm, open-label, uncontrolled pilot study	Diarrhea-predominant and alternating bowel habit IBS patients	-	2 wk	a small, but significant increase in bowel movement frequency ↓ in straining, abdominal pain, bloating, flatulence, and global IBS symptoms well-tolerated	[54]

Iberogast (STW 5)	Iberis amara (whole plant) Chelidonium majus (root) Silybum marianum (fruit) Melissa officinalis (leaf) Carum carvi (fruit) Glycyrrhiza glabra (root)	*In vitro*	Human intestinal mucosa/submucosa preparations human epithelial cell line T84 human enteric neurons			A dose-dependent increase in ion secretion in human tissue and T84 cells evoke an increased spike discharge in 51% of human submucous neurons	[45]
	Angelica sinensis (root) Matricaria recutita (flower) Mentha piperita (leaf)	*In vivo*	Wistar rats	-	Single dose	↑Afferent discharge to 5-HT and bradykinin dose-dependently	[47]
		Double-blind, randomized, placebo-controlled, multicentre trial	IBS patients	-	4 wk	Significant improvement in IBS symptom scale and abdominal pain scale in STW 5 group compared with placebo	[44]
STW 5-Ⅱ	Iberis amara (whole plant) Melissa officinalis (leaf) Carum carvi (fruit) Glycyrrhiza glabra (root) Matricaria recutita (flower) Mentha piperita (leaf)	Double-blind, randomized, placebo-controlled, multicentre trial	IBS patients	-	4 wk	Significant improvement in IBS symptom scale and abdominal pain scale in STW 5-Ⅱ group compared with placebo	[44]
Padma Lax	Aloe barbadensis A. ferox (extract) Jateorhiza palmata (root) Marsdenia condurango (bark), Rhamnus frangula (bark) Gentiana lutea (root) Inula helenium (rhizome) Terminalia chebula (fruit) Piper longum (fruit) Rhamnus purshiana. (bark) Rheum palmatum (root) Strychnos nux-vomica (seed) Zingiber officinale (root)	Randomized, double-blind, placebo-controlled trial	Constipation predominant-IBS patients	-	3 mo	Significant improvement compared to placebo in constipation, severity of abdominal pain, incomplete evacuation, abdominal distension and flatus/flatulence Significantly more Padma Lax patients compared to placebo rated the current treatment superior to previous therapies tried for IBS Laboratory parameters displayed no clinically significant changes	[43]
TXNG	Paeonia lactiflora (root) Atractylodes macrocephala (rhizome) Citrus reticulate (green unripe exocarp)	Prospective, randomized, double-blind, placebo-controlled trial	Diarrhea predominant-IBS patients	-	3 wk	↓IBS-related pain in the TXNG group compared with the placebo ↓Frequency and the duration of abdominal pain between the TXNG group and the placebo Improvement of IBS-related stool in form or appearance in the TXNG group in comparison with the placebo	[52]

	Allium macrostemon (bulb)					↓Stool frequency in the TXNG group compared with the placebo Improvement of stool passage (urgency or Feeling of incomplete rectal emptying) in the TXNG group compared with the placebo. Improvement in IBS-related diarrhea in the TXNG group compared to placebo No statistical difference in either the effective time of IBS-related pain or the effective time of IBS-related diarrhea between the two groups ↓IBS-related pain alleviation time and the IBS-related diarrhea alleviation time in the TXNG group compared to those in the placebo group	
TXYF	Atractylodes macrocephala (rhizome)	*In vivo*	Maternal separation-induced visceral hypersensitivity rats	-	2 wk	↓Pain threshold pressure and abdominal withdrawal reflex scores in a dose-dependent manner	[54]
	Paeonia lactiflora (root) Citrus sinensis (dried old peel) Ledebouriella divaricata (root)					↓ 5-HT levels in serum ↓Corticotrophin releasing factor concentrations in the brain Visceral hypersensitivity alleviation was dependent on the substance P expression in the colon mucosa	
		Randomized placebo-controlled trial	Diarrhea predominant-IBS patients	Miyarisan	4 wk	No significant difference between two groups in terms of the total efficacy or the scores of symptoms before and after treatment ↓The number of activated mast cells in the intervention	[53]
TCM	Atractylodes macrocephala (rhizome)	Randomized placebo-controlled trial	Diarrhea predominant-IBS patients	-	16 wk (8 wk drug administration +8 wk follow up)	No significant difference in the proportion of patients with global symptom improvement between the TCM and placebo groups at week 8 and at week 16	[51]
	Astragalus membranaceus (root) Paeonia lactiflora (peeled root, fried) Atractylodes chinensis (rhizome) Bupleurum chinense (root) Citrus reticulata (peel) Saposhnikovia divaricata (root) Paniculata (twigs) Punica granatum (rind) Portulaca oleracea (above-ground parts) Coptis chinensis (rhizome)					No difference in individual symptom scores and the quality-of-life assessment between the two groups at all time points	

CHM: Chinese herbal medicine; TXNG: Tong-xie-ning; TXYF: Tong-Xie-Yao-Fang; TCM: Traditional Chinese Medicine; 5-HT: Serotonin; spp.: Schisandra chinensis.

tract in male Wistar rats. Following the different doses of serotonin and bradykinin, the peak in afferent nerve discharge was always reduced after pretreatment with STW 5 compared to controls. The ramp distension of the intestinal loop stimulated a rise in intestinal afferent nerve discharge that was lower in the STW 5 pretreated group compared to controls[47]. STW 5 decreased acetylcholine- and histamine-induced contraction of guinea pig ileum. This was also true for extracts of some constituents of this compound formula including Mentha piperita leaf, Matricaria recutita flower and Glycyrrhiza uralensis root. Extract from Iberis amara, however, showed no spasmolytic action; on the contrary, it increased the basal resting tone and contraction of atonic ileal segments. These data may explain, at least in part, the clinically observed therapeutic efficacy of STW 5 in both hypotonic and spastic

dysmotility symptoms of IBS[48]. Another study on STW 5 and its components showed that STW 5 evoked a relaxation of the proximal stomach but increased antral motility whereas both effects are myogenic. The extracts of Angelica sinensis root, Matricaria recutita flower and Glycyrrhiza uralensis root mimicked the inhibitory effects in the proximal stomach whereas the extracts of Chelidonium majus, Melissa officinalis leaf, Carum carvi fruit and Iberis amara increased motility of the proximal stomach. All extracts increased motility in the antrum comparable to the effects of STW 5[49]. These data justify the differential effect of STW 5 which is a result of the combined actions of its individual components explaining the clinically observed therapeutic efficacy of STW 5 in both hypotonic and spastic dysmotility symptoms of IBS. Moreover, STW 5 reduced inflammation-induced alterations in ileum/jejunum segments. The effects were associated with a restoration of the disturbed acetylcholine-induced contraction, pathohistological protection and inhibition of tumor necrosis factor (TNF)-α[50].

A traditional Chinese medicine

Therapeutic efficacy of a traditional Chinese medicine (TCM) (Table 2) was investigated in patients with diarrhea-predominant IBS. There was no significant difference in the proportion of patients with global symptom improvement between the TCM and placebo groups. Moreover, there was no difference in individual symptom scores and the quality-of-life assessment between the two groups[51].

Tong-xie-ning

Tong-xie-ning (TXNG) is a traditional Chinese medicine composed of four different herbs. The efficacy of this preparation was evaluated in diarrhea predominant-IBS patients by a prospective, randomized, double-blind, placebo-controlled trial. IBS-related pain measured by the numeric pain intensity scale in the TXNG group, significantly decreased as compared with the placebo group. A total of 82.7% of the patients reported a reduction in IBS-related pain in the TXNG group compared with 39.3% in the placebo group. Furthermore, there was a significant reduction in the frequency and the duration of abdominal pain between the TXNG group and the placebo group. In addition, IBS-related stools improved in form or appearance in the TXNG group in comparison to the placebo group. The stool frequency was significantly decreased in the TXNG group compared with the placebo group. Moreover, stool passage (urgency or feeling of incomplete rectal emptying) in the TXNG group was significantly improved when compared with the placebo group. There was a 20.7% and 42.9% loss of appetite in the TXNG and placebo groups, respectively. An observable improvement in IBS-related diarrhea was seen in 86.2% of subjects in the TXNG group and 42.9% of subjects in the placebo group. There was no statistical difference in either the effective time of IBS-related pain or the effective time of IBS-related diarrhea between the two groups. However, the IBS-related pain alleviation time and the IBS-related diarrhea alleviation time in the TXNG group were markedly shorter than those in the placebo group[52].

Tong-Xie-Yao-Fang

Tong-Xie-Yao-Fang (TXYF) is a prescription in TCM, prepared from four herbs (Table 2). A study was done to compare the efficacy of this formulation with Myarisan, a probiotic formulation, in treating diarrhea-predominant IBS. No significant difference between the two groups in terms of the total efficacy or the scores of symptoms before and after treatment was found. In this study, the number of activated mast cells was decreased in the TXYF group after treatment, showing a significant difference as compared with that before treatment as well as with that in the Myarisan group after treatment. This result suggested that the mechanism of action of TXYF might be through adjustment of mast cells activation to decrease visceral hypersensitivity[53]. This product has been investigated in experimental visceral hypersensitivity models that showed a dose-dependent analgesic effect. It significantly decreased serotonin levels in serum and CRF concentrations in the brain. Moreover, it was found that visceral hypersensitivity alleviation by TXYF was dependent on substance P (SP) expression in the colon mucosa[54].

C-IBS and DA-IBS formulations

The efficacy and tolerability of C-IBS (Table 2), a formula designed to treat constipation-predominant IBS, and DA-IBS, a formula designed to treat diarrhea-predominant and alternating bowel habit IBS, were evaluated in an uncontrolled study. Ingestion of the DA-IBS formula was associated with a small, but significant, increase in bowel movement frequency. Reductions in straining, abdominal pain, bloating, flatulence, and global IBS symptoms were also demonstrated in patients using this formula. Subjects in the C-IBS group experienced a 20% increase in bowel movement frequency and significant reductions in straining, abdominal pain, bloating, and global IBS symptom severity, as well as improvements in stool consistency. Both formulas were well-tolerated[55].

CONCLUSION

In this paper, different herbal preparations investigated for the management of IBS and their possible mechanisms of action were reviewed. Among the single preparations, the most evidence for efficacy in IBS patients was found for essential oil of Mentha piperita. Some single preparations including Aloe vera, Curcuma xanthorriza, Fumaria officinalis showed no benefit in IBS. There are conflicting results for the efficacy of Hypericum perforatum in IBS. Among compound preparations, most studies had been performed with STW 5, a formula containing hydroethanolic extract of 9 herbs. Hopeful results come from the efficacy of this herbal preparation

in the management of IBS with different mechanisms of action such as anti-inflammatory, prosecretory activity, and affecting gastrointestinal motility. Because of multifactorial nature of the pathophysiology of IBS, it seems that compound preparations can be more efficacious than single ones. Despite the wide prevalence of IBS, there are few studies on the use of herbal medicine in IBS and most studies in this area have focused on the use of peppermint oil and STW 5; while it seems that more effective herbal preparations can be found. For example, in traditional Iranian medicine (TIM) many single and compound herbal preparations have been introduced for the management of various gut disorders such as IBS[56]. Among the herbal contents of this preparations, oleogumresin from Boswellia carterii, fruit of Trachyspermum amum, flower of Eugenia caryophyllata, gall of Quercus infectoria, seed of Nigella sativa, fruit of Cuminum cyminum, tabasheer (a hard, whitish, translucent substance extracted from the joints of different Bambusa species), and fruit of Cucurbita pepo can be stated[57-59]. There is evidence in modern phytotherapy for the beneficial effects of these plants in IBS. Boswellia carterii has shown anti-inflammatory[60,61] and immunomodulatory activity[62]. An *in vitro* study demonstrated that Trachyspermum copticum has a beneficial effect on intestinal microbiota by inhibiting the growth of potential pathogens[63]. Eugenia caryophyllata has anti-inflammatory[64], immunomodulatory[65], and antimicrobial[66] properties. Gall of Quercus infectoria possesses anti-inflammatory[67] and antibacterial[68] activity. Nigella sativa showed anti-inflammatory, antimicrobial, and immunomodulatory activity[69]. Antimicrobial properties were reported for Cuminum cyminum[70] and Cucurbita pepo[71]. However, more studies are required to get more conclusive results about the efficacy of these herbs in IBS.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.601

World J Gastroenterol 2012 February 21; 18(7): 601-608
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions in the colon

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Received: June 2, 2011 Revised: July 8, 2011
Accepted: July 15, 2011
Published online: February 21, 2012

Abstract

Epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions are well established biological events which have an important role in not just normal tissue and organ development, but in the pathogenesis of diseases. Increasing evidence has established their presence in the human colon during colorectal carcinogenesis and cancer invasion, chronic inflammation-related fibrosis and in the course of mucosal healing. A large body of evidence supports the role for transforming growth factor-β and its downstream Smad signaling, the phosphatidylinositol 3'-kinase/Akt/mTOR axis, the Ras-mitogen-activated protein kinase/Snail/Slug and FOXC2 pathway, and Hedgehog signaling and microRNAs in the development of colorectal cancers *via* epithelial-to-mesenchymal transition. C-met and Frizzled-7, among others, seem to be the principle effectors of mesenchymal-to-epithelial transition, hence have a role not just in mucosal regeneration but in the progression of colonic wall fibrosis. Here we discuss a role for these pathways in the initiation and development of the transition events. A better understanding of their induction and regulation may lead to the identification of pathways and factors that could be potent therapeutic targets. The inhibition of epithelial-to-mesenchymal transition using mTOR kinase inhibitors targeting the ATP binding pocket and which inhibit both mTORC1 and mTORC2, RNA aptamers or peptide mimetics, such as a Wnt5A-mimetic, may all be useful in both cancer treatment and delaying fibrosis, while the induction of mesenchymal-to-epithelial transition in induced pluripotent stem cells may enhance epithelial healing in the case of severe mucosal damage. The preliminary results of the current studies are promising, but more clinical investigations are needed to develop new and safe therapeutic strategies for diseases of the colon.

Key words: Epithelial-to-mesenchymal transition; Mesenchymal-to-epithelial transition; Colorectal cancer; Fibrosis; Mucosal healing

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Sipos F, Galamb O. Epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions in the colon. *World J Gastroenterol* 2012; 18(7): 601-608 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/601.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.601

INTRODUCTION

Human colonic diseases are some of the most common diseases worldwide, and their incidence is increasing[1,2]. Although the diversity of the etiologic and pathophysiologic factors of colonic diseases is very wide, there exist two biological processes whose presence is indispensable in the progression or healing phase of these conditions.

One of these processes is the epithelial-to-mesenchymal transition (EMT), which has a significant role in the

development of the human body. EMT is also involved in the initial step, acquisition of migratory and invasive capability of colorectal cancer (CRC), and even has an important role in tissue fibrosis[3].

The other process is a reverse phenomenon, namely the mesenchymal-to-epithelial transition (MET). MET is also essential for normal tissue and organ development, but it is also involved in colorectal carcinogenesis, and it seems to have an important role in colonic mucosal regeneration[4,5].

The induction and regulation of these complex, reversible biological programs are not fully understood. Therapeutically, the influence of EMT and MET is recognized, but, unfortunately, the exact description of these biological phenomena is still missing. As the understanding of the steps of EMT and MET is of great clinical importance, and, at the same time, data about their complex induction and regulation, as well as their role in the pathogenesis of colonic diseases are scarce in the scientific literature, we aimed to summarize the current knowledge in this review.

EPITHELIAL-TO-MESENCHYMAL TRANSITION

EMT is a physiological mechanism which is present during development, including mesoderm formation and neural tube formation, and is also encountered in several pathological situations, such as renal interstitial fibrosis, endometrial adhesion, and cancer metastasis[3].

Cells that undergo EMT exhibit dramatic shape changes during which they can lose many of their epithelial characteristics, such as the loss of apico-basal polarity and cell adhesion, the repression of E-cadherin, occludin, tight junction protein 1, or cytokeratin expression, and increased cell mobility[6]. At the same time, elevated expression of tyrosine kinases or their activation, upregulation of N-cadherin, vimentin, fibronectin, zinc-finger domain proteins (SNAI1/SAIL, SNAI2/SLUG, ZEB2/SIP1), and matrix metalloproteinases, as well as basic helix-loop-helix domain protein Twist1 expression are often linked to a mesenchymal-like phenotype[7,8].

EMT INDUCTION AND REGULATION

Several oncogenic pathways (i.e., peptide growth factors, Src, Ras, integrin, Wnt/β-catenin, Notch) may induce EMT (Figure 1).

Transforming growth factor-β (TGF-β) is a potent inducer of EMT. TGF-β directly activates the expression of transcription factors such as SNAI1/2, Twist and ZEB1/2. These factors are the key regulators of the EMT program[9].

The Src SH3 and SH2 domains cooperate with extracellular signal-regulated kinase (ERK), MEK (ERK kinase), myosin light chain kinase (MLCK), and Rho-dependent protein kinase (ROCK) signaling to accumulate phosphomyosin at the colon cancer cell periphery and promote a mesenchymal-like phenotype[6]. It was recently shown that actomyosin contractility is a key determinant of EMT[6].

Ras-mitogen-activated protein kinase has been shown to activate two related transcription factors, namely Snail and Slug, both of which are transcriptional repressors of E-cadherin, and their expression induces EMT[10].

Recently, activation of the phosphatidylinositol 3'-kinase (PI3K)/Akt axis has emerged as a central feature of EMT[11]. Activation of PI3K/Akt signaling is associated with growth and progression of CRC. It was reported that the mTOR kinase, a downstream effector of PI3K/Akt signaling, regulates tumorigenesis in CRC[12,13]. Increased expression of mTOR, Raptor, and Rictor mRNA was noted with advanced stages of CRC, suggesting that mTOR signaling may be associated with CRC progression and metastasis[14]. Gulhati *et al*[14] showed that the inhibition of mTORC1 and mTORC2 attenuated migration and invasion of colon cancer cells concomitant with altered cytoskeletal rearrangement and decreased activation of RhoA and Rac1. The inhibition of mTORC1 and mTORC2 induces changes reminiscent of mesenchymal-to-epithelial transition, and it was also shown that the establishment of metastasis *in vivo* was completely abolished upon targeted inhibition of mTORC1 and mTORC2. Based on these results, one may propose that mTORC1 and mTORC2 regulate motility of colon cancer cells *via* RhoA and Rac1 signaling[11]. Twist, another transcription factor, has been shown to possibly induce EMT too, and is also implicated in the regulation of metastasis[7,8].

Expression of FOXC2, an important element of embryonic development is supposed to induce EMT and regulate metastasis. In addition, the expression of FOXC2 is induced when epithelial cells undergo EMT by Snail, Twist, Goosecoid, and TGF-β1[15-17].

The majority of human colon cancers carry mutations that lead to the activation of Wnt signaling, a pathway that also has a pivotal role in intestinal stem cell biology[18]. Despite the underlying genetic background, cells within individual tumors display differential Wnt signaling, suggesting further regulation by the microenvironment. A local loss of basement membrane at the invasive edge has been suggested to expose cancer cells to a different microenvironment, which promotes Wnt signaling (nuclear β-catenin expression), EMT-like changes and loss of differentiation[19]. Type 1 collagen is a known component of the microenvironment at the host–tumor interface in CRC[20], and is more highly expressed in tumors displaying infiltrative growth compared with those with expansive growth[21]. Type 1 collagen also reduces CDX2 expression, an early marker of epithelial commitment, in human CRC cell lines *in vitro*[20], and enhances tumorigenicity in human CRC cells in xenografts[22]. Integrin α1β2 has a central role in type 1 collagen-induced EMT[23].

Hedgehog signaling cascade cross-talks with Wnt, epithelial growth factor/fibroblast growth factor, and TGFβ/Activin/Nodal/bone morphogenic protein signaling cascades, which are implicated in EMT through

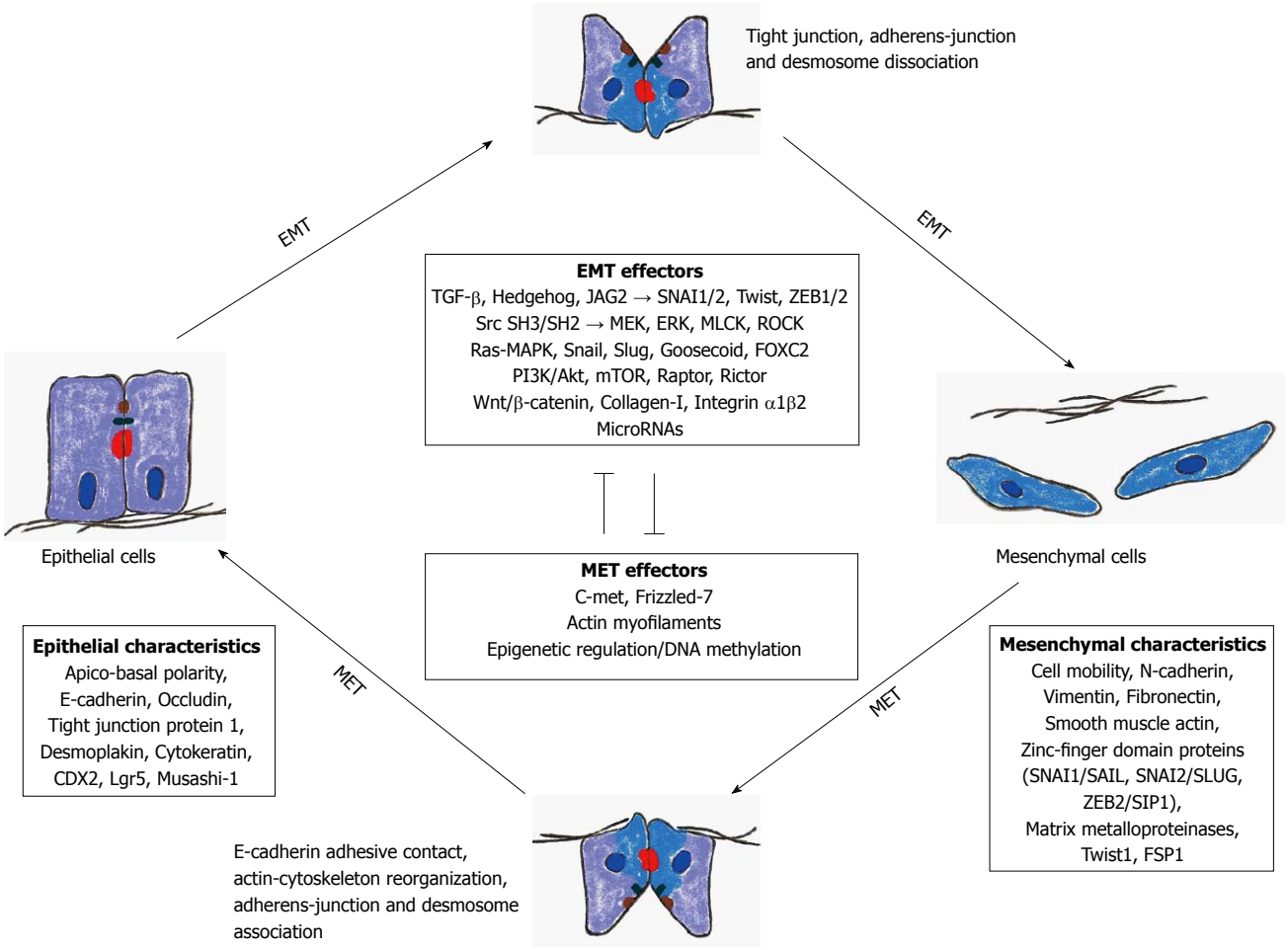

Figure 1 Schematic view of the effector pathways associated with epithelial-to-mesenchymal transition and mesenchymal-to-epithelial transition. EMT: Epithelial-to-mesenchymal transition; MET: Mesenchymal-to-epithelial transition; TGF-β: Transforming growth factor-β; MAPK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase; MEK: MAPK/ERK kinase; MLCK: Myosin light chain kinase; ROCK: Rho-dependent protein kinase.

E-cadherin repression[8,24,25]. Although the Hedgehog signaling cascade induces the SNAI1 upregulation, there is no evidence for its direct SNAI1 transcriptional activation. On the other hand, Hedgehog signals induce JAG2 upregulation, and TGF-β1 secretion to promote motility and invasiveness of cancer cells[26]. JAG2 signaling induces transition of the Notch receptor to the Notch intracellular domain (NICD). NICD is then associated with the CSL transcription factor in the nucleus to induce SNAI1 upregulation[27]. TGF-β1 activates the TGF-β receptor for nuclear factor-κB-mediated transcriptional upregulation of ZEB1 and ZEB2 (zinc-finger transcription factors)[28], and also for the SMAD-Sp1-mediated transcriptional upregulation of mesenchymal markers, such as vimentin. Together these facts indicate that the Hedgehog signals indirectly induce EMT through the upregulation of multiple EMT regulators *via* Notch and TGF-β signaling cascades[29].

The 20-22 bp nucleotide noncoding RNAs, the microRNAs (miRNAs), regulate gene expression at post-transcriptional levels. Earlier profiling experiments have identified cohorts of miRNAs whose levels undergo significant changes upon TGF-β induced EMT, suggesting possible involvements of miRNAs in this process[30]. The miR 200 family has been linked to inhibition of EMT (promotion of the epithelial phenotype) through inhibition of ZEB1/2, known transcriptional repressors of the human E-cadherin gene[31].

In LIM 1863 colon carcinoma cells, the upregulation of miR-21 and miR-31 had been reported during EMT[32]. Overexpression as well as inhibition experiments support the contributions of both miR-21 and miR-31 not only in the TGF-β-induced morphological changes, but also in cell motility and invasion. It was also shown that T lymphoma and metastasis gene 1 (*TLAM1*) is a direct target of both miR-21 and miR-31, and that the suppression of *TLAM1* is important for the pro-migration and invasion activities of miR-21 and miR-31. Based on these results, miR-21 and miR-31 were indentified as positive regulators of the EMT in colon carcinoma cells[32].

Interestingly, it was recently shown[33] that nicotine enhanced the expression level of fibronectin, an important EMT-related maker, in a dose-dependent manner. Furthermore, an α7-nicotinic acetylcholine receptor antagonist and siRNA reversed the nicotine-enhanced fibronectin expression in both SW480 and DLD-1 cells[33].

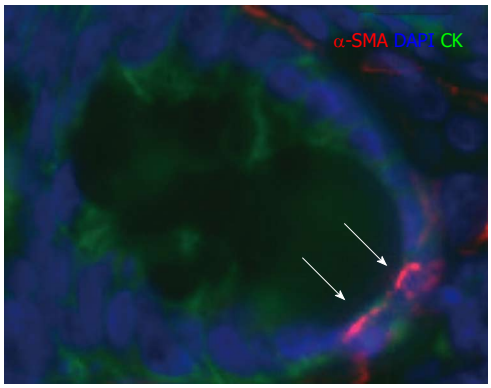

Figure 2 Chronic, fibrotizing phase of inflammatory bowel disease. The arrowed cytokeratin (CK) positive epithelial cells in a colonic crypt show α-smooth muscle actin (α-SMA) positivity. The initiation of the epithelial-to-mesenchymal transition is possible. (α-SMA: Red; CK: Green; nuclear counter-staining: Blue; fluorescence immunohistochemistry, taken by virtual microscope). DAPI: 4',6'-diamidino-2-phenylindole hydrochloride.

ROLE OF EMT IN PATHOLOGICAL CIRCUMSTANCES

The switch between epithelial and mesenchymal phenotypes occurs during the advanced stages of cancer development. As a general rule, epithelial cancer cells that have undergone EMT are thought of as being more migratory, which may contribute to the invasive or metastatic phenotype.

In adult organisms, it has been proposed that restrictive mechanisms repress EMT and MET[34]. During tumor development, these mechanisms appear to fail, allowing EMT as described in metastasis generation[35]. Colonic stroma tissue, including subepithelial lymphoid aggregates surrounding the cancer cells, plays an important role in both EMT regulation and tumor behavior. Mesker *et al*[36] analyzed the expression of markers involved in pathways related to stroma production and EMT (β-catenin, TGF-β-R2, Smad4) in high-risk CRC patients, and found that patients with high stroma and Smad4 loss are at high risk. The anti-EMT effect of Smad4 was also proven in colon carcinoma cells[37].

Besides colon cancer cell migration and invasion, EMT is also involved in organ fibrosis. The epithelium has been proposed to be a significant source of matrix-producing fibroblasts and of myofibroblasts[38]. Tissue accumulation of myofibroblasts shows strong correlation with the severity and progression of colonic fibrosis[39]. TGF-β1 has been long known as the chief inducer not just of EMT, but fibrosis, and myofibroblast generation. Accordingly, receptor Smads (Smad2 and particularly Smad3), the direct targets of the activated TGF-β receptor have been implicated as critical mediators in fibrogenesis and EMT[40,41] (Figure 2).

THERAPEUTIC ASPECTS OF EMT

Inhibition of EMT would be an ideal choice for the treatment of CRC, as well as colonic fibrosis developed on the basis of chronic inflammation. Despite the incontrovertible rationale for treating diseases belonging to PI3K/Akt/mTOR signaling with rapamycin, clinical results have been disappointing. One proposed mechanism of resistance to rapamycin arises from its inability to inhibit mTORC2[14,42]. The findings of Gulhati *et al*[11] support a role for elevated mTORC1 and mTORC2 activity in regulating EMT and metastasis of CRC. Taken together with previous results, showing that both mTORC1 and mTORC2 contribute to CRC tumorigenesis[14], it may be hypothesized that the inherent redundancy in functions of both complexes may allow mTORC2 to compensate for loss of mTORC1 activity upon rapamycin treatment, thereby leading to rapamycin resistance. Based on these data, Gulhati *et al*[11] provides the rationale for including mTOR kinase inhibitors targeting the ATP binding pocket, which inhibit both mTORC1 and mTORC2 more completely, as part of the therapeutic regimen for treating CRC patients.

miRNAs targeted to mRNAs, encoding stem cell signaling components or EMT regulators, are also potent drug targets. miRNAs inducing proliferative, anti-apoptotic, pro-angiogenic, or pro-metastatic effects on tumor cells could be downregulated for cancer therapy, while those with proapoptotic, anti-angiogenic, or anti-metastatic effects could be applied for synthetic miRNA[43,44]. An RNA aptamer is a short RNA oligonucleotide with a stable 3D structure[44]. RNA aptamers binding to the extracellular region of Patched1 could be utilized for drug delivery to cancer cells with Hedgehog signaling activation. RNA aptamers binding to the cytoplasmic region of Smoothened, and those binding to Fused or GLI1 could be utilized as Hedgehog signaling inhibitors. Peptide mimetics, resembling Wnt and fibroblast growth factor family members, have been developed[45,46]. Because Wnt5A is involved in the non-canonical signaling cascade for the induction of EMT partly through SNAI1 upregulation[47-50], a Wnt5A mimetic is able to suppress invasion and metastasis of cancer cells. On the other hand, great care should be taken before clinical application of these technologies, as the miRNA and siRNA off-target effects are serious problems.

MESENCHYMAL-TO-EPITHELIAL TRANSITION

Mesenchymal-to-epithelial transition is a reversible biological process that involves the transition from motile, multipolar or spindle-shaped mesenchymal cells to polarized epithelial cells. MET, just like EMT, also takes place during normal development in processes such as somitogenesis, kidney development, cardiogenesis, hepatogenesis and celomic cavity formation[4,51,52], moreover MET occurs in cancer metastasis, induced pluripotent stem cell reprogramming and mucosal healing[5].

While the mechanism in which MET occurs during each organ morphogenesis is similar, in that epithelium-associated genes are upregulated and mesenchymal ones

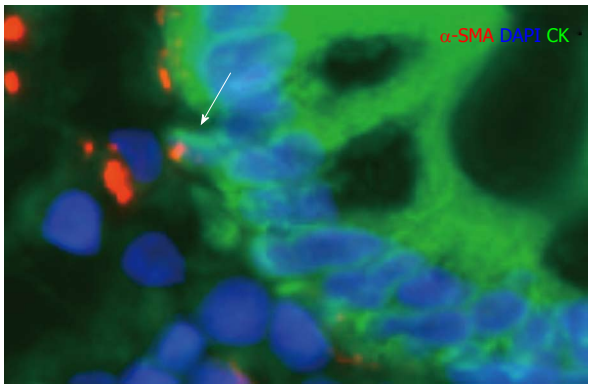

Figure 3 Regenerative phase of ulcerative colitis. The arrowed α-SMA positive pericryptic cell shows cytokeratin (CK) expression. The presence of the mesenchymal-to-epithelial transition is possible. (α-SMA: Red; CK: Green; nuclear counter-staining: Blue; fluorescence immunohistochemistry, taken by virtual microscope). DAPI: 4',6'-diamidino-2-phenylindole hydrochloride.

are downregulated, each process has a unique signaling pathway to induce MET and related changes in gene expression.

Though our knowledge about the mechanism and regulation of EMT in the colon is increasing nowadays, the reverse phenomenon, MET, is still not a well understood mechanism (Figure 1).

MET INDUCTION AND REGULATION

MET during carcinogenesis has been shown to be induced by the c-met proto-oncogene[53-55]. C-met, also known as hepatocyte growth factor receptor, is a receptor tyrosine kinase for hepatocyte growth factor and its increased expression leads to epithelial differentiation[56,57]. In addition to epithelial specification by C-met, 5-azacytidine, a DNA methyltransferase inhibitor with broad spectrum epigenetic effects, has been used to induce MET *in vitro*[58]. Recent research on the transcription factor Snail has been linked to aberrant DNA methylation of the epithelial specific E-cadherin promoter in association with EMT, and stable RNA interference of Snail expression in carcinoma cell lines induced a complete MET[59-61].

Frizzled-7 (FZD7) is necessary for MET in the LIM1863-Mph CRC model. The loss of FZD7 in cancer cells results in the persistence of a mesenchymal state (increased SNAI2/decreased E-cadherin)[62]. Moreover, FZD7 is also required for migration of the LIM1863-Mph monolayer cells. This suggests that FZD7 induced either migratory or epithelialization events depending on the context.

The role of DNA methylation in MET induction and induced pluripotent stem (iPS) cell reprogramming had been also highlighted[63].

In the inflamed colon, signs of MET can be detected in the subepithelial lymphoid follicles[64-66]. These data suggest that migrating stem cells undergoing MET may be sensitive to the chemokine/cytokine milieu of the inflammatory environment.

ROLE OF MET IN PATHOLOGICAL CIRCUMSTANCES

Regarding the colon, the need for MET is high when severe mucosal damage is present. In inflammation, MET can also be altered because mesenchymal stem cells are mobilized to the site of injury and consequently subjected to the inflammatory response[67]. Bone marrow-derived stem cells could differentiate into mature-appearing epithelial cells in response to tissue damage[68]. It was recently published that versican, a large chondroitin sulfate proteoglycan, mediates MET[69]. The results of Hirose *et al*[70] indicate that versican can bind specific chemokines through its chondroitin sulfate chains and by doing so, it tends to downregulate the chemokine function. This raises the possibility that versican is a potent regenerative factor in the colonic mucosa.

In the inflamed colon, the presence of CDX2- and cytokeratin-positive subepithelial cells in the marginal zone of subepithelial lymphoid follicles also suggests that MET may have a role in colonic mucosal regeneration[64]. Presumably the mesenchymal cells committed to an epithelial fate are sensitive for the regeneration-associated paracrine cytokine and chemokine environment in the inflammatory stroma (Figure 3).

THERAPEUTIC ASPECTS OF MET

Induced pluripotent stem cells can be derived from somatic cells by the induction of a small number of genes, like POU5F1, MYC, KLF4 and SOX2[71-74]. Originating from an individual's own tissue, iPS cells offer considerable therapeutic promise, avoiding both immunologic and ethical barriers to their use. Upon induction, mouse fibroblasts must undergo MET to successfully begin the initiation phase of reprogramming. Epithelial-associated genes (i.e., *E-cadherin*, *Claudin-3*, *-4*, *-7*, *-11*, *occludin*, epithelial cell adhesion molecule, *Crumbs homolog 3*) were all upregulated before the turning on of Nanog, a key transcription factor in maintaining pluripotency. Additionally, mesenchymal-associated genes like Snail, Slug, Zeb-1/2, and N-cadherin were downregulated[75].

Epigenetic changes, DNA methylation, seems to be also involved in MET regulation, which may also have therapeutic significance[63].

CONCLUSION

According to our current knowledge, both EMT and MET are highly significant biological events, not just in physiological, but in pathological circumstances. A better understanding of their induction and regulation may lead to the identification of pathways and factors that can be potent therapeutic targets.

The inhibition of EMT seems to be a useful technique to avoid CRC cell invasion and metastasis genera-

tion, and chronic inflammation-related colonic wall fibrosis may be also delayed, or even reversed.

The induction of MET is also of major clinical importance. In the case of severe mucosal damage, the time for complete epithelial regeneration may be reduced by enhancing the phenotype change of mesenchymal stem cells to epithelial cells.

The preliminary results of ongoing studies are promising, but more investigations are needed to develop new therapeutic strategies that can be safely used in the near future.

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doi:10.3748/wjg.v18.i7.609

World J Gastroenterol 2012 February 21; 18(7): 609-615
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Adamantiades-Behcet's disease-complicated gastroenteropathy

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Received: July 2, 2011 Revised: October 12, 2011
Accepted: December 31, 2011
Published online: February 21, 2012

Abstract

Adamantiades-Behcet's disease (ABD) is a chronic, relapsing, systemic vasculitis of unknown etiology. It is more prevalent in populations along the ancient Silk Road from Eastern Asia to the Mediterranean Basin, and most frequently affects young adults between the second and fourth decades of life. ABD-complicated gastroenteropathy is a significant cause of morbidity and mortality, with abdominal pain as the most common symptom. The ileocecal region is affected predominantly, with ulcerations that may lead to penetration and/or perforation, whereas other parts of the gastrointestinal system including the esophagus and stomach can also be affected. Endoscopy is useful to locate the site and extent of the lesions, and tissue biopsy is often warranted to examine the histopathology that is often suggestive of underlying vasculitis of small veins/venules or, alternatively in some cases, nonspecific inflammation. Bowel wall thickening is the most common finding on computed tomography scan. Treatment is largely empirical since well-controlled studies are difficult to conduct due to the heterogeneity of the disease, and the unpredictable course with exacerbation and remission. Corticosteroids with or without other immunosuppressive drugs, such as cyclophosphamide, azathioprine, sulfasalazine, tumor necrosis factor α antagonist or thalidomide should be applied before surgery, except in emergency.

Key words: Adamantiades-Behcet's disease; Gastroenteropathy; Ulceration; Vasculitis

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Wu QJ, Zhang FC, Zhang X. Adamantiades-Behcet's disease-complicated gastroenteropathy. *World J Gastroenterol* 2012; 18(7): 609-615 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/609.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.609

INTRODUCTION

Adamantiades-Behcet's disease (ABD) is a systemic inflammatory vasculitis of unknown etiology, characterized by relapsing episode of oral aphthous ulcers, genital ulcers, cutaneous and ocular lesions and other manifestations, including vascular, neurological and gastrointestinal involvements[1].

ABD usually occurs between the second and fourth decades of life. Recent epidemiological surveys indicate that male and female are equally affected in ABD[2-4]. ABD is particularly prevalent in ancient "Silk Route" populations from Far East to Middle East to Mediterranean, albeit it has global distribution. The marked geographic predilection of ABD can be explained by its genetics background and/or environmental triggers[1].

ABD-complicated gastroenteropathy (ABD-GE) varies in different populations, being much more frequent in the Far East than in the Middle East and Mediterranean. Several nationwide surveys and large-scale case series

show that ABD-GE is frequent in Japan (16%)[5], Germany (12%)[6], Iran (7.4%)[7], Korea (7.3%)[8] and China (6.5%)[9], but rare in Turkey (1.4%)[10].

The clinical manifestation of ABD-GE may also vary greatly, from mild symptom to life-threatening complications, including perforation, infarction, and massive bleeding resulting from vasculitis and/or thrombosis. Prompt treatment with corticosteroid plus immunosuppressive agents rather than surgery can alleviate the clinical symptoms and improve prognosis of ABD-GE patients. According to the International Study Group Criteria for ABD, diagnosis of ABD requires the presence of recurrent oral ulcers and two of the followings: Genital ulcers, typical eye lesions, typical skin lesions, and positive pathergy test[11]. However, in clinical practice, it is often challenging to make prompt and correct diagnosis when gastroenteropathy is presented as the initial or predominant manifestation in ABD patients, and sometimes is misdiagnosed as inflammatory bowel disease or other disorders. This paper systematically reviews the clinical manifestation and treatment of ABD-GE for the purpose of better managing this life-threatening complication.

MANIFESTATIONS OF GASTROINTESTINAL COMPLICATION

Among the 136 reported gastrointestinal (GI) endoscope cases in the Japanese patients with ABD who required surgery, abdominal pain was found in 92%, abdominal mass in 21%, and melena in 17%[12]. Almost all Japanese juvenile ABD-GE manifested with abdominal pain, bloody stool, high fever, and anal lesions[13]. The main manifestations of ABD-GE are described as follows (Table 1).

Mouth lesions

Almost all patients have recurrent oral ulceration. This is often the first symptom and occurs long before other manifestations appear. Minor aphthous ulcer (< 10 mm in diameter) is the most common type (85%), whereas major (> 10 mm in diameter) and herpetiform ulcers are less frequent. Lesions are usually painful, occur as single ulcer or in crops, with a round or oval appearance with erythematous border (pouched out), covered with grayish-white pseudo-membrane or a central yellowish fibrinous base. They usually heal without scarring. The most commonly involved sites are gingival and buccal mucosa, tongue and lips, although ulcers can also appear in the soft and hard palate, pharynx and tonsils. Histological findings suggest vasculitis with monocyte and lymphocyte infiltration at the early stage of the disease and neutrophils at later stage. Other disorders such as fibrinoid necrosis, endothelial swelling, and perivascular infiltration can also occur in ABD. Although aphthae is often multiple and occurs more frequently in ABD, they are indistinguishable from those of recurrent oral ulcers due to other causes, such as malnutrition, viral infections, inflammatory bowel disease, and Reiter's syndrome[14].

Esophageal lesions

Symptomatic esophageal involvement is considered very rare[15,16], usually associated with other GI manifestations[15-19]. Only 45 retrospective case reports of esophageal involvement in ABD were found in literature. Most of the patients had symptoms severe enough to warrant upper gastrointestinal endoscopic examination[20]. The middle or lower part of the esophagus is often involved, causing substernal pain, dysphagia, and in rare case, hematemesis. Morphological findings include erosion, aphthous, linear or perforating ulcer (Figure 1), and widely distributed esophagitis[15,16]. Occasionally, there may be perforation, dissection, penetration, fistula formation, or pharyngeal stenosis causing dysphagia and dyspnea[21-25]. Esophageal varices may occur in association with superior vena cava obstruction, and portal hypertension due to portal or hepatic vein thrombosis[26]. Although some prospective studies suggest that the prevalence of asymptomatic esophageal involvement in ABD is rather high, it does not lead to severe abnormalities, and esophagogastro-duodenoscopy is recommended only in those with clinical symptoms[20,25]. Histological findings reveal nonspecific inflammation with lymphocytic or neutrophilic infiltration rather than vasculitis. The esophageal lesions in ABD often respond to corticosteroid instead of treatment with a proton pump inhibitor[27,28]. Viral or candida esophagitis should be excluded before initiation of corticosteroid.

Gastric and duodenal lesions

The stomach is the least frequently involved part of ABD, and aphthous ulcers are the most common findings. It was reported that 45% of Taiwanese patients with ABD had gastric and/or duodenal ulcers[29]. There may be pyloric stenosis due to edematous hypertrophy of pyloric ring[30,31] or Dieulafoy's ulcer[32]. ABD patients with gastric and/or duodenal lesions usually present with epigastric pain. Duodenal ulcers may be resistant to antiulcer medications[31], but whether corticosteroid should be used is unclear. Such ulcers can resolve spontaneously, and corticosteroid may inhibit its healing process, so that the effect of corticosteroid is uncertain[33]. There was also report suggesting that *Helicobacter pylori* treatment could diminish the oral and genital ulcers of ABD patients[34].

Small and large intestinal lesions

There are two forms of intestinal involvement: small vessel disease with mucosal inflammation causing ulcer (Figure 2) and large vessel disease resulting in intestinal ischemia and infarction. Mucosal ulceration is most commonly seen in the ileocecal region, and it was found in 88% patients in a study[35], usually on the antimesenteric side[36], followed by involvement of other part of colon, but rarely rectum or anus. The ulcers may be aphthous or, alternatively, deep and round with pouched-out appearance. Longitudinal ulcers are rare. The ulcers may penetrate the intestinal wall, resulting in perforation (often at multiple sites), fistula formation, or bleeding. Ulcers may resolve with medical therapy, but recur later[37]. In

Table 1 Summary of major gastrointestinal manifestations of Adamantiades-Behcet's disease reported in the literatures

Ref.	Type of manifestation	Symptom	Frequency	Complication	Therapy	Outcome
Zhang *et al*[9] Tursen *et al*[10]	Recurrent oral ulcer	Painful ulcer	Almost 100%	Rare	Topical measures	Excellent
Mori *et al*[15] Yi *et al*[16]	Esophageal ulcer/ esophagitis	Substernal pain, dysphagia	Very rare	Very rare	Corticosteroid	Excellent
Ning-Sheng *et al*[29]	Gastric ulcer and/or duodenal ulcer	Epigastric pain	Variable (very rare to 45%)	Very rare	Uncertain	Excellent
Choi *et al*[35] Köklü *et al*[45]	Small and/or large intestinal ulcer	Abdominal pain, hematochezia	Variable (1.4% up to 16%)	Rare (perforation, massive bleeding)	Sulfalazzine, corticosteroid azathioprine, tumor necrosis factor α antagonist, thalidomide	Good
Bayraktar *et al*[53] Chubachi *et al*[54]	Large artery aneurysm/thrombosis in abdomen	Infarction, ischemia	Very rare	Very rare	Corticosteroid azathioprine, cyclophosphamide	Poor
Bismuth *et al*[56]	Large vein thrombosis in abdomen	Budd-Chiari syndrome	Very rare	Very rare	Corticosteroid azathioprine	Poor

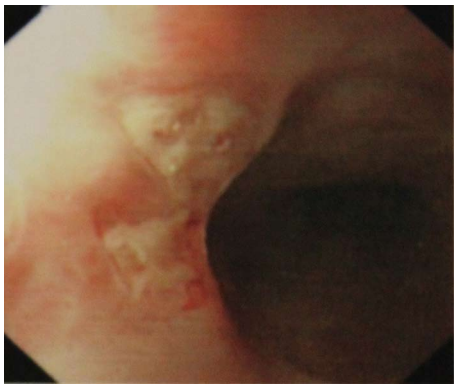

Figure 1 Esophageal involvement of Adamantiades-Behcet's disease. Endoscopic examination reveals two small punched-out, active ulcerations in the middle esophagus of the patient.

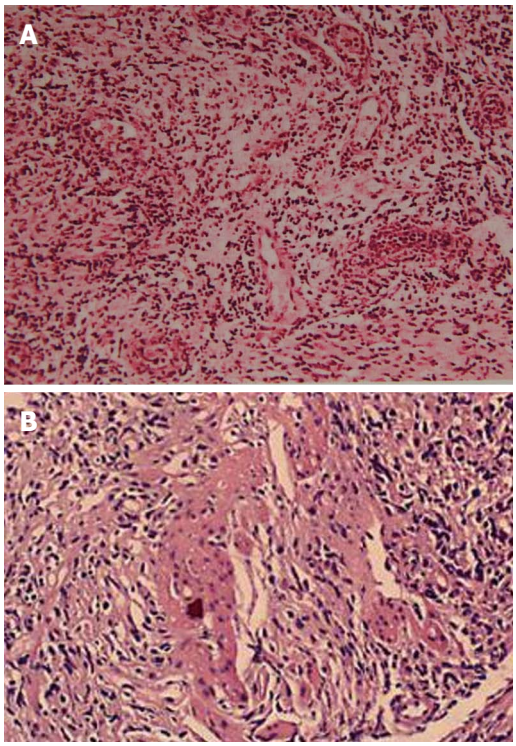

Figure 2 Ileum biopsy reveals lymphocyte-predominant inflammatory cells infiltration (A: HE, × 150) and fibrinoid vasculitis (B: HE, × 200). HE: Hematoxylin and eosin.

those ABD patients who underwent surgery while their disease was still active, the lesions tended to recur at the anastomotic site, especially along the ileal side of ileocolic anastomoses[38]. Although it is rare, there are also case reports of toxic megacolon without any precipitating factors[39] and severe proctitis with rectovaginal fistula[40].

Barium studies demonstrate single or multiple discrete ulcers with considerable thickening of the surrounding mucosal folds[18,38]. Other nonspecific findings include cecal contraction, widening of the ileocecal valve, fold thickening in the terminal ileum, and an apparent ileocecal mass with ulceration[41,42]. Barium study is a noninvasive and helpful examination to locate the GI lesions of ABD, but has limitations in defining the characteristics of lesions, and may not be applied to some cases of intestinal ABD that are inclined to penetrate the intestinal wall. In such situation, computed tomography (CT) scan and magnetic resonance imaging (MRI) will yield valuable information. CT scan shows concentric or uneven bowel wall thickening that remarkably enhances after administration of contrast agent[18,38]. This enhancement suggests stasis of blood due to vasculitis or perivasculitis affecting mainly the veins and venules of the ulcerous submucosa[38], which is supported by histopathological findings with diffuse vascular dilatation and perivascular lymphocyte infiltration. Polypoid lesions, sometimes with central ulceration, should be differentiated from malignancy by their marked contrast enhancement and involvement of both the terminal ileum and cecum. Complications such as bowel perforation and peritonitis, occur more frequently in those with a thickened bowel wall and severe perienteric infiltration than in those with polypoid lesions. MRI is helpful in revealing bowel wall thickening and increased contrast enhancement as well as extraluminal manifestations such as mesenteric infiltration around the involved bowel[43].

Colonoscopy is usually warranted for patients with clinical symptoms. The most common colonoscopic findings are localized single or multiple ulcers in the ileocecal

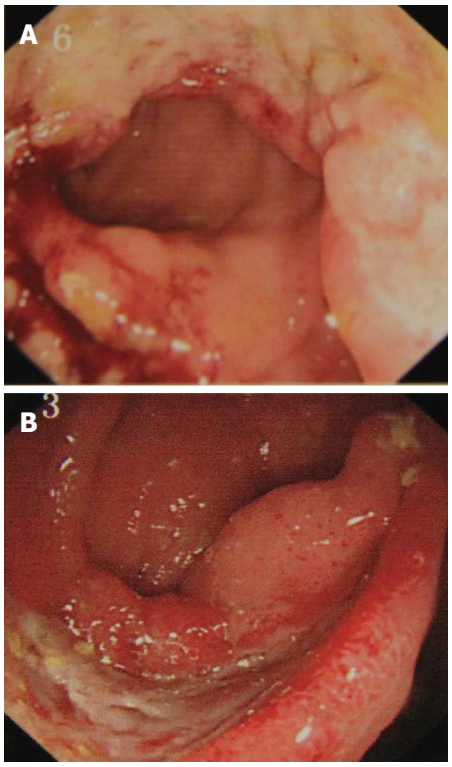

Figure 3 Typical colonoscopic findings of intestinal Behçet's disease. A: A single, large, and oval-shaped ulceration in the ileocecal region; B: A single annular-shaped ulcer in the ileum.

region (Figure 3), and only 4% has a diffuse distribution of lesions[44,45]. The characteristics of ileocecal ulcer of ABD also vary in different regions, with multiple superficial ulcers localized prominently in the terminal ileum in Turkish patients[46], and single deep ulcer with distinct borders in the Far East[44,45]. The colonic ulcers have been classified as volcano, geographic, and aphthous types. Volcano-type ulcers are defined as well-demarcated penetrating ulcers with nodular margins, converging folds, or pseudopolyps. Geographic-type ulcers are defined as shallow ulcers with sharp edges, and aphthous-type ulcers are small, pouched-out shallow ulcers similar to oral aphthous ulceration. Volcano-type ulcers have the least favorable response to medical treatment, and are at more frequent requisition for surgery, and are more likely to recur than the other two types[47]. Therefore, volcano-type ulcers should be treated vigorously and followed up closely. In rare cases, there are vesicular lesions or single rectal ulcers with otherwise normal mucosa[48].

Wireless capsule endoscopy is also useful in the investigation of gastrointestinal symptoms in ABD. It is particularly helpful for those patients in whom conventional investigations (such as esophago-gastro-duodenoscopy, colonoscopy, and barium study) are normal or fail to account for symptoms and signs[49,50].

The diagnosis of ABD is mainly based on the typical clinical findings, no specific serum markers or pathological features are available. Diagnosis of ABD-GE can be made if there is a typical oval-shaped large ulcer in the terminal ileum, or there are ulcerations or inflammation in the small or large intestines, and clinical findings meet the diagnostic criteria of ABD. Crohn's disease (CD), tuberculosis, vasculitis and other diseases that mimic ABD-GE should be excluded before diagnosis of ABD-GE is established[51]. Like CD, ABD-GE manifests as discrete ulcers and discontinuous bowel involvement with relative sparing of the rectum. The two diseases share extraintestinal manifestations, such as uveitis and arthritis. Unlike CD, ABD-GE is characterized by vasculitis of the small veins and venules with deep ulcerations, generally without granulomas or cobblestoning. However, both diseases may have chronic nonspecific inflammation with normal intervening mucosa. Perforation is more common in ABD-GE than in CD since the latter is characterized by intense fibrosis. Scalloping, ulceronodular patterns, and abscess formation are not observed in ABD-GE[46]. Unlike ulcerative colitis, colonic ABD consists of multiple aphthous ulcers with preservation of haustra and involvement primarily of the terminal ileum and proximal colon. Both intestinal tuberculosis and ABD-GE mainly manifest ulcerations in the ileocolonic region. Unlike ABD-GE, intestinal tuberculosis is characterized by granuloma with positive acid-fasting staining. Systemic lupus erythematosus (SLE) and ABD also share some common features, such as oral ulcerations, arthritis, and central nervous system involvement. Unlike ABD, SLE presents serum markers, such as anti-nuclear antibody, anti-ds-DNA and anti-Sm antibodies.

Vascular lesions in abdomen

ABD abdominal vasculitis is more likely to affect small veins and venules than arteries or arterioles. There can be intense inflammation around the vasa vasorum, resulting in destruction of the media and fibrous thickening of the intima and adventitia[52]. Pseudoaneurysms are the most common arterial manifestation, mainly involving the aorta, the pulmonary, and the femoral arteries. Thrombosis of the superficial and deep veins is more common than thrombosis and aneurysms of the large arteries in the abdominal cavity in ABD[53], albeit there was case report showing ABD patient manifested with a large aneurysm of the superior mesenteric artery causing occlusion and ischemic enteritis[54]. Mesenteric ischemia and infarction may be due to large vessel occlusion or to mucosal disease secondary to vasculitis of the small vessels[52].

Large vessel thrombosis occurs in 11% of ABD cases, of which 26% have hepatic vein or the inferior vena cava (IVC) thrombosis. In fact, ABD is the most common cause of Budd-Chiari syndrome in Turkey, especially in young male patients[55]. It may present with hepatomegaly, ascites, and involvement of other large vessels, mostly venous. Obstruction of the IVC due to extension of the thrombus to the ostium of the hepatic veins was found in most ABD patients with Budd-Chiari[56]. It could lead to acute hepatic failure and rapid death in one-third of the patients in one series. The major predicting factor for survival was the extent of vascular thrombosis in the IVC. ABD may also contribute to development of cavernous transformation of the portal vein[57], portal vein

thrombosis with splenomegaly, and superior vena cava thrombosis[58].

Other rare lesions

Hepatobiliary complications include fatty liver or congestion, acute and chronic hepatitis, cholelithiasis and cholecystitis, primary biliary cirrhosis, and hepatic abscesses[59-61]. The liver alkaline phosphatase level was elevated in 11% of ABD patients and correlated with disease activity[61]. Splenic involvement occurred in 37 of 170 autopsies in Japanese ABD patients with splenitis, splenomegaly, hemosiderosis, infarction, and auto-splenectomy, and pancreatic involvement was found in 2.9% of the ABD cases[60].

Acute ABD pancreatitis responded to treatment with corticosteroids[62]. Chronic pancreatitis was reported in an ABD patient with an alcohol history[63]. Due to the rarity of pancreatitis in ABD, other causes such as gallstone disease should be excluded.

Other rare complications include hepatic artery aneurysm causing hemobilia[64] as well as pylephlebitis and septic thrombophlebitis of the portal vein[65]. Type AA amyloidosis can also complicate with ABD, manifesting as diarrhea and malabsorption. It can also affect the kidneys with proteinuria and progress to nephrosis and renal failure. It has a 50% mortality rate after an average duration of 3.4 years[66-68].

TREATMENT AND PROGNOSIS

Treatment is largely empirical since well-controlled studies are difficult to conduct due to the heterogeneity of the disease, and the unpredictable course with exacerbation and remission. Therefore, there lacks evidence-based treatment recommended for the management of ABD-GE. Agents such as corticosteroids, sulfasalazine, azathioprine, cyclophosphamide, TNFα antagonist or thalidomide should be tried first before surgery, except in emergency[69,70]. Retrospective studies suggest that corticosteroids, sulfasalazine and azathioprine are effective in achieving remission without the need for surgery in a large proportion of patients. One study reported that azathioprine could decrease re-surgery rates and suggested that it should be used as maintenance therapy in patients whose gastrointestinal complication requires emergent surgery. Mesalazine may reduce the total dose of corticosteroids required to treat intestinal disease and esophageal ulcers[71,72]. Anti-TNFα therapy such as infliximab and thalidomide were also reported useful in treating intestinal lesions in case studies[22,73], which highlighted the role of large amounts of TNFα secreted by γδ T cells in ABD patients[74].

ABD-GE can sometimes resolve spontaneously, making it difficult to precisely evaluate the effectiveness of the therapy[33,36]. On the other hand, corticosteroids may prolong the healing process, provoke colonic perforation, and worsen the pancreatitis[33,75]. In one report, perforation was noted in 41% of patients who received corticosteroids and 33% of those who did not[12].

There is also no firm evidence to guide the management of major abdominal vessel disease in ABD. For the treatment of acute deep vein thrombosis in ABD, immunosuppressive agents such as corticosteroids, azathioprine, cyclophosphamide, or cyclosporine A are recommended[69,70]. For management of arterial aneurysms, cyclophosphamide and corticosteroids are recommended[69,70]. There is no evidence that anticoagulation is beneficial and it may in fact lead to fatal pulmonary hemorrhage in those with pulmonary arteritis and aneurysm formation[76].

Patients who have a history of intestinal perforation or fistula formation have a high probability of recurrence after surgery[35]. The recommended length of resection is controversial. Some advocate wide surgical margins, while others recommend removal of only the grossly affected bowel[12,35,77]. In one report, the incidence of recurrence was 18% with resection of the right colon and 35% with resection of just the ileocecal region[12]. In another report, the length of resection did not affect the rate of recurrence or reoperation[35]. Intraoperative endoscopy, especially of the small bowel, can help determine the length of resection during surgery. Follow-up endoscopy and barium studies should be done to evaluate the anastomotic site.

ABD usually has a chronic, unpredictable course with exacerbation and remission which decrease in frequency and severity over time. Death is mainly due to major vessel disease and neurological involvement. The prognosis is worst among young males[78]. Complete remission was achieved in 38% of ABD patients with gastrointestinal involvement after 8 wk of medical treatment[35]. The rate of recurrence after surgery has been reported to be 40%-56%[12,42]. Recurrence was found in 49% of patients at 5 years, especially in those with intestinal perforation or fistula formation[35]. Recurrent lesions were at or near the anastomosis in 81% of patients. Of those who underwent surgery, 75% recurred within 2 years and were associated with a higher rate of complications such as ocular and ileal lesions than the nonsurgical group[79]. The incidence of postoperative recurrence was lower in those with normal intraoperative endoscopy than in those with observed lesions[80].

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S- Editor Gou SX **L- Editor** Ma JY **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.616

World J Gastroenterol 2012 February 21; 18(7): 616-626
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Psychosocial determinants of irritable bowel syndrome

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Received: January 17, 2011 Revised: September 22, 2011
Accepted: January 18, 2012
Published online: February 21, 2012

Abstract

From a pure motor disorder of the bowel, in the past few years, irritable bowel syndrome (IBS) has become a multifactorial disease that implies visceral hypersensitivity, alterations at the level of nervous and humoral communications between the enteric nervous system and the central nervous system, alteration of the gut microflora, an increased intestinal permeability and minimum intestinal inflammation. Psychological and social factors can interfere with the communication between the central and enteric nervous systems, and there is proof that they are involved in the onset of IBS and influence the response to treatment and outcome. There is evidence that abuse history and stressful life events are involved in the onset of functional gastrointestinal disorders. In order to explain clustering of IBS in families, genetic factors and social learning mechanisms have been proposed. The psychological features, such as anxiety, depression as well as the comorbid psychiatric disorders, health beliefs and coping of patients with IBS are discussed in relation to the symptoms and outcome.

Key words: Anxiety; Depressive symptoms; Irritable bowel syndrome; Personality; Psychosocial factors; Sexual abuse; Stressful events

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Surdea-Blaga T, Băban A, Dumitrascu DL. Psychosocial determinants of irritable bowel syndrome. *World J Gastroenterol* 2012; 18(7): 616-626 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/616.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.616

INTRODUCTION

Irritable bowel syndrome (IBS) is a frequent diagnosis in clinical practice for gastroenterologists and primary care physicians. It is a burden to society through total direct costs, reduced social functioning and quality of life impairment. IBS has a multifactorial etiology, involving altered gut reactivity and motility, altered pain perception, and alteration of the brain-gut axis. In addition, psychological and social factors can influence digestive function, symptom perception, illness behavior and outcome[1]. Psychological distress and major life events are frequently present in IBS, and are responsible, at least in part, for some outcomes. Whether they are also risk factors for IBS, is still uncertain. Since the biopsychosocial model of IBS was developed[2,3] the number of papers on IBS has skyrocketed. Accordingly, there has been a constant growing interest on the influences of psychosocial factors on the pathogenesis, course, severity and outcome in IBS. This review will highlight the place of environmental and psychosocial stressors in the biopsychosocial model of

IBS, and their role in the onset and course of symptoms.

BIOPSYCHOSOCIAL MODEL OF IRRITABLE BOWEL SYNDROME

The idea that emotions may influence the sensorimotor function of the gastrointestinal tract emerged at the beginning of the 19th century, and a lot of the evidence from research during that period is still valid[4]. Nevertheless, the vivid part of the history of IBS began only three decades ago, when the concept of the biopsychosocial model of illness and disease was developed[5]. This model integrates all possible accountable factors for the pathogenesis and clinical expression in IBS. The biopsychosocial approach allows for symptoms to be both determined and modified by psychological and social influences[3]. The link between psychosocial factors and gastrointestinal function (motility, sensation, inflammation) is through the brain-gut axis. This implies a bidirectional connection system between the gastrointestinal tract and the brain, through neural, neuroimmune and neuroendocrine pathways[6].

Psychosocial factors influence every component of the biopsychosocial model (Figure 1). Early in life, genetics and environmental factors (e.g., family influences, abuse, major losses), may affect one's psychosocial development (psychological state, coping skills) and/or the development of gut dysfunction. Gut dysfunction and dysregulation of the brain-gut axis can lead to IBS. During life, psychosocial factors (stressful life events, psychological distress), may influence digestive function, symptom perception, illness behavior, and consequently health outcome, daily function and quality of life[3]. Conversely, visceral pain can affect central pain perception, mood and behavior[7]. The psychological and social factors which may influence IBS are listed in Table 1.

ENVIRONMENTAL INFLUENCES IN IBS

Patients with IBS frequently report a positive family history of IBS, ranging from 33%[8] to 42%[9]. A constant research question is whether the clustering of IBS in families is due to common environmental risk factors or due to a common IBS specific set of genes.

Familial aggregation of IBS and twin studies showed that the concordance for IBS in monozygotic twins is significantly higher than in dizygotic twins, supporting the role of genetics in the etiology of IBS[10]. In the last decade, a lot of genetic studies looked for associations between gene functions (such as IL-10, the serotonin transporter, α-2 adrenergic receptors, and G protein) and gastrointestinal and colonic physiology in IBS patients, and searched for interactions between genotype and phenotypes[11]. So far, a definitive disease-causing gene or set of genes for IBS hasnot been identified.

There is a higher prevalence of IBS among subjects who have a family member with a history of abdominal pain, bowel dysfunction[12] or inflammatory bowel disease (IBD)[13]. An interesting fact is that subjects whose spouses had abdominal complaints did not report IBS symptoms more often than controls[12]. Only 5.4% of IBD patients' spouses reported IBS symptoms, *vs* 10.8% of first

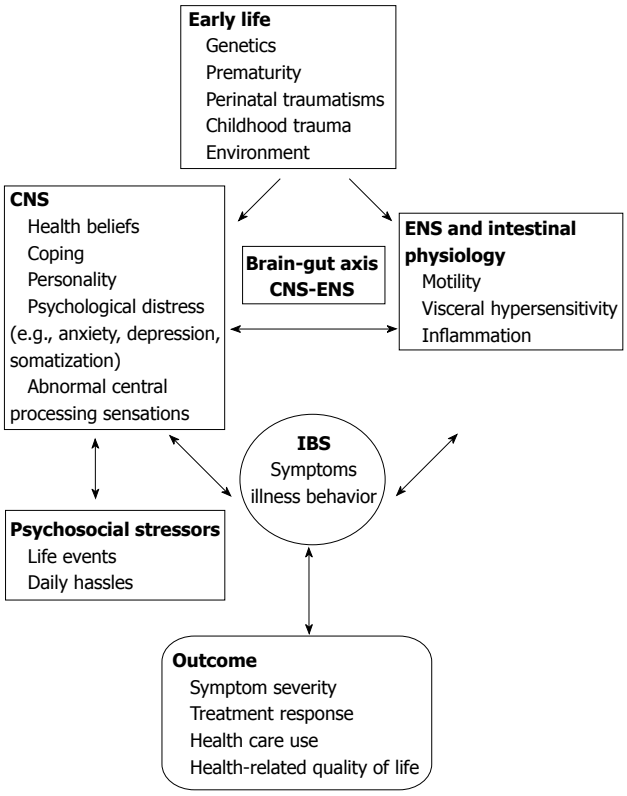

Figure 1 The biopsychosocial model of irritable bowel syndrome: Pathogenesis and clinical expression in relation to psychosocial factors. CNS: Central nervous system; ENS: Enteric nervous system; IBS: Irritable bowel syndrome.

Table 1 Psychological and social factors in irritable bowel syndrome

Environmental factors
Early life events
Upbringing environment
Incentives
Family function
Abuse history
Psychosocial stressors
Life events (divorce, unemployment, death of a close relative)
Daily hassles
Personality traits
Neuroticism, agreeableness, conscientiousness
Alexithymia
Health beliefs
Hypochondriacal beliefs
Illness representation
Perceived susceptibility
Coping strategies
Maladaptive coping (catastrophyzing, self-blame, substance abuse)
Negative emotions and psychiatric disorders
Mood disorders (major depression and dysthymic disorder)
Anxiety disorders (generalized anxiety disorder, panic disorder, post-traumatic stress disorder)
Somatization and somatoform disorders
Neurasthenia

degree relatives of IBD patients. Even if these observations support the idea that the inherited pathophysiological mechanisms are more important in IBS clustering in families, the effects of the common environment cannot be excluded.

Prenatal traumatic events may contribute to the development of IBS[14]. For example, exposure to severe wartime conditions in early life (in the first two years of life) was associated with an increased risk of developing IBS. To what extent this is attributable to the stressful environment of war, to severe undernutrition, or to the increased prevalence of infectious diseases is, however, unclear[15]. A large Norwegian population-based twin study[10] (on 12 700 twins) assessed the influence of nutrition in fetal life on the development of IBS, using birth weight as an indicator. The twins with a birth weight below 1500 g were significantly more likely to develop IBS. In addition, weight below 1500 g influenced the age at onset: IBSappeared 7.7 years earlier than in higher weight groups.

A recent study on children who had pyloric stenosis suggested that early stressful life events, such as gastric surgery, and perioperative nasogastric tube placement represent risk factors in the development of chronic abdominal pain in children at long-term follow-up[16].

The role of environmental factors in IBS was studied in a pediatric population in China. Adolescents and children who lived in a single-parent household, children exposed to low temperature environmental conditions, had a higher prevalence of IBS. Dietary habits (such as excessive intake of pepper and cold food) and personal habits (like alcohol consumption and smoking) were also associated with higher rates of IBS symptoms among children and adolescents[17]. On the other hand, two studies reported that an affluent childhood social class was associated with an increased risk of IBS[18,19]. One study concluded that privileged childhood living conditions (childhood living density < 1 person per room) was an important risk factor for IBS[18]. Howell *et al*[19] showed that there is a linear decrease in the odds of IBS across decreasing levels of social class.

Data regarding the effects of family environment are scarce. One study reported that by the age of 15 years, in 333 IBS patients, 31% had lost a parent through death, divorce or separation; 19% had an alcoholic parent, and 61% reported unsatisfactory relationships with, or between their parents[20]. Therefore, childhood deprivation may have an important influence on the etiology of IBS.

The clustering of IBS in families could partially be the result of social learning during childhood. The children of mothers with IBS have more non-gastrointestinal (GI) as well as GI symptoms (especially stomach aches), more school absences, and more physician visits for GI symptoms than children of non-IBS mothers[21]. In addition, children whose mothers made solicitous responses to illness complaints reported more severe stomach aches and they also had more school absences for stomach aches. Thus, children may learn abnormal illness behavior from their parents through social learning from parental reactions to symptom complaints[14].

ABUSE HISTORY

The role of abuse (especially childhood abuse) in IBS patients is still unclear. So far, authors have tried to determine (1) whether abuse is a risk factor for IBS; (2) whether there is a higher prevalence of abuse among IBS patients compared with other gastrointestinal diseases or healthy controls; (3) the effects of abuse on clinical outcome; and (4) the relationship between abuse and psychological distress, as a possible explanation of abuse-IBS association.

The prevalence of abuse history among patients with functional gastrointestinal disorders (FGIDs) varies widely. When comparing different data, we should consider at least three aspects: cultural differences which can lead to lower or higher self-report rates; the measurement methods of abuse used in different studies; and the type of abuse considered: physical, emotional/verbal or sexual. Also, the type of sexual act that is considered abusive varies from exhibitionism, fondling or contact abuse, to penetration or rape[22]. Given these facts, the prevalence of sexual and emotional abuse in IBS varies from 26%[23] to 50.8%[24], but lower values have also been reported. In other studies only 17% of abused patients had reported the abuse[25], therefore, the actual prevalence of abuse is usually higher.

In our experience, of 125 female patients with IBS, only one admitted she had "an undesired sexual contact", leading to a less than 1% reported frequency of sexual abuse. On the other hand, of 15 abused children from a specialized care center, seven met Rome Ⅲ criteria for IBS. We consider that the cultural differences between Romania and Western European countries are responsible for this under-recognition of abuse in female IBS patients[26].

There are studies which show that the prevalence of abuse history in patients with functional bowel disorders is greater than that in patients with organic bowel disorders and healthy control subjects[25,27,28]. Other studies failed to reinforce this observation[29]. Nevertheless, a constant finding is that abused individuals express higher levels of psychological distress[28,29] and higher levels of somatization[25,30], suggesting that previous abuse experience might lead to psychiatric disorders. There are significant data showing that anxiety disorders, depression[31] and somatization[32], are risk factors for IBS.

Two recent studies concluded that a lifetime history of a broad range of trauma and abuse, either in childhood or in adult life are independently associated with an increased IBS risk[30,33]. In a study that included women veterans[33], who are at increased risk of occupational trauma, including sexual trauma, the prevalence of IBS was 33.5%, and the most frequently reported trauma was sexual assault (38.9%). Even when depression and post-traumatic stress disorder were significantly more com-

Table 2 Environmental factors associated with irritable bowel syndrome

Prenatal traumatic events (e.g., nutrition in fetal life)
Early stressful life events (surgery, emotional, physical or sexual abuse)
Upbringing environment (low temperature, affluent childhood social class)
Family function (divorce, death of a parent)
Family history of abdominal pain, bowel dysfunction, inflammatory bowel diseases
Social learning (modeling)
Abuse history either in childhood or during adult life

mon in IBS cases than controls, neither explained the association between trauma and increased IBS risk.

Even if the role of abuse in IBS remains unclear, it was proven that abuse leads to increased psychological distress. Most probably, as a consequence, abuse is associated with greater impairment of functioning in daily lives, more visits to the doctor[25] and a poorer health outcome[34].

There are more and more data supporting the inherited component of IBS, but at the same time, the influences of the upbringing environment cannot be disregarded. IBS remains a multifactorial, complex disorder, caused by both environmental (Table 2) and genetic risk factors[9,11].

PSYCHOSOCIAL STRESSORS

Lazarus[35] divided stressors into life events and daily hassles. Life events refer to major events such as divorce, unemployment, or death of a close relative. Daily hassles are events which everybody experiences daily and frequently[36]. His assumption is that "(daily) hassles appear to be better predictors of health outcomes than life events". The results of a recent prospective study support this theory: there was a significant increase in stressor score (daily hassles) just before progression from IBS non-patient to IBS patient[37]. The majority of the subjects in this study however, were young college students, so factors rated as relatively stressful life events, were not very common.

The current data regarding the role of life events in the onset of IBS are the result of observations from the 80s. For example, Creed *et al*[38] showed that the most frequent events reported by patients with functional abdominal pain (FAP) (including IBS patients) during 38 wk prior to onset of symptoms, were a major disruption of close relationships, a marital separation, a family member leaving home, or break-up of a serious girl/boyfriend relationship. In addition, marked personal relationship difficulties such as severe marital problems or extreme family or household tensions, were much more frequently recorded among the FAP patients than in the organic GI diseases group (such as ulcer disease) or community subjects.

Recent data support the role of major life events in IBS. Childhood trauma was associated with an increased

Table 3 Psychosocial stressors and their relation to irritable bowel syndrome

Daily hassles, major life events (divorce, unemployment, or death of a close relative), and major social events (surviving holocaust, revolution, social changes) determine the onset of irritable bowel syndrome symptoms in susceptible individuals
Psychosocial stressors determine symptom exacerbation and health care seeking

vulnerability for multiple somatic symptoms of which IBS is one subset[30]. The items reported significantly more frequently in IBS than in healthy controls were: seeing someone being murdered, death or illness of a parent, failing to be understood by parents and having someone in the family with a psychiatric illness.

Holocaust survivors are another example of the impact of stressful life events on the development of IBS. The prevalence of IBS, duration of suffering, and frequency of GI symptoms were significantly higher in Holocaust survivors[39], when compared to controls with the same demographic background, but who had not been exposed to extreme mental and physical hardships during the war. From our personal experience[40], the stress developed by dramatic events presented live on television, during the uprising in Romania in 1989, led to an increased number of IBS symptoms within the first month.

Sometimes, what is considered to be a major life event is difficult to determine. For instance, a sudden cultural change (such as moving from a rural to an urban area) increased the prevalence of IBS in one study[41].

The experience of stressful life events can also determine symptom exacerbation among adults with IBS and frequent health-care seeking[7,42]. Thus, the severity of abdominal pain was higher in patients exposed to emotional stress[43], and stress exacerbated abdominal distension in one third of IBS patients[44]. In addition, recent data showed that environmental factors and psychosocial stressors (for example history of being psychologically abused, less than 6 h of sleep and irregular diet) influenced the progression from an IBS non-consulter to an IBS patient[37].

Based on these data we can say that psychosocial stressors, either during childhood or later in life are involved in the onset of IBS symptoms in susceptible individuals, and these factors influence the clinical course of IBS (Table 3).

PERSONALITY TRAITS

Personality is also considered to play a role in the etiology of IBS and in the decision to seek medical help. Personality traits have been defined as a "dynamic organization, inside the person (…) that create a person's characteristic patterns of behavior, thoughts and feelings"[45]. There are still debates regarding the major determinants of personality, and which personality traits should be included in psychometric questionnaires. However, very often,

Table 4 Personality traits and irritable bowel syndrome

Neuroticism and alexithymia are common in irritable bowel syndrome patients
Neuroticism is a predictor of illness perception and influences coping strategies
Examples of measurement tools: Toronto Alexithymia Scale (TAS-20), Neuroticism Extraversion Openness Personality Inventory[46]

personality is assessed based on five dimensions: extraversion (talkativeness, assertiveness, activity *vs* silence, passivity and reserve); agreeableness (kindness, trust, and warmth *vs* selfishness and distrust); conscientiousness (organization, thoroughness, and reliability *vs* carelessness, negligence and unreliability); neuroticism (tendency to experience negative emotions, such as anger, anxiety, or depression; also called emotional instability); and openness to experience (imagination, curiosity, and creativity *vs* shallowness and imperceptiveness)[46,47].

The majority of studies on this topic supports the idea that IBS patients have higher levels of neuroticism both when compared with the general population[48], and with patients with similar GI complaints (IBD patients)[49]. Neuroticism might influence coping strategies, being associated with escaping the problem or blaming oneself[50]. Neuroticism is also a significant predictor of illness perception and treatment beliefs in IBS[49].

Data are sometimes discordant with regard to the other dimensions of personality. The level of conscientiousness in IBS patients was high in some studies[48], while only average when compared with FD in other studies[51]. The differences may come from the fact that in some studies patients with comorbid psychiatric disorders were excluded[48].

Alexithymia is a stable personality trait, which is also frequently observed in IBS patients. Alexithymia is defined as a difficulty in identifying feelings and distinguishing between feelings and bodily sensations, difficulty describing feelings to other people, a markedly constricted imaginative process, and externally oriented thinking[52,53]. The Toronto Alexithymia Scale (TAS-20)[54] is the most widely used questionnaire to measure a person's alexithymia level. Subjects with a TAS-20 score > 61 (the score ranges from 20 to 100) are considered alexithymic. In the general population, less than 10% of patients are alexithymic[55]. In an Italian study, IBS patients had higher TAS-20 scores than healthy controls (59.1 *vs* 40.5). In addition, 43% of IBS patients had a TAS-20 score > 61, while only 2% of healthy subjects were alexithymic[56]. Our personal data on a female IBS population showed similar results[57]. Alexithymic individuals may misinterpret the somatic sensations associated with emotional arousal as symptoms of disease. It follows that, alexithymia is often associated with somatization, also a frequent finding in IBS patients[58]. Table 4 summarizes the association between personality traits and IBS.

HEALTH BELIEFS AND COPING WITH STRESS

A lot of patients with IBS believe that their chronic gut symptoms indicate a serious illness or even cancer. In addition, patients describe IBS not only as symptoms but mainly as it affects daily function, thoughts, feelings and behaviors. Patients report the sense of losing freedom, spontaneity and social contacts, as well as feelings of fearfulness, shame, and embarrassment. All these could lead to changes in their behavior such as avoidance of activities and many adaptations in routine in an effort for patients to gain control[59].

The most extreme form of preoccupation with an illness is hypochondriasis, included in the group of somatoform disorders. Hypochondriasis is the excessive fear of a serious illness, despite medical testing and reassurance to the contrary[60]. In a study from the 90s, patients with IBS expressed more hypochondriacal attitudes when compared with healthy subjects or patients with organic GI diseases. Patients with IBS had high scores on bodily preoccupation, hypochondriacal beliefs and disease phobia[61]. No other data on this subject are available. We cannot recommend screening for hypochondriacal attitudes, but in everyday practice, we can encounter IBS patients with excessive illness-related fear. The presence of hypochondriacal attitudes can be measured using the Illness Attitudes Scales (IAS) questionnaire. This questionnaire was developed in 1986[62], but even now remains a valid tool[63].

Psychological assessment of IBS patients revealed that there are differences regarding how individuals with IBS respond to their illness. In other words, IBS patients do adopt different coping strategies compared with patients with organic diseases or healthy controls. Coping is defined as "constantly changing cognitive and behavioral efforts to manage specific external and/or internal demands that are appraised as taxing or exceeding the resources of the person"[36]. The authors divided coping strategies into two main categories: problem-focused coping targeting directly the causes of stress, such as information seeking, constructive way of reducing/solving the problem and planning, and emotion-focused coping used to handle negative emotions evoked by the situation (such as an avoiding feeling, trying to escape the problem or blaming oneself).

There are a number of questionnaires which assess coping strategies, such as the Ways of Coping Questionnaire (WCQ)[64], Coping Strategies Questionnaire (CSQ)[65] and Coping Inventory for Stressful Situation (CISS)[66]. All of these include the main coping strategies mentioned. The CISS has proved so far to have very good psychometric properties and was validated in several languages[67].

Using WCQ, Drossman *et al*[50] showed that patients with IBS and other FGIDs, didnot use positive reap-

Table 5 Health beliefs and coping with stress in relation to irritable bowel syndrome

Health beliefs in IBS may be irrational, leading to hypochondriacal attitudes
Coping strategies can be inefficient in IBS patients, patients often adopt maladaptive coping strategies such as catastrophyzing
Patients with a high degree of catastrophyzing report more severe pain
Measurement methods: CSQ, CISS, WCQ

IBS: Irritable bowel syndrome; CSQ: Coping strategies questionnaire; CISS: Coping inventory for stressful situation; WCQ: Ways of coping questionnaire.

praisal as often as patients with organic disorders (such as IBD, acid peptic disease, pancreatico-biliary diseases). In a polish study, IBS patients had a high emotional-oriented coping style[51].

The CSQ focuses primarily on coping in response to painful conditions. The interest in using the CSQ in patients with IBS is related to the subscale measuring catastrophyzing (e.g., "When I am in pain, I feel I can't stand it anymore" or "it's awful and I feel it overwhelms me"). Catastrophyzing is a maladaptive coping strategy defined as "a negative cognitive process of exaggerated negative rumination and worry"[68]. Patients with IBS are more likely to catastrophize than patients with organic disorders[50]. In addition, catastrophyzing mediates the relationship between depression and pain severity. This relationship was suggested by the following observations: patients with IBS and a high degree of catastrophyzing have a tendency to report more severe pain; catastrophyzing and depression are associated[69]; depression did not predict symptom severity[70]. Patients with IBS who experience higher levels of depression engage in more catastrophic thinking, and partly through this thinking style experience more intense pain and greater activity limitations due to pain[69].

The CSQ also measures the overall effectiveness of coping strategies (the amount of control over symptoms and self-perceived ability to decrease symptoms). IBS patients were less likely to feel in control of symptoms and to feel able to decrease symptoms than patients with organic disorders[50], suggesting that coping strategies are not very efficient in IBS patients.

When speaking about coping styles in IBS patients it is difficult to draw a general conclusion. The studies mentioned above used different questionnaires to assess coping strategies in IBS. The results are not contradictory, but at the same time didnot point out a specific coping strategy in IBS patients (see also the summary in Table 5). Further research is necessary to establish the role of coping in symptom perception and control, and clinical outcome in IBS patients.

NEGATIVE EMOTIONS AND COMORBID PSYCHIATRIC DIAGNOSIS

Psychiatric symptoms and psychiatric disorders are more common in patients seen in referral practices than primary care or in the community of nonconsulters. The most frequent psychiatric diagnosis in IBS is mood disorders (major depression and dysthymic disorder), anxiety disorders and somatoform disorders[7].

Depression is the most common psychiatric diagnosis in IBS patients[71,72]. Patients with IBS have higher scores of depression than healthy controls[73,74], but lower than the psychiatric population[7,73]. However, depressive disorders are more common in clinic patients with IBS compared to patients with similar symptoms and organic GI diseases and compared to healthy controls[72,75]. In the study by Whitehead *et al*[72], the prevalence of depression in IBS was 31.4%, 21.4% in IBD and 17.5% in controls. Another study reported a lower prevalence of depression in IBS than in patients with chronic C hepatitis[76]. An interesting fact is that the number of self-reported depressive symptoms is not significantly higher in patients who seek medical care for their GI complaints compared with those who do not consult[75].

Anxiety and anxiety disorders, such as panic disorder (PD), generalized anxiety disorder (GAD) and posttraumatic stress disorder (PTSD) are also often observed in IBS patients. Anxiety tends to precede IBS onset, particularly if diarrhea predominates. This indicates that the psychiatric disorder cannot be regarded as a response to the functional GI disorder. It seems more likely that the psychiatric symptoms, especially anxiety, play a role in the development of IBS[31].

Anxiety is more common in IBS patients than in the general population[77,74]. In a community study, GAD was found in 16.5% of subjects with IBS symptoms, while in the group of subjects without these symptoms the prevalence was 3.3%[77]. Whitehead *et al*[72]reported similar results (15.8%) regarding the frequency of anxiety among IBS patients. However, other studies reported higher values. For example, 47% of patients with IBS met Hospital Anxiety and Depression Scale (HADS) screening criteria for anxiety in a South Australian study[76].

This significant difference in prevalence may have at least two causes. The first one refers to the sample of subjects taken into account-some studies were conducted on samples of patients seeking medical care, mostly in tertiary care settings; other studies investigated community subjects. The second explanation may be that the authors used different scales or criteria to establish the presence of anxiety. Lee *et al*[77] used the diagnosis criteria for GAD according to the Diagnostic and Statistical Manual of Mental Disorders[60]. HADS is a widely used instrument developed as a screening tool for detecting states of depression and anxiety in hospital settings[78]. Studies have also shown that HADS is valid when used in community settings and primary care medical practice[79]. HADS should be used only to estimate the likely prevalence of anxiety and depression, and not to establish a firm diagnosis. The latter can only be made by a psychologist or a psychiatrist using a structured interview based on the Diagnostic and Statistical Manual of Mental

Disorders (DSM)-Ⅳ.

Anxiety related to GI sensations, symptoms or the context in which these may occur is referred to as gastrointestinal specific anxiety (GSA). GSA influences symptom severity and quality of life in patients with IBS[80]. Patients with IBS have more severe GSA when compared with healthy subjects. In addition, IBS patients with severe GI symptoms have more severe GSA scores[81]. GSA can be assessed using The Visceral Sensitivity Index (VSI), a 15-item scale developed for this purpose[80]. The main information from measuring GSA, is related to the fact that the GSA score predicts GI symptom severity[81].

Data regarding the frequency of PD in IBS are scarce. Panic disorder is characterized by repeated, unexpected panic attacks, and is more common in women. Sufferers experience unexpected episodes of intense fear and associated cardiorespiratory, GI, neurological, and cognitive symptoms[60]. A study conducted in several secondary and tertiary gastroenterology clinics, reported that 12% of IBS patients had PD, 14% had GAD and 29% had depressive disorder[71]. The frequency of GAD and depressive disorder are similar to those mentioned above, even if the criteria used for the diagnosis of psychiatric disorders were different. Studies in female veterans, showed that IBS is more common in patients with PTSD, and PTSD represents an independent risk factor for IBS[33,82].

Somatization disorder (SD) is included, according to DSM-Ⅳ, in the larger group of somatoform disorders. SD is defined as a chronic condition in which "the individual experiences physical symptoms that suggest the presence of a general medical condition, but a medical workup cannot establish an etiological general medical condition that adequately explains the problem"[60]. Several studies conducted on tertiary care IBS patients reported an excessive somatization tendency in patients with IBS, the prevalence of SD reaching 25%[83,84]. However, a review on SD, mentioned that in primary care and in population-based samples, SD is very rare, with a lifetime prevalence of only 0.1% to 0.2%[85].

High scores on somatization questionnaires in patients with functional gastrointestinal disorders (FGIDs) were observed both in population-based studies and in clinical studies[86]. Somatization is frequently associated with anxiety and depression, and explains the frequent "extra-gastrointestinal symptoms" such as musculoskeletal complaints, urinary symptoms, sexual symptoms, headaches, and constant fatigue observed in patients with IBS[84,87]. It is also associated with a poor health-related quality of life, and predicts a poor response to treatment[88-90].

Recent data endorse these assumptions and also suggest that somatization is a risk factor for IBS. In a study conducted in a tertiary care setting on functional dyspepsia patients, somatization was found to be a common risk factor for co-morbid IBS and chronic fatigue-like symptoms[32]. A community-based prospective study[91], showed that psychosocial factors indicative of somatization (such as illness behavior scores, anxiety, sleep problems and somatic symptoms) are independent risk markers for the development of IBS, in a group of subjects previously free of IBS. After one year follow-up, 3.5% of the followed subjects developed IBS. Those who reported all four of these markers at baseline were six times more likely to report IBS when compared to those who were exposed to none or one marker.

Those who have SD use health services frequently, and have twice the annual medical care cost of people without SD[92]. This finding is also true for IBS patients, as patients with IBS and SD have a significantly greater number of medical consultations, telephone calls to physicians, urgent care visits, medication changes, missed work days and benzodiazepine use[84]. In addition, IBS patients with probable or definite somatization report more abnormal illness behaviors than those without SD[83].

Neurasthenia is also frequently reported in IBS patients, up to 35% in severe IBS patients[71]. It is very similar to chronic fatigue syndrome, being characterized by persistent and distressing fatigue after mental or physical effort associated with muscular aches, dizziness, sleep disturbance, irritability and mild depressive symptoms[60,93]. This entity is currently contested by some authors[93]. It is still included in the World Health Organization's International Classification of Diseases, but not in DSM-Ⅳ, due to its overlap with anxiety and depression.

Patients with IBS very often have more than one psychiatric disorder, and this finding is more common in severe IBS. For example in 74 patients with depressive disorder and severe IBS[71], 16 had PD, 6 had hypochondriasis and 41 had neurasthenia. Other psychiatric diagnoses reported in this study were dysthymia (7%), phobias (15%), undifferentiated somatoform disorder (9%) and drug or alcohol problems (8%).

Psychological distress is seen more often in IBS patients than in the general population. It also holds true that in patients with psychiatric disorders, IBS symptoms are more frequently reported than in the general population. In a community study, IBS was 4.7 times more common among patients with GAD than in the general population (22% *vs* 4.7%)[77]. Frequent abdominal pain, diarrhea, constipation, dyspepsia or IBS were present in 54% of subjects with depressive symptoms and in only 29% of non-depressed controls[86]. Patients with PD also have high rates of IBS symptoms[94-96], varying from 26.3%[96] to 46.3%[94], most probably due to different diagnostic criteria for IBS.

Numerous instruments have been developed to assess the presence of psychiatric symptoms or specific psychiatric disorders in IBS patients. The complexity of these questionnaires is clearly related to the purpose, which can either be screening or diagnosis, or research. One can obtain a general idea about a patient's psychological distress in IBS using the Symptom Checklist-90-Revised (SCL-90-R), developed by Derogatis[97]. The SCL-90-R has 9 subscales, including subscales for somatization, depression and anxiety, and provides an overview of symptom intensity at a specific point in time. From the three global indices determined, the Global Severity Index (GSI) mea-

Table 6 Comorbid psychiatric diagnoses and their relation to irritable bowel syndrome

Psychiatric symptoms and psychiatric diseases are frequent in IBS, especially in severe forms
Depression is the most common psychiatric disorder in IBS (approximately 30% of patients)
Generalized anxiety disorder is present in about 15% of patients
High gastrointestinal specific anxiety predicts symptom severity
High levels of somatization determine frequent use of health care services, a poor response to treatment and a poor health-related quality of life
Other psychiatric disorders in IBS patients: posttraumatic stress disorder, panic disorder, hypochondriasis, dysthymia, phobias, undifferentiated somatoform disorder, drug or alcohol problems
Patients with severe IBS may have more than one psychiatric disorder
Measurement methods: Symptom checklist-90-revised for overall psychological distress; state-trait anxiety inventory, beck depression Inventory

IBS: Irritable bowel syndrome.

sures overall psychological distress. The State-trait anxiety inventory[98] (STAI) and the Beck depression inventory[99] are another two questionnaires commonly used to assess the presence of anxiety and depression in IBS patients.

In the general population, half of patients with presumed IBS have psychiatric symptoms compared with one third in controls[100]. In several systematic reviews published at the beginning of the 2000's, the proportion of patients who met the criteria for any psychiatric diagnosis ranged from 40% to 94%[42,101,102]. We should take into account that some data are the results of studies conducted on tertiary care patients who are likely to be more distressed than other patients. Some studies did not evaluate the prevalence of psychiatric disorders in other chronic GI diseases, in the same setting. In addition, the criteria used to diagnose psychiatric disorders differed from one study to another. In a study by Creed *et al*[103] in severe IBS patients, 42% of patients had a concurrent psychiatric disorder (depressive, panic or generalized anxiety disorder). Even if this study didnot purposely determine the frequency of psychiatric disorders in IBS patients, it showed that even in the most severe IBS patients, a comorbid psychiatric disorder is found in less than half of the patients. In summary (Table 6), half or more IBS patients commonly have psychiatric symptoms or psychiatric disorders and their frequency is higher than for other medical patients in the same clinics.

CONCLUSION

Environmental and psychosocial factors are an important part of the biopsychosocial model of IBS, being involved in dysregulation of the brain-gut axis, leading to the onset of IBS, persistence of symptoms or abnormal illness behavior. There is a wide range of environmental and psychosocial stressors, acting at different moments in one's life; a number of people submit to these factors, but only susceptible individuals will develop IBS. Other major elements of the biopsychosocial model such as personality traits and psychiatric disorders are probably the elements which make one susceptible to the development of IBS.

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S- Editor Shi ZF **L- Editor** Webster JR **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.627

World J Gastroenterol 2012 February 21; 18(7): 627-636
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Differential effects of energy balance on experimentally-induced colitis

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Received: January 14, 2011 Revised: April 9, 2011
Accepted: April 16, 2011
Published online: February 21, 2012

Abstract

AIM: To characterize the influence of diet-induced changes in body fat on colitis severity in SMAD3-/- mice.

METHODS: SMAD3-/- mice (6-8 wk of age) were randomly assigned to receive a calorie restricted (30% of control; CR), control (CON), or high fat (HF) diet for 20 wk and were gavaged with sterile broth or with *Helicobacter hepaticus* (*H. hepaticus*) to induce colitis. Four weeks after infection, mice were sacrificed and the cecum and colons were processed for histological evaluation.

RESULTS: Dietary treatment significantly influenced body composition prior to infection ($P < 0.05$), with CR mice having less (14% ± 2%) and HF-fed mice more body fat (32% ± 7%) compared to controls (22% ± 4%). Differences in body composition were associated with alterations in plasma levels of leptin (HF > CON > CR) and adiponectin (CON > HF ⩾ CR) ($P < 0.05$). There were no significant differences in colitis scores between CON and HF-fed mice 4 wk post-infection. Consistent with this, differences in proliferation and inflammation markers (COX-2, iNOS), and infiltrating cell types ($CD3^+$ T lymphocytes, macrophages) were not observed. Unexpectedly, only 40% of CR mice survived infection with *H. hepaticus*, with mortality observed as early as 1 wk following induction of colitis.

CONCLUSION: Increased adiposity does not influence colitis severity in SMAD3-/- mice. Importantly, caloric restriction negatively impacts survival following pathogen challenge, potentially due to an impaired immune response.

Key words: SMAD3; Colitis; Adipokine; Obesity; Calorie restriction

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McCaskey SJ, Rondini EA, Langohr IM, Fenton JI. Differential effects of energy balance on experimentally-induced colitis. *World J Gastroenterol* 2012; 18(7): 627-636 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/627.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.627

INTRODUCTION

Adipose tissue (AT) is increasingly recognized as an active endocrine organ modulating a number of physiological processes. AT is a key regulator of insulin resistance[1,2]

and contributes to systemic inflammation through production of a variety of proteins, hormones and cytokines collectively referred to as adipokines[3,4]. Many of these secretory products play important roles in energy homeostasis and the immune response[5]. Several pro-inflammatory cytokines, including interleukin (IL)-6, C-reactive protein (CRP) and leptin, are released from AT even in the absence of acute injury or inflammation, and their production is increased in proportion to AT mass[6-10]. Such altered production of these cytokines contributes to a number of pathophysiological processes including peripheral insulin resistance, inflammation, vascular disease, and immune dysfunction commonly observed in obesity[2,11].

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic conditions characterized by remittent inflammation resulting in extensive damage to the gastrointestinal tract[12-14]. CD can affect any part of the intestine[12], whereas UC is confined to the colon[14]. Although certain clinical features differ between these two conditions[13], both are thought to result from a dysregulated immune response in susceptible individuals[15].

Altered local and systemic levels of cytokines including tumor necrosis factor (TNF)-α, leptin, and adiponectin have been observed in individuals with IBD and are suggested to contribute to the disease pathogenesis[16]. Leptin is a 16-kDa product of the ob gene and is produced primarily by adipocytes[17]. Circulating levels of leptin are increased in obesity and show a positive correlation to body mass index[18,19]. Although leptin regulates energy metabolism by inhibiting food intake and increasing energy expenditure[20], it also has important immunomodulatory roles[21-23]. Increases in leptin levels in the serum[24], mesenteric AT[25], and in the colonic lumen[26] have been reported during the active stage of IBD. Leptin is also associated with susceptibility to experimental colitis in mice[27]. Colonocytes express the leptin receptor, and luminal administration of leptin induces epithelial wall damage and neutrophil infiltration, suggesting a local pro-inflammatory role for this protein[26].

Adiponectin is a high molecular weight protein secreted by AT that contributes to glucose homeostasis by increasing peripheral insulin sensitivity and reducing hepatic gluconeogenesis[28]. Pro-inflammatory mediators, including TNF-α and IL-6, suppress adiponectin secretion and serum levels are markedly reduced in obese individuals[29-31]. Adiponectin is generally considered anti-inflammatory due to antagonistic effects on cytokine signaling[32-34]. However, increased levels have been detected in serum and hypertrophied mesenteric AT in patients with active IBD[35,36]. A direct role for adiponectin during experimental colitis in animals has produced inconsistent results[37-39].

There is currently insufficient evidence to support a causal relationship between obesity and IBD; however, the conditions share similar inflammatory characteristics. Recent studies indicate that the constant low-grade inflammation associated with excess AT, including elevated serum levels of CRP, IL-6 and TNF-α, may contribute to the severity of IBD[40]. Additionally, overweight and/or obese individuals with CD were found to have more complications and more frequent disease relapses than normal weight individuals[41], providing a potential link between excessive AT and pathogenesis of IBD.

In the current study we evaluated the influence of adiposity on colitis severity in SMAD3-/- mice. SMAD3-/- mice have defective transforming growth factor (TGF)-β signaling and develop mild colitis within 4 wk following infection with *Helicobacter spp*. Dysfunctions in TGF-β signaling are commonly observed in human IBD and during colon cancer development. Maggio-Price *et al*[42] demonstrated that SMAD3-/-, but not SMAD3+/- mice develop chronic colitis and colon cancer in response to a bacterial infection. In the SMAD3-/- mouse model of colon cancer, initiation and progression is induced by a bacterial infection *Helicobacter hepaticus* (*H. hepaticus*). The bacterium colonizes the cecum and proximal colon persistently, low grade inflammation and immune cell infiltration observed eventually lead to mucinous adenocarcinoma formation at 15-30 wk post infection[42]. Importantly, these lesions are flatter, more aggressive and harder to diagnose in humans. It is widely hypothesized that chronic low levels of inflammation, whether induced by a pathogen or not, leads to cancer promotion and progression. Therefore, this model is highly relevant to the process of human colon carcinogenesis. Specifically, the SMAD model is very similar to the development of specific human cancers where a pathogen is necessary (but not sufficient) to cause dysplasia and tumor formation. Examples include hepatitis and liver cancer, *Helicobacter pylori* and stomach cancer, and human papillomavirus and cervical cancer. The contribution of this research was to understand how energy balance differentially modulates promotion/progression of inflammation and pathogen-induced cancers.

Mice were submitted to one of three dietary treatments (control, 30% caloric restriction, or high fat diet) to induce differing levels of adiposity after 20 wk, and were then infected with *H. hepaticus* to induce colitis. Plasma leptin and adiponectin were measured pre-infection and histological scoring was performed on cecum and colon tissue 4 wk post-infection.

MATERIALS AND METHODS

Animal husbandry

Mice (129-*Smad3*^{tm1Par}/J, referred to hereafter as SMAD3-/-) were generously donated by Lillian Maggio-Price at the University of Washington. The mouse colony was developed by pairing SMAD3-/- males with SMAD3+/- females. Weaning and genotyping of subsequent litters was performed as described below at approximately 21 d after birth; only SMAD3-/- mice were used in this study. All mice were housed in 60 square inch plastic cages with micro-isolator lids and maintained in temperature and humidity controlled rooms with a 12-h light-dark cycle. Harlan Teklad 22/5 Rodent Diet 8640 (22% crude protein, 5% crude fat) was given *ad libitum* prior to the start

of the study. All mouse procedures were approved by the Michigan State University Institutional Animal Care and Use Committee.

Genotyping

Ear tissue samples were obtained and DNA extracted with REDExtract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO) according to manufacturer's recommendations. Four primers were used for polymerase chain reaction: 1271 (GGATGGTCGGCTGCAGGTGTCC) and 1272 (TGTTGAAGGCAAACTCACAGAGC) to recognize SMAD sequences and give a 130 bp product, and 506 (CGGCGAGGATCTCGTCGTGACCCA) and 507 (GCGATACCGTAAAGCACGAGGAAG) to recognize vector sequences. Thermal cycling of the samples was conducted with an initial denaturation at 94 ℃ for 3 min, 40 cycles of denaturation-annealing-extension (respectively 20 s at 94 ℃, 30 s at 58 ℃, and 1 min at 72 ℃), and a final extension of 72 ℃ for 3 min. Polymerase chain reaction products were then evaluated on a 2% agarose gel and visualized under UV transillumination.

Helicobacter hepaticus culture

Isolates of *H. hepaticus* (strain 3B1, ATCC 51449) were kindly donated by Vince Young at University of Michigan. Bacteria were streaked onto sheep blood agar plates and incubated at 36 ℃ for 24-48 h under anaerobic conditions using GasPak™ pouch systems (BD, Franklin Lakes, NJ). After incubation, cultures were collected by the addition of Bacto™ Tryptic Soy Broth (BD, Franklin Lakes, NJ) and the optical density was assessed using a Bio-Tek Synergy HT multi-mode microplate reader (Bio-Tek, Winooski, VT) to ensure a constant bacterial population ($\geqslant$ 1.8 at 600 nm wavelength).

Dietary treatments and experimental procedures

SMAD3-/- mice (6-8 wk of age) were randomly assigned to one of three Open Source diets (Research Diets Inc, New Brunswick, NJ): control (CON; formula D12450B: 20% protein, 70% carbohydrate, 10% fat), 30% calorie-restricted (CR; formula D03020702B: 27% protein, 54% carbohydrate, 6% fat) or high fat (HF; formula D12492: 20% protein, 20% carbohydrate, 60% fat) to induce differing levels of adiposity as previously described[43]. Mice were weighed weekly to assess body weight changes. Body composition was also assessed after 20 wk using an EchoMRI-100™ quantitative nuclear magnetic resonance machine (Echo Medical Systems, Houston, TX).

In a pilot study (data not shown; n = 73 mice), weight differences between dietary treatments were found to be maximal around 20 wk, therefore this time frame was chosen for induction of colitis. Mice were thus fed diets (n = 37 CON, 19 CR, and 36 HF) for 20 wk and then gavaged with 0.3 mL dosages of either bacteria-free control Tryptic Soy Broth or *H. hepaticus*, one dosage per day on two consecutive days. Continued weight monitoring was conducted on the gavaged mice and any animal that exhibited a weight loss of > 20% from one week to the next was euthanized. Four weeks after infection, mice were euthanized *via* carbon dioxide asphyxiation. Terminal bleeds were performed *via* cardiac puncture and blood was collected in a heparin-coated syringe. Blood samples were centrifuged at 12 000 × g for 15 min at 4 ℃, and plasma was collected and frozen at -80 ℃ until further use.

Histopathology

The entire lower gastrointestinal tract was isolated and removed. Ceca were incised and cleared of fecal material with ice-cold phosphate buffered saline (PBS). Colons were similarly cleared, rinsed with PBS and sectioned. Cecum and colon samples were fixed for 24 h in a 10% formalin solution and then processed, stained, and scored by a board certified pathologist (Dr. Ingeborg Langohr) blinded to treatment for degree of colitis and dysplasia[44]. Grades were on a 1 to 4 scale both for inflammation (1, no inflammation; 2, mild inflammation; 3, moderate inflammation; 4, marked inflammation) and dysplasia (1, no dysplasia; 2, low grade dysplasia; 3, high grade dysplasia; 4, high grade dysplasia with invasion/adenocarcinoma). Briefly, low-grade dysplasia was characterized by thickened mucosa with elongated crypts with reduced numbers of goblet cells, but maintenance of cell polarity and nuclear morphology. High-grade dysplasia was characterized by thickened mucosa with elongated, irregularly branching glands, cytological and nuclear atypia including loss of differentiation and polarity, closely aggregated nuclei, and numerous mitotic figures. The two scores for colon and two scores for cecum tissue in each animal were combined such that a score of 4 indicated no inflammation or dysplasia and a score of 16 reflected maximal inflammation and neoplasia.

Quantification of serum adipokines by enzyme linked immunoabsorbance assay

Adiponectin and leptin were quantified by enzyme linked immunoabsorbance assay in plasma samples from mice prior to infection according to the manufacturer's instructions (R and D Systems; Minneapolis, MN). Plasma (n = 5 per group) was diluted 1:10 for leptin and 1:10 000 for adiponectin in reagent diluent. Upon completion of the assay, the plate was read at 450 nm wavelength using a Synergy® HT plate reader (Bio-Tek; Winooski, VT).

Immunohistochemistry

Antibodies specific for cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), and F4/80 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), CD3 from AbCam (Cambridge, MA) and Ki67 from Novus Biologicals (Littleton, CO). A rat ABC detection kit (sc-2019) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and remaining secondary antibodies from DAKO (DAKO Co., Carpinteria, CA). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Five-micron thick sections of formalin-fixed paraffin-embedded colon tissue were mounted on coated slides,

and dietary differences in macrophage (F4/80) and T lymphocyte cell infiltration (CD3), proliferation (Ki67), and expression of COX-2 and iNOS were evaluated using peroxidase biotin-streptavidin immunohistochemistry. Epitope retrieval was carried out either by heating sections (92-95 ℃) in 10 mmol/L citrate buffer (pH 6.0) for COX-2, iNOS, CD3, and Ki67 or with proteinase K digestion (Roche biochemicals) for F4/80. Slides were subsequently washed, treated with 3% H_2O_2 and incubated in 2.5% bovine serum albumin to reduce non-specific binding of antibody. Sections were incubated overnight at 4 ℃ with the primary antibody diluted in blocking buffer. After washing, sections were treated with appropriate biotinylated immunoglobulins followed by peroxidase-conjugated streptavidin at room temperature for 45 min each. Antigen-linked peroxidase was detected with the chromagen 3-3'-diaminobenzidine (DAB; 0.5 mg/mL) diluted in 10 mmol/L PBS (pH 7.2) containing 0.015% H_2O_2.

For quantification of $Ki67^+$ cells, a researcher blinded to treatments evaluated 10-20 full-length crypts/animal. The total number of nuclei ($Ki67^+$ and Ki67$^-$) lining one side of the crypt and extending from the base of the crypt to the lumen was recorded. Proliferative index (number of $Ki67^+$ cells/total cells) was then calculated and analyzed. Colonic staining of COX-2, iNOS, CD3, and F4/80 were quantified using Nikon software and an inverted light microscope (Nikon; Kanagawa, Japan) equipped with a color camera (DS-U2, Nikon; Kanagawa, Japan). Using a 20 × objective, areas surrounding full length crypts in the proximal colon were traced and the positive stained area (total number of pixels) was quantified. Data are expressed as a percentage of positive stained area in relation to the total surface area. For each stain, at least 10 measurements/animal were taken.

Statistical analysis

Data for body weight and composition, colitis scores, and plasma adiponectin and leptin levels were analyzed with analysis of variance using Prism software (Graph Pad; San Diego, CA). Prior to analysis, normal distribution of the data was tested and when appropriate, data were transformed prior to statistical analysis. When statistical differences were detected, individual comparisons were made using Bonferroni's multiple comparison test.

RESULTS

Effect of dietary treatment on body fat composition and plasma adipokines

Dietary treatments significantly influenced AT stores in mice prior to initiation of *H. hepaticus* infection. HF-fed mice weighed significantly more and had a higher percent body fat than CON or CR mice (Table 1). Conversely, CR mice weighed less than both HF and CON mice primarily due to lower amount of AT.

Dietary treatment also affected plasma levels of metabolic hormones (Figure 1). Adiponectin was significantly lower in CR and HF mice compared to CON mice (Figure 1B, $P < 0.05$) whereas plasma concentrations of leptin were lowest in CR and highest in HF mice (Figure 1A, $P < 0.05$).

Table 1 Pre-infection body weights and body composition of SMAD3-/- mice after 20 wk on dietary treatment

Diet	Weight change (%)	Lean tissue (%)	Adipose (%)
CON	35 ± 17.8	68 ± 5.0	22 ± 4.0
CR	32 ± 11.8	73 ± 3.0[a]	14 ± 2.0[a]
HF	45 ± 18.4	59 ± 4.0[d]	32 ± 7.0[d]

[a]$P < 0.05$ *vs* control animals; [d]$P < 0.01$ *vs* control and calorie restricted animals. CON: Control; CR: Calorie restricted; HF: High fat.

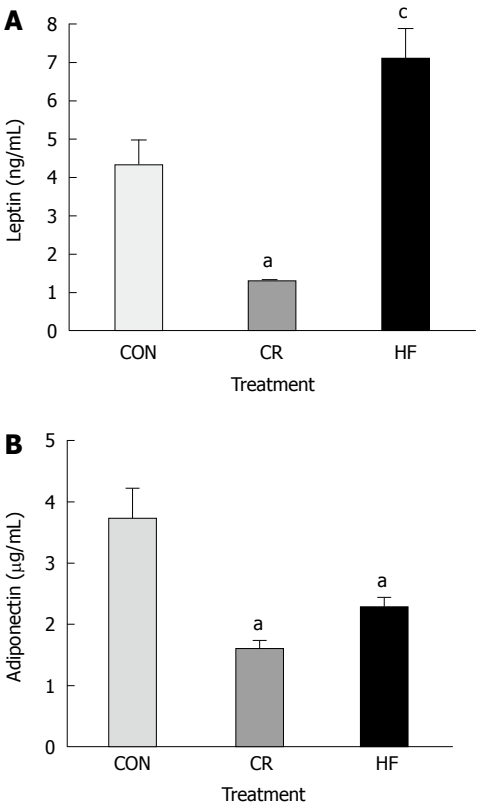

Figure 1 Plasma adipokines in SMAD3-/- mice after 20 wk on dietary treatment. A: Average plasma concentrations of leptin between diet groups prior to infection. Calorie restricted (CR) mice had significantly lower concentrations, whereas high fat (HF) mice had significantly higher concentrations of leptin compared to control (CON) mice ($P < 0.01$); B: Average plasma concentrations of adiponectin between diets prior to infection. Both CR and HF diet mice had significantly lower concentrations of adiponectin than mice on CON diet ($P < 0.05$). [a]$P < 0.05$ *vs* control; [c]$P < 0.05$ *vs* all other groups.

Effect of diet treatment on colitis and dysplasia scores 4 wk post-infection

After 20 wk on dietary treatment, mice were infected with *H. hepaticus* to determine if pre-infection adiposity would influence colitis scores 4 wk following infection. Unexpectedly, we found that CR mice had higher mortality rates beginning at 1 wk post-infection (Figure 2). Because only 40% of CR mice ($n = 2$) survived the infec-

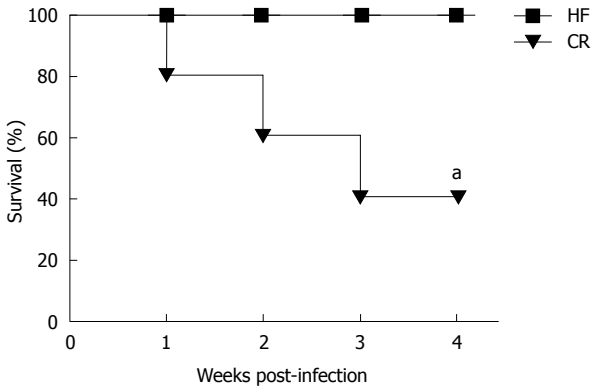

Figure 2 Survival curve of high fat, control and calorie restricted mice after infection with *Helicobacter hepaticus*. Calorie restricted (CR) mice experienced increased mortality after infection, with only 40% of CR mice surviving 4 wk post-infection. $^{a}P < 0.05$ *vs* baseline. HF: High fat.

tion period, we were unable to obtain enough tissue to appropriately evaluate colitis in this group.

HF and CON mice were sacrificed 4 wk following infection and dietary differences in colitis and dysplasia were scored in the cecum and colon. Compared to the uninfected SMAD3-/- mice, CON and HF-fed mice exhibited significant infiltration of immune cell populations into the lamina propria following infection (Figure 3A). We found no significant differences in combined scores between CON (7.9 ± 1.9) and HF (7.8 ± 2.1) mice following infection (Figure 3B). In order to examine the potential effect of time, we allowed some mice to remain infected for up to 6 wk, and compared scores at 4, 5 and 6 wk post-infection. However, there were no further significant changes between or within dietary treatments across time (Figure 3C).

Differences in pre-infection body weight on colonic proliferation and inflammatory markers

Immunohistochemistry analysis on proliferation and inflammatory markers in colon sections from CON-fed uninfected mice and CON and HF-fed mice 4 wk post-infection are presented in Figure 4. There was no significant difference in percentages of colon epithelia positively stained for Ki67, a marker of cellular proliferation, or F4/80 macrophages between CON or HF diet mice at 4 wk post-infection as compared to uninfected mice. A higher percentage of iNOS and COX-2 immunoreactivity ($P > 0.05$) and significantly higher levels of $CD3^{+}$ lymphocytes ($P < 0.05$) was observed after infection in CON and HF fed mice compared to broth-treated mice, consistent with the increased colitis scores; however, there was no difference between infected animals on either diet (Figure 4).

DISCUSSION

Local and systemic alterations in adipokines are implicated in the pathogenesis of IBD[16]. In the current study, we investigated whether diet-induced changes in adiposity prior to induction of colitis would influence inflammatory changes in the colon of SMAD3-/- mice. After 20 wk on dietary treatment, significant changes in body composition were observed, with CR mice having the least and high fat-fed mice the most body fat compared to controls. Consistent with differences in adiposity, plasma concentrations of adipokines were significantly altered. Leptin is secreted in proportion to white AT mass whereas plasma adiponectin concentrations are markedly reduced in obese individuals[45]. In the current study, leptin levels were 1.5-fold higher in HF mice and 4.5 lower in CR mice, whereas low plasma adiponectin concentrations were observed in both HF and CR mice. Significantly lower levels of adiponectin were reported in individuals with anorexia and bulimia prior to treatment, which was restored following refeeding[46], suggesting a critical fat mass may be necessary for secretion[46].

We next evaluated whether differences in adiposity and adipokine levels would influence severity of colitis. We found that restricting caloric intake to 70% that of control animals significantly impacted survival of mice following *H. hepaticus* infection, with only 40% of mice surviving the full infection period. These results were somewhat unexpected as moderate calorie restriction delays or reduces severity of autoimmune disorders[47,48], prolongs life span[49], as well as inhibiting tumorigenesis at several different sites[50,51]. Additionally, Shibolet *et al*[52] found that calorie restricted mice were protected from chemically-induced colitis, which was associated with a decrease in pro-inflammatory cytokine release and an increase in NK1.1 + T lymphocytes. However, in experimental models of infection, CR increases susceptibility to bacterial[53] and parasitic[54] infections, as well as viral infections[55-57], consistent with our findings.

Leptin is recognized to play a pivotal role in both innate and adaptive immune responses by stimulating T cell proliferation[58], chemotaxis of neutrophils[59], NK cell maturity and activation[60], differentiation of dendritic cells[61], eicosanoid synthesis and cytokine release by monocytes and macrophages[62-64], as well as in preventing thymocyte apoptosis[65]. The increased mortality observed in this study was not investigated and is the aim for future projects. However, in a parallel study, we found baseline NK cell populations are reduced in the SMAD3-/- compared to SMAD3+/- mice (Fenton, JI, unpublished observations). Therefore, it is possible that reduced immune cell populations combined with lower circulating leptin in CR mice contributed to immune suppression and reduced capability to mount a response to *H. hepaticus*. In support of this, Clinthorne *et al*[56] recently reported that short term refeeding restored leptin in CR mice, improved survival, and attenuated the decline in NK cell function following influenza infection. Low circulating leptin in tuberculosis patients was also associated with increased disease severity[66], suggesting a causal relationship between adiposity, leptin, and immune response.

The development of IBD results from a complex interaction between genetic, immune and environmental

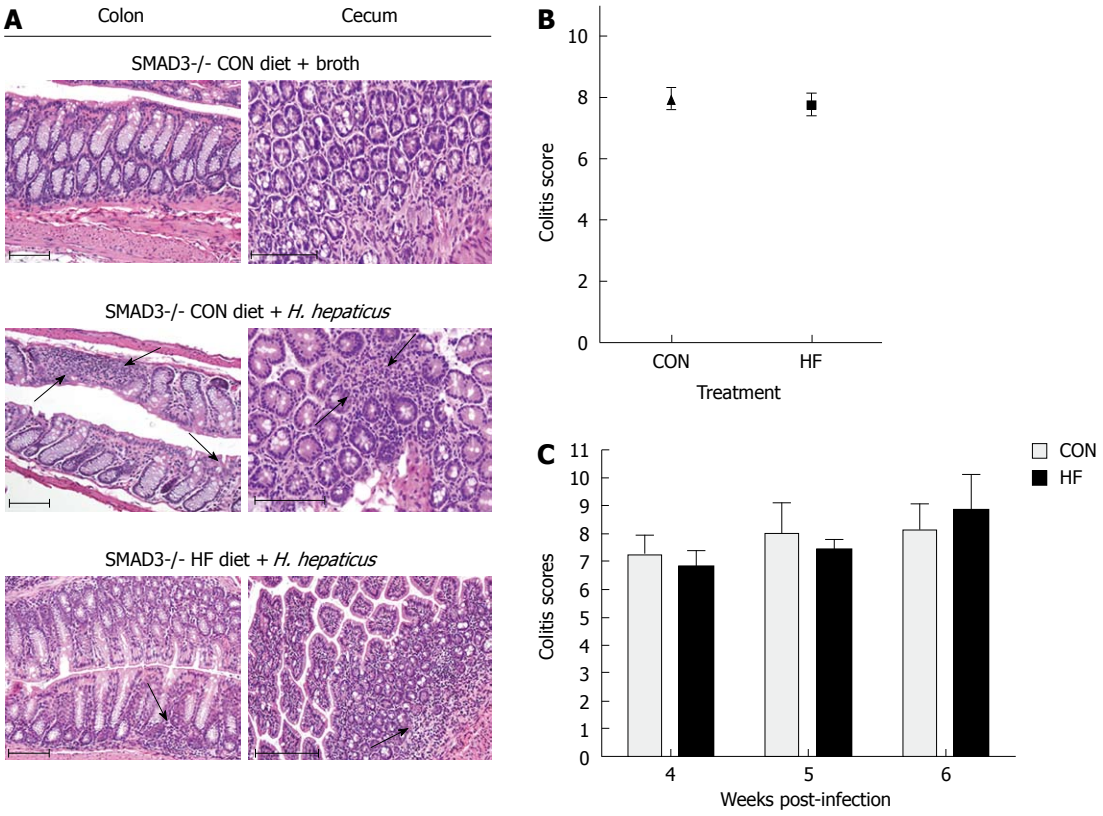

Figure 3 Effect of dietary treatment on colitis severity in SMAD3-/- mice. A: HE stained sections from the colon and cecum of SMAD3-/- control (CON)-fed mice treated with broth and SMAD3-/- CON- or high fat (HF)-fed mice 4 wk post-infection. Four weeks following infection, the number of inflammatory cells in the lamina propria is increased in the colon and cecum of both CON and HF diet animals, consistent with mild inflammation (arrows denote). Scale bars represent 100 μm; B: Average combined colitis and dysplasia scores between diet groups at 4 wk post-infection. There was no significant difference in theses scores between the CON and HF diet treatment groups; C: Average combined colitis and dysplasia scores between CON and HF mice at 4 wk, 5 wk and 6 wk post-infection. There was no difference between CON and HF diet groups at any point, and there was no effect of time on the scores between diets. *H. hepaticus*: *Helicobacter hepaticus*; HE: Hematoxylin and eosin.

factors. Diet is an important environmental factor in IBD pathogenesis; diets high in dairy products, refined sugar and fast food are associated with an increased risk of developing IBD[67,68]. However, there is little conclusive epidemiological evidence for a causal relationship between dietary intake and onset of IBD[68]. Importantly, after the onset of IBD, malnutrition (resulting from decreased food intake, malabsorption and increases in both nutrient loss and energy requirements) is common[69]. These changes in intake and energy expenditure may result from circulating inflammatory mediators associated with the pathophysiology of IBD, such as TNF-α, IL-1 and IL-6. These cytokines can increase catabolism and lead to anorexia[68,70]. Malnutrition is also associated with adverse outcomes in IBD progression, exacerbating immunodeficiency, perpetuating malabsorption and increasing risk of infections, particularly *via* bacterial translocation. This differs somewhat from what is modeled in this study. The caloric restriction diet met 100% of all nutrient needs and was only deficient by 30% of energy. The model caloric restriction of experimental-colitis is consistent with increased mortality in IBD related to energy deficit but not malnutrition[70-73]. Given the importance of leptin and immune function, our data do imply that reduced AT and leptin production (directly related to fat cell size and number) may further impair innate immune response to a pathogen. Similar mortality effects were observed in caloric restriction and influenza infection in mice discussed above.

Although low body fat stores and reduced circulating levels of leptin may impair immune responses to infectious stimuli, elevated leptin and peripheral leptin resistance is commonly observed in obese individuals. Previous studies suggest that obesity exacerbates colonic inflammation[40,74-77]. Increased mesenteric fat and fat creeping were also observed in inflamed intestinal regions in patients with CD[78]. Additionally, overweight and/or obese individuals with CD have more complications from and more frequent disease relapses than normal weight individuals[41]. In the current study, we did not observe any overall changes in colitis severity between control and HF mice, despite differences in body fat and serum adipokines. To determine whether general inflammatory markers were altered, we stained for COX-2 and iNOS, which are induced by pro-inflammatory cytokines in a variety of pathological conditions including UC in humans[79,80]. Further, we examined colons for T lymphocyte and macrophage infiltration to determine whether higher body fat

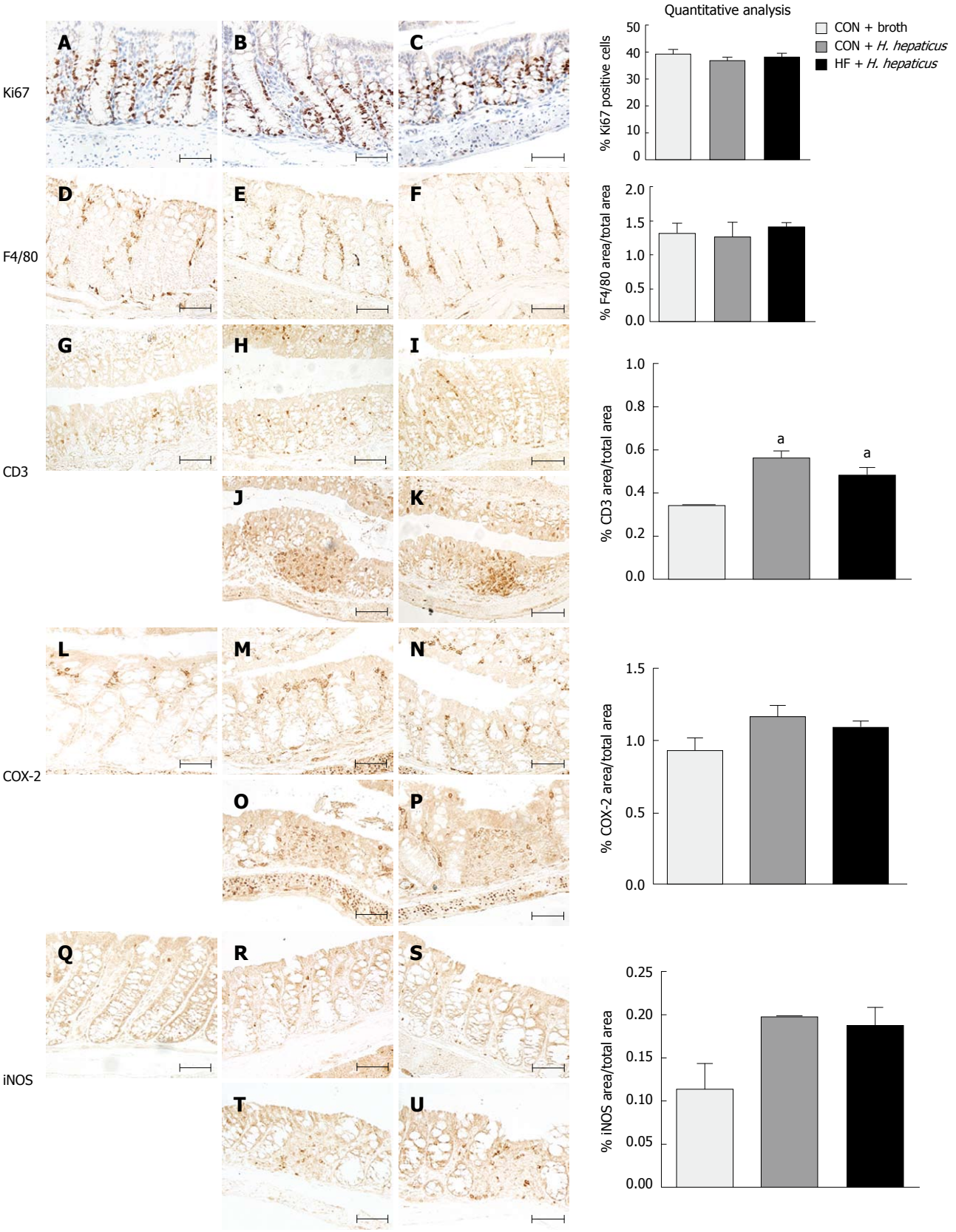

Figure 4 Effect of diet on proliferation and inflammatory markers following infection with *Helicobacter hepaticus* in SMAD3-/- mice. Immunohistochemical staining for Ki-67 (A-C), F4/80 (D-F), CD3 (G-K), COX-2 (L-P) and iNOS (Q-U) in proximal colon sections of SMAD3-/- mice fed control (CON) diet and treated with broth (A, D, G, L, Q), CON-fed mice treated with *Helicobacter hepaticus* (*H. hepaticus*) (B, E, H, J, M, O, R, T), or fed a high fat (HF) diet and treated with *H. hepaticus* (C, F, I, K, N, P, S, U) 4 wk post-infection. Normal appearing proximal colon segments (A-I, L-N, Q-S) and inflamed colon segments with lymphoid infiltrate (J, K, O, P, T, U). Scale bars represent 100 μm. There were no significant differences in proliferation indices or in macrophage infiltration. CON and HF diet mice had slightly increased staining for $CD3^+$ T lymphocytes, cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS) post-infection compared to broth-treated controls, but averages were not statistically significant between diets ($P > 0.05$). $^aP < 0.05$ *vs* CON animals.

would influence specific subsets of inflammatory cells. Both control and high fat mice treated with *H. hepaticus* had moderately elevated levels of $CD3^+$ T lymphocytes, as well as COX-2 and iNOS immunoreactivity in epithelia-associated myofibroblasts and macrophages, compared to broth-treated mice. However no further changes were observed between dietary treatments. We also did not observe any differences in the proliferative index in the proximal colon segments between treatments, demonstrating that higher body fat does not influence disease severity in our model.

Results from the current study suggest that moderately increased adiposity induced by high fat feeding does not influence colitis severity in SMAD3-/- mice despite changes in plasma adipokines. Although we were able to induce a body fat percentage of 32% in the SMAD3-/- mice, this percentage body fat may be insufficient to induce chronic inflammation associated with obesity observed in other mouse strains that approach 50%-60% AT. More importantly, we found that calorie restricted mice had a higher mortality in response to infection with *H. hepaticus*. Future studies examining the association between percent body fat, leptin, and immune responses to infectious stimuli leading to IBD are warranted.

COMMENTS

Background

Recent studies indicate that the constant low-grade inflammation associated with obesity or excess adipose tissue (AT), including elevated serum levels of C-reactive protein, interleukin-6 and tumor necrosis factor-α, may contribute to the severity of inflammatory bowel diseases (IBD). Additionally, overweight and/or obese individuals with Crohn's disease were found to have more complications and more frequent disease relapses than normal weight individuals, providing a potential link between excessive AT and pathogenesis of IBD.

Research frontiers

There is currently insufficient evidence to support a causal relationship between obesity and IBD; however, the conditions share similar inflammatory characteristics. In this study, the authors demonstrate that moderately increased adiposity with associated changes in adipokines does not influence colitis severity in SMAD3-/- mice.

Innovations and breakthroughs

Previous studies suggest that obesity exacerbates colonic inflammation. Malnutrition is associated with adverse outcomes in IBD progression, exacerbating immunodeficiency, perpetuating malabsorption and increasing risk of infections. We report that moderate obesity did not influence the severity of colon inflammation in experimentally-induced colitis. Importantly, caloric restriction negatively influences survival following pathogen challenge, potentially due to an impaired immune response.

Applications

Further studies should examine the role of energy balance, both positive and negative, on the pathogenesis of IBD. Understanding how caloric restriction increases mortality in this preclinical model of experimentally-induced colitis may lead to therapeutic strategies for bacterially-driven IBD in humans.

Terminology

In the current study,the authors evaluated the influence of adiposity on colitis severity in SMAD3-/- mice. SMAD3 is a transcription factor for transforming growth factor (TGF)-β. TGF-β is a protein that controls proliferation and cellular differentiation, both in normal cells and in the early stages of carcinogenesis. SMAD3-/- mice have defective TGF-β signaling and develop mild colitis within 4 wk following infection with *Helicobacter spp*. Dysfunctions in TGF-β signaling are commonly observed in human IBD and during colon cancer development.

Peer review

It is a relevant paper dealing with a demanding issue too much neglected in the biologic-era treatment of IBD. The suggestion is implementing discussion with some speculation on the potential negative influence of malnutrition in IBD.

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S- Editor Tian L **L- Editor** Rutherford A **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.637

World J Gastroenterol 2012 February 21; 18(7): 637-645
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Identification of colorectal cancer metastasis markers by an angiogenesis-related cytokine-antibody array

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Supported by A grant of "Department of Health, Government of Navarra, Spain (23/2009)"
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Received: May 16, 2011 Revised: July 4, 2011
Accepted: July 11, 2011
Published online: February 21, 2012

Abstract

AIM: To investigate the angiogenesis-related protein expression profile characterizing metastatic colorectal cancer (mCRC) with the aim of identifying prognostic markers.

METHODS: The expression of 44 angiogenesis-secreted factors was measured by a novel cytokine antibody array methodology. The study evaluated vascular endothelial growth factor (VEGF) and its soluble vascular endothelial growth factor receptor (sVEGFR)-1 protein levels by enzyme immunoassay (EIA) in a panel of 16 CRC cell lines. mRNA VEGF and VEGF-A isoforms were quantified by quantitative reverse-transcription polymerase chain reaction (Q-RT-PCR) and vascular endothelial growth factor receptor (VEGFR)-2 expression was analyzed by flow cytometry.

RESULTS: Metastasis-derived CRC cell lines expressed a distinctive molecular profile as compared with those isolated from a primary tumor site. Metastatic CRC cell lines were characterized by higher expression of angiopoietin-2 (Ang-2), macrophage chemoattractant proteins-3/4 (MCP-3/4), matrix metalloproteinase-1 (MMP-1), and the chemokines interferon γ inducible T cell α chemoattractant protein (I-TAC), monocyte chemoattractant protein I-309, and interleukins interleukin (IL)-2 and IL-1α, as compared to primary tumor cell lines. In contrast, primary CRC cell lines expressed higher levels of interferon γ (IFN-γ), insulin-like growth factor-1 (IGF-1), IL-6, leptin, epidermal growth factor (EGF), placental growth factor (PlGF), thrombopoietin, transforming growth factor β1 (TGF-β1) and VEGF-D, as compared with the metastatic cell lines. VEGF expression does not significantly differ according to the CRC cellular origin in normoxia. Severe hypoxia induced VEGF expression up-regulation but contrary to expectations, metastatic CRC cell lines did not respond as much as primary cell lines to the hypoxic stimulus. In CRC primary-derived cell lines, we observed a two-fold increase in VEGF expression between normoxia and hypoxia as compared to metastatic cell lines. CRC cell lines express a similar pattern of VEGF isoforms ($VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$) despite variability in VEGF expression, where the major transcript was $VEGF_{121}$. No relevant expression of VEGFR-2 was found in CRC cell lines, as compared to that of human umbilical vein endothelial cells and sVEGFR-1 expression did not depend on the CRC cellular origin.

CONCLUSION: A distinct angiogenesis-related expression pattern characterizes metastatic CRC cell lines. Factors other than VEGF appear as prognostic markers and intervention targets in the metastatic CRC setting.

Key words: Colorectal cancer metastasis; Cytokine-antibody array; Angiogenesis; Vascular endothelial growth factor; Biomarkers

Peer reviewers: Marek Bebenek, MD, PhD, Department of Surgical Oncology, Regional Comprehensive Cancer Center, pl. Hirszfelda 12, 53-413 Wroclaw, Poland; Lucia Ricci Vitiani, Dr., Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Viale Regina Elena 299, Rome 00161, Italy

Abajo A, Bitarte N, Zarate R, Boni V, Lopez I, Gonzalez-Huarriz M, Rodriguez J, Bandres E, Garcia-Foncillas J. Identification of colorectal cancer metastasis markers by an angiogenesis-related cytokine-antibody array. *World J Gastroenterol* 2012; 18(7): 637-645 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/637.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.637

INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths. The prognosis of CRC is dependent upon the extent of disease and approximately 60% of patients develop metastases after surgical resection. With a 5-year survival rate of less than 10% in patients with distant metastatic disease, targeting the metastatic process and sites should provide an effective treatment[1]. The progressive growth of colon cancer and subsequent metastatic process is dependent on an angiogenic network[2,3]. Thus, anti-angiogenic strategies have emerged as effective therapies in patients with colon cancer, especially in the metastatic setting of the disease[4-6]. Yet, differences in the magnitude of survival benefit point to alternative pathways in the tumor microenvironment as responsible for inconsistent outcomes[7].

Angiogenesis is a complex process dependent on the angiogenic factors secreted by the tumor and stroma cells[8]. Vascular endothelial growth factor is considered the major pro-angiogenic factor[9]. The vascular endothelial growth factor (VEGF) gene encodes for six alternatively spliced isoforms[10] with differential diffusion potential and binding to receptors[11]. The question currently consists of understanding the significance of VEGF/vascular endothelial growth factor receptor (VEGFR) signaling in cancer cells[12,13]. The VEGF isoforms and VEGF receptor expression pattern would drive the activity and functionality of the VEGF/VEGFR pathway in both tumor and endothelial cells. The multistep process of angiogenesis accompanies the multistage development of a tumor[14]. The switch into the metastatic phenotype brings a number of changes within the tumor microenvironment, including acquisition of hypoxia-tolerance mechanisms[15]. While up-regulation of VEGF expression is activated mainly under hypoxia[9], recent reports reflect on the question of whether metastatic tumors rely as much on angiogenesis and VEGF as primary tumors[15].

Other studies report that tumors in more advanced stages do not rely on a unique angiogenesis driver[2]. A network of multiple cytokines and growth factors create a crosstalk within the tumor microenvironment which ultimately drives tumor angiogenesis[2,16]. The mediators of vessel wall remodeling matrix metalloproteinases, macrophage chemoattractant proteins and angiopoietin, involved in invasion and metastasis processes, exert pro-angiogenic signals[8,17]. Chemokines such as interleukin (IL)-1α and IL-8 play an important role in colon cancer progression and angiogenesis[18], and IL-8 up-regulates MMPs[19]. VEGF expression actually determines the activity of Ang-1/Ang-2 and the expression of MCPs[20,21].

Great efforts have been made to characterize biomarkers in CRC[22]. However, the question of biomarkers of CRC metastasis remains currently unresolved. On this basis, the aim of this study was to characterize the protein factors behind the angiogenic potential of CRC cell lines of metastatic origin.

Table 1 Colorectal cancer cell lines origin

Cell line	Type/origin
SW620	Colon adenocarcinoma. Derived from: metastasis to lymph node
T84	Colon carcinoma. Derived from metastasis to lung
LoVo	Derived from metastatic site: left supraclavicular region
SW480	Colon adenocarcinoma
WiDr	Colon adenocarcinoma
RKO	Colon carcinoma
HT29	Colon adenocarcinoma
HCT15	Colon adenocarcinoma
HCT116	Colon carcinoma
SW1116	Colon adenocarcinoma
SW1417	Colon adenocarcinoma
LS174T	Colon adenocarcinoma
LS513	Colon carcinoma
Caco2	Colon adenocarcinoma
DLD-1	Colon adenocarcinoma
LS411N	Colon adenocarcinoma
Colo320	Colon adenocarcinoma

MATERIALS AND METHODS

Cell cultures and conditioned media

We used 16 CRC cell lines: HT29, WiDr, HCT116, RKO, SW480, Colo320, Caco2, SW1116, LS174T, SW1417, DLD-1, LS513, HCT15, SW620, LoVo and T84 (all from American Type Culture Collection, Manassas, VA) (Table 1). The cell lines were maintained in the recommended growth media supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (GIBCO). For harvesting conditioned media, CRC lines cells were grown approximately to 70% confluence in serum free media. The conditioned media were collected after 24 h of incubation, centrifuged and kept frozen.

VEGF and sVEGFR1 protein detection by quantitative immunoassay

VEGF-A in supernatant was determined using the Human VEGF Quantikine® EIA kit (R and D Systems) and soluble vascular endothelial growth factor receptor (sVEGFR)-1 was quantified by EIA (Human sVEGF

Table 2 Primer and probe sequences for vascular endothelial growth factor isoforms quantitative reverse-transcription polymerase chain reaction

	Sense primer	Antisense primer	Taqman probe	Amplicon size (bp)
VEGF end-point and cloning	ACTGCCATCCAATCGAGACC	GATGGCTTGAAGATGTACTCGATCT		
GAPDH end-point and cloning	TGGTATCGTGGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTCAGC		189
$VEGF_{121}$ mRNA	CAAGGCCAGCACATAGGAGA	CTCGGCTTGTCACATTTTTC	CTTCCTACAGCACAACAAATGT-GAATGCAGA	101
$VEGF_{165}$ mRNA	TGTGAATGCAGACCAAAGAAAGA	TGCTTTCTCCGCTCTGAGC	AGAGCAAGACAAGAAAATCCCT-GTGGGC	74
$VEGF_{189}$ mRNA	CGCAAGAAATCCCGGTATAAGT	TGCTTTCTCCGCTCTGAGC	AGGCCCACAGGGAACGCTCCAG	65
GAPDH	TGGTATCGTGGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTCAGC	CCCAGAGACTGTGGATGGCCCC	189

VEGF: Vascular endothelial growth factor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

R1/Flt-1 Quantikine®, R and D Systems), according to the manufacturer's instructions. We normalized VEGF and sVEGFR-1 protein levels per number of cells. Results are the average of replicates.

Total VEGF and isoforms mRNA determination by quantitative reverse-transcription polymerase chain reaction

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA). Single strand DNA was synthesized from 1 μg total RNA using the cDNA Archive kit (Applied Biosystems). Quantitative reverse-transcription polymerase chain reaction (Q-RT-PCR) for total VEGF was performed using primers and probes purchased from Applied Biosystems (Hs00900054_m1). RNA18s (Hs99999901_s1) was used as an endogenous control and data obtained was represented as 2-ΔCt.

VEGF isoforms were determined by Q-RT-PCR using primers designed specifically for $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control (Table 2). The relative quantification of samples was performed using a standard curve by dilution of a specific plasmid for each isoform (ranging from 10 pg to 1 fg). Human VEGF cDNA for each isoform and GAPDH were cloned from total RNA isolated from lung cancer resection as follows. PCR products were run through a 1% agar gel and bands of the size expected for $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ were isolated and purified. Each VEGF isoform was cloned into the pCRII vector (Invitrogen) and sequenced (ABI PRISM Big Dye Terminator Cycle Sequencing reaction kit; ABI Protocol, Gene Amp 9600, Applied Biosystems) to verify its identity.

Time course hypoxia-normoxia

The cell lines were maintained in the recommended growth media supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (GIBCO). After washing with phosphate buffered saline (PBS), serum-free medium was added and the cells exposed to normoxic or hypoxic conditions for 6 h, 12 h, 24 h, 36 h, 48 h and 72 h. Hypoxic conditions were achieved by culturing cells in a modulator incubation chamber (Sanyo MCO-18 M) gassed with 1% O_2, 50 mL/L CO_2, and 94% N_2. VEGF protein secretion was measured in the supernatant by enzyme immune-assay (EIA) and VEGF mRNA levels by Q-RT-PCR. Cell proliferation was evaluated by the Trypan Blue exclusion method.

VEGFR-2 expression in colorectal cancer cell lines by flow cytometry

The expression of VEGFR-2 (KDR) in CRC cell lines was determined by flow cytometry (FacScan, Becton-Dickinson). After trypsinization, cells were incubated in medium for 12 h on a rocker platform to enable regeneration of the receptors. Cells were Fc-blocked by treatment with 100 μL of AB human serum for 15 min at room temperature prior to staining with 10 μL of PE-conjugated anti-VEGFR-2 antibody (Becton Dickinson Biosystems) for 30 min at 4 ℃. Following the incubation, unbounded anti-VEGFR-2 antibody was removed by washing the cells twice in 4 mL PBS buffer. The human umbilical vein endothelial cells (HUVEC) cell line was used as a positive control.

Secreted angiogenic profile by cytokine antibody-array

The secretion of angiogenic factors by CRC cell lines was evaluated in duplicate using a protein array method (RayBio® Human Angiogenesis Antibody Array, RayBiotech C Series 1000, RayBiotech, Inc Norgross, GA). This assay is capable of simultaneously detecting 44 different angiogenic factors (spotted in sub-arrays I and II) with high specificity. The sensitivity of the antibodies present in the arrays ranged from 1-2000 pg/mL. Conditioned media was obtained after the incubation of 2×10^5 cells in serum-free medium for 20 h at 37 ℃ and 5% CO_2. Each array was incubated with 1.2 mL of medium at 4 ℃ overnight, and bound antigens were detected according to the manufacturer's instructions. To determine the relative concentrations of angiogenic factors in the media, the densities of individual spots were measured using Imagene 4.1 software (Biodiscovery Inc., Marina Del Rey, United States) for image capture and analysis.

Statistical analysis

Statistical analysis was carried out with SPSS 13.0 soft-

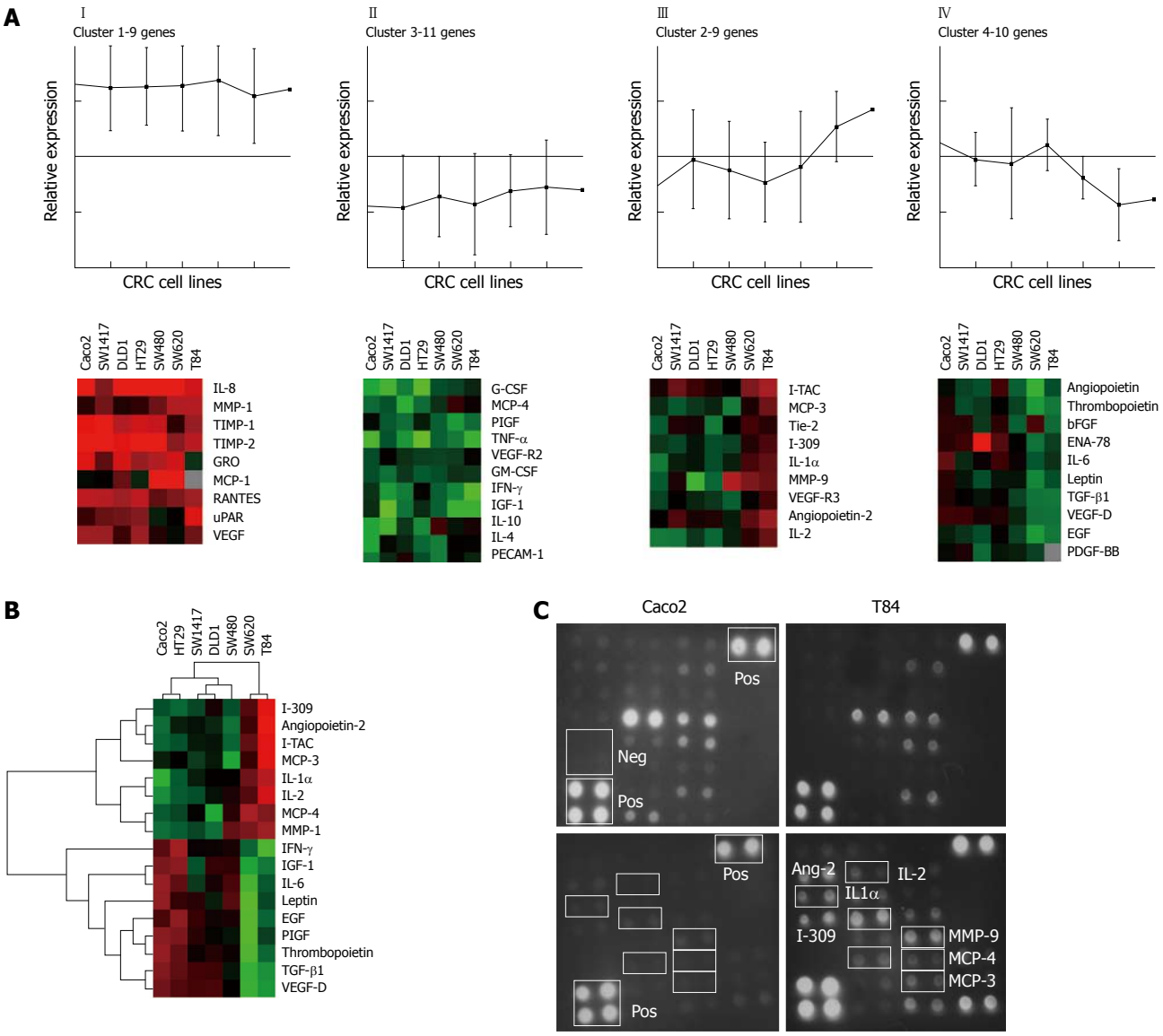

Figure 1 Angiogenesis-related factors expression profile in colorectal cancer cell lines as determined by cytokine antibody-array. A: K-means (n = 4) clustering grouped the angiogenesis-related proteins according to level of expression; B: Unsupervised-hierarchical clustering of the factors with a significantly different expression in primary and metastatic colorectal cancer (CRC) cell lines; C: Images of subarrays Ⅰ and Ⅱ of the primary Caco2 and the metastatic T84 CRC cell lines after detection and processing. IL: Interleukin; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinases; GRO: Growth related oncogene; MCP: Macrophage chemoattractant proteins; RANTES: Regulated upon activation normally T-expressed and secreted; uPAR: Urokinase-type plasminogen activator-receptor; G-CSF: Granulocyte colony-stimulating factor; PIGF: Phosphatidylinositol glycan, class F; TNF-α: Tumor necrosis factor-α; GM-CSF: Granulocyte macrophage colony-stimulating factor; IFN-γ: Interferon γ; IGF: Insulin-like growth factor; PECAM: Platelet-endothelial cell adhesion molecule; I-TAC: Inducible T cell α chemoattractant protein; ENA: Epithelial neutrophil activating protein; EGF: Epidermal growth factor; PDGF-BB: Platelet-derived growth factor, β polypeptide; TGF-β1: Transforming growth factor β1; Neg: Negative control; Pos: Positive control.

ware (SPSS Inc.). Associations between VEGF expression and VEGF isoforms pattern were determined with the Spearman correlation. Differences between groups were determined by the Mann-Whitney *U* test. The level of two-tailed statistical significance was 0.05.

CRC cell line angiogenesis cytokine antibody-arrays raw data were normalized to the global median [BRB Array Tools 3.6.0 (NCI)] of signals detected as per manufacturer's instructions. GENESIS software (Institute for biomedical engineering, Graz University of Technology, Graz, Austria) was used for the analyses of clustering of samples and genes and K-means and hierarchical unsupervised clustering analyses were performed to determine cytokine profiles.

RESULTS

Distinct angiogenesis-related expression pattern in primary and metastatic colorectal cancer cell lines

To identify the angiogenesis-related "secretome" of CRC cell lines in normoxia, we analyzed 44 angiogenesis-related cytokines and growth factors by an antibody-array in primary (Caco2, SW1417, DLD1, HT29 and SW480) and metastatic (SW620 and T84) CRC cell lines. K-means analysis classified CRC cell line angiogenesis-related secreted factors according to their level of secretion (Figure 1A). Cluster I showed a homogeneous high expression of the pro-angiogenic IL-8, MMP-1, MCP-1, growth related oncogene (GRO)-α, regulated upon activation,

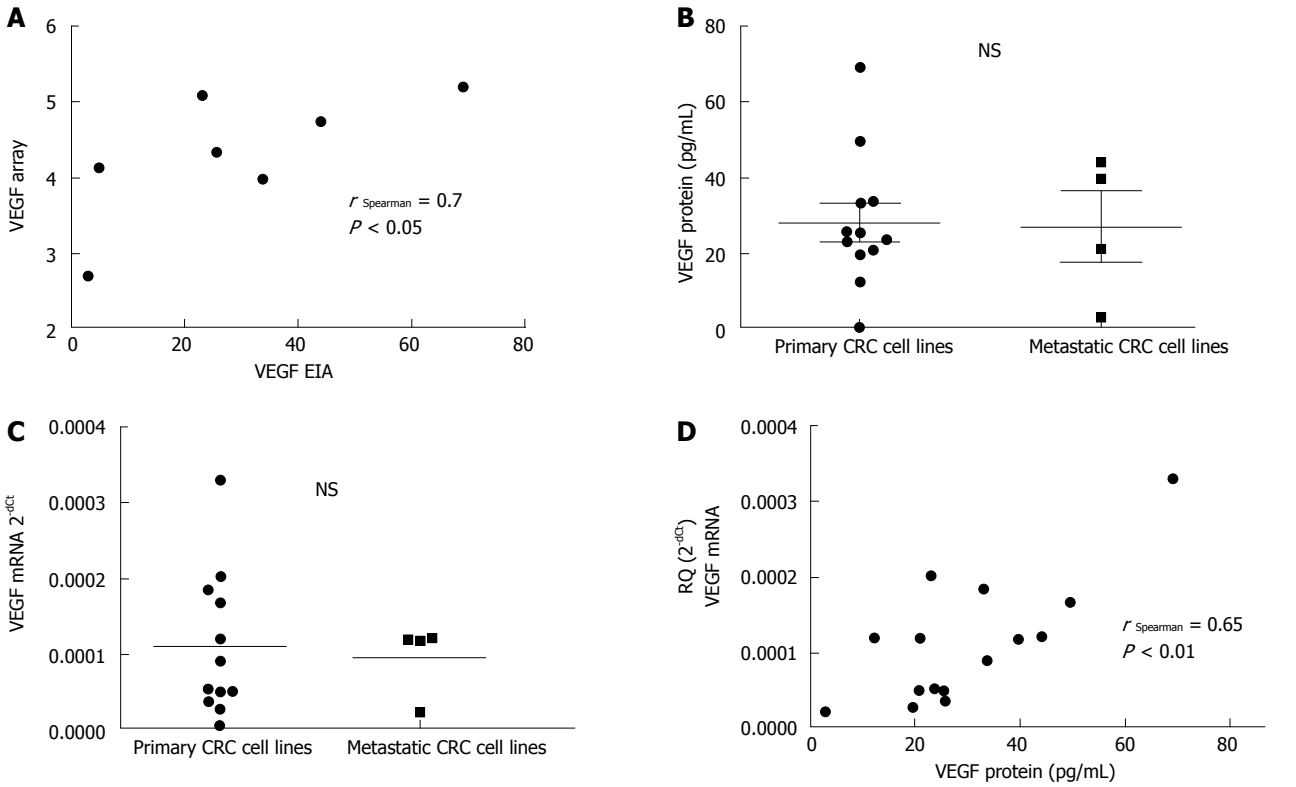

Figure 2 Vascular endothelial growth factor expression in colorectal cancer cell lines. A: A statistically significant positive correlation is found between vascular endothelial growth factor (VEGF) protein as determined by antibody-array and by enzyme immunoassay (EIA), validating the array method; B and C: Colorectal cancer (CRC) cell lines exhibit variability in VEGF protein (B) and mRNA (C) expression according to their primary or metastatic origin (not statistically significant); D: A statistically significant positive correlation is found between VEGF protein by EIA and VEGF mRNA, suggesting the major role of transcriptional mechanisms in the regulation of VEGF expression. NS: Not significant.

normal T-cell expressed, and secreted protein (RANTES), urokinase-type plasminogen activator-receptor (uPAR) and VEGF; and the anti-angiogenic tissue inhibitor of metalloproteinases tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2 (Figure 1A, cluster Ⅰ). Cluster Ⅱ integrated angiogenic factors not secreted by CRC cell lines in normoxia, including VEGF family proteins placental growth factor (PlGF) and sVEGFR-2 and inflammatory cytokines with pro-angiogenic properties granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ, tumor necrosis factor-α (TNF-α) (Figure 1A, cluster Ⅱ). Primary tumor- and metastasis-derived CRC cell lines were characterized by a distinct angiogenesis-related molecular pattern in normoxia (Figure 1A, cluster Ⅲ and Ⅳ). Figure 1B shows the unsupervised hierarchical clustering of the antibody-array proteins significantly differing in expression according to their cellular origin. One-way ANOVA ($P < 0.05$) grouped primary and metastatic cell lines according to their differential molecular expression pattern. Metastasis-derived cell lines were characterized by higher expression of Ang-2, MCP-3, MCP-4, MMP-1 and the chemokines I-TAC, I-309, IL-2 and IL-1α ($P < 0.05$), and a trend was found for MMP-9, as compared to primary tumor cell lines (Figure 1B). On the other hand, CRC cell lines isolated from a primary tumor site were clustered together according to the higher expression of IFN-γ, IGF-1, IL-6, leptin, EGF, PlGF, thrombopoietin, TGF-β1 and VEGF-D ($P < 0.05$), as compared with the metastatic ones (Figure 1B). Interestingly, VEGF-A (VEGF) was not found among the proteins differentially expressed according to the cellular source of isolation. Figure 1C illustrates processed antibody-arrays and the images captured of Caco2 (primary CRC cell line) and T84 (metastatic CRC cell line).

VEGF expression in primary and metastatic colorectal cancer cell lines

The antibody array data showed no significant changes in VEGF secretion between primary and metastasis-derived CRC cell lines (Figure 1B). To validate the antibody array results, we analyzed VEGF levels by EIA. The results were confirmed by a statistically significant positive correlation between VEGF protein as determined by the antibody-array and by EIA ($r_{Spearman} = 0.7$, $P < 0.05$) (Figure 2A).

In a second step, VEGF secretion by EIA and VEGF mRNA expression was analyzed in a larger panel of 16 CRC cell lines. As shown in Figure 2B and C, we did not detect any significant difference in VEGF expression according to the primary or metastatic CRC cell lines (mean of 28.9 pg/mL and 22.7 pg/mL VEGF protein; 0.011 and 0.009 (relative quantification) VEGF mRNA, respectively). Further, a strong correlation ($r = 0.65$, $P <$

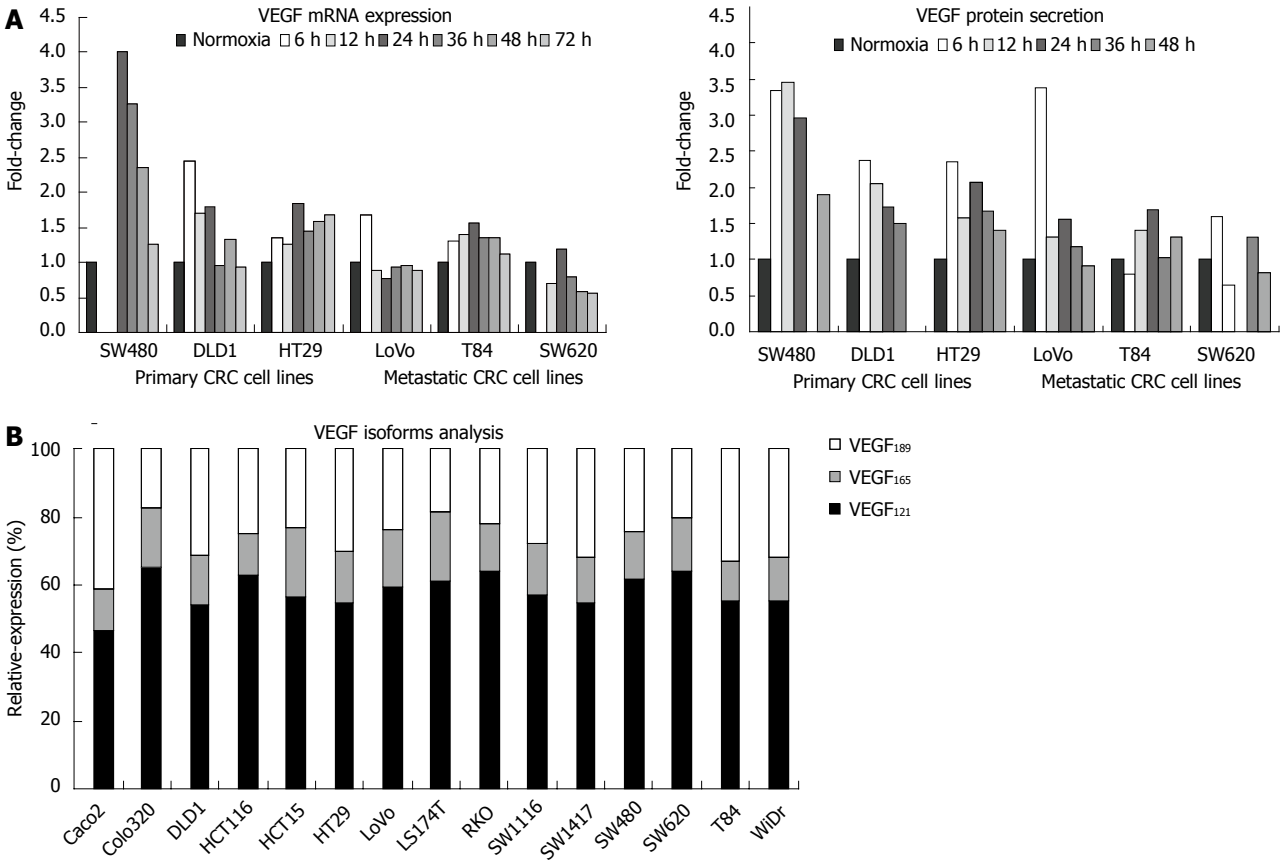

Figure 3 Vascular endothelial growth factor expression regulation. A: Modulation of vascular endothelial growth factor (VEGF) expression (mRNA and protein) in response to severe hypoxia in primary and metastatic colorectal cancer (CRC) cell lines; B: Expression of VEGF isoforms 121, 189 and 165 by CRC cells in normoxia.

Table 3 Association between vascular endothelial growth factor mRNA isoforms and vascular endothelial growth factor protein secretion

	VEGF protein	$VEGF_{121}$ mRNA	$VEGF_{165}$ mRNA
$VEGF_{121}$ mRNA	$r = 0.55$ $P = 0.034$		
$VEGF_{165}$ mRNA	$r = 0.67$ $P = 0.007$	$r = 0.93$ $P = 0.000$	
$VEGF_{189}$ mRNA	$r = 0.69$ $P = 0.005$	$r = 0.95$ $P = 0.000$	$r = 0.92$ $P = 0.000$

VEGF: Vascular endothelial growth factor.

0.01) was detected between VEGF protein (by EIA) and VEGF mRNA expression (Figure 2D) in CRC cell lines, indicative of the major role of transcriptional mechanisms in the regulation of VEGF expression[23]. A similar correlation was observed in hypoxia between VEGF protein (by EIA) and VEGF mRNA expression (Figure 3A). Severe hypoxia induced different levels of VEGF expression up-regulation depending on the CRC cellular origin. Surprisingly, the fold change normoxia-hypoxia in VEGF expression of metastatic CRC cell lines was ≤ 1.5 in the majority of time points tested, as compared with the > 1.5-4.0 fold change in primary cell lines for both protein and mRNA VEGF (Figure 3A).

VEGF isoforms have differential angiogenic and tumorigenic activities and their expression pattern may also define the CRC cell angiogenic capacity[24]. Primary and metastatic CRC cell lines had a similar expression pattern of the three major isoforms $VEGF_{121}$, $VEGF_{169}$ and $VEGF_{185}$, despite variability in VEGF expression (Figure 3B), implying a similar mechanism of regulation. $VEGF_{121}$ was the predominant isoform expressed by CRC cell lines (58.23% ± 5.05% of total VEGF mRNA), as compared to$VEGF_{165}$ and $VEGF_{189}$ (15.13% ± 2.71% and 26.6% ± 6.5% of VEGF transcripts, respectively). In line with a previous study on tumor tissue[25], the expression of the three isoforms was significantly associated with total VEGF protein; $r = 0.55$, $P < 0.05$ for $VEGF_{121}$ and furthermore, $VEGF_{165}$ and $VEGF_{189}$ showed higher correlation ($r = 0.67$ and $r = 0.69$, $P < 0.01$, respectively) (Table 3).

VEGFR expression in colorectal cancer cell lines

While the role of the VEGF/VEGFR pathway in endothelial cells is well characterized, its functionality and expression by tumor cells is still controversial[13]. Soluble VEGFR-1 was quantified in CRC cell line supernatants at a lower range than VEGF (mean 8.3 and 27.8 pg/mL respectively) and no differences were found according to the cellular origin (7.57 ± 2.12 and 10.67 ± 3.1, in primary and metastatic CRC cell lines, respectively) (Figure 4A). In agreement with other studies[26], a trend was observed for

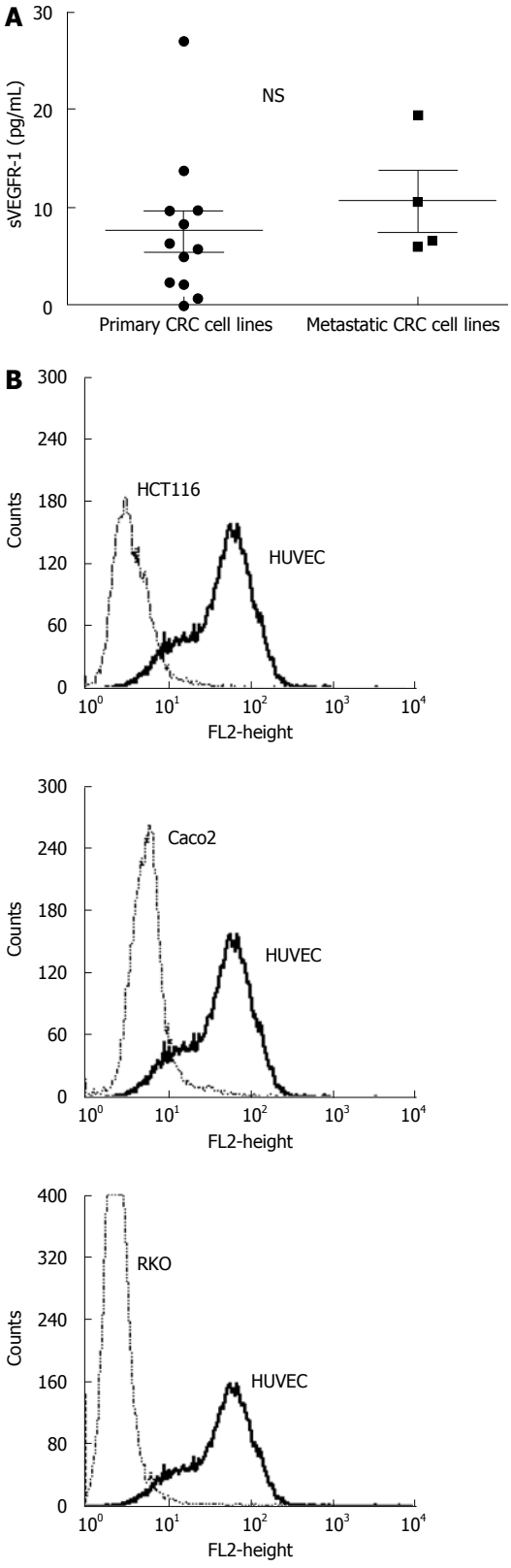

Figure 4 Vascular endothelial growth factor receptors expression in colorectal cancer cell lines. A: Soluble vascular endothelial growth factor receptor (sVEGFR)-1 expression measured by EIA is not significantly different between primary and metastatic colorectal cancer (CRC) cell lines; B: Flow cytometry of the surface expression of vascular endothelial growth factor receptor (VEGFR)-2 in human umbilical vein endothelial cells (HUVEC) and the primary CRC cell lines HCT116, Caco2 and RKO under normoxic conditions reveals a general lack of VEGFR-2 expression on the surface of CRC cells as compared to HUVEC. NS: Not significant.

an inverse correlation between sVEGFR-1 and VEGF expression (data not shown), indicative of the angiogenesis inhibiting role of sVEGFR-1[13].

In our CRC cell lines panel, the antibody array data showed a lack of expression of sVEGFR-2 (Figure 1A). Given the hypothesis that earlier tumor stages are more dependent on the VEGF/VEGFR signaling pathway[15], we analyzed surface VEGFR-2 expression in CRC cells of primary origin. Flow cytometry revealed a general lack of surface VEGFR-2 expression in CRC cells of medium to high VEGF expression, as compared to HUVEC cell line (Figure 4B). These findings add to the stock of controversial results to date[27,28].

DISCUSSION

Identifying the proteins responsible for the different behavior of more advanced CRC tumors seems warranted in order to more effectively use current treatment options. Furthermore, there is a need to characterize definite biomarkers of CRC metastasis to serve as prognostic indicators and novel interventional targets. As derived from our findings in vitro, the tumor microenvironment of CRC metastases would be different to that of primary tumors, because of the effect of the CRC cells secreted factors. Metastatic CRC cell lines are characterized by a greater expression of cytokines majorly involved in metastasis, migration and invasion, while being proven pro-angiogenic effectors. MMP-1 plays an important role in CRC tumor invasion and metastasis[29] and MMP-9 has proved to be of prognostic value in stage Ⅱ colon cancer patients, where tumors with higher protein expression had a higher recurrence rate[30]. The monocyte attractant chemokine I-309 has been shown to stimulate chemotaxis and invasion of endothelial cells and the roles of IL-1α in colon cancer angiogenesis and of IL-2 in inflammation and apoptosis, seem also consistent with the metastatic phenotype[18,31,32].

Hypoxia is widely recognized as the major transcription effector for VEGF expression[9]. However, the greater (two-fold increase) induction of VEGF expression in hypoxia observed in primary CRC as compared to metastatic cell lines is an interesting finding which agrees with recent hypotheses. Tolerance to hypoxia is frequently acquired by tumor cells progressing towards more advanced phenotypes[15]. Our finding suggests the metastatic CRC molecular phenotype provides some intrinsic resistance to the hypoxic induction of VEGF expression. Some authors have shown that hypoxia would select more malignant metastatic cells, less sensitive to anti-angiogenic treatment[33], to yield poorer patients outcomes[34,35]. The community still agrees that angiogenesis is a hallmark of cancer in metastatic stages[36]. However, given the broad angiogenic network in the tumor microenvironment, research should move in the direction of investigating the mechanisms by which metastatic tumors depend on VEGF, since they seem to be different to those exploited by primary tumors[15]. Furthermore, with the objective of individualized care in mCRC, the distinct metastatic "sec-

retome" proteins emerge as alternative targets to consider in the management of advanced disease.

Further to the VEGF expression profile, the pattern of VEGF isoforms represents the next step to identifying intrinsic differences to guide treatment choice. However, the similar expression of VEGF isoforms across cell lines does not offer clarification. Further to this finding, it would be of interest to explore how VEGF transcription factors modulate the ratio of VEGF isoforms as disease progresses, given the changes on VEGF dependence. Interestingly, a novel class of VEGF isoforms, VEGFxxxb, generated through alternative splicing of exon 8, has been recently described[37]. Studies suggest anti-angiogenic or weak angiogenic properties for these isoforms[38,39]. Not exempt from controversy, this discovery will help in further defining the role of VEGF/VEGFR signaling in CRC, yet still the testing techniques need refinement in specificity between the two classes.

Emerging data suggest VEGF to be a growth factor also for tumor cells and VEGF/VEGFR signaling to regulate their expression. However, this hypothesis remains unproven until consolidated results on VEGF receptor expression on tumor cells become available[12,28]. Extensive work has been done on the activity of VEGF/VEGFR-1 signaling in CRC cells showing that it mediates cell motility and invasiveness but not cell proliferation[13]. While this would involve VEGF/VEGFR-1 in CRC progression and metastatic processes, sVEGFR-1 secretion was not found of significant relevance in metastasis-derived CRC cells. In contrast, not so much is known about the activity of VEGF/VEGFR-2 in cancer cells. Reports suggest an involvement in the sensitivity of CRC cells to inhibition of VEGF-related survival pathways[40]. However, controversial results on the VEGFR-2 expression on tumor cells to date[27,28], to which our results add, do not help to resolve this question. Definite confirmation of the expression and functionality of this pathway is necessary in order to shed more light on the mechanism of action of anti-VEGF therapies[40].

Consistent with the key role of VEGF in the "angiogenic switch" and the hypoxia-resistance mechanisms in metastatic stages, CRC cell dependence on VEGF in more advanced settings seems attenuated in favor of other cytokines in the progression of metastasis. Further investigation of these findings and testing the significance of the distinct "secretome" of CRC metastases at the clinic side seems warranted given the implications for patient outcomes.

ACKNOWLEDGMENTS

We thank Dr. Paul Miller for English editorial work.

COMMENTS

Background

Identifying the proteins responsible for the different behavior of more advanced colorectal cancer is necessary in order to more effectively use current treatment options. The progressive growth of colon cancer depends on the blood vessels (angiogenesis) network within the tumor. Therapies targeting angiogenesis have emerged in the field; however, variances in the magnitude benefit lead to great amount of research to explain inter-individual differences. It is thought that different proteins or biomarkers in the tumor microenvironment are responsible for these facts.

Research frontiers

The lack in understanding of biomarkers of colorectal cancer metastasis led the authors to set up this work. Using a novel cytokine antibody array technique, this work identifies the differences in angiogenesis-related protein expression of colorectal cancer cell lines of primary and metastatic origin. This is the first step prior to translation into a clinic setting, where these differences are to be corroborated in patients with colorectal cancer.

Innovations and breakthroughs

The distinct profile of metastatic cell lines comprises eight proteins with different cellular properties, including favoring the growth of those tumor blood vessels. Interestingly, the classical angiogenesis marker vascular endothelial growth factor is not in such a profile, indicating that tumors in more advanced phases tend to rely on different mechanisms for their growth.

Applications

The findings of this work show that a number of markers might be of value when determining the course of disease in colorectal cancer. Furthermore, these proteins arise as novel intervention targets in the metastatic colorectal cancer setting.

Peer review

The researchers intent was to investigate the angiogenesis-related protein expression profile characterizing metastatic colorectal cancer with the aim of identifying prognostic markers. The subject of biomarkers of colorectal cancer (CRC) metastasis is not well understood up to this time. Because of that, efforts of authors to characterize the protein factors behind the angiogenic potential of CRC cell lines of metastatic origin is of great importance. This work is a next step forward to identify the proteins responsible for the different behavior of metastatic colorectal cancers and for developing new treatment options.

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S- Editor Lv S **L- Editor** O'Neill M **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.646

World J Gastroenterol 2012 February 21; 18(7): 646-653
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Thermotherapy enhances oxaliplatin-induced cytotoxicity in human colon carcinoma cells

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Supported by The Guangdong Provincial Science and Technology Programs, China, No. 2009A030301013; the Scientific Funds of Guangzhou Medical College, China, No. 2010A29; National Natural Science Foundation of China, No. 30700398 and 81172831; Natural Science Foundation of Guangdong Province, No. 10151008901000200
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Received: June 11, 2011 Revised: September 28, 2011
Accepted: October 5, 2011
Published online: February 21, 2012

Abstract

AIM: To observe the synergistic effects of hyperthermia in oxaliplatin-induced cytotoxicity in human colon adenocarcinoma Lovo cells.

METHODS: The human colon adenocarcinoma cell line Lovo was obtained from Sun Yat-Sen University. Cells were sealed with parafilm and placed in a circulating water bath, and was maintained within 0.01 ℃ of the desired temperature (37 ℃, 39 ℃, 41 ℃, 43 ℃ and 45 ℃). Thermal therapy was given alone to the negative control group while oxaliplatin was administered to the treatment group at doses of 12.5 μg/mL and 50 μg/mL. Identification of morphological changes, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, flow cytometry and Western blotting were used to investigate the effect of thermochemotherapy on human colon adenocarcinoma Lovo cells, including changes in the signal pathway related to apoptosis.

RESULTS: A temperature-dependent inhibition of cell growth was observed after oxaliplatin exposure, while a synergistic interaction was detected preferentially with sequential combination. Thermochemotherapy changed the morphology of Lovo cells, increased the inhibition rate of the Lovo cells ($P < 0.05$) and enhanced cellular population in the G_0/G_1 phase (16.7% ± 4.8 % in phase S plus 3.7% ± 2.4 % in phase G_2/M, $P < 0.05$). Thermochemotherapy increased apoptosis through upregulating *p53*, *Bax* and downregulating *Bcl-2*. Protein levels were elevated in p53, Bax/Bcl-2 in thermochemotherapy group as compared with the control group ($P < 0.05$).

CONCLUSION: Thermochemotherapy may play an important role in apoptosis *via* the activation of *p53*, *Bax* and the repression of *Bcl-2* in Lovo cells.

Key words: Colorectal cancer; Oxaliplatin; Thermochemotherapy; Mitochondrial apoptotic pathway

Peer reviewers: Luis Bujanda, PhD, Professor, Departament of Gastroenterology, CIBEREHD, University of Country Basque, Donostia Hospital, Paseo Dr. Beguiristain s/n, 20014 San Sebastián, Spain; Haruhiko Sugimura, MD, PhD, Professor, Department of Pathology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan

Zhang XL, Hu AB, Cui SZ, Wei HB. Thermotherapy enhances oxaliplatin-induced cytotoxicity in human colon carcinoma cells. *World J Gastroenterol* 2012; 18(7): 646-653 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/646.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.646

INTRODUCTION

Colorectal cancer (CRC) is one of the most common types of cancer worldwide[1,2]. Over the past decades, the incidence of CRC has been increasing in China, with an increased mortality due to early metastases[3]. Despite surgical resection, more than 40% of the CRC patients die of the metastases ultimately[4-6]. The poor prognosis is associated with difficulties in early diagnosis at a curable stage. Therefore, the urgent need to develop new therapeutic strategies in order to significantly improve the prognosis of the metastatic CRC patients is not overstated.

Oxaliplatin, a new third-generation platinum coordination complex of the 1,2-diaminocyclohexane family, has shown promising activity for CRC[7-9]. Its spectrum of antitumor activities in tumor models differs from that of cisplatin and carboplatin. Oxaliplatin is active in a broad range of cancer cell lines and does not produce cross-resistance of cisplatin and carboplatin Its side effects are also distinct from other platinum drugs-it induces no renal or hepatic toxicity but causes both a reversible acute, cold-related dysesthesia and a dose-limiting cumulative peripheral sensory neuropathy that usually rapidly regresses after treatment withdrawal[10].

It is well known that hyperthermia is a strong factor that increases tumor sensitivity to chemotherapy[11,12]. Cells in the DNA synthetic phase of the cell cycle are relatively resistant to chemotherapy, but are especially sensitive to hyperthermia. Thus, a combined treatment may, in some circumstances, be an advantage[13]. Previous data have shown that the cytotoxicity and anti-tumor effect of cisplatin are greatly enhanced at elevated temperatures[14]. It is also reported that the effect of oxaliplatin on colon cells is thermally enhanced at 42 ℃ when compared with 37 ℃[15].

A key mechanism associated with cancer cell growth is the control of apoptosis. Apoptosis is defined as programmed cell death, which occurs in response to disruption of normal homeostatic mechanisms. It is a critical parameter in tumor surveillance of abnormal cells[16]. Induction of apoptosis of cancer cells is considered a potentially new treatment for colon cancer[17].

However, whether the antitumor effect of oxaliplatin in colon cancer has a linear relationship with temperature is not known. Therefore, we explored the effect of thermal treatment in colon cancer cells and investigated the optimal temperature for the inhibitory effect of oxaliplatin in colon cancer cell lines.

MATERIALS AND METHODS

Major reagents

Newborn calf serum and Dulbecco's modified Eagle's medium (DMEM, low glucose) were purchased from GIBCO Corporation, penicillin/streptomycin was from Gibco-BRL (Germany), while 0.25% trypsin digest and trizol reagent were purchased from Invitrogen Corporation. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma Corporation. Primary antibodies (applied for 1 h at room temperature or overnight at 4 ℃) were: anti-Bcl-2, anti-Bax and anti-p53 monoclonal antibody all purchased from Santa Cruz Biotechnology (Heidelberg, Germany), goat anti-rabbit IgG (secondary) and prestained protein molecular weight marker were purchased from Cell Signaling Technology (United States).

Cell culture

The human colon adenocarcinoma cell line Lovo was obtained from the Cell Bank of Sun Yat-Sen University. All cell culture operations were carried out in a sterile class II biological safety cabinet (Thermo Fisher, United States). The Lovo cell lines were cultured in DMEM containing 10% fetal bovine serum (GIBCO Corporation, Carlsbad, CA, United States), 50 U/mL of penicillin and 50 mg/mL of streptomycin (Invitrogen, Carlsbad, California, United States). The cells were grown in 25-mL and 75-mL flasks (Corning, New York, United States) in a humidified incubator at 37 ℃ with 5% CO_2 atmosphere (Thermo Fisher, United States). Cells were stained with Trypan blue (Sigma-Aldrich, St Louis, Missouri, United States) and then counted on a hemacytometer.

Hyperthermia and oxaliplatin treatment

Cells cultured in 60-mm dishes were sealed with parafilm and placed in a circulating water bath (Yiheng Corporation, Shanghai, China), and was maintained within 0.01 ℃ of the desired temperatures (37 ℃, 39 ℃, 41 ℃, 43 ℃, 45 ℃).Thermal therapy was given alone to the control group while oxaliplatin was administered to the treatment group at doses of 12.5 μg/mL and 50 μg/mL. The duration of thermal therapy and chemotherapy was 1 h, and the cells were cultured in a humidified incubator at 37 ℃ with 5% CO_2 atmosphere for 24 h.

Cellular morphological evaluation

The cells were observed under inverted phase contrast microscope (Olympus, Japan) after completion of thermal therapy or thermochemotherapy.

MTT assay

Measurement of cell growth inhibition by MTT assay was described previously[18]. Lovo cells were seeded in 96-well plates (5×10^3 cells/well) and were treated with 12.5 μg/mL or 50 μg/mL oxaliplatin at 37 ℃, 39 ℃, 41 ℃, 43 ℃ or

45 ℃. Negative control wells contained only CRC cells but not oxaliplatin. The experiment was repeated 3 times. Untreated and treated cells were cultured at 37 ℃ with 5% CO_2 for 24 h. MTT was added to 50 μL of cell suspension for 4 h. After supernatant was removed, DMSO (150 μL) was added to each well and mixed at a low speed for 10 min to fully dissolve the blue crystals. Absorbance was measured at 570 nm (A_{570}) and the percentage of growth inhibition of Lovo cells was calculated at each time point and for each concentration of oxaliplatin according to the following formula: % cell survival = (Lovo oxaliplatin group - Lovo blank)/(Lovo negative - Lovo blank) × 100% and % cell growth inhibition = 1 - % cell survival.

The fraction for each concentration of oxaliplatin was calculated. Model parameters included the concentration to inhibit 50% of cell growth (IC_{50}). At least three independent experiments were carried out to test the relationship between temperature of drug exposure and the concentration of oxaliplatin.

Flow cytometric analysis

Cells were plated at 1 × 10^6 cells/well in 6-well plates, incubated for 24 h, and then treated with different concentrations of oxaliplatin for 1 h. Trypsinized cells were washed with PBS and fixed in 70% ethanol. After fixation, the cells were incubated for 30 min with 200 mg/mL of RNase A and stained with 25 μg/mL propidium iodide (PI). The stained cells were analyzed using a flow cytometry cell sorter (Becton Dickinson, NJ, United States). Samples were analyzed using a FACScan flow cytometer (Becton Dickinson) according to the manufacturer's protocol. Experiments were performed in triplicate.

Apoptosis was measured according to the manufacturer's instructions, using an annexin V-FITC kit (BD Biosciences, San Jose, CA, United States). The cells were collected after drug delivery, washed twice with PBS and then centrifuged. The cell pellet was resuspended in ice-cold binding buffer. The annexin V-FITC and PI solutions were added to the cell suspension and mixed gently. The samples were then incubated for 15 min in the dark before flow cytometric analysis[19]. The analysis of the apoptotic cells was performed by flow cytometry (FACScan, Becton Dickinson, NJ, United States).

Western blotting analysis

Lovo cells (cultured in 6-well plate at 1.5 × 10^5 cells/well) were treated with 12.5 μg/mL or 50 μg/mL oxaliplatin at 37 ℃, 39 ℃, 41 ℃, 43 ℃ or 45 ℃ and total proteins were extracted. Protein samples were separated by SDS-PAGE and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore, United States). The membrane was blocked overnight at 4 ℃ in TBS-Tween 20 (TBST) buffer containing 5% skimmed milk powder. The membrane was washed with TBST (3 × 8 min). Membranes were then incubated overnight at 4 ℃ in primary antibody (125 μL/cm^3; diluted 1:1000) with gentle shaking. The membranes were washed with TBST (3 × 8 min) and incubated for 1 h at room temperature in horse radish peroxidase (HRP)-conjugated secondary antibody (125 μL/cm^3; diluted 1:2500). The membranes were washed with TBST (3 × 8 min) and protein signals were detected by chemiluminescence kit (Cell signaling Technology, United States). Primary antibodies (applied for 1 h at room temperature, or overnight at 4 ℃) were: anti-Bcl-2, anti-Bax and anti-p53 monoclonal antibody all purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Statistical analysis

Normally distributed continuous variables were compared by one-way analysis of variance (ANOVA). Statistical analyses were performed using SPSS 13.0 statistical software (SPSS Inc, Chicago, IL). When a significant difference between groups was apparent, multiple comparisons of means were performed using the Holm-Bonferroni procedure with type-I error adjustment. Data were presented as means ± SD. All statistical assessments were two-sided and evaluated at the 0.05 level of significant difference.

RESULTS

Cellular morphology

Lovo cells were treated with oxaliplatin (12.5 μg/mL or 50 μg/mL) at different temperatures for 1 h and then cultured under normal conditions for 24 h. There were disparities after treatment between the control group and the thermochemotherapy group (Figure 1). Thermotherapy alone caused the inhibition of cell growth by inducing cell apoptosis and cell cycle arrest. However, thermochemotherapy caused a greater decrease of Lovo cells as compared with the control group, especially in the 50 μg/mL group. The effect of thermochemotherapy on tumor growth was observed as early as 24 h after treatment.

Thermotherapy enhances oxaliplatin-induced inhibition of cytotoxicity

First, we used the MTT assay to detect the effect of temperature on Lovo cell proliferation. With increasing temperature, the inhibition rate of cell growth became higher and higher. The cytotoxicity of Lovo cells exposed to 50 μg/mL oxaliplatin for 1 h was higher than those exposed to 12.5 μg/mL oxaliplatin at the same temperature ($P < 0.05$). And 43 ℃ was the optimal temperature to inhibit cell proliferation when the cells were exposed to 50 μg/mL oxaliplatin ($P < 0.05$, Figure 2).

Thermotherapy enhances oxaliplatin-induced cell cycle arrest and apoptosis

To elucidate the mechanism of action of thermotherapy and oxaliplatin, we used flow cytometry to determine cell cycle distribution and apoptosis in Lovo cells exposed to different temperatures.

A significant increase in the number of G_0/G_1 phase cells and a decrease in the number of S and G_2/M phase

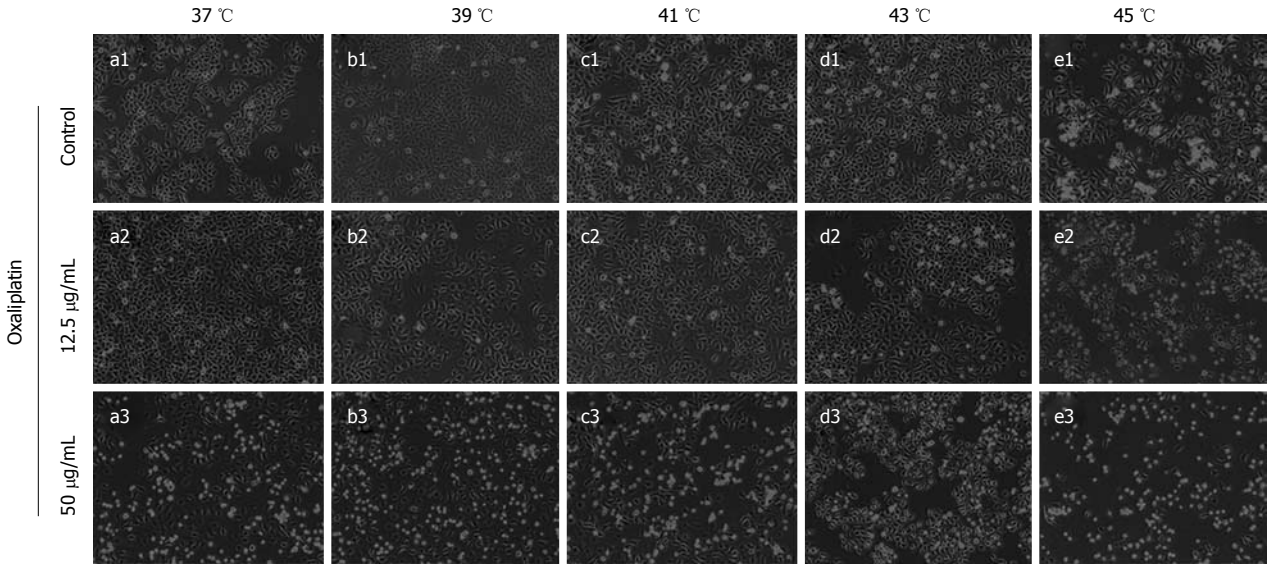

Figure 1 Effect of thermochemotherapy on human colon carcinoma Lovo cells. Cells were treated with oxaliplatin (12.5 μg/mL and 50 μg/mL) at different temperatures (37 ℃, 39 ℃, 41 ℃, 43 ℃ or 45 ℃) for 1 h and then cultured under normal conditions for 24 h. The cells were observed under inverted phase contrast microscope (Olympus, Japan) after completion of thermal therapy or thermochemotherapy.

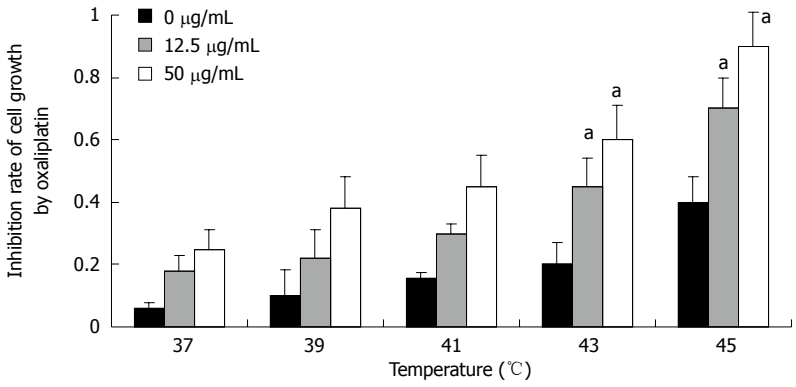

Figure 2 Effect of temperature on the proliferation of Lovo cells by MTT assay. Lovo cells were treated with oxaliplatin (12.5 μg/mL and 50 μg/mL) at 37 ℃, 39 ℃, 41 ℃, 43 ℃ or 45 ℃. The control group only used thermal therapy but not oxaliplatin. Statistically significant differences were observed between the two groups. Lovo cell viability was determined by MTT assay. The white bars are the results of the oxaliplatin treatment for 1 h at 37 ℃, 39 ℃, 41 ℃, 43 ℃, and 45 ℃ followed by 24 h recovery. The results of three independent experiments are reported as mean ± SD. $^{a}P < 0.05$ *vs* 43 ℃ or 45 ℃ *vs* 37 ℃.

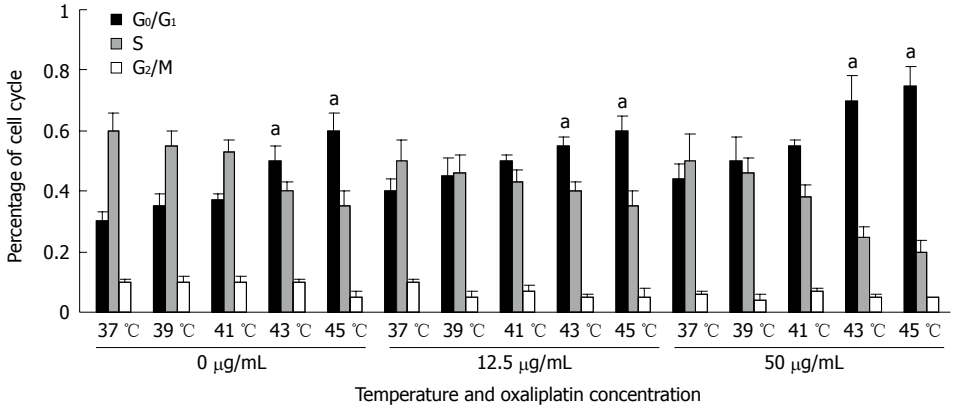

Figure 3 Effect of temperature on cell cycle and apoptosis detected by flow cytometry. Lovo cells were treated with oxaliplatin (12.5 μg/mL and 50 μg/mL) at 37 ℃, 39 ℃, 41 ℃, 43 ℃ or 45 ℃ for 1 h. The different bars represent the percentage of Lovo cells at different phases of the cell cycle at different temperature points. $^{a}P < 0.05$ *vs* 43 ℃ or 45 ℃ *vs* 37 ℃.

cells after 1 h of oxaliplatin treatment were observed. There was a linear relationship between the cell cycle

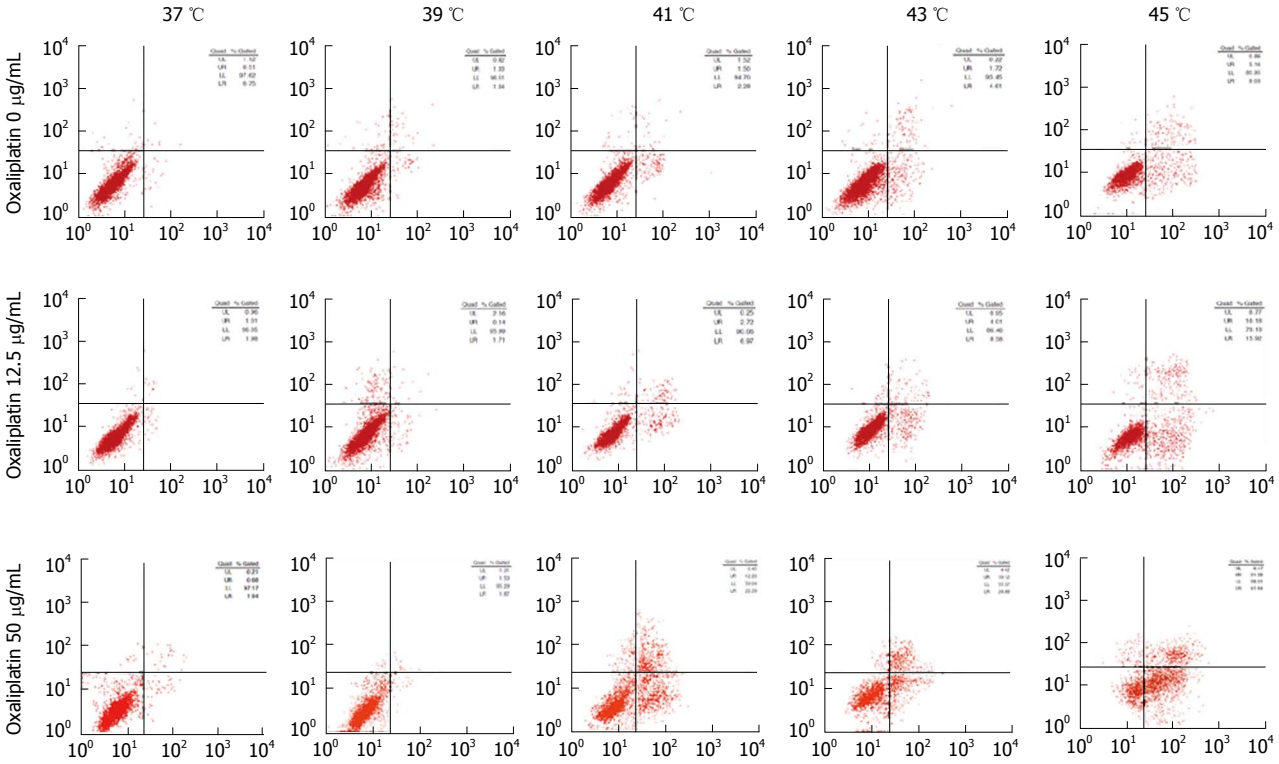

Figure 4 Measurement of Lovo cell apoptosis using apoptosis detection kit. Data are presented as dot plots in which the vertical axis represents propidium iodide (PI)-positive cells and the horizontal axis annexin V-positive cells. The upper left quadrant region contains necrotic (PI-positive) cells, the upper right region contains the late stage of apoptotic and necrotic (PI- and annexin V-positive) cells, the lower left region contains viable non-apoptotic (PI- and annexin-V-negative) cells, and the lower right region contains early apoptotic (PI-unstained and annexin-V-positive) cells.

and the temperature ($P < 0.05$). The proliferation and proportions of cells in different phases of the cell cycle were analyzed at 24 h by the incorporation of PI. DNA histogram analysis revealed that thermal therapy induced a temperature-dependent increase in the number of cells within the G_0/G_1 phase. This increase was accompanied by a decrease in the percentage of proliferating cells (16.7% ± 4.8% in phase S and 3.7% ± 2.4% in phase G_2/M) ($P < 0.05$, Figure 3). Accumulation of 12.5 μg/mL oxaliplatin-treated cells at any phase was less remarkable than that of the 50 μg/mL-treated cells ($P < 0.05$).

We also used PI staining to show that thermotherapy induced apoptosis of Lovo cells in a temperature-dependent manner (Figure 4).

Hyperthermia enhances oxaliplatin induced-regulation of p53 and Bax/Bcl-2

It is well known that reduction of intra-cellular apoptotic molecules, such as p53 and Bax/Bcl-2, sensitizes Lovo cells to thermotherapy. We therefore investigated whether changes in the amounts of apoptotic proteins were associated with the promotion of hyperthermia and oxaliplatin.

p53 stimulated the mitochondrial apoptotic pathway, thus enabling direct protein interaction or inhibition of the Bcl-2 protein family. p53 can also induce the pro-apoptotic Bcl-2 proteins by transcripting or inhibiting the transcription of anti-apoptotic Bcl-2 proteins. We examined the effect of thermal therapy on the expression of the Bcl-2 protein group.

The results showed a thermal-dependent increase in Bax expression over temperature and a concomitant decrease in the expression of Bcl-2. The maximal levels of Bax reached a peak at 43 ℃. As for Bcl-2, there was a marked decrease when compared 43 ℃ and 45 ℃ with 37 ℃. These levels later increased somewhat but always remained lower than that in the control group ($P < 0.05$). Both Bax activation and Bcl-2 inhibition were required for the release of mitochondrial apoptotic factors and the activation of the intrinsic apoptotic route.

Finally, we detected the possible signal pathway involved in the effects of oxaliplatin on Lovo cells. There was an increase in the expression of p53 and Bax protein in cells treated with oxaliplatin for 1 h. Compared with the cells of the control group, a gradual decrease in Bcl-2 levels was found at an increasing temperature, with the most significant reduction at 43 ℃ (Figure 5).

DISCUSSION

Thermotherapy combined with immediate intraperitoneal delivery of adjuvant anti-cancer drugs is a novel cancer treatment strategy to improve prognosis and prolong the overall survival of the patients with advanced CRC. The aim of this study was to assess the role of hyperthermia in oxaliplatin-induced cytotoxicity, to find an appropriate temperature using *in vitro* studies in maximizing anti-tumor activities and discuss the possible mechanism of thermochemotherapy. Preclinical studies have suggested

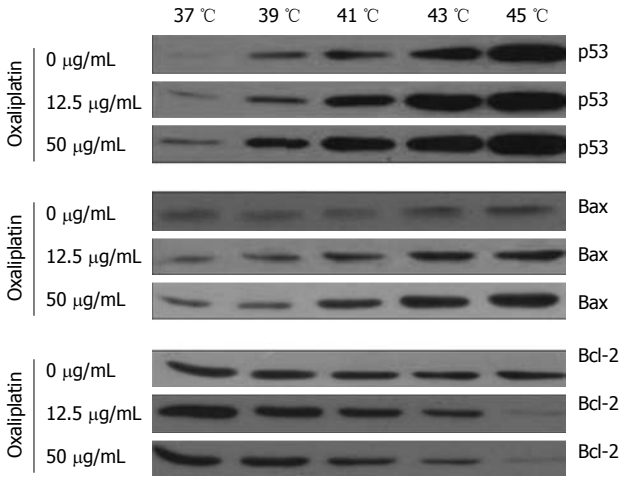

Figure 5 Effect of thermal therapy on p53, Bax and Bcl-2 detected by Western blotting. Lovo cells were treated with 12.5 μg/mL or 50 μg/mL oxaliplatin for 1 h at 37 ℃, 39 ℃, 41 ℃, 43 ℃ or 45 ℃. Cells were harvested, and total proteins were extracted and immunoblotted for p53, Bax and Bcl-2. The values represent means ± SD of at least three separate experiments. Beta-actin was used as loading control (data not shown).

that oxaliplatin may offer therapeutic advantages in a variety of malignancies with either intrinsic or acquired cisplatin resistance[20]. The primary mechanism of oxaliplatin has been shown to be mediated by the formation of intrastrand DNA cross-links[21]. Rietbroek *et al*[22] reported that thermotherapy at 43 ℃ enhanced the formation of DNA cross-links and concluded that a large portion of enhanced cytotoxicity may be attributed to the increased cross-links. Other factors such as an increased drug uptake at the elevated temperature are also suggested[23]. Evidence that oxaliplatin exerts a specific anti-tumor effect strongly suggests that oxaliplatin may be a promising new compound for the treatment of gastrointestinal tumors[24-26].

The *in vitro* exposure of human Lovo cells at clinical concentration of oxaliplatin with thermotherapy exerted a strong anti-proliferative effect and induced apoptosis of Lovo cells in this study. Our results highlight the potential clinical value of thermochemotherapy in the treatment of colon cancer. The exposure to 43 ℃ or above augmented the cytotoxicity of the oxaliplatin-treated Lovo cells in the thermochemotherapy group in a temperature-dependent fashion. This effect was observed by the MTT assay which demonstrated a linear increase in cytotoxicity with thermotherapy. After exposure to 43 ℃, the activity of oxaliplatin markedly and rapidly increased, indicating its potential inhibition ability in Lovo cells. Consistently, previous studies have demonstrated that oxaliplatin treatment with different concentrations (7.5-39.7 μg/mL) for 5-150 min can enhance cell death in various cell lines[27,28].

The exact mechanisms of thermochemotherapy remain unclear. Exposure to high temperatures can alter the fluidity of cell membranes, inhibit protein synthesis, and destroy DNA synthesis enzymes. Regulation of the cell cycle and apoptosis is a major strategy to inhibit the progression of a number of cancers. A critical role of p53, a sequence-specific DNA-binding protein, has been demonstrated to execute apoptosis. Either thermotherapy or oxaliplatin can cause cell cycle arrest at the G_0/G_1 phase and can induce apoptosis of human colon cancer cells.

Some studies have reported that p53 changes cell-cycle arrest and apoptosis induction by regulating the expression of different proteins such as p21, Bax and Bcl-2[29,30]. The *Bcl-2* gene product functions as an anti-apoptotic signal, suppressing apoptosis induced by chemotherapeutic drugs. The exact mechanism of Bcl-2 in preventing apoptosis is still not clear. Others have described *p53* expression in response to genotoxicity. It has been proposed that *p53* may be involved in the cellular response to DNA damage, producing arrest in the G_0/G_1 phase of the cell cycle to allow efficient repair of DNA before entry into S phase[31,32]. For these reasons, we inferred that the effects of thermotherapy in Lovo cells were mediated partly by the expression of *p53*.

Our results showed that thermotherapy could induce apoptosis and cell cycle arrest in Lovo cell lines in a temperature-dependent manner. Thermochemotherapy significantly induced cell cycle of G_0/G_1 phase arrest by upregulating *p53* and *Bax* expression and downregulating Bcl-2 in Lovo cells. We also detected the expression of Bcl-2 and Bax in Lovo cells to understand the impact of thermotherapy on the mitochondrial pathway of apoptosis. Down-regulation of Bcl-2 and up-regulation of p53 and Bax synthesis showed that apoptosis induced by thermochemotherapy may be mediated by the mitochondrial pathway[33,34]. Our findings were consistent with other studies[35,36] that thermotherapy caused G_0/G_1 cell cycle arrest and promoted apoptosis of Lovo cells by upregulating p53, Bax expression and downregulating Bcl-2[37,38].

In conclusion, our findings indicate that oxaliplatin is a promising agent for the treatment of human Lovo cells. Thermotherapy exerted synergistic interaction with oxaliplatin especially at 43 ℃ or above, inhibiting the survival of Lovo cells *in vitro*. Apparently, the observed synergism between thermotherapy and oxaliplatin results in mutual completion and enhancement of anticancer activity that may be extrapolated to animal models of colon cancer and to clinical use. The results of the present study suggest that thermochemotherapy might be a useful future strategy for treating colon cancers.

ACKNOWLEDGMENTS

We thank Dr. Shi-Hui Xing for his careful revision of this manuscript and the assistance in the figure and data analyses.

COMMENTS

Background

Oxaliplatin is reported to be active in a broad range of cancer cell lines and hyperthermia is a strong factor that increases tumor sensitivity to chemotherapy. However, whether the antitumor effect of oxaliplatin in colon cancers has a linear relationship with temperature and the optimal temperature for the inhibitory

effect of oxaliplatin in colon cancer cell lines remains elusive.

Research frontiers

Thermochemotherapy may play an important role in the inhibition of the Lovo cells and the optimal temperature is 43 ℃. In addition, down-regulation of Bcl-2 and up-regulation of p53 and Bax may be essential in inducing apoptosis in human colon cancer cell lines.

Innovations and breakthroughs

Thermal therapy and oxaliplatin can inhibit colon cancer cells through inducing apoptosis and regulating the cell cycle. Suppression of Bcl-2 and up-regulation of p53 and Bax might contribute to the regulation of human Lovo cell lines.

Applications

This study may help clarify the mechanism of thermochemotherapy for colon cancer and choose the appropriate therapeutic strategy in clinical practice.

Terminology

Thermochemotherapy is the combination of thermal therapy and chemotherapy.

Peer review

This is a study using a single cell line. The experiments are straightforward and the data are short and succinct.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.654

World J Gastroenterol 2012 February 21; 18(7): 654-661
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

(Z)-5-(4-methoxybenzylidene)thiazolidine-2,4-dione protects rats from carbon tetrachloride-induced liver injury and fibrogenesis

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Zhi-Zhi Chen, Zheng-Lin Wang, Chong-Yang Deng, Hao Zheng, Xian-Huo Wang, Liang Ma, Xia Ye, Ying-Hua Ma, Cai-Feng Xie, Li-Juan Chen, Yu-Quan Wei, State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, Chengdu 610041, Sichuan Province, China

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Supported by National Natural Science Foundation of China and National Key Technologies R and D Program of the 11th five-year plan, No. 2009ZX09501-015

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Received: December 19, 2010 Revised: April 19, 2011
Accepted: April 26, 2011
Published online: February 21, 2012

Abstract

AIM: To evaluate the hepatoprotective roles of (Z)-5-(4-methoxybenzylidene)thiazolidine-2,4-dione (SKLB010) against carbon tetrachloride (CCl_4)-induced acute and chronic liver injury and its underlying mechanisms of action.

METHODS: In the first experiment, rats were weighed and randomly divided into 5 groups (five rats in each group) to assess the protective effect of SKLB010 on acute liver injury. For induction of acute injury, rats were administered a single intraperitoneal injection of 2 mL/kg of 50% (v/v) CCl_4 dissolved in olive oil (1:1). Group 1 was untreated and served as the control group; group 2 received CCl_4 for induction of liver injury and served as the model group. In groups 3, 4 and 5, rats receiving CCl_4 were also treated with SKLB010 at doses of 25, 50 and 100 mg/kg, respectively. Blood samples were collected at 6, 12 and 24 h after CCl_4 intoxication to determine the serum activity of alanine amino transferase. Tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) were determined using enzyme-linked immunosorbent assay. At 24 h after CCl_4 injection,liver fibrogenesis was evaluated by hematoxylin-eosin (HE) staining and immunohistochemical analyses. Cytokine transcript levels of TNF-α, IL-1β and inducible nitric oxide synthase in the liver tissues of rats were measured using a reverse transcriptase reverse transcription-polymerase chain reaction technique. In the second experiment, rats were randomly divided into 2 groups (15 rats in each group), and liver injury in the CCl_4-administered groups was induced by a single intraperitoneal injection of 2 mL/kg of 50% (v/v) CCl_4 dissolved in olive oil (1:1). The SKLB010-treated groups received oral 100 mg/kg SKLB010 before CCl_4 administration. Five rats in each group were sacrificed at 2 h, 6 h, 12 h after CCl_4 intoxication and small fortions of livers were rapidly frozen for extraction of total RNA, hepatic proteins and glutathione (GSH) assays. In the hepatic fibrosis model group, rats were randomly divided into 2 groups (5 rats each group). Rats were injected intraperitoneally with a mixture of CCl_4 (1 mL/kg body weight) and olive oil [1:1 (v/v)] twice a week for 4 wk. In the SKLB010-treated groups, SKLB010 (100 mg/kg) was given once daily by oral gavage for 4 wk after CCl_4 administration. The rats were sacrificed one week after the last injection and the livers from each group were harvested and fixed in 10% formalin for HE and immunohistochemical staining.

RESULTS: In this rat acute liver injury model, oral administration of SKLB010 blocked liver tissue injury by down-regulating the serum levels of alanine ami-

notransferase, suppressing inflammatory infiltration to liver tissue, and improving the histological architecture of liver. SKLB010 inhibited the activation of NF-κB by suppressing the degradation of IκB, and prevented the secretion of pro-inflammatory mediators such as tumor necrosis factor-α, interleukin-1β, and the reactive free radical, nitric oxide, at the transcriptional and translational levels. In this chronic liver fibrosis model, treatment with 100 mg/kg per day SKLB010 attenuated the degree of hepatic fibrosis and area of collagen, and blocked the accumulation of smooth-muscle actin-expressed cells.

CONCLUSION: These results suggest that SKLB010 is a potent therapeutic agent for the treatment of CCl_4-induced hepatic injury.

Key words: Anti-inflammatory effects; Anti-oxidative effects; (Z)-5-(4-methoxybenzylidene) thiazolidine-2; 4-dione (SKLB010) against carbon tetrachloride; Fibrogenesis; Hepatitis; Nuclear factor-κB; SKLB010

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Chen ZZ, Wang ZL, Deng CY, Zheng H, Wang XH, Ma L, Ye X, Ma YH, Xie CF, Chen LJ, Wei YQ. (Z)-5-(4-methoxybenzylidene)thiazolidine-2,4-dione protects rats from carbon tetrachloride-induced liver injury and fibrogenesis. *World J Gastroenterol* 2012; 18(7): 655-661 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/655.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.655

INTRODUCTION

Hepatic damage, i.e., acute liver injury and chronic liver fibrogenesis, has become a severe health problem worldwide. Despite considerable and continuous efforts, effective treatment strategies against this disease resulting in fewer side effects are still lacking.

Carbon tetrachloride (CCl_4), is an acknowledged hepatotoxin and can cause acute or chronic liver injury characterized by centrilobular necrosis, inflammatory cell infiltration, centrilobular fatty changes, and apoptosis[1]. CCl_4-induced hepatic inflammatory response was found to be mediated by the action of cytokines, especially tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and the free radical, nitric oxide (NO)[2]. CCl_4 also induced the peroxidation of lipids or lipid membranes, and up-regulated the serum level of alanine aminotransferase (ALT). Sustained hepatic inflammation provoked by long-term exposure to CCl_4 is believed to induce hepatic fibrosis through ongoing hepatocytic necrosis and the production of fibrogenic cytokines acting on fibroblasts [e.g., activated hepatic stellate cells (HSC)][3].

On the other hand, oxidative stress is capable of

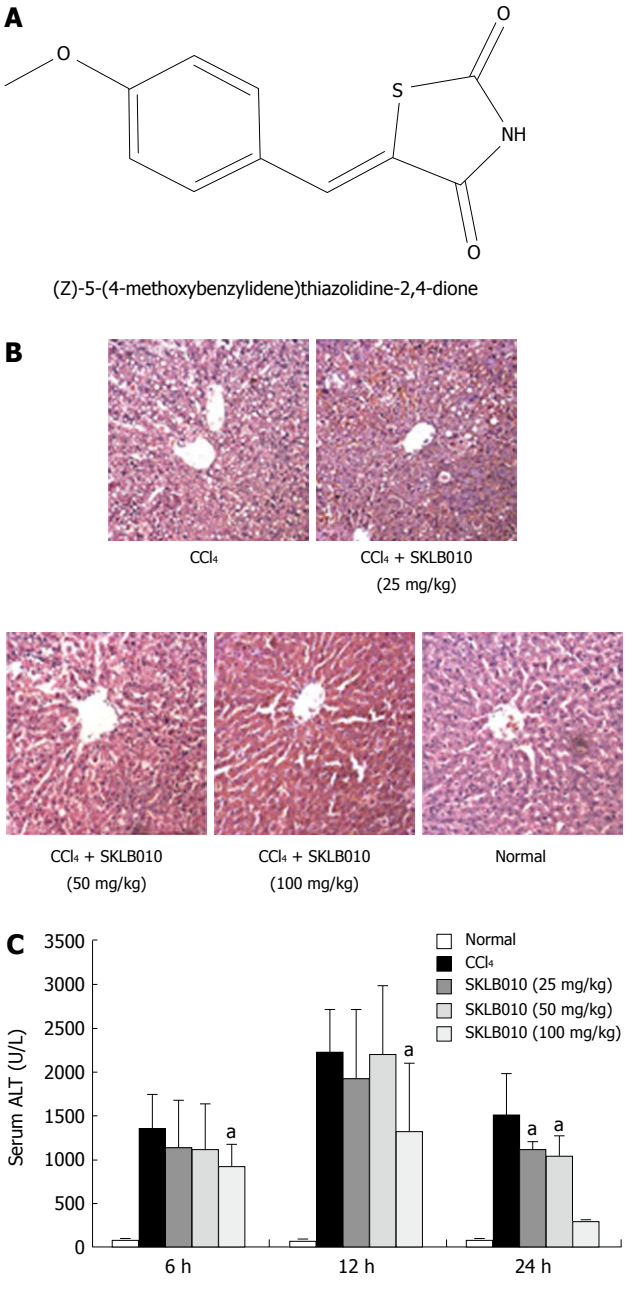

Figure 1 Effect of (Z)-5-(4-methoxybenzylidene)thiazolidine-2,4-dione on carbon tetrachloride-induced hepatitis. A: The structure of (Z)-5-(4-methoxybenzylidene)thiazolidine-2,4-dione (SKLB010); B: Representative liver histology of the rats in each group at 24 h after carbon tetrachloride (CCl_4) injection, liver sections were stained with hematoxylin-eosin (original magnification 200 ×); C: Time course of hepatocyte necrosis after CCl_4 challenge, alanine aminotransferase (ALT) was measured in serum collected at time points as indicated. Values are presented as mean ± SD of the mean. (n = 5, $^aP < 0.05$ *vs* the level in the model group).

stimulating nuclear factor-κB activation (NF-κB)[4]. NF-κB is an inducible transcription factor whose activity is primarily regulated by phosphorylation and degradation of IκB[5]. CCl_4-induced oxidative stress can stimulate the phosphorylated effect of IκB, and degradation of IκB leads to the translocation of NF-κB to the nucleus[5]. This process further regulates the expression of inducible inflammatory cytokines (such as TNF-α, NO, and IL-1β)[6]. The up-regulation of TNF-α and IL-1β con-

versely activates the expression of NF-κB. Therefore, the up-regulation of these inflammatory mediators induces a vicious cycle, which eventually alters the structure of hepatocytes and impairs their biological functions[7]. Consequently, prolonged activation of NF-κB results in the perpetuation of inflammatory responses. Hence, it is regarded as the potential target for the treatment of anti-inflammatory disease including hepatic damage[8].

In our previous study, we found that several analogues of thiazolidinediones (TZDs) showed high inhibitory effects on the chemotaxis of RAW264.7 cells. We observed that (Z)-5-(4-methoxybenzylidene)thiazolidine-2,4-dione (SKLB010) exhibited the strongest inhibitory activity against chemotaxis in MCP-1-stimulated macrophage-like RAW 264.7 cells with an IC_{50} value of 0.72 μmol/mL, and evaluated its protective effects on Con A-induced acute hepatitis[9]. In the present study, we further investigate the therapeutic effect of SKLB010 on CCl_4-induced acute hepatitis and chronic liver fibrosis, and explore its underlying mechanisms.

MATERIALS AND METHODS

Reagents and animals

SKLB010 (Figure 1A) was synthesized with purity of more than 99.5% in our lab, and dissolved in saline containing 5% (v/v) Tween-80 (Sigma-Aldrich, St. Louis, MO, USA). The structure and purity were identified by high performance liquid chromatography, a Q-TOF Premier Mass Spectrometer (Waters, Milford, MA, United States) and nuclear magnetic resonance (Bruker Avance 400 NMR system).

Female Sprague-Dawley rats from Western China Experimental Animal Center were maintained under controlled conditions and had free access to standard chow and water. All rats received human care according to the National Institutes of Health Guidelines of China until their weight was 200 g to 220 g after which they were used in the experiments.

Animal treatment

In the first experiment, rats were weighed and randomly divided into 5 groups (five rats in each group) to assess the protective effect of SKLB010 on acute liver injury. For induction of acute injury, rats were administered a single intraperitoneal injection of 2 mL/kg of 50% (v/v) CCl_4 dissolved in olive oil (1:1)[10]. Group 1 was untreated and served as the control group; group 2 received CCl_4 for induction of liver injury and served as the model group. In groups 3, 4 and 5, rats received CCl_4 to induce liver injury and were also treated with SKLB010 at doses of 25, 50 and 100 mg/kg, respectively.

In groups 2 to 5, blood samples were collected at 6, 12 and 24 h after CCl_4 intoxication to determine the serum activity of ALT using an Olympus AU2700 multifunctional biochemistry analyzer (Olympus, Tokyo, Japan).

At 24 h after CCl_4 injection, rats were killed and the livers were removed. A small portion of the liver was used for hematoxylin-eosin (HE) staining studies following fixation with 10% formalin and subsequent embedment in paraffin.

In the second experiment, rats were randomly divided into 2 groups (15 rats in each group), and liver injury in the CCl_4-administered groups was induced by a single intraperitoneal injection of 2 mL/kg of 50% (v/v) CCl_4 dissolved in olive oil (1:1). The SKLB010-treated groups received oral 100 mg/kg SKLB010 before CCl_4 administration. Five rats in each group were sacrificed at 2 h, 6 h, 12 h after CCl_4 intoxication and the livers were cut into pieces and rapidly frozen with liquid nitrogen for extraction of total RNA, hepatic proteins and glutathione (GSH) assays.

In the hepatic fibrosis model group, rats were randomly divided into 2 groups (5 rats each group). Rats were injected intraperitoneally with a mixture of CCl_4 (1 mL/kg body weight) and olive oil [1:1 (v/v)] twice a week for 4 wk[11]. In the SKLB010-treated groups, SKLB010 (100 mg/kg) was given once daily by oral gavage for 4 wk after CCl_4 administration. The rats were sacrificed one week after the last injection and the livers from each group were harvested and fixed in 10% formalin for HE and immunohistochemical staining.

Histochemistry and immunohistochemistry

In the study of acute liver injury, liver sections of 3 μm thickness were stained with HE using a standard procedure and analyzed by light microscopy to determine histological changes in tissue structure assessed using an optical microscope.

During the study of chronic liver fibrogenesis, liver tissues fixed in 10% formalin were embedded in paraffin, cut into 4-μm sections, and stained with HE to evaluate liver injury under an optical microscope. Sections were stained with Masson's Trichrome Staining to detect collagen deposition. An immunohistochemistry method was used as previously described. Briefly, liver sections were treated with 3% H_2O_2/PBS and incubated overnight at 4℃ with an anti-CD11b antibody (Cell Signaling, United States) used as the primary antibody for detecting macrophages in liver tissue and a rabbit-anti-mouse-α smooth-muscle actin (SMA) antibody (Thermo, Immunohistochemistry Specific, United States) used as the primary antibody for analyzing the activity of HSCs. The proportion of tissue stained with picrosirius red was assessed by morphometric analysis with MetaView software (Universal Imaging, Downingtown, PA, United States). Collagen staining was quantitated in random sections (under × 400 magnification, 10 fields each from sample).

GSH determinations in liver

Levels of hepatic GSH (γ-glutamyl-cysteinylglycine, GSH) were determined using the enzyme immune assay kit GSH (Jiancheng Bioengineering, Nanjing, China), following the protocol provided by the manufacturer.

Detection of serum mediators and mRNA levels

Detection of serum TNF-α, IL-1β was performed using enzyme-linked immunosorbent assay (ELISA) kits

(RandD Systems, Minneapolis, MN, United States), according to the manufacturer's instructions. Detection of serum NO was performed using the NO assay kit (nitrate reductase progress; Jiancheng Bioengineering, Nanjing, China), which reduces nitrate to nitrite as an index of NO, and was assayed colorimetrically at 450 nm.

Cytokine transcript levels of TNF-α, IL-1β and inducible nitric oxide synthase (iNOS) in the liver tissues of rats were measured using a reverse transcriptase reverse transcription-polymerase chain reaction (RT-PCR) technique. Total RNA was isolated using Trizol reagents (Invitrogen, Carlsbad, CA, United States). The RNA was reverse transcribed and PCR-amplified by the prime script one-step RT-PCR kit (TaKaRa, Japan). The results were expressed as a ratio of the number of copies of the goal gene to the number of copies the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, from the same RNA samples. The sequences of primers for the cytokine genes are as follows: TNF-α, sense 5'-CGGGGGCCACCACGCTCTTC-3' and antisense 5'-GGCAAATCGGCTGACGGTGTG-3'; IL-1β, sense 5'-TCAAGGCATAACAGGCTCATC-3' and antisense 5'-CCACGGGCAAGACATAGGTAG-3'; iNOS, sense 5'-CCCTTCCGAAGTTTCTGGCAGCAG-3', and anti-sense 5'-GGGCTCCTCCAAGGTGTTGCCC-3'.

GADPH, sense 5'-GTGCTGAGTATGTCGTGGAGTCT-3' and anti-sense 5'-GTGGAAGAATGGGAGTTGCTGT-3'.

Electrophoretic mobility shift assay

Nuclear extracts were prepared according to the method of Hentze[12]. The oligonucleotide probes used for this experiment were sense, 5'-AGTTGAGGGGACTTTCCCAGGC-3'; antisense, 5'-GCCTGGGAAAGTCCCCTCAACT-3'; and NF-κB mutant, sense 5'-AGTTGAGGCGACTTTCCCAGGC-3', anti-sense, 5'-GCCTGGGAAAGTCGCCTCAACT-3'.

Western blotting

Forty micrograms of protein from each sample, where the concentration was measured by the Bradford assay, was separated on 10% SDS-polyacrylamide gel and electrotransferred to nitrocellulose membranes (Schlecher-Schuell, Dassel, Germany). Membranes were blocked for 1 h at room temperature with 5% nonfat dry milk in TBST buffer. The reactions were then incubated at 4℃ overnight with a 1:1000 dilution of anti-IκB or anti-β-actin antibody (Abcam, United States) in blocking buffer. After the membranes were washed three times, they were further incubated with a 1:4000 dilution of suitable secondary antibody (Cell Signaling Technology, United States) for 1 h at room temperature. The signals were normalized to the protein levels of the housekeeping gene β-actin.

Statistical analysis

SPSS 16.0 was used to determine the significance of differences between normal and experimental groups. All results were expressed as mean ± SD. Differences between groups were evaluated using an independent-samples *T* test. The results were considered significantly different at $P < 0.05$.

Table 1 SKLB010 increases the levels of hepatic glutathione in the carbon tetrachloride rat model (mean ± SD)

	Normal	6 h		12 h	
		CCl_4	CCl_4 + SKLB010	CCl_4	CCl_4 + SKLB010
GSH	5.24 ± 0.15	2.99 ± 0.01	3.26 ± 0.06[b]	4.07 ± 0.16	4.70 ± 0.21[d]

$n = 5$. [b]$P < 0.01$ *vs* 6 h after carbon tetrachloride (CCl_4) injection; [d]$P < 0.01$ *vs* 12 h after CCl_4 injection.

RESULTS

SKLB010 exerts therapeutic effects in acute hepatic injury induced by CCl_4

As shown in Figure 1B, SKLB010 effectively prevented the development of CCl_4-induced liver injury as demonstrated by the reduction in serum ALT release. The ALT level was elevated after administration of CCl_4 and peaked at 12 h. In contrast, oral administration of SKLB010 dose- and time-dependently down-regulated the level of ALT, and SKLB010 showed the most potent reduction at the dose of 100 mg/kg.

Histological changes in liver tissue shown by HE staining also confirmed the hepatoprotective effect of SKLB010 against CCl_4-induced liver injury. The histopathological changes in liver tissue in CCl_4-intoxicated rats at 24 h after CCl_4 injection are shown in Figure 1C. Compared to the normal group, the group exposed to CCl_4 exhibited extensive inflammatory cells infiltration, centrilobular fatty changes, apoptosis and widespread hepatocellular necrosis. Following treatment with SKLB010, inflammatory infiltration and the necrotic region in liver sections were reduced in a dose-dependent manner.

SKLB010 increases anti-oxidative activity

GSH is an indicator of oxidative stress at the hydrophilic level in the liver[13]. Hence, the content of GSH was measured at 6 h and 12 h after CCl_4 injection. As shown in Table 1, the liver content of GSH in the normal group was 5.24 mg/g protein. However, SKLB010-treated hepatic GSH was enhanced from 2.99 mg/g to 3.26 mg/g protein at 6 h, and from 4.07 mg/g to 4.7 mg/g protein at 12 h compared with the CCl_4-induced group, respectively. We conclude that administration of SKLB010 blocked the decrement in GSH induced by CCl_4.

SKLB010 reduces pro-inflammatory mediator levels and mRNA expression in vivo

To investigate the ability of SKLB010 to modulate the production of inflammatory cytokines, the serum levels

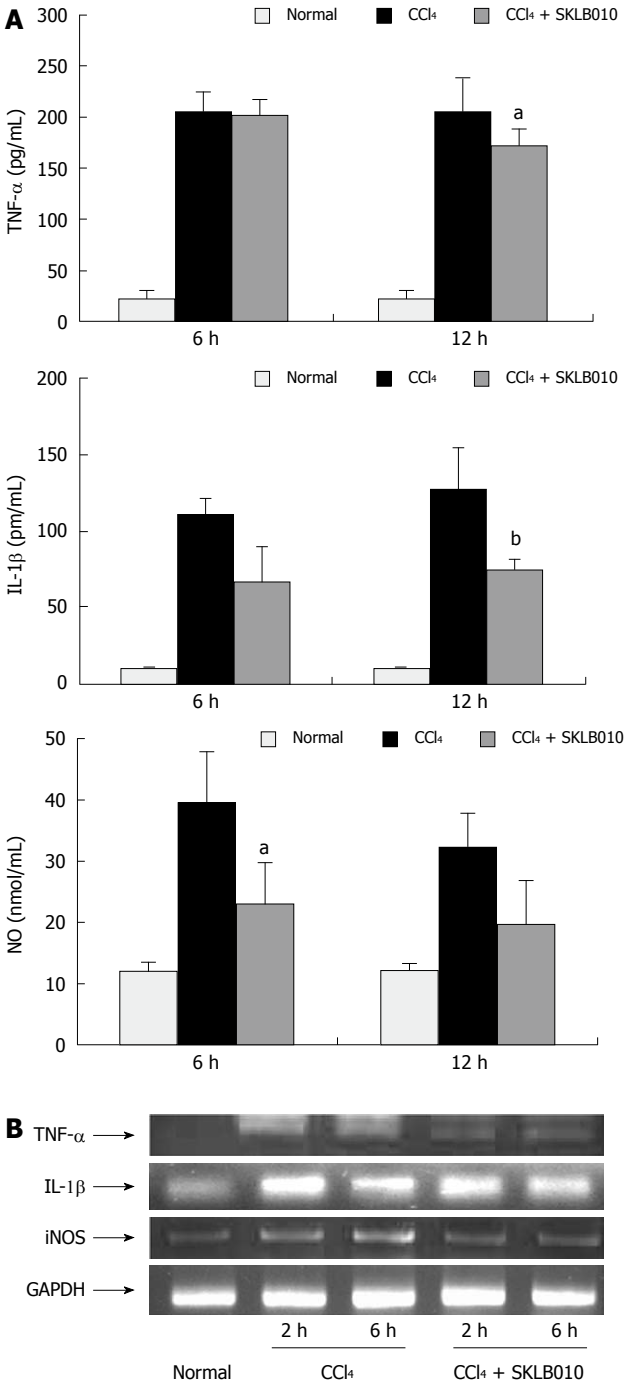

Figure 2 SKLB010 reduces the production of inflammatory mediators *in vivo*. A: Blood was collected at different time points (5 rats per time point). Tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) were assayed in serum using an enzyme linked immunosorbent assay (mean ± SD). Nitric oxide (NO) was assayed in serum using a NO assay kit $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the level of carbon tetrachloride (CCl_4) administration at each time point; B: Total RNA was prepared at different time points for the reverse transcription-polymerase chain reaction (RT-PCR) analysis of TNF-α, IL-1β and iNOS gene expression. IL-1β-specific sequences (387 bp), TNF-α-specific sequences (351 bp) and inducible nitric oxide synthase (iNOS)-specific sequences (474 bp) were detected by agarose gel electrophoresis, as described in Methods. PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed to verify that the contents of the samples were similar.

of TNF-α, IL-1β, and NO production and their corresponding liver mRNA expression were determined in rats

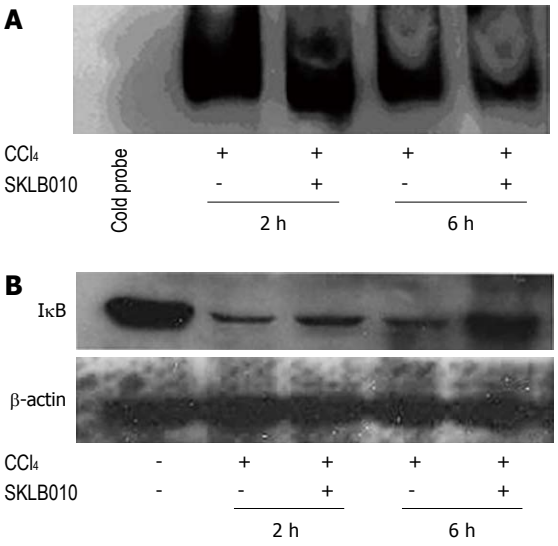

Figure 3 SKLB010 prevented the nuclear translocation of nuclear factor-κB by inhibiting degradation of IκB. A: The inhibition of nuclear factor-κB (NF-κB)-DNA binding. Carbon tetrachloride (CCl_4)-mediated activation of NF-κB resulted in an increase in DNA-binding activity of NF-κB within 2 h, followed by inactivation of NF-κB at 6 h after stimulation which was demonstrated by a lower DNA-binding activity; B: SKLB010 inhibited the CCl_4-induced degradation of IκB. Total tissue proteins were analyzed by Western blotting at different time points for IκB-α using specific IκB antibodies. β-actin was used as an internal control.

with CCl_4-induced hepatitis.

As shown in Figure 2A, treatment with SKLB010 reduced the serum levels of TNF-α and IL-1β at 12 h and NO production at 6 h compared with the CCl_4-injected groups. In order to investigate whether the reduction in serum levels of inflammatory mediators was due to the changes in mRNA expression, we examined liver mRNA expression of TNF-α, IL-1β, and iNOS both at 2 h and 6 h after CCl_4 injection. It can be seen from Figure 2B, that the administration of SKLB010 reduced the mRNA expression of TNF-α, IL-1β, and iNOS in contrast to the CCl_4-induced groups.

SKLB010 inhibits activation of NF-κB

Electrophoresis mobility shift assays (EMSA) were performed in order to examine whether SKLB010 suppressed the activation of NF-κB after CCl_4 injection. Correspondingly, a NF-κB-DNA binding assay was also carried out using nuclear extracts from acutely damaged liver stimulated by CCl_4 in the presence or absence of SKLB010. Indeed, CCl_4-mediated activation of NF-κB resulted in an increase in DNA-binding activity within 2 h, followed by inactivation of NF-κB at 6 h after stimulation which was demonstrated by a lower DNA-binding activity, suggesting that administration of SKLB010 reduced the DNA binding activity of NF-κB (Figure 3A).

As reported, degradation of IκB was required to activate NF-κB[5]. We investigated the effect of SKLB010 on the cytoplasmic level of IκB by Western blot analysis both at 2 h and 6 h after CCl_4 injection. As shown in Figure 3B, treatment with SKLB010 inhibited the degradation of IκB

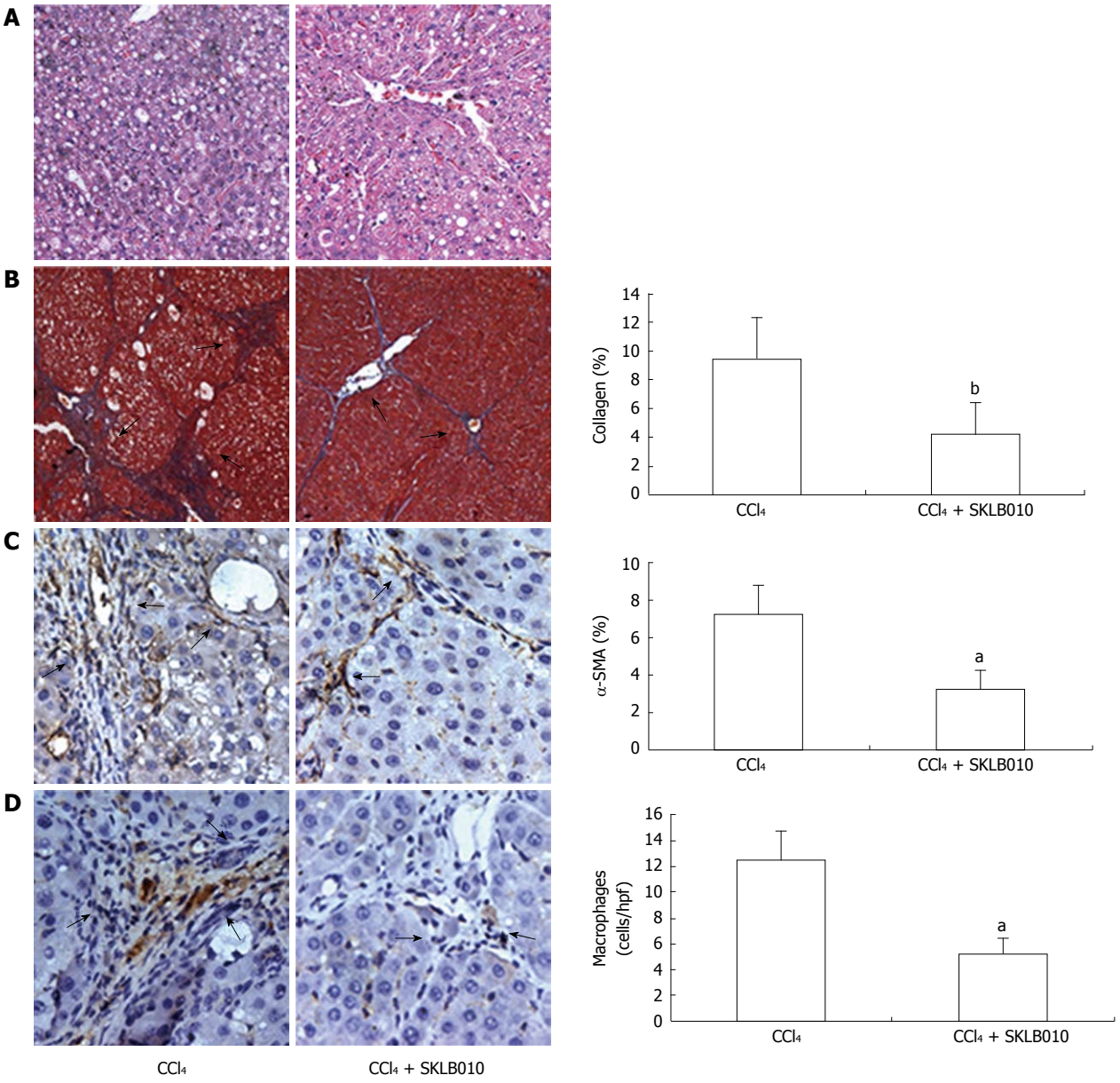

Figure 4 SKLB010 improves liver function and causes a marked reduction in liver fibrosis. Arrows points to positive direction. $^{a}P < 0.05$, $^{b}P < 0.01$. A: Hematoxylin-eosin (original magnification 200 ×); B: Masson (original magnification 100 ×) and digital image analysis quantification of collagen; C: α-smooth-muscle actin (SMA) original magnification 400 ×) and digital image analysis quantification of α-SMA; D: CD-11b (original magnification 400 ×) and digital image analysis quantification of macrophages. CCl_4: Carbon tetrachloride.

induced by the administration of CCl_4.

SKLB010 improves histology of the liver and causes a marked reduction in liver fibrosis

The hepatoprotective effects of SKLB010 on liver injury and fibrosis were initially evaluated by histological analyses. The results from HE staining showed that oral administration of SKLB010 daily for 4 wk significantly reduced the level of steatosis and necrosis, and suppressed hepatic fibrogenesis (Figure 4). Masson staining revealed that rats treated with SKLB010 showed histological improvement with a marked reduction in fibrosis and decreased expression of collagen I and IV. As a unique marker for activated HSC, α-SMA-positive cells around the fibrotic septa were significantly increased by CCl_4. In contrast, the number of α-SMA-positive cells was reduced in the livers of SKLB010-treated rats compared with those from cirrhotic rats. These findings indicate that SKLB010 efficiently reduced the presence of activated HSC in liver fibrogenesis. Furthermore, immunohistochemical analysis of CD11b revealed fewer macrophages in the fibrotic septa between nodules after treatment with SKLB010.

DISCUSSION

In the present study, oral administration of SKLB010 improved CCl_4-induced liver injury *via* the inhibition of NF-κB followed by suppression of the degradation of IκB. Treatment with SKLB010 also showed therapeutic effects

on liver fibrogenesis. In the acute liver injury study, oral treatment with SKLB010 decreased ALT enzyme levels. Histopathological findings revealed reduced levels of inflammatory cell infiltration, centrilobular fatty changes, apoptosis, and necrosis in SKLB010-treated rats. Results on the hepatoprotective effects of SKLB010 on CCl_4-induced chronic liver fibrogenesis showed that SKLB010 possessed potent ability to alleviate the development of fibrogenesis by reducing the level of steatosis and necrosis, expression of collagen I and Ⅳ, activated HSC, and macrophage infiltration of liver tissue.

SKLB010 is a novel analogue of TZDs. Recent evidence has shown that TZDs participate in the regulation of inflammation, especially in modulating the production of inflammatory mediators (such as TNF-α, IL-1β, inducible iNOS), by decreasing the activation of NF-κB while increasing the expression of IκB[1,14].

We suggest that the most likely underlying mechanism by which SKLB010 regulates the expression of proinflammatory cytokines production is through the inactivation of NF-κB.

GSH and the antioxidative enzyme system play important roles in protecting liver cells against oxidative stress[15]. CCl_4-induced liver injury was associated with decreased hepatic GSH. In our experiments, treatment with SKLB010 increased the levels of GSH which were greater than those observed for the CCl_4 control. Following exposure to CCl_4, oxidative stress was attenuated by treatment with SKLB010. Thus, we supposed that SKLB010 possessed the properties to enhance GSH and decrease reactive metabolite formation in CCl_4-induced liver injury.

These inflammatory mediators (e.g., TNF-α, IL-1β, and NO), play key roles in the induction and perpetuation of inflammation in macrophages. Our recent study using an ELISA and RT-PCR assay revealed that these mediators were inhibited by SKLB010 treatment both at the serum and mRNA level. Furthermore, the decrease in TNF-α expression occurred before the decrease in IL-1β. This is because the macrophage membrane contains the TNF-α receptor, hence the decrease in TNF-α may stimulate the expression of IL-1β. The fact that TNF-α levels initially increased rapidly and later decreased compared with the SKLB010-treated group was in accordance with the lower inactivation of NF-κB at 6 h which was able to control TNF-α production.

The expressions of these inflammatory mediators have been shown to be dependant on NF-κB activation[16,17]. Therefore, the possibility that the extract inhibited the activity of NF-κB needs to be examined further.

NF-κB, a transcription factor that regulates the expression of proinflammatory cytokines and proteins, is activated in response to several extracellular stimuli, and oxidative stress. NF-κB is sensitive to the oxidation of a particular cysteine at position 62 in p50, which is essential for DNA binding[18]. Evidence suggests that unregulated NF-κB-related gene expression in Kupffer cells contributes to CCl_4-induced liver injury[19]. To support this hypothesis, the development of a pharmacological strategy to suppress the activation of NF-κB is required.

The entry of NF-κB from the cytosol to the nucleus is regulated by IκB, and its induction and binding to NF-κB which prevents its translocation into the nucleus was estimated[20].

Since phosphorylated IκB is degraded by a multisubunit protease complex, i.e., proteasome[21], we hypothesized that SKLB010 prevented the nuclear translocation of NF-κB by inhibiting degradation of IκB through inhibition of some of the proteases in the proteasome.

The EMSA assay revealed that the subsequent NF-κB-DNA binding was inhibited in the nucleus by pretreatment with SKLB010 compared to the CCl_4-control group. CCl_4-mediated activation of NF-κB resulted in a peak of DNA-binding activity at 2 h, followed by inactivation of NF-κB at 6 h which was confirmed by lower DNA-binding activity. These results were in accordance with previous reports on the NF-κB pathway. NF-κB translocates to the nucleus and activates a series of gene transcriptions including the IκB gene which can transform NF-κB from the activated state to the inactivated state. In addition, in the study on IκB, a lower level of degradation of IκB demonstrated that SKLB010 prevented CCl_4-induced degradation of IκB. These results proved that SKLB010 inhibited the CCl_4-induced activation of NF-κB by suppressing the degradation of IκB.

In summary, based on the above results, SKLB010 had significant potent inhibitory effects on CCl_4-induced acute liver injury by reducing the activity of serum ALT, and improving the histology of liver tissue. Furthermore, the development of CCl_4-induced chronic fibrogenesis was alleviated by treatment with SKLB010, which was demonstrated by histological staining. Importantly, the underlying mechanisms of action were proved, in that SKLB010 possessed properties to enhance GSH and anti-oxidative activity, and attenuated inflammatory mediators such as TNF-α, IL-1β, and NO through inactivation of NF-κB. These observations may have potential therapeutic value in the treatment of hepatitis.

COMMENTS

Background

Hepatic damage, including acute liver injury and chronic liver fibrogenesis, constitute a major problem to human health worldwide. Despite extensive efforts, effective treatment strategies resulting in fewer side effects are still lacking.

Research frontiers

Our previous study demonstrated that (Z)-5-(4-methoxybenzylidene)thiazolidine-2,4-dione (SKLB010), a derivative of thiazolidinediones (TZDs) exhibits protective effects on Con A-induced acute hepatitis and adjuvant-induced arthritis without side effects.

Innovations and breakthroughs

SKLB010 inhibited the carbon tetrachloride (CCl_4)-induced activation of nuclear factor-κB (NF-κB) by suppressing degradation of IκB. A dose- and time-dependent relationship was found. The protection effects of SKLB010 on liver injury and fibrogenesis were initially evaluated using histological analyses.

Applications

These observations may contribute to the therapeutic value of novel small molecule drugs in the treatment of hepatitis.

Terminology

NF-κB is a transcription factor that regulates the expression of proinflammatory cytokines and proteins.

Peer review

In this manuscript, the author examined the role of SKLBO10, a derivative of TZDs, in protecting the liver against CCl_4 induced acute and chronic liver injury in rats. They found that orally administration of SKLBO10 at relatively high dose (100 mg per kg body mass per 24 h) protect the liver from acute injury (reduce serum alanine aminotransferase activity and inflammatory infiltration as well as improve histological architecture of the liver). Moreover, they have shown that SKLB010 inhibit: (1) degradation of $I_\kappa B$; (2) secretion of proinflammatory mediators; and (3) NO synthesis.

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S- Editor Sun H **L- Editor** Webster JR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.662

World J Gastroenterol 2012 February 21; 18(7): 662-665
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Clinical outcomes of lung metastasectomy in patients with colorectal cancer

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Received: May 20, 2011 Revised: July 14, 2011
Accepted: July 26, 2011
Published online: February 21, 2012

Abstract

AIM: To investigate prognostic factors of survival following curative, non-palliative surgical removal of lung metastases secondary to colorectal cancer (CRC).

METHODS: Between 1999 and 2009, a radical metastasectomy with curative intent was performed on lung metastases in 21 patients with CRC (15 male and 6 female; mean age: 57.4 ± 11.8 years; age range: 29-74 years) who had already undergone primary tumour resection.

RESULTS: The mean number of lung metastases ranged from one to five. The mean overall survival was 71 ± 35 mo (median: 25 mo). After adjusting for potential confounders, multivariable Cox regression analyses predicted only the number of lung metastases (1 *vs* ⩾ 2; hazard ratio: 7.60, 95% confidence interval: 1.18-17.2, P = 0.03) as an independent predictor of poor survival following lung resection for metastatic CRC.

CONCLUSION: Resection of lung metastases is a safe and effective treatment in selected CRC patients with single lung metastases.

Key words: Lung metastases; Colorectal cancer; Metastasectomy; Prognostic factors; Survival

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Olmez OF, Cubukcu E, Bayram AS, Akcali U, Evrensel T, Gebitekin C. Clinical outcomes of lung metastasectomy in patients with colorectal cancer. *World J Gastroenterol* 2012; 18(7): 662-665 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/662.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.662

INTRODUCTION

The lung is one of the most frequently affected metastatic sites in patients with colorectal cancer (CRC)[1]. Indeed, lung metastases may be detected sequentially or simultaneously in approximately 10% of patients with this malignancy[2,3]. Since Blalock first described pulmonary resection for metastases of colorectal carcinoma[4], several studies have demonstrated the efficacy of lung metastasectomy in CRC patients[5-14]. As the safety of the operation has improved over time, more patients may be able to undergo this surgery. In a recent single-center retrospective study, Maeda *et al*[15] reported an analysis

of patients with pulmonary metastases from colorectal carcinoma who underwent surgical resection. The overall 5-year survival rate was 74%. Importantly, the number of pulmonary metastases and prethoracotomy carcinoembryonic antigen (CEA) levels were significant independent predictors of survival after the first pulmonary metastasectomy[15]. Earlier studies reported wide ranges of survival percentages from 27% to 40.5% and tried to identify independent factors associated with clinical outcomes[5-14]. This knowledge is clinically helpful for defining a subset of patients who are most likely to benefit from surgical resection.

Here, we report our experience in lung metastasectomy in patients with primary CRC who were referred to a Turkish tertiary hospital during the past 10 years. A number of prognostic factors were analyzed to identify their impact on survival.

MATERIALS AND METHODS

This study was designed as a single-center retrospective investigation at the Department of Oncology, Uludag University Medical School, Bursa, Turkey. Retrospective analysis of the patient data was approved by the local ethics committee.

Patients

Between 1999 and 2009, a radical metastasectomy with curative purposes was performed in 21 patients with CRC (15 males and 6 females). These patients had already undergone primary tumor resection. The pulmonary lesions were also evaluated with conventional chest computed tomography. In addition, all patients underwent bronchoscopy, pulmonary function tests, and endoscopic evaluations before the operation. Indications for pulmonary resection of metastatic CRC were as follows: (1) completely resectable lung lesions diagnosed by preoperative imaging; (2) ability of the patient to tolerate the required surgical procedure and to carry out a normal life with the remaining respiratory function; and (3) surgically controllable extrapulmonary disease, including the primary lesion. All thoracotomy specimens were processed according to standard procedures and histologically confirmed to contain cancer consistent with CRC origin. All resected lung metastasis specimens had pathological tumor-free margins.

Clinical data and follow-up

The medical charts of all patients were reviewed and examined for sex, age, tumor differentiation, history of previous liver metastasis, prethoracotomy CEA levels, disease-free interval between the colorectal resection and the first pulmonary resection, number of metastases, location of metastases, type of resection, history of hilar or mediastinal node resection, size of lung metastases, and administration of postoperative chemotherapy after pulmonary resection. Clinical data and follow-up information were obtained from the medical records and were further complemented using telephone contacts with patients, family members, and physicians.

Statistical analysis

The primary endpoint was the time from lung resection to the time of death. Univariate and multivariable Cox proportional hazards models were used to identify predictors of survival. The appropriateness of the proportional hazards assumption was verified using graphical methods and tested as described by Grambsch and Therneau[16]. The assumption of linearity for the Cox models was examined by visual inspection, and no violation was found. All tests were two-sided, and $P < 0.05$ was considered statistically significant. All calculations were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Characteristics of CRC patients

The mean age was 57.4 ± 11.8 years, with a range of 29 years to 74 years. All patients had adenocarcinoma. Regarding localization at the time of primary tumor diagnosis, 11 of 21 patients had rectal cancer, and the other 10 had colon cancer. Tumor differentiation grades were as follows: G1, one patient; G2, 18 patients; and G3, two patients. Seven patients had a history of previous liver metastases. There were no operative or hospital deaths. The median preoperative CEA level was 3.6 ng/mL (range: 1.2-77.0 ng/mL).

Characteristics of pulmonary metastatic lesions

The mean disease-free interval (interval between initial treatment and onset of pulmonary metastasis) was 28 ± 21 mo (range: 3-75 mo). A solitary metastatic lesion was found in 11 patients. Multiple metastases were found in the other 10 patients, that is, two metastatic lesions in five patients, three metastatic lesions in one patient, four metastatic lesions in two patients, and five metastatic lesions in two patients. In patients with a solitary metastasis, left lung metastasis was found in four patients, and right lung metastasis in seven. In patients with multiple metastases, left lung metastases were found in two patients, right lung metastases were found in five, and metastases were found in both lungs in three patients. Wedge resection was performed in eight patients (five with a solitary lesion, one with two lesions, one with four lesions, and one with five lesions); segmentectomy in eight patients (three with a solitary lesion, two with two lesions, one with three lesions, one with four lesions, and one with five lesions); lobectomy in four patients (two with a solitary lesion and two with two lesions); and pneumectomy in one patient with a solitary lesion. Four patients underwent either hilar or mediastinal lymph node dissection. The pulmonary metastatic tumor size was obtained for all patients and ranged from 5 mm to 41 mm (mean size: 18 ± 10 mm). Nineteen patients received postoperative chemotherapy after pulmonary resection.

Table 1 Univariate analysis of prognostic factors

Risk factors	*P* value[1]
Sex (male *vs* female)	0.52
Age (yr)	0.39
Tumor differentiation (G1 and 2 *vs* G3)	0.71
History of previous liver metastasis (yes *vs* no)	0.04
Preoperative CEA levels (below *vs* above median)	0.04
Disease-free interval (mo)	0.42
Number of metastases (1 *vs* ≥ 2)	0.01
Location of metastasis (left *vs* others)	0.82
Type of resection (wedge *vs* others)	0.74
Hilar or mediastinal node resection (yes *vs* no)	0.51
Lung metastatic tumour size (mm)	0.43
Postoperative chemotherapy (yes *vs* no)	0.89

[1]Calculated with univariate Cox regression. CEA: Carcinoembryonic antigen.

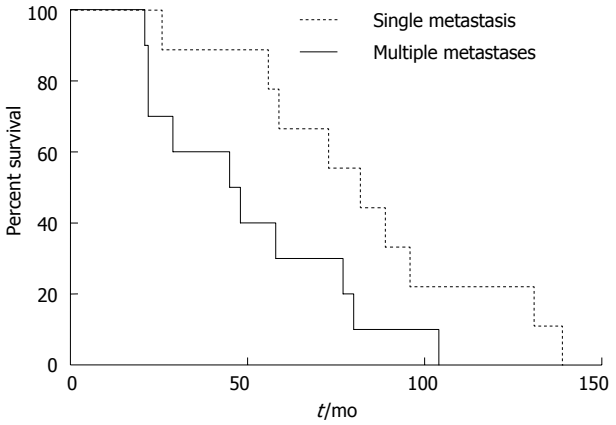

Figure 1 Kaplan-Meier plot for patients with single and multiple lung metastases from colorectal cancer.

Survival data

Survival was calculated from the time of lung resection for metastasis, and the primary end point was death. The mean overall survival was 71 ± 35 mo (median: 25 mo), ranging from 21 mo to 139 mo. The actuarial overall survival following lung metastasectomy for CRC was 71.4% at 3 years and 47.6% at 5 years.

Prognostic factors

Several prognostic factors were analyzed to identify their impact on survival. The factors that were associated with poor survival outcome following univariate analysis were the number of lung metastases ($P = 0.01$), history of previous liver metastasis ($P = 0.04$), and preoperative CEA levels ($P = 0.04$) (Table 1). No other factors had an influence on survival (Table 1). After adjusting for potential confounders, multivariable Cox regression analyses showed that only the number of lung metastases (1 *vs* ≥ 2; hazard ratio: 7.60, 95% confidence interval: 1.18-17.2, $P = 0.03$) was an independent predictor of poor survival following lung resection for CRC (Figure 1).

DISCUSSION

The results from this study indicate that the presence of a single pulmonary metastasis is the main indication for a metastasectomy in Turkish patients with CRC. The lung is a key site of metastases from CRC, and several previous studies have attempted to identify significant prognostic factors for pulmonary metastasectomy. Previously identified prognostic factors include the number of pulmonary metastases, hilar and/or mediastinal lymph node metastasis, prethoracotomy CEA levels, time of appearance of metastasis, liver metastasis before thoracotomy, mode of operation, and location of pulmonary metastases[5-14]. In our study, there were no prognostic factors except for the number of pulmonary metastases. The overall 5-year survival rate in our series was largely in agreement with previous reports[5-14], and we suggest that surgery should remain the mainstay for treating patients with single pulmonary metastases from CRC. In agreement with our current findings, Pfannschmidt *et al*[9] have reported solitary metastases as a significant prognostic factor; the post-resection outcome of patients with two or more lesions was significantly worse than that of patients with a single metastasis. Prethoracotomy CEA levels may serve as a biochemical marker for post-resection outcome[14]. CEA participates in intracellular recognition and metastasis by functioning as an attachment factor[14]. CEA levels may therefore reflect the highly malignant nature of cancer cells that undergo systemic dissemination. In our current study, an abnormal CEA level was identified as a prognostic factor in univariate but not multivariate analysis. The observation that CEA levels did not serve as a significant independent prognostic factor suggests that concentrations of this molecule simply reflect the number of lung metastases observed in our patients. A history of previous liver metastases had prognostic significance in univariate but not multivariate analysis. Our results are in agreement with numerous previous reports[17,18]. In this regard, it has been suggested that selected patients with simultaneous presentation with technically resectable liver and lung metastases may benefit from an aggressive surgical approach[19]. We cannot, however, exclude the possibility that these favorable results may be found only in selected patients.

The limitations of this study are the small patient population and the retrospective nature of the study, although the data were collected prospectively. The other drawback is that the patients included in the study may be a selected group and may not represent the general population of patients with lung metastases from CRC. These limitations notwithstanding, our results suggest a favorable outcome in Turkish CRC patients with solitary lung metastases.

In summary, the results from this study indicate that lung resection for metastatic CRC appears to be valuable in selected patients showing isolated lesions, with acceptable long-term survival. This study demonstrated that the presence of multiple lung metastases is associated with poor survival. Surgery should not be considered as the first modality of treatment for patients with multiple

lung metastases, because it is associated with a relatively poor outcome. The presence of previous liver metastases should not be considered a contraindication for lung metastasectomy. Large multicenter studies are, however, required to confirm these results.

COMMENTS

Background

The lung is one of the most frequently affected metastatic sites in patients with colorectal cancer (CRC). Several studies have demonstrated the efficacy of lung metastasectomy in CRC patients.

Research frontiers

Earlier studies have reported outcomes that varied widely and have tried to identify independent factors associated with clinical outcomes. This knowledge is clinically helpful for defining the subset of patients who are most likely to benefit from surgical resection.

Innovations and breakthroughs

Here, we report our experience concerning lung metastasectomy in patients with primary CRC referred during the past 10 years. A number of prognostic factors were analyzed to identify their impact on survival.

Applications

The results from this study indicate that lung resection for metastatic CRC appears to be valuable in selected patients with single lesions, providing acceptable long-term survival. The presence of multiple lung metastases is associated with poor survival.

Peer review

The paper is a nice addition to a growing body of literature illustrating the potential benefits of lung metastasectomy for CRC patients. This was a small series of 21 patients, yet multivariate analysis correctly identified the number of lung metastases (1 *vs* $\geqslant$ 2) as a significant predictor of survival. The message is therefore clear, and the small number of patients is not a problem. The paper is concise and well written, the results are clearly presented, and the survival data (71% and 47% at 3 years and 5 years, respectively) are in accordance with previously published series. These results also suggest that we deal with a selected population of CRC patients (mostly young individuals with resectable metastatic disease), but this is not a surprise.

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S- Editor Lv S **L- Editor** Kerr C **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.666

World J Gastroenterol 2012 February 21; 18(7): 666-672
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Outcome of patients who have undergone total enteroscopy for obscure gastrointestinal bleeding

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Received: January 16, 2011 Revised: April 20, 2011
Accepted: April 27, 2011
Published online: February 21, 2012

Abstract

AIM: To assess the diagnostic success and outcome among patients with obscure gastrointestinal bleeding who underwent total enteroscopy with double-balloon endoscopy.

METHODS: Total enteroscopy was attempted in 156 patients between August 2003 and June 2008 at Hiroshima University Hospital and achieved in 75 (48.1%). It is assessed whether sources of bleeding were identified, treatment methods, complications, and 1-year outcomes (including re-bleeding) after treatment, and we compared re-bleeding rates among patients.

RESULTS: The source of small bowel bleeding was identified in 36 (48.0%) of the 75 total enteroscopy patients; the source was outside the small bowel in 11 patients (14.7%) and not identified in 28 patients (37.3%). Sixty-one of the 75 patients were followed up for more than 1 year (27.2 ± 13.3 mo). Four (6.6%) of these patients showed signs of re-bleeding during the first year, but bleeding did not recur after treatment. Although statistical significance was not reached, a marked difference was found in the re-bleeding rate between patients in whom total enteroscopy findings were positive (8.6%, 3/35) and negative (3.8%, 1/26) (3/35 *vs* 1/26, $P = 0.63$).

CONCLUSION: A good outcome can be expected for patients who undergo total enteroscopy and receive proper treatment for the source of bleeding in the small bowel.

Key words: Double-balloon endoscopy; Small bowel; Obscure gastrointestinal bleeding; Total enteroscopy; Outcome

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Shishido T, Oka S, Tanaka S, Imagawa H, Takemura Y, Yoshida S, Chayama K. Outcome of patients who have undergone total enteroscopy for obscure gastrointestinal bleeding. *World J Gastroenterol* 2012; 18(7): 666-672 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/666.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.666

INTRODUCTION

Approximately 1%-5% of cases of gastrointestinal (GI) bleeding cannot be diagnosed by upper-GI and lower-GI endoscopic examination[1,2]. In such cases, the small bowel is the suspected source of obscure GI bleeding (OGIB)[3]. Until recently, push enteroscopy[4,5] and intraoperative enteroscopy[6,7] have been the standard modalities for diagnosing OGIB. However, push enteroscopy does not reach deep into the small bowel because of the short length of the scope, and intraoperative enteroscopy

is an invasive procedure that requires general anesthesia. Double-balloon endoscopy (DBE) was developed by Yamamoto *et al*[8-10] in 2001, and it has been clinically available since 2003. DBE allows examination of the entire small bowel (total enteroscopy) through a combined oral and anal approach, providing also for diagnosis and therapeutic interventions in cases of small bowel lesions. There are many reports regarding diagnoses, therapeutic interventions, and outcomes in patients with OGIB who have undergone DBE[11-22]. However, there are no reports regarding the diagnosis of small bowel lesions in patients with OGIB based on total enteroscopy. We undertook a retrospective study to assess diagnoses and long-term outcomes in patients who have undergone total enteroscopy for OGIB.

MATERIALS AND METHODS

Patients

The study involved 156 patients in whom DBE was performed for OGIB at Hiroshima University Hospital during the period August 2003 through June 2008. Total enteroscopy was achieved in 75 (48.1%). All patients had already undergone standard upper-GI and lower-GI endoscopy at our hospital or elsewhere. OGIB was defined by the following American Gastroenterological Association criteria[3]: overt (*vs* occult) GI bleeding and recurrent fecal occult bleeding with iron-deficiency anemia that was not identifiable by upper-GI and lower-GI endoscopy, for example, hematemesis, hematochezia, or melena.

Methods

We evaluated whether sources of bleeding in the small bowel were diagnosed by DBE, whether treatment was performed during DBE, and whether complications ensued. Preliminary diagnosis was made on the basis of endoscopic findings and clinical history. Final diagnosis was made on the basis of histologic examination of biopsy specimens and/or surgically resected specimens. If a detected lesion did not explain a patient's complaints, (for example, a single small lymphangioma), the lesion was not identified as the source of the OGIB.

We also evaluated clinical outcomes. Clinical outcome was described as either continued bleeding or complete resolution of the bleeding, regardless of whether treatment was performed. Re-bleeding was defined as hematochezia, melena, or the need for blood transfusion during follow-up. In the patients who underwent total enteroscopy, clinical variables were assessed over a 1-year follow-up period. These included general condition, bleeding episodes, hemoglobin level, treatment, and blood transfusion. For patients in whom re-bleeding occurred, the source of re-bleeding, final diagnosis, treatment method, and clinical outcome after the second treatment were evaluated.

DBE procedure

The DBE system (Fujifilm, Kanagawa, Japan) consisted of a videoendoscope with a working length of 200 cm, a flexible overtube with a total length of 145 cm, and a pressure-controlled pump system. The EN-450P5 endoscope with the TS-12140 overtube, or the EN-450T5 endoscope with the TS-13140 overtube, was used; both systems have a balloon attached to the tip of the endoscope and another attached to the tip of the overtube. The balloons can be inflated and deflated with a single touch by using a specially designed pump, while the balloon pressure is accurately monitored.

Total enteroscopy was first performed by anal approach, and tattoo injection was performed at the most proximal site reached by the endoscope. DBE was then carried out by oral approach to examine the remaining area. DBE by oral approach was performed within 2 d after DBE by anal approach. For both approaches, intestinal looping was checked fluoroscopically. DBE by anal approach was performed after bowel preparation with an oral electrolyte lavage as for regular lower-GI endoscopy. DBE by oral approach was performed after overnight fasting. Patients were sedated with midazolam and pethidine or pentazocine, if necessary. Blood pressure, heart rate, and oxygen saturation were monitored during the DBE procedures.

Total enteroscopy was considered successful when the enteroscope reached the tattoo mark made during the prior approach.

Follow-up

Cases were excluded from the study if the source of bleeding was determined to be outside the small bowel. All patients underwent follow-up examinations at our hospital or an affiliated hospital, and data were obtained from the patients' medical records or by correspondence with the affiliated hospital.

Statistical analysis

Continuous data are shown as mean ± SD and range. Differences in re-bleeding rates were analyzed by Fisher's exact probability test or χ^2 test. $P < 0.05$ was considered statistically significant. JMP software, version 5.01J (SAS Institute Inc., Cary, NC, United States), was used for all statistical analyses.

RESULTS

DBE-based diagnosis and treatment of OGIB

Clinical characteristics of the 75 patients who underwent total enteroscopy are summarized in Table 1. The source of bleeding was identified in the small bowel in 36 of these patients (48.0%) and outside the small bowel in 11 of these patients (14.7%). The source of bleeding was not traced to the digestive tract in 28 patients (37.3%). The sources of bleeding identified in the small bowel were as follows: tumor (n = 7), vascular lesion (n = 5), ulcerative lesion (n = 23), and Meckel's diverticulum (n = 1) (Table 2). Specific treatments were performed in 27 patients (75.0%) in whom the source of bleeding was identified in the small bowel by total enteroscopy. Specific treatments were as follows: medical treatment (n = 9); en-

Table 1 Clinical characteristics of patients (n = 75) who underwent total enteroscopy for obscure gastrointestinal bleeding at our hospital, August 2003 to June 2008 (mean ± SD) n (%)

Sex ratio (M/F)	44/31
Age (yr)	62.8 ± 16.9
Comorbid illness	
Cardiovascular disease	16 (21.3)
Chronic renal disease	9 (12.0)
Chronic liver disease	6 (8.0)
Cerebrovascular disease	4 (5.3)
Chronic respiratory disease	4 (5.3)
Use of anticoagulants	14 (18.7)
Use of non-steroidal anti-inflammatory drugs	11 (14.7)
Bleeding type	
Overt	56 (74.7)
Occult	19 (25.3)
Time from last bleeding episode to DBE (d)	33.2 ± 18.0
Blood transfusion	18 (24.0)
Hb before DBE (g/dL)	10.9 ± 2.5

Number of patients is shown unless otherwise indicated. Hb: Hemoglobin; DBE: Double-balloon endoscopy.

Table 2 Identification and treatment of bleeding source in the small bowel by total enteroscopy

Source of bleeding	n	Bleeding type overt/occult	Specific therapy
Tumor			
Hamartoma	2	1/1	Endoscopic resection (2)
Lipoma	1	1/0	Endoscopic resection (1)
Gastrointestinal stromal tumor	3	3/0	Surgery (3)
Leiomyosarcoma	1	1/0	Surgery (1)
Vascular lesion			
Angioectasia	4	3/1	Endoscopic hemostasis (4)
Arteriovenous malformation	1	1/0	Endoscopic hemostasis (1)
Ulcerative lesion			
Drug-induced ulcer	9	5/4	Medication (4), clinical observation (5)
Anastomotic ulcer	5	3/2	Endoscopic hemostasis (5)
Nonspecific erosion	4	4/0	Clinical observation (4)
Enteric tuberculosis	3	2/1	Medication (3)
Crohn's disease	1	1/0	Medication (1)
Radiation enteritis	1	1/0	Endoscopic hemostasis (1)
Other			
Meckel's diverticulum	1	1/0	Medication (1)

Number of patients is shown unless otherwise indicated.

doscopic hemostasis (clip placement, n = 7; argon plasma coagulation and/or injection therapy, n = 4); endoscopic resection (endoscopic mucosal resection, n = 1; polypectomy, n = 2); and surgery (n = 4). Non-specific treatment was performed for the other 9 patients.

Table 3 Outcome of patients who underwent total enteroscopy for obscure gastrointestinal bleeding (mean ± SD) n (%)

Number of follow-up cases	61
Observation period after DBE (mo)	27.2 ± 13.3
Re-bleeding rate	4 (6.6)
Time period from DBE to re-bleeding episode (mo)	7.0 ± 4.2
Number of patients who underwent transfusion	3 (4.9)
Outcome	
Survival	60
Death	1

Number of patients is shown unless otherwise indicated. DBE: Double-balloon endoscopy.

Complications of DBE

Two patients experienced complications from the diagnostic DBE. One suffered aspiration pneumonia and the other suffered acute pancreatitis. These 2 patients received medical treatment and recovered within a few days. One patient experienced a complication after therapeutic DBE: bleeding occurred after polypectomy for lipoma in the jejunum, and endoscopic hemostasis with clip placement and blood transfusion was required.

Clinical outcomes and re-bleeding rates

Data were obtained for 61 of the patients with OGIB who were followed up for more than 1 year after total enteroscopy. Excluded were the 11 patients in whom the source of bleeding was outside the small bowel and 1 patient who was lost to follow-up. The outcome and clinical course of these cases are shown in Table 3 and Figure 1, respectively. Four patients (6.6%) (4 women; mean age, 67.8 ± 7.0 years) showed signs of re-bleeding, such as hematochezia, melena, or a need for blood transfusion during this follow-up period (Table 4). The average observation period until the re-bleeding episode after total enteroscopy was 7.0 ± 4.2 mo. In 3 of the 4 patients, the re-bleeding derived from the same lesion identified upon initial total enteroscopy: 2 enteric tuberculosis lesions and 1 angioectasia. Medical treatment without endoscopic hemostasis and clinical observation was continued for the 2 patients with enteric tuberculosis. The patient in whom angioectasia was found underwent endoscopic hemostasis after epinephrine injection and argon plasma coagulation during DBE. In 1 patient in whom re-bleeding occurred, the source of the bleeding had not been identified during initial total enteroscopy. This patient was discharged from our hospital after the initial DBE examination, but severe hematochezia developed 1 mo later. Blood transfusion was performed, and DBE performed immediately thereafter revealed an arteriovenous malformation as the bleeding source. Endoscopic hemostasis with argon plasma coagulation and clip placement was performed. However, the hemorrhage did not stop, so angiography and surgical intervention were undertaken. Bleeding did

Table 4 Patients with obscure gastrointestinal bleeding in whom re-bleeding occurred after total enteroscopy

Patient	Bleeding type	Hb (g/dL)	Initial diagnosis	Initial treatment	Time after DBE (mo)	Hb (g/dL) after treatment	Final diagnosis	Second treatment	Transfusion	Re-bleeding after second treatment
78-yr-old woman	Overt	12.3	Enteric tuberculosis	Medication	10	6.6	Enteric tuberculosis	Medication	No	No
66-yr-old woman	Occult	10.1	Enteric tuberculosis	Medication	7	7.5	Enteric tuberculosis	Medication	Yes	No
62-yr-old woman	Overt	10.6	Angioectasia	EH	10	8.3	Angioectasia	EH	Yes	No
65-yr-old woman	Overt	6.5	No source of bleeding	No treatment	1	6.0	Arteriovenous malformation	EH→ angiography	Yes	No

Hb: Hemoglobin; EH: Endoscopic hemostasis; DBE: Double-balloon endoscopy.

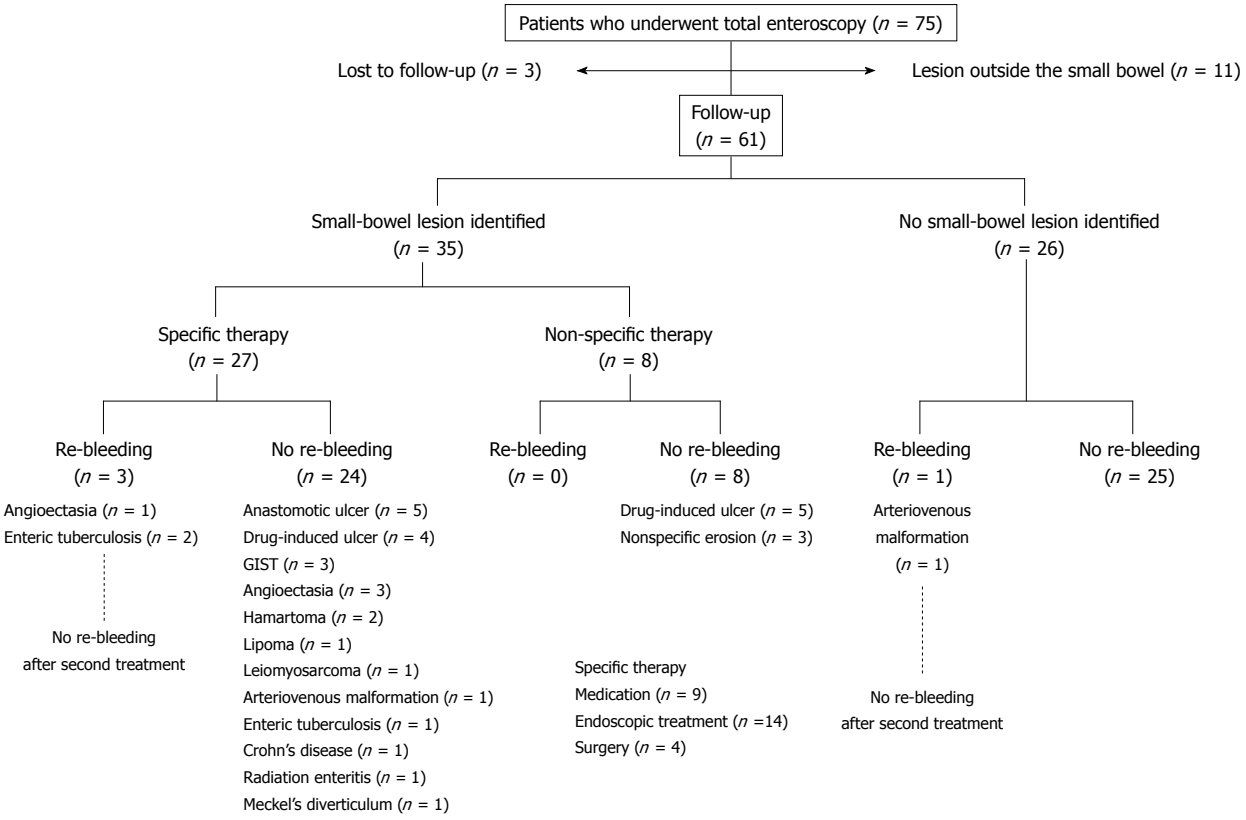

Figure 1 Clinical course after total enteroscopy for obscure gastrointestinal bleeding. GIST: Gastrointestinal stromal tumor.

not recur after the second treatment in any of the 4 patients, and they remain healthy. The re-bleeding rate for patients with positive total enteroscopy findings was 8.6% (3/35), and that for patients with negative total enteroscopy findings was 3.8% (1/26). The difference between these 2 groups was not significant ($P = 0.63$). One patient died from leiomyosarcoma with multiple metastatic liver tumors 26 mo after the initial DBE examination.

DISCUSSION

There have been several reports concerning diagnoses and outcomes in patients with OGIB in which the small bowel was the source of bleeding[23-29]; however, the entire small bowel was not examined by DBE in these patients. Ours is the first report on the prognosis of OGIB originating from a small bowel lesion that was identified through total enteroscopy performed by means of DBE. Overall, the source of bleeding, whether within or outside the small bowel, was identified in 62.7% of cases in which total enteroscopy was performed by DBE. The source of bleeding was found to be outside the small bowel in 14.7% of patients. Arakawa *et al*[29] reported lesions outside the small bowel in 9.3% of patients. It has been reported that identification of lesions responsible for OGIB by means of DBE is successful in only 50%-76% of cases[24,30-32]; however, the reports include cases of small lesions that were unlikely to be the source

of bleeding and cases in which the interior of the entire small bowel could not be observed. Therefore, the reports do not describe the true diagnostic performance of total enteroscopy by means of DBE in patients with OGIB.

Re-bleeding occurred in 8.6% of our patients in whom the source of bleeding was identified by initial total enteroscopy and proper treatment was performed. This rate is lower than the rates reported by Hadithi *et al*[23] (14.3%), by Hsu *et al*[25] and Madisch *et al*[28] (20.0%), and by Kameda *et al*[26] (19.0%). These reports do not clarify whether the sources of bleeding were identified by initial DBE or whether total enteroscopy was achieved. Re-bleeding from a small bowel lesion occurred in 3.8% (n = 1) of our patients in whom the source of bleeding was not identified by initial total enteroscopy. This rate is lower than rates reported previously (8.7%-80.0%)[23-25,28]. Possible reasons are as follows: patients in whom the entire small bowel was not observed were included in our study; lesions outside the small bowel were included; and only lesions that were believed to be the clear source of bleeding were included.

Our data show that identifying a lesion in the small bowel and administering proper treatment results in a decrease in the re-bleeding rate, as well as a good clinical outcome. According to Arakawa *et al*[29], vascular lesions account for approximately 80% of cases of re-bleeding. Vascular lesions were the cause in 2 of our 4 patients with re-bleeding. In 1 of our patients, there was a problematic arteriovenous malformation that was not discovered during initial total enteroscopy. It is possible that the hemorrhage had stopped spontaneously by the time DBE was performed. In general, however, there is little possibility of re-bleeding from the small bowel because, if no lesions are identified in the small bowel by total enteroscopy, only exceptional minute vascular lesions would cause re-bleeding.

Among our patients, the average follow-up period was 27.2 mo after total enteroscopy, but the average period until re-bleeding was 7.0 mo, and all re-bleedings occurred within 1 year. In the Fujimori *et al*[24] and Kameda *et al*[26] series, 75.0% and 83.3% of patients, respectively, experienced re-bleeding within 1 year. Thus, we believe it is necessary to monitor patients for at least 1 year after the source of bleeding has been identified and treatment has been performed. Moreover, performing total enteroscopy in patients with OGIB is helpful in administering proper treatment for the source of bleeding and for diagnosing previously unidentified GI lesions.

One of the issues of DBE is insertability. The reported rate at which total enteroscopy is achieved by means of DBE falls between 40% and 86%[12,33-36]; however, from multicenter studies in Western countries, a much lower rate of 15%-16% was reported[37-39]. Insertion of the endoscope into a deeply situated small bowel is difficult, particularly in postoperative cases and cases of adhesion. The usefulness of capsule endoscopy (CE)[40] has been reported for such cases. Total enteroscopy has been achieved by CE in 74%-83% of cases[36,41-44]. Moreover, CE is a safe modality for diagnosing OGIB[41-44]. Although CE is suitable as an initial screening for OGIB, one disadvantage of CE is that submucosal tumors are overlooked[45-48], and CE does not provide for histopathologic biopsy or endoscopic treatment when a lesion is detected. Also, there are no reports concerning diagnostic agreement between DBE and CE in patients who have undergone total enteroscopy for OGIB. Thus, DBE is an essential modality for both diagnosing and treating small bowel lesions. Efforts are needed to improve instruments to increase insertability.

We assessed the prognosis of OGIB in patients for whom a small bowel lesion was identified as the source of bleeding by total enteroscopy. Total enteroscopy was performed by DBE, and when no source of bleeding was identified or proper treatment was administered for an identified source of bleeding, the outcome was satisfactory. However, considering the possibility of re-bleeding, a follow-up period of 1 year seems necessary for patients who have undergone treatment.

Our data lead us to conclude that total enteroscopy by DBE is advantageous over general DBE for patients with OGIB. A good outcome can be expected for such patients when the entire small bowel is examined by DBE and proper treatment is given for the source of bleeding in the small bowel.

COMMENTS

Background

There are many reports regarding diagnoses, therapeutic interventions, and outcomes in patients with obscure gastrointestinal bleeding (OGIB) who have undergone double-balloon endoscopy (DBE). However, there are no reports regarding the diagnosis of small bowel lesions in patients with OGIB based on total enteroscopy.

Research frontiers

The authors undertook a retrospective study to assess diagnoses and long-term outcomes in patients who had undergone total enteroscopy for OGIB.

Innovations and breakthroughs

The study found that total enteroscopy by DBE is advantageous over general DBE for patients with OGIB.

Peer review

The authors have presented a well written documentation of total enteroscopy for obscure gastrointestinal bleeding.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.673

World J Gastroenterol 2012 February 21; 18(7): 673-678
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Nutritional risk index as a predictor of postoperative wound complications after gastrectomy

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Received: April 9, 2011 Revised: July 14, 2011
Accepted: July 21, 2011
Published online: February 21, 2012

Abstract

AIM: To investigate the correlation between the nutritional risk index (NRI) and postoperative wound complications.

METHODS: From January 2008 through June 2008, 669 patients who underwent curative gastrectomy for gastric cancer were included in a retrospective study. Medical records of consecutive patients were collected and analyzed to determine postoperative wound complication rates. The NRI was assessed on the fifth postoperative day and other possible risk factors for the incidence of wound complications were analyzed to identify the factors affecting postoperative wound complications. Patients with other postoperative complications were excluded from the study.

RESULTS: On the 5th postoperative day, the NRI showed a malnutrition rate of 84.6% among postoperative patients. However, postoperative wound complications occurred in only 66/669 (9.86%) patients. Of the patients with wound complications, 62/66 (94%) belonged to the malnourished group (NRI < 97.5), and 4/66 (6%) patients to the non-malnourished group (NRI $\geqslant$ 97.5). The only factor correlated with wound complications was the NRI on the 5th postoperative day (odds ratio of NRI $\geqslant$ 97.5 *vs* NRI < 97.5: 0.653; 95% confidence interval: 0.326-0.974; P = 0.014) according to univariate analysis as well as multivariate analysis.

CONCLUSION: This study suggests that malnutrition immediately after surgery may play a significant role in the development of wound complications.

Key words: Nutritional risk index; Wound complication; Gastric cancer

Peer reviewers: Wei-Dong Tong, Dr., MD, PhD, Associate Professor, Daping Hospital, Third Military Medical University, Chongqing 400042, China; Takaaki Arigami, MD, PhD, Field of Oncology, Department of Surgical Oncology and Digestive Surgery, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 891-0175, Japan

Oh CA, Kim DH, Oh SJ, Choi MG, Noh JH, Sohn TS, Bae JM, Kim S. Nutritional risk index as a predictor of postoperative wound complications after gastrectomy. *World J Gastroenterol* 2012; 18(7): 673-678 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/673.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.673

INTRODUCTION

Gastric cancer is the second most common cancer in the world, the most common cancer in South Korea, and the third common cause of cancer death in South Korea[1,2]. A successful outcome after surgery is highly dependent on the incidence and severity of postoperative complications, and malnutrition has been reported to be an important risk factor for perioperative morbidity and mortality[3]. Bozzetti *et al*[4] found that low serum albumin was one of the factors correlated with postoperative complications. Weight loss has also been shown to identify patients at risk of postoperative complications[5].

Wound infection has been one of the most common postoperative complications associated with surgical treatment of patients[6,7]. Its occurrence may significantly contribute to increased postoperative morbidity and longer duration of hospitalization. Typically reported risk factors for wound breakdown were duration of surgery, obesity, diabetes, patient age, coincident infection, and poor nutrition[8,9].

In a previous study, it was demonstrated that low serum albumin on the 5th day after surgery was well correlated with postoperative short-term complications[10]. It was also shown that a total of 13.45% patients developed postoperative complications, of which 55% were related to wound complications. Based on these previous data, it was assumed that malnourished patients might have a high rate of postoperative wound complications, as well as other postoperative complications. It is vital to detect and treat malnutrition in patients undergoing major surgery, because malnutrition results in poor clinical outcomes if unrecognized and not treated properly.

Thus, a proper assessment of the nutritional status in these patients should be performed. Numerous researchers have sought a reliable, valid scoring system that can identify patients with poor nutritional status[11]. Many nutrition risk scores to predict nutrition-related complications in gastrointestinal surgery are available, including the nutrition risk index (NRI), the nutrition risk score, and the bioelectrical impedance analysis[3]. None of these is generally accepted as the gold standard. Among them, the NRI, which is based on the serum albumin concentration and weight loss, has been shown to identify patients at risk for postoperative complications[3]. NRI is based on a mathematical equation, can easily be scored after one laboratory test, does not require subjective judgment, and can be safely applied in the clinical setting, with no significant effect on the predictive value[11]. On the other hand, other nutritional assessment techniques are based on clinical and subjective assessments, so can be variable between observers.

For this reason, the NRI was chosen as the index reflecting the nutritional state of the postoperative patient in this study. We aimed to evaluate the correlation of the NRI with wound complications in gastric cancer patients undergoing curative gastrectomy, and to determine strategies to support postoperative patients' nutritional status in order to reduce postoperative complications, including wound complications.

Table 1 Types and definitions of wound complications

Types	Definitions
Seroma	Serous fluid collection in the absence of infection
Hematoma	Subcutaneous blood in the absence of infection
Wound infection	Displayed two or more of the following characteristics: drainage of purulent material, erythema, tenderness, induration or fever
Wound dehiscence	(1) Hematoma, seroma, or infection that required that the incision be opened, evacuated, and/or irrigated and debrided; (2) Status that required packing and healing by secondary intention

From Chelmow *et al*[9] 2004.

MATERIALS AND METHODS

Patients

From January 2008 through June 2008, 669 patients who underwent curative gastrectomy for gastric adenocarcinoma at Samsung Medical Center were enrolled in a retrospective study, with data collected prospectively. Medical records of consecutive patients were analyzed to determine the postoperative wound complication rates and to examine the effect of other possible risk factors of wound complications. Gender, age, disease progression, types of operation, comorbid disease and nutritional status were reviewed and analyzed as the possible risk variables. Postoperative wound complications which developed during the hospital stay, and the types of wound complications were classified according to Table 1. The patients with other postoperative complications were excluded because these might affect the occurrence of postoperative wound complications and could act as confounding factors.

Nutritional assessment

The nutritional assessment of the patients was performed using the NRI on the 5th postoperative day. The NRI was calculated using the formula: NRI = (1.489 × serum albumin, g/L) + (41.7 × current weight/usual weight)[3]. The usual weight was defined as the stable weight 6 mo or more before illness[11], but in this study, the weight measured at admission was used as the usual weight. The present weight was also determined on the 5th postoperative day using a calibrated balance. It has been suggested that a NRI > 100 indicated that the patient is not malnourished, while 97.5-100 indicated mild malnourishment, 83.5-97.5 indicated moderate malnourishment, and < 83.5 indicated severe malnourishment[11,12]. However, in this study, the cutoff points suggested by Buzby *et al*[13] were used to define malnutrition for the NRI. Therefore, patients with a score $\geqslant$ 97.5 were classified as well nourished, between 83.5 and 97.5 as moderately malnourished, and below 83.5 as severely malnourished. For analysis, the patients were stratified into two groups: a well-nourished group with a NRI score $\geqslant$ 97.5 and a moderately or severely malnourished group with a NRI score below 97.5. The

Table 2 Characteristics of patients according to the nutritional risk index on the 5th postoperative day

Characteristic		NRI			*P* value
		< 97.5 (*n* = 566, 84.6%)	⩾ 97.5 (*n* = 103, 15.4%)	All (*n* = 669)	
Gender, *n* (%)	Male	346 (61)	86 (83)	432	0.000[1]
	Female	220 (39)	17 (17)	237	
Age, mean ± SD		57.15 ± 11.7	54.70 ± 9.69	56.77 ± 11.44	0.035[2]
Disease progression, *n* (%)	EGC	307 (54)	72 (70)	379	0.003[1]
	AGC	259 (46)	31 (30)	290	
Type of operation, *n* (%)	B-Ⅰ	355 (63)	89 (86.4)	444	0.000[1]
	B-Ⅱ	61 (11)	5 (4.9)	66	
	TG	150 (26)	9 (8.7)	159	
Comorbid diseases, *n* (%)	Hypertension	130 (23)	29 (28.1)	159	0.365[1]
	Diabetes mellitus	66 (11.7)	20 (19.4)	86	
	Pulmonary tuberculosis	22 (3.9)	7 (6.8)	29	
	Cardiovascular diseases	13 (2.3)	3 (2.9)	16	
	Hepatitis	40 (7.1)	5 (4.8)	45	
	Others	18 (3)	1 (1)	19	
	None	277 (50)	38 (36.9)	315	
Postoperative hospital stay (d, mean ± SD)		12.76 ± 4.02	12.01 ± 2.45	12.64 ± 3.82	0.160[2]
Postoperative wound complication, *n* (%)		62 (10.9)	4 (3.8)	66 (9.86)	0.027[1]

NRI: Nutrition risk index; EGC: Early gastric cancer; AGC: Advanced gastric cancer; B-Ⅰ: Subtotal gastrectomy with Billroth-Ⅰ reconstruction; B-Ⅱ: Subtotal gastrectomy with Billroth-Ⅱ reconstruction; TG: Total gastrectomy. [1]Fisher's exact test; [2]Mann-Whitney *U* test.

NRI characteristics of patients, as well as the postoperative wound complication rate (%), were compared between the two groups and evaluated for their association with wound complications. To clarify the factors affecting postoperative wound complication, logistic regression analysis were performed.

Statistical analysis

The characteristics of patients, operation types, medical comorbid disease, length of postoperative hospital stay, and wound complications were compared based on the NRI classification using Fisher's exact test (Chi-squared) and the Mann-Whitney *U* test. In addition, these variables were compared between the groups with wound complications and those without wound complications using the same statistical methods and a repeated analysis of variance test, where appropriate. A difference was considered significant at $P < 0.05$. All statistical analyses were performed using the statistical software package PASW 17.

RESULTS

A total of 669 patients who had undergone curative gastrectomy for gastric cancer were enrolled in the study. The mean age was 56.8 ± 11.4 years. The ratio of males to females was 1.8:1 (432/237). The majority of the patients, 379 (56.7%) had early gastric cancers, while 290 (43.7%) had advanced gastric cancers. Early gastric cancer is defined as tumor invasion limited to the mucosa and submucoa, irrespective of the presence of a lymph node metastasis, and advanced gastric cancer is defined as tumor invasion above the muscularis propria[14].

Most patients [*n* = 444 (66.4%)] underwent subtotal gastrectomy with Billroth-Ⅰ reconstruction, 66 (9.8%) underwent subtotal gastrectomy with Billroth-Ⅱ reconstruction, and 159 (23.8%) patients underwent total gastrectomy. Co-morbid diseases were present in 354 (52.9%) patients, with the most common being hypertension (23.7%), followed by diabetes mellitus (12.9%), hepatitis (6.7%), pulmonary tuberculosis (4.3%), and cardiovascular diseases (2.3%).

The clinical demographic characteristics are described in Table 2. Patients were stratified on the basis of the NRI on the 5th postoperative day (NRI < 97.5, malnourished or NRI ⩾ 97.5, not malnourished). Malnutrition rates on the 5th postoperative day were 84.6% (566/669), with 3% (21/669) of the patients showing severe malnutrition (NRI < 83.5), 81.5% (545/669) showing moderate malnutrition (83.5 ⩽ NRI < 97.5).

As shown in Table 2, there were statistically significant differences in gender, age, disease progression, types of operation, and postoperative wound complication rates between NRI classes. However, there were no statistically significant differences in comorbid diseases or length of postoperative hospital stay between NRI classes. Postoperative wound complications occurred in 66 (9.86%) of the 669 patients. Among these patients, 62 (94%) belonged to the malnourished group (NRI < 97.5), and 4 (6%) patients to the non-malnourished group (NRI ⩾ 97.5).

Table 3 shows the patient characteristics, or risk factors, that correlate with wound complications. The only risk factor that correlated with wound complications was the NRI on the 5th postoperative day. By contrast, there were no differences in the rates of wound complications with respect to gender, age, disease progression, type of operation, or comorbid diseases. As expected, in patients who developed wound complications, length of postoperative hospital stay was prolonged.

Table 3 Relationship between patient characteristics and occurrence of wound complications

Characteristic		Wound complication			*P* value
		(-) (*n* = 603)	(+) (*n* = 66)	All (*n* = 669)	
Gender, *n* (%)	Male	393 (65.2)	39 (59.1)	432	0.327[1]
	Female	210 (34.8)	27 (40.9)	237	
Age, mean ± SD		56.57 ± 11.2	58.62 ± 13.2	56.77 ± 11.44	0.164[2]
Disease progression, *n* (%)	EGC	346 (57.4)	33 (50)	379	0.251[1]
	AGC	257 (42.6)	33 (50)	290	
Type of operation, *n* (%)	B- Ⅰ	404 (67)	40 (60.6)	444	0.253[1]
	B- Ⅱ	61 (10)	5 (7.6)	66	
	TG	138 (23)	21 (31.8)	159	
Comorbid diseases, *n* (%)	Hypertension	142 (23.5)	17 (25.8)	159	0.722[1]
	Diabetes mellitus	73 (12.1)	20 (30.3)	93	
	Pulmonary tuberculosis	26 (4.3)	7 (10.6)	33	
	Cardiovascular diseases	16 (2.7)	0 (0)	16	
	Hepatitis	40 (6.6)	5 (7.6)	45	
	Others	17 (2.8)	2 (3)	19	
	None	289 (48)	15 (22.7)	304	
Postoperative hospital stay (d, mean ± SD)		12.24 ± 3.25	16.38 ± 6.104	12.64 ± 3.82	0.000[2]
NRI, *n* (%)	NRI < 97.5	504 (83.6)	62 (93.9)	566	0.027[1]
	NRI ⩾ 97.5	99 (16.4)	4 (6.1)	103	

NRI: Nutrition risk index; EGC: Early gastric cancer; AGC: Advanced gastric cancer; B- Ⅰ: Subtotal gastrectomy with Billroth- Ⅰ reconstruction; B- Ⅱ: Subtotal gastrectomy with Billroth- Ⅱ reconstruction; TG: Total gastrectomy. [1]Fisher's exact test; [2]Mann-Whitney *U* test.

Table 4 Distribution of types of wound complications according to the nutritional risk index

	NRI	
	< 97.5 *n* = 62	⩾ 97.5 *n* = 4
Seroma	36	0
Hematoma	4	0
Wound infection	9	0
Wound dehiscence	13	4
Total	62	4

NRI: Nutrition risk index.

Table 5 Multivariate analysis of characteristics of patients with wound complications by logistical regression

	Odds ratio (95% CI)	*P* value
Age (⩾ 56.77 *vs* < 56.77)	1.299 (0.829-2.035)	0.253
Gender (male *vs* female)	1.253 (0.795-1.976)	0.330
Disease progression (AGC *vs* EGC)	1.363 (0.873-2.127)	0.157
Surgical methods:		0.400
B- Ⅱ *vs* B- Ⅰ	0.839 (0.365-1.930)	0.679
TG *vs* B- Ⅰ	1.511 (0.920-2.483)	0.103
Comorbid diseases		NS
NRI (⩾ 97.5 *vs* < 97.5)	0.653 (0.326-0.974)	0.014

NRI: Nutrition risk index; EGC: Early gastric cancer; AGC: Advanced gastric cancer; B- Ⅰ: Subtotal gastrectomy with Billroth- Ⅰ reconstruction; B- Ⅱ: Subtotal gastrectomy with Billroth- Ⅱ reconstruction; TG: Total gastrectomy; NS: Not significant; CI: Confidence interval.

As shown in Table 4, the most common wound complication was seroma. It was encountered in 36 (54.5%) patients, all of whom were malnourished. Seventeen (25.8%) of the patients developed wound dehiscence, which required gauze packing and healing by secondary intention. Thirteen (70%) of these patients belonged to the malnourished group. Nine patients (13.6%) had wound infection, which required draining of purulent material in all cases and treatment with antibiotics in some cases. Three (13.6%) of the patients had hematoma that required evacuation. These patients were all malnourished.

When wound complications were correlated with the six possible risk factors by logistic regression analysis, the only factor that significantly increased the rate of wound complication was the 5th postoperative NRI of the malnourished group (Table 5).

DISCUSSION

It has been well known that malnutrition is a significant risk factor for postoperative complications in major abdominal surgery[15]. The reported prevalence of malnutrition in gastrointestinal surgery patients ranges from 30% to 50%[3,16]. Kuzu *et al*[11] reported that the level of malnourishment was directly correlated with both the severity and the frequency of postoperative complications. Beattie also suggested that perioperative nutritional support in malnourished patients decreased postoperative complications such as wound infections and sepsis[16].

It was reported that serum albumin on the 5th day after surgery was correlated with postoperative short-term complications[10]. It was also shown that 13.45% of the patients developed postoperative complications and that 24% of these patients had hypoalbuminemia on the 5th postoperative day. Furthermore, 55% of the postoperative complications were wound complications. Gorman *et al*[17] suggested that malnutrition was an important risk factor for postoperative infections and wound com-

plications in patients undergoing major surgery.

An accurate and reliable method for identifying patients who are malnourished or at risk for malnutrition may be beneficial in preventing postoperative infections and wound complications[11]. Many simple nutritional assessment or screening tools have been developed in recent years and have been validated[18]. Among them, the NRI, based on serum albumin concentration and weight loss, has been shown to identify patients at risk of postoperative complications. Its value, however, is limited by non-nutritional factors that affect albumin synthesis and the fact that the score does not represent specific, disease-related nutritional risk, such as in cancer. Nonetheless, it is simple, reliable, and does not require subjective judgment[11].

In the present study, it was found that the NRI on the 5th postoperative day identified 566 (84.6%) malnourished patients and that 10.9% of these malnourished patients developed wound complications. The NRI on the 5th postoperative day was the only risk factor for wound complications which was a new finding in gastric surgery.

However, patients with severe malnutrition [serum albumin < 3.0 g/dL or weight loss > 10% or body mass index (BMI) < 18.5 kg/m^2] did not show a significantly higher incidence of postoperative complications[4]. Additionally, weight loss and hypoalbuminemia were not associated with an increased risk of mortality and morbidity in patients who underwent surgery for gastric cancer[15]. Some recent evidence suggested that overweight and obesity rather than malnutrition were significant risk factors for postoperative complications in major abdominal surgery[19]. With respect to wound complications, Shin *et al*[20] reported that overweight (BMI > 25.0 kg/m^2) patients that underwent gastrectomy had a higher wound complication rate than normal body weight (BMI ≤ 25.0 kg/m^2) patients. Furthermore, Jaibati reported that overweight at the time of surgery, as measured by increased BMI, was a significant risk factor for an increased wound complication rate following abdominoplasty[21]. In addition, Vastine *et al*[22] reported that for abdominoplasties, 80% of obese patients had complications compared with the borderline and non-obese patients, and Hester *et al*[23] showed that in abdominoplasty procedures, obesity was a significant factor in predicting major morbidity.

Bearing in mind that other factors in addition to nutritional status may be involved in the development of postoperative complications, analyses were conducted in this study to identify possible risk factors, but no other factors were correlated with wound complications. In particular, and in contrast to common expectations that diabetes mellitus may be related to wound complications, there was no association between comorbidities, including diabetes mellitus, and wound complications, which contradicted the reports related to other surgical procedures. For example, Hensel *et al*[24] found that in abdominoplasty, complications were significantly increased in patients with diabetes and/or hypertension, and Jabaiti[21] found a negative correlation between diabetes mellitus and wound complications. It was also suggested that malnutrition was not always the cause of postoperative complications, but that both malnutrition and complications were the result of the underlying disease or other factors, because malnutrition and underlying disease were inextricably interwoven.

In conclusion, it was clearly shown that malnutrition had a high correlative relationship with postoperative wound complications, and NRI on the 5th postoperative day was a very good predictor of wound complications in gastric resection.

COMMENTS

Background

It was supposed that malnutrition might be related with postoperative wound complication. Nutritional risk index (NRI) was used as an evaluation tool of nutritional status in this study because it was easy and convenient to be applied.

Research frontiers

Surgical metabolism and clinical nutrition are important areas in the research field related with this article.

Innovations and breakthroughs

NRI on the 5th postoperative day was a good predictor of postoperative wound complications.

Applications

Preoperative or postoperative nutritional support, especially with attention to serum level of albumin may reduce wound complications.

Terminology

NRI is calculated with serum albumin level and body weight to evaluate nutritional status of patients.

Peer review

Authors showed that NRI on the 5th postoperative day was useful to predict postoperative wound complications.

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S- Editor Sun H **L- Editor** Cant MR **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.679

World J Gastroenterol 2012 February 21; 18(7): 679-684
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Glycyrrhizin attenuates HMGB1-induced hepatocyte apoptosis by inhibiting the p38-dependent mitochondrial pathway

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Supported by Samsung Biomedical Research Institute grant, No. SBRI C-A8-219-1
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Received: February 26, 2011 Revised: June 9, 2011
Accepted: June 16, 2011
Published online: February 21, 2012

Abstract

AIM: To examine how high-mobility group box 1 (HMGB1) regulates hepatocyte apoptosis and, furthermore, to determine whether glycyrrhizin (GL), a known HMGB1 inhibitor, prevents HMGB1-induced hepatocyte apoptosis.

METHODS: A human hepatocellular carcinoma cell line stably transfected with a bile acid transporter (Huh-BAT cells), were used in this study. Apoptosis was quantified using 4',6-diamidino-2-phenylindole dihydrochloride staining and the APO Percentage apoptosis assay, and its signaling cascades were explored by immunoblot analysis. Kinase signaling was evaluated by immunoblotting and by using selective inhibitors. It is also tried to identify hepatocyte apoptosis affected by the HMGB1 inhibitor, GL.

RESULTS: HMGB1 increased cellular apoptosis in Huh-BAT cells. HMGB1 led to increased cytochrome c release from mitochondria into the cytosol, and induced the cleavage of procaspase 3. However, it did not affect the activation of caspase 8. HMGB1-induced caspase 3 activation was significantly attenuated by the p38 inhibitor SB203580. GL significantly attenuated HMGB1-induced hepatocyte apoptosis. GL also prevented HMGB1-induced cytochrome c release and p38 activation in Huh-BAT cells.

CONCLUSION: The present study demonstrated that HMGB1 promoted hepatocyte apoptosis through a p38-dependent mitochondrial pathway. In addition, GL had an anti-apoptotic effect on HMGB1-treated hepatocytes.

Key words: High-mobility group box 1; Hepatocyte; Apoptosis; Glycyrrhizin; p38; Mitochondria

Peer reviewers: Filip Braet, Associate Professor, Australian Key Centre for Microscopy and Microanalysis, Madsen Building (F09), The University of Sydney, Sydney NSW 2006, Australia; Richard A Rippe, Dr., Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7038, United States

Gwak GY, Moon TG, Lee DH, Yoo BC. Glycyrrhizin attenuates HMGB1-induced hepatocyte apoptosis by inhibiting the p38-dependent mitochondrial pathway. *World J Gastroenterol* 2012; 18(7): 679-684 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/679.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.679

INTRODUCTION

High-mobility group box 1 (HMGB1) is an evolutionarily conserved protein present in the nucleus of almost all eukaryotic cells, where it is involved in DNA replication, repair and transcription[1-3]. This molecule is known to be released by cells undergoing necrosis as well as being

secreted by activated macrophages. While early studies of HMGB1 demonstrated its role as a late mediator of sepsis[4], HMGB1 has been more recently implicated as a putative danger signal involved in the pathogenesis of a variety of non-infectious inflammatory conditions including autoimmunity, cancer, trauma, and hemorrhagic shock, and ischemia-reperfusion injury (IRI)[5-11]. So far, HMGB1 has been studied in a number of organs including liver, lung, breast and prostate[7-9,11].

In the liver, the importance of HMGB1 signaling has been largely identified in cases of IRI, during which tissue levels of HMGB1 were elevated following reperfusion and neutralizing antibodies against HMGB1 ameliorated the damage resulting from IRI in a toll-like receptor (TLR)4-dependent manner[11]. The pathogenetic role of HMGB1 in liver disease was also clarified by studying the inflammatory response to viral infection[12]. Following hepatocyte death by hepatitis B virus-specific cytotoxic T lymphocytes in a mouse model of hepatitis, HMGB1 directs the intrahepatic recruitment of neutrophils and all other non-antigen specific inflammatory cells (natural killer cells, T cells, B cells, monocytes, macrophages and dendritic cells).

Apoptosis, a stereotyped morphologic form of cell death, is an event that contributes to liver injury in a wide range of acute and chronic liver diseases[13]. However, it is not clear whether HMGB1 contributes to apoptotic cell death in the liver. Furthermore, the regulatory mechanism of HMGB1 in hepatocyte apoptosis remains largely undefined.

Glycyrrhizin (GL) is a major active constituent of licorice root that is commonly used in Asia to treat patients with chronic hepatitis[14-16]. This compound has been associated with numerous pharmacologic effects, including anti-inflammatory, anti-viral, anti-tumor, and hepatoprotective activities[17]. Recently, GL was recognized by Sitia *et al*[18] as an HMGB1 inhibitor, which binds directly to both HMG boxes in HMGB1.

Thus, the aim of this study was to provide *in vitro* evidence and a potential theoretical basis for HMGB1 regulation of hepatocyte apoptosis in order to further elucidate the molecular mechanism of HMGB1 involvement in various pathologic conditions that can affect the liver. Furthermore, we attempted to determine whether GL attenuates HMGB1-induced hepatocyte apoptosis and, if so, to identify the signaling cascades responsible for this modulation.

MATERIALS AND METHODS

Cell line and culture

Several human hepatoma cell lines were chosen for this study: Huh-7 cells stably transfected with a bile acid transporter[19] derived from a well-differentiated hepatocellular carcinoma (HCC)[20] (Huh-BAT), HepG2 and SNU-475 cells derived from a poorly differentiated HCC[21]. All cells were cultured in Dulbecco's Modified Eagle medium supplemented with 10% fetal bovine serum, 100 000 U/L penicillin and 100 mg/L streptomycin. In all experiments, cells were serum-starved for 12 h in order to avoid the effects of serum-induced signaling.

Materials and reagents

HMGB1 (human, recombinant expressed in *E. coli*) was synthesized by Sigma-Aldrich, Inc. (St. Louis, MO, United States) at a purity of > 90%. The MAPK inhibitors [SB203580 for p38 mitogen activated protein kinase (MAPK), U0126 for p42/44 MAPK or extracellular signal-regulated kinase, and SP600125 for c-Jun N-terminal kinase (JNK) and GL] were also obtained from Sigma-Aldrich, Inc.

Preparation of mitochondrial and cytosolic extracts

Cells were washed twice with phosphate-buffered saline, and mitochondrial and cytosolic extracts were isolated using a mitochondria/cytosol fractionation kit (BioVision, Inc., Mountain View, CA, United States) according to the manufacturer's instruction.

Immunoblot analysis

Huh-BAT cells were lysed for 20 min on ice with lysis buffer (50 mol/L Tris-HCl, pH 7.4; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mol/L NaCl; 1 mol/L EDTA; 1 mol/L PMSF; 1 μg/mL aprotinin, leupeptin, pepstatin; 1 mol/L Na_3VO_4; and 1 mol/L NaF) and centrifuged at 14 000 × *g* for 10 min at 4 ℃. Proteins in the lysates were resolved by 10% or 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and probed using the following primary antibodies: mouse anti-caspase 8 (1:500 dilution) from Cell Signaling Technology (Danvers, MA, United States); rabbit anti-caspase 3 (1:1000 dilution) from Cell Signaling Technology; rabbit anti-ACTIVE® p42/p44 (1:2000 dilution), anti-ACTIVE® p38 (1:1000 dilution), and anti-ACTIVE® JNK (1:1000 dilution) specific for the phosphorylated forms of p42/p44 MAPK, p38 MAPK, and JNK, respectively, from Cell Signaling Technology; mouse anti-cytochrome c (1:500 dilution) from BD Pharmingen (San Jose, CA, United States), and goat anti-actin (1:1000 dilution) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, United States). Twenty μg of protein was used for each well in Western blotting. Primary antibody binding was detected with appropriate peroxidase-conjugated secondary antibodies (Biosource International, Camarillo, CA). Bound antibodies were visualized using a chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, United States) and the blots were exposed to Kodak X-OMAT film.

The signals in the Western blotting were quantified by densitometric scanning and normalized by using the intensity of corresponding protein band relative to the actin band.

Quantification of apoptosis

Quantitative detection of apoptotic cells was performed using two different methods: the nuclear binding dye DAPI and fluorescence microscopy, and the APO Per-

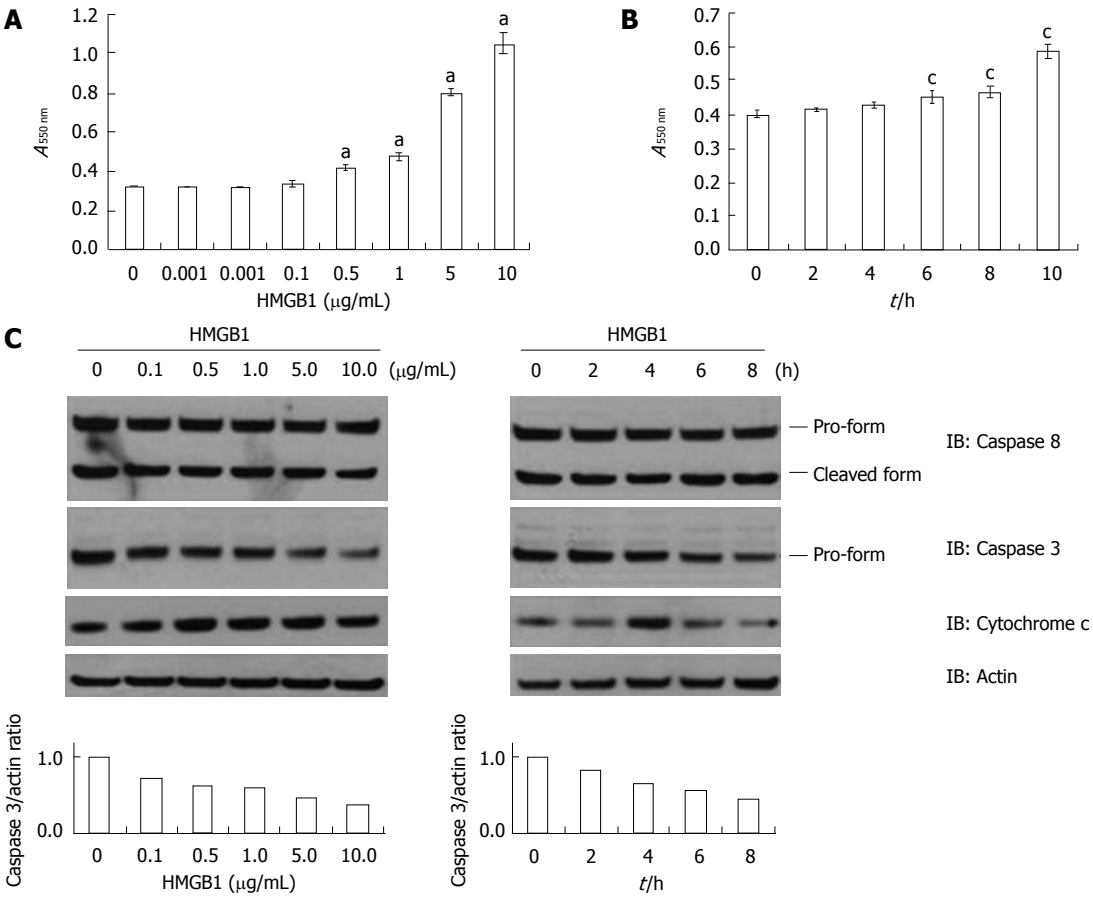

Figure 1 High-mobility group box 1 enhances hepatocyte apoptosis *via* a mitochondrial pathway. A: Huh-BAT cells were treated with high-mobility group box 1 (HMGB1) (0, 0.001, 0.01, 0.1, 0.5, 1, 5 and 10 μg/mL) for 6 h. Apoptosis was quantified using an APO Percentage apoptosis assay kit. Data are expressed as the mean ± SD of three individual experiments. $^{a}P < 0.05$, *vs* HMGB1 0 μg/mL; B: Huh-BAT cells were treated with 10 μg/mL of HMGB1 for the indicated time periods. $^{c}P < 0.05$, *vs* 0 h; C: Huh-BAT cells were treated with HMGB1 (0 μg/mL, 0.1 μg/mL, 0.5 μg/mL, 1, 5 μg/mL and 10 μg/mL) for 6 h (left column), or with 10 μg/mL of HMGB1 for the indicated time periods (right column). Cells were lysed at the indicated time points, and immunoblot analysis was performed using anti-caspase 8 and anti-caspase 3 antibodies. Mitochondrial and cytosolic extracts were also isolated, and equivalent amounts of cytosolic protein were immunoblotted with an anti-cytochrome c antibody. Immunoblot analysis using an anti-actin antibody was performed as a control for protein loading.

centage apoptosis assay kit (Biocolor Ltd., Belfast, Northern Ireland). For the APO Percentage apoptosis assay, the cells were seeded at 10^4 cells per well in a 96-well plate and processed according to the manufacturer's instructions.

Statistical analysis

All data were from at least three independent experiments using cells from a minimum of three separate isolations, and are expressed as the mean ± SD. Differences between the groups were compared using a two-tailed Student's *t* tests or the Mann-Whitney *U* test as appropriate. *P* values of < 0.05 were considered to be statistically significant.

RESULTS

HMGB1 significantly increased cellular apoptosis in Huh-BAT cells in a dose- and time-dependent manner (Figure 1A and B). We repeated the same experiments in the other two hepatoma cell lines (HepG2 and SNU-475 cells) and observed the same effects (data not shown). We next identified the pro-apoptotic signaling pathways induced by HMGB1 treatment. HMGB1 increased cytochrome c release from mitochondria into cytosol and induced the cleavage of procaspase 3. However, it did not affect the activation of caspase 8, an initiator caspase downstream of death receptor activation (Figure 1C).

Since the MAPK family signaling cascades regulate apoptotic pathways, we next evaluated whether HMGB1 modulates MAPK activation. HMGB1 induced the activation of MAPKs such as p42/44, p38 MAPK, and JNK in Huh-BAT cells (Figure 2A). In order to explore the role of individual MAPKs in apoptotic signaling, the cells were then treated with HMGB1 either in the presence or absence of various inhibitors: U0126 for p42/44, SB203580 for p38, and SP600125 for JNK. When the cells were treated with the p38 inhibitor, HMGB1-induced caspase 3 activation was significantly attenuated whereas treatment with inhibitors of p42/44 or JNK did not affect caspase 3 cleavage (Figure 2B).

Pretreatment with GL significantly attenuated HMGB1-induced hepatocyte apoptosis in a dose-dependent manner (Figure 3A). GL also attenuated cytochrome c release from the mitochondria into cytosol (Figure 3B). Finally, pretreatment with GL decreased HMGB1-induced p38

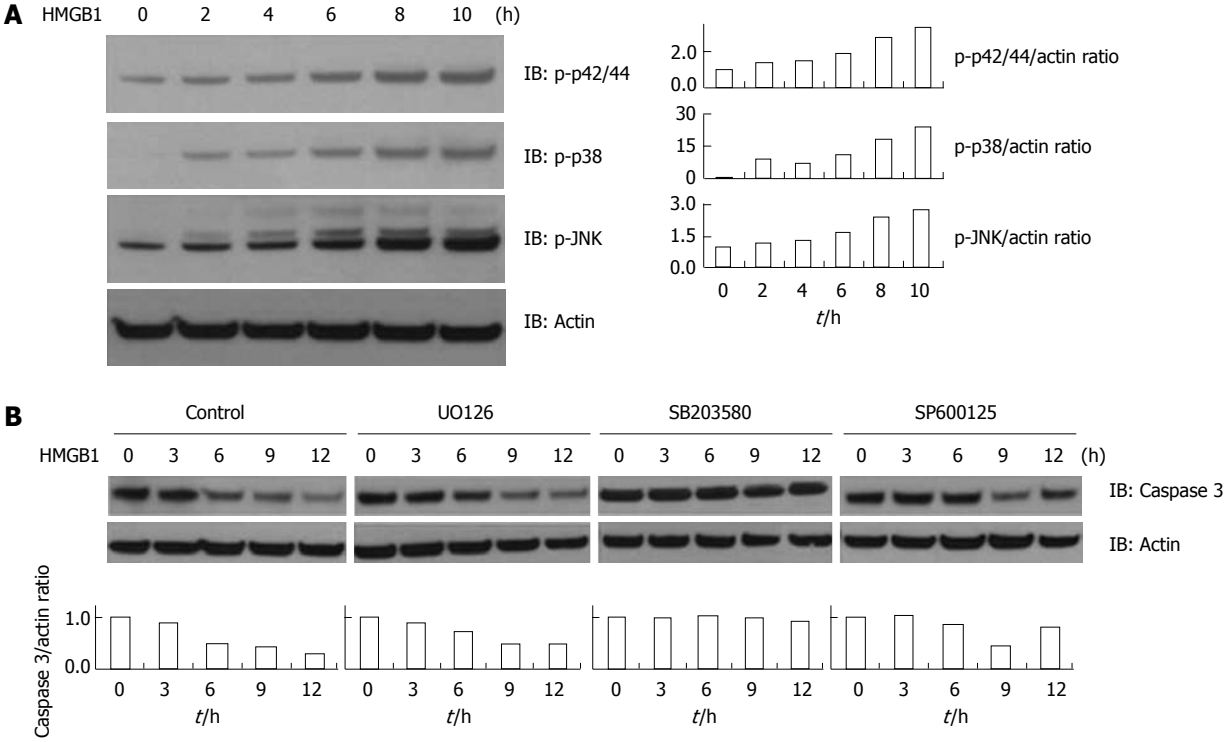

Figure 2 High-mobility group box 1-induced hepatocyte apoptosis is p38-dependent. A: Huh-BAT cells were treated with 10 μg/mL of high-mobility group box 1 (HMGB1) for the indicated time periods. Cells were lysed at the indicated time points, and immunoblot analysis was performed on cell lysates using antibodies specific for the phosphorylated forms of p42/p44, p38, or c-Jun N-terminal kinase (JNK); B: Huh-BAT cells were pretreated with mitogen activated protein kinase inhibitors U0126 (30 μmol/L), SB203580 (10 μmol/L), or SP600125 (20 μmol/L), or media (control) for 12 h. Cells were then treated with 10 μg/mL of HMGB1. Cells were lysed at the indicated time points, and immunoblot analysis was performed on cell lysates using anti-caspase 3 and anti-actin antibodies.

activation in Huh-BAT cells (Figure 3C). Taken together, all of the findings from our study indicate that HMGB1 induces hepatocyte apoptosis through a p38-dependent mitochondrial pathway which was inhibited by GL.

DISCUSSION

In virtually all human liver diseases, hepatocytes undergo cell death by apoptosis[13]. Thus, therapeutic modulation of apoptosis has the potential to alter the course of human liver disease. Apoptosis may occur *via* two fundamental pathways: (1) the death receptor or extrinsic pathway; and (2) the mitochondrial or intrinsic pathway. Caspases, representing the family of cysteine proteases, play a critical role in both pathways. Both pathways can either directly or indirectly converge to activate the "effector caspase", namely caspase-3, which induces DNA fragmentation and other morphological changes characteristic of apoptotic cell death[22]. In the present study, HMGB1 activated caspase 3 without affecting caspase 8, an initiator caspase downstream of death receptor activation. These findings suggest that the mitochondrial pathway is responsible for HMGB1-induced hepatocyte apoptosis, which was further supported by the findings that HMGB1 increased cytochrome c release from mitochondria into the cytosol.

MAPKs, which include p42/44, p38, and JNK, are involved in pro-apoptotic signal transduction as well as cell growth and differentiation[23]. It has been previously shown that the p38 MAPK cascade promotes either cell death or cell survival[23,24] depending on the cell type and the kinase isoforms activated by various stress stimuli[25]. There is abundant evidence that p38 participates in cellular apoptosis[26,27] with one mechanism being the modulation of Bcl-2 protein family members to maintain an apoptotic checkpoint for mitochondrial dysfunction and cytochrome c release[28,29]. Likewise, in the present study HMGB1-induced hepatocyte apoptosis occurred through a mitochondrial pathway which was p38-dependent.

GL, a triterpene glycoside extracted from licorice root (*Glycyrrhiza glabra*), consists of one molecule of 18b-glycyrrhetinic acid and two molecules of glucuronic acid having the structure 18-b-glycyrrhetinic acid-3-O-b-D-glucuronopyranosyl-(1/2)-b-D-glucuronide. It is known that this molecule has a variety of hepato-protective properties in terms of anti-inflammatory, antiviral, and anti-tumor effects[17]. In an animal model of acute liver injury induced by carbon tetrachloride, GL reduced the serum tumor necrosis factor-alpha (TNF-α) level and alleviates acute liver injury[30]. Moreover, GL has anti-inflammatory effects on lipopolysaccharide (LPS)-induced acute liver injury through inhibition of TNF-α release[31]. Recently, Ikeda *et al*[32] reported that GL reduced the number of TUNEL-positive cells in cases of acute hepatitis induced by LPS/D-galactosamine (GalN)-treatment. However, in a mouse model treated with LPS/D-GalN, anti-apoptotic effects of GL were found to be caspase-independent, and probably achieved

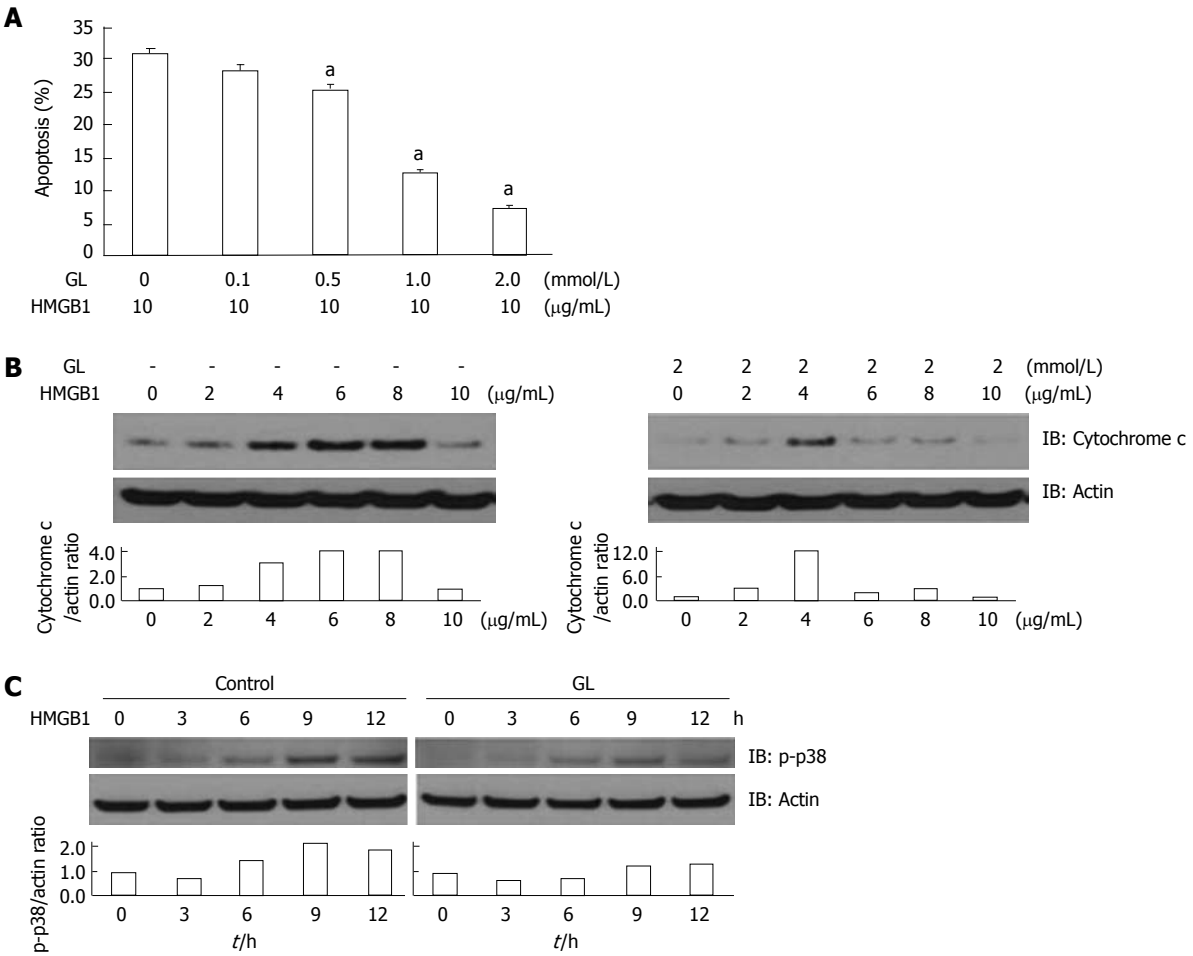

Figure 3 Glycyrrhizin attenuates high-mobility group box 1-induced hepatocyte apoptosis. A: Huh-BAT cells were pretreated with glycyrrhizin (GL) (0, 0.1, 0.5, 1.0 and 2.0 mmol/L) for 12 h. Cells were then treated with 10 μg/mL of high-mobility group box 1 (HMGB1) for 6 h. Apoptosis was quantified by 4',6-diamidino-2-phenylindole staining and fluorescence microscopy. Data are expressed as the mean ± SD of three individual experiments. $^{a}P < 0.05$, *vs* GL 0 mmo/L; B: Huh-BAT cells were pretreated with GL (2.0 mmol/L) for 12 h prior to adding HMGB1 (0-10 μg/mL). Mitochondrial and cytosolic extracts were isolated after 6 h of HMGB1 treatment, and equivalent amounts of cytosolic protein were immunoblotted with an anti-cytochrome c antibody. Immunoblot analysis using an anti-actin antibody was performed as a control for protein loading; C: Huh-BAT cells were pretreated with GL (2.0 mmol/L) for 12 h prior to being incubated with HMGB1. Cells were then lysed at the indicated time points, and immunoblot analysis was performed using antibodies specific for the phosphorylated forms of p38 and actin.

through the prevention of an interleukin-18-mediated inflammatory response. In the present study, we demonstrated that GL attenuated HMGB1-induced hepatocyte apoptosis by blocking the p38-dependent mitochondrial pathway. Therefore, it is likely that the hepato-protective effects of GL are attributed to various mechanisms.

In summary, the present study demonstrated that HMGB1 participated in hepatocyte apoptosis through a p38-dependent mitochondrial pathway. In addition, GL had an anti-apoptotic effect on hepatocytes treated with HMGB1. Therefore, HMGB1 inhibitors, including GL, might be therapeutically efficacious in treating HMGB1-mediated liver injury such as viral hepatitis, hepatic ischemia-reperfusion injury and sepsis-related liver injury.

COMMENTS

Background

High mobility group box 1 (HMGB1) is an evolutionarily conserved protein present in the nucleus of almost all eukaryotic cells, where it is involved in DNA replication, repair and transcription. Glycyrrhizin (GL) is a major active constituent of licorice root that is commonly used in Asia to treat patients with chronic hepatitis. This compound has been associated with numerous pharmacologic effects, including anti-inflammatory, anti-viral, anti-tumor, and hepatoprotective activities. Recently, GL was recognized as an HMGB1 inhibitor, which binds directly to both HMG boxes in HMGB1.

Research frontiers

The authors provide *in vitro* evidence and a potential theoretical basis for HMGB1 regulation of hepatocyte apoptosis in order to further elucidate the molecular mechanism of HMGB1 involvement in various pathologic conditions that can affect the liver. Furthermore, they attempted to determine whether GL attenuates HMGB1-induced hepatocyte apoptosis and, if so, to identify the signaling cascades responsible for this modulation.

Innovations and breakthroughs

Present study demonstrated that HMGB1 participated in hepatocyte apoptosis through a p38-dependent mitochondrial pathway. In addition, GL had an anti-apoptotic effect on hepatocytes treated with HMGB1.

Applications

HMGB1 inhibitors, including GL, might be therapeutically efficacious in treating HMGB1-mediated liver injury such as viral hepatitis, hepatic ischemia-reperfusion injury and sepsis-related liver injury.

Peer review

This is an interesting study where the authors show that HMGB1 induces hepatocyte apoptosis which is mediated by p38. In addition, glycyrrhizin was shown to inhibit HMGB1-induced apoptosis as well as activation of p38 in the

cultured hepatocyte cell line. The study is well conducted and the manuscript is well written.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.685

World J Gastroenterol 2012 February 21; 18(7): 685-691
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Gastric mucosa-associated lymphoid tissue lymphomas and *Helicobacter pylori* infection: A Colombian perspective

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Received: May 6, 2011 Revised: August 18, 2011
Accepted: August 27, 2011
Published online: February 21, 2012

Abstract

AIM: To assess the significance of chromosome translocation t(11;18)(q21;q21), B-cell lymphoma 10 (BCL-10) protein and *Helicobacter pylori* (*H. pylori*) infection in gastric mucosa-associated lymphoid tissue (MALT) lymphoma in Colombia.

METHODS: Fifty cases of gastric MALT lymphoma and their respective post-treatment follow-up biopsies were examined to assess the presence of the translocation t(11;18)(q21;q21) as identified by fluorescence *in situ* hybridization; to detect protein expression patterns of BCL10 using immunohistochemistry; and for evaluation of tumor histology to determine the correlation of these factors and resistance to *H. pylori* eradication.

RESULTS: Infection with *H. pylori* was confirmed in all cases of gastric MALT lymphoma in association with chronic gastritis. Bacterial eradication led to tumor regression in 66% of cases. The translocation t(11;18)(q21;q21) was not present in any of these cases, nor was there evidence of tumor transformation to diffuse large B-cell lymphoma. Thirty-four percent of the patients showed resistance to tumor regression, and within this group, 7 cases, representing 14% of all those analyzed, were considered to be t(11;18)(q21;q21)-positive gastric MALT lymphomas. Protein expression of BCL10 in the nucleus was associated with the presence of translocation and treatment resistance. Cases that were considered unresponsive to therapy were histologically characterized by the presence of homogeneous tumor cells and a lack of plasmacytic differentiation. Responder cases exhibited higher cellular heterogeneity and a greater frequency of plasma cells.

CONCLUSION: Both t(11;18)(q21;q21)-positive MALT lymphoma cases and those with nuclear BCL10 expression are considered resistant to *H. pylori* eradication. It is suggested that chronic antigenic stimulation is not a dominant event in resistant cases.

Key words: Mucosa-associated lymphoid tissue lymphoma; *Helicobacter pylori*; Treatment; t(11;18)(q21;q21); B-cell lymphoma 10

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Yepes S, Torres MM, Saavedra C, Andrade R. Gastric mucosa-associated lymphoid tissue lymphomas and *Helicobacter pylori*

infection: A Colombian perspective. *World J Gastroenterol* 2012; 18(7): 685-691 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/685.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.685

INTRODUCTION

Gastric B-cell lymphoma (BCL) of mucosa-associated lymphoid tissue (MALT) is an indolent B-cell non-Hodgkin lymphoma that arises from the mucosal lymphoid tissue and is normally acquired as a reaction to *Helicobacter pylori* (*H. pylori*) infection[1-3]. This disease is considered one of the best models reflecting how genetic events lead to oncogenesis, determine tumor biology, dictate clinical behavior and represent viable therapeutic targets.

Gastric MALT lymphoma pathogenesis is a multistep process initiated by infection with *H. pylori*, which induces genetic abnormalities and subsequent malignant transformation. Gene alterations include trisomy of chromosomes 3, 7, 12 or 18 and the disease-specific chromosome translocations t(1;14)(p22;q32), t(14;18)(q32;q21), t(11;18)(q21;q21) and t(3;14)(p13;q32) resulting in *IGH-BCL10*, *IGH-MALT1*, *API2-MALT1* and *IGH-FOXP1* rearrangements respectively. Notably, these events are associated with the activation of nuclear factor-κB (NF-κB)[4,5].

Various studies of gastric MALT lymphoma have shown that t(11;18)(q21;q21) is present in approximately 30% of cases. The clinical importance of the translocation is well-characterized: positive cases are usually unresponsive to *H. pylori* eradication, which is the generally accepted first-choice therapy[6,7]. The translocation is often present in advanced cases of the disease but rarely undergoes high-grade transformation to diffuse large B-cell lymphoma (DLBCL)[8-10].

However, complete tumor regression is known to occur in 20% of patients with t(11;18)(q21;q21)-positive disease. Therefore, all patients who have *H. pylori*-positive gastric MALT lymphoma should undergo eradication therapy, regardless of their t(11;18)(q21;q21) status[11]. On the other hand, new evidence has shown that translocations can be found at approximately equivalent frequencies in both gastric MALT lymphomas and gastric DLBCLs[12].

Although most patients with gastric MALT lymphoma experience an indolent clinical course[13], there is considerable individual variability in the extent of the disease response to treatment, relapse and event-free survival. Consequently, optimal management is changing, and there is a need for an improved understanding of the genetic abnormalities underlying gastric MALT lymphoma and for new genetic biomarkers that are able to guide prognosis and management.

The incidence of chromosomal translocations in gastric MALT lymphoma has been described mainly from patients in Western countries[14-17]. The prevalence of t(11;18)(q21;q21)-positive MALT lymphoma has been reported to be within a range of 15%-24% in Europe[14,15,18] but is considered relatively rare in the United States (5%)[17].

The incidence of MALT lymphoma and its genetic background has not been established and validated in Latin America; likewise, the ideal prognosis and management of the Colombian patient population is unclear given the complex genetic background of the disease.

In Colombia, the identification of MALT lymphoma resistant to *H. pylori* eradication therapy is not typically considered in initial diagnoses and patient classification is possible only after repeated follow-up assessments involving endoscopy and histology. This situation warrants validation and a search for biomarkers of treatment response to direct the treatment regimen.

In the present study, we attempted to evaluate the t(11;18)(q21;q21) status and BCL10 staining patterns in patients with gastric MALT lymphomas and the relationship among these factors with the response to the eradication of *H. pylori* in a sample of patients from Colombia.

MATERIALS AND METHODS

Cases and diagnostic criteria

Fifty cases of gastric MALT lymphomas were retrieved from the archives of the Department of Pathology, Fundación Santa Fe de Bogotá. All of these cases had hematoxylin and eosin-stained sections and immunohistochemistry (IHC)-marked slides available for reevaluation. Cases selected must have had previous histopathological diagnoses, available archival material and complete information about diagnosis and disease monitoring.

Diagnosis of MALT lymphoma was made on the basis of morphological and immunophenotypic examination according to the criteria described by Isaacson *et al*[19] and subsequently included in the Revised European American Lymphoma/World Health Organization (WHO) classification. Other factors evaluated included the accompanying epithelium (assessment of gastritis, atrophy and metaplasia) and a characterization of the tumoral infiltrate (large cells, centrocyte-like/small lymphocytes, monocytoid cells, plasma cell differentiation, residual follicles, colonization follicles and Dutcher's bodies). Detection of *H. pylori* was performed by histology and/or histochemistry using a Genta stain on all follow-up biopsies.

Assessment of the response to H. pylori eradication treatment

All 50 cases analyzed were treated with triple antibiotic therapy as the first-choice measure. This consisted of a proton pump inhibitor (such as omeprazole, lansoprazole or pantoprazole) plus two antibiotics (amoxicillin and clarithromycin) administered according to the manufacturer's recommended dose for 14 d. To confirm *H. pylori* eradication, a first endoscopic mucosal biopsy was conducted 2-3 mo after the cessation of antibiotic treatment, followed by endoscopy every 6 mo for at least 2 years at the discretion of the treating physician.

Surveillance of patients was performed by serial gastric biopsies to allow the histological evaluation of the tumor's response to treatment. Histological responses were graded using the Wotherspoon histological score. This system assigns a score and diagnosis based on histological characteristics of the tissue: a score of zero represents a normal diagnosis with scattered plasma cells in the lamina propria; score of one represents chronic active gastritis with small clusters of lymphocytes in the lamina propria, a lack of lymphoid follicles and lymphoepithelial lesions; a score of two represents chronic active gastritis with florid lymphoid follicle formation, prominent lymphoid follicles with surrounding mantle zone and plasma cells, and the absence of lymphoepithelial lesions; a score of three represents a likely reactive suspicious lymphoid infiltrate with lymphoid follicles surrounded by small lymphocytes that infiltrate diffusely into the lamina propria and occasionally into the epithelium; a score of four represents a suspicious lymphoid infiltrate, likely lymphoma, and lymphoid follicles surrounded by marginal zone cells that infiltrate diffusely into the lamina propria and the epithelium in small groups; a score of five represents MALT lymphoma with the presence of dense diffuse infiltrates of marginal zone cells into the lamina propria with prominent lymphoepithelial lesions[20].

Lymphoma remission was investigated by regular endoscopic examinations, including multiple biopsies, conducted at 6-mo intervals. Histological responses were graded using the Wotherspoon histological score, considering scores 0-2 to be a complete lymphoma regression (CR), score 3 to be a partial remission and scores 4-5 to be no response (no change). A persistence score of 5, mainly characterized by the presence of lymphoepithelial lesions after antibiotic treatment, was the criterion for tumor resistance to therapy[20].

Fluorescence in situ hybridization

The authors performed manual microdissection of tumor tissue from each of the biopsies with initial diagnoses of gastric MALT lymphoma and subsequently organized the tissues for inclusion in a new block, generating tissue microarrays to perform fluorescent in-situ hybridization (FISH) and IHC for BCL10 expression. The FISH for t(11;18)(q21;q21) was performed using standardized protocols appropriate for paraffin-embedded tissue and the commercial Dual Color probe LSI API2-MALT1 (Vysis, Downers Grove, IL, United States).

The cut-off point for translocation-positive status was determined through the analysis of 6 control samples of hyperplastic tonsils fixed in neutral formalin and embedded in paraffin, and was defined as the arithmetic mean count of 100 nuclei plus three standard deviations obtained for each sample. Assessments were performed using an Olympus BX51 fluorescence microscope with a 100-watt bulb for each 100 cells examined.

Immunohistochemistry

Immunohistochemical analysis using standardized procedures for CD20, CD3, CD43, BCL2, cytokeratin and kappa and lambda light chain immunoglobulin antibodies was performed to confirm the MALT lymphoma diagnosis and in follow-up biopsies when necessary. Expression of BCL10 was also determined using the immunoperoxidase technique in paraffin-embedded sections with a monoclonal mouse anti-human BCL10 antibody (DakoCytomation, Glosturp, Denmark). Briefly, 4-μm-thick paraffin sections were placed on silanized slides, dewaxed in xylene and hydrated through graded solutions of alcohol. Slides were then immersed in 0.01 mol/L citrate buffer (pH 6.0) and epitope retrieval was performed using a pressure cooker. The sections were then incubated with the diluted (1:40) primary antibodies and staining was performed using the EnVision system (DakoCytomation) according to the manufacturer's recommendations. Hyperplastic tonsils that were positive for cytoplasmic expression of the protein were used in each experiment as a control. Samples were considered positive for nuclear expression of BCL10 when detected in more than 5% of the nuclei of tumor cells.

Table 1 Definition of groups with respect to the presence of the *API2-MALT1* rearrangement and response to treatment (n = 50)

Gastric malt lymphoma	Treatment response	*API2-MALT1* positive	*API2-MALT1* negative
A group	Responders	0	33
B group	Non-responders	7	
C group	Non-responders		10

MALT: Mucosa-associated lymphoid tissue.

RESULTS

Characteristics of gastric MALT lymphoma in terms of response to the eradication of H. pylori, t(11;18)(q21;q21) status and BCL10 expression

H. pylori infection was confirmed in all cases of gastric MALT lymphoma in association with chronic gastritis. Ninety percent of cases showed the presence of *H. pylori* in the histological analysis of early gastric biopsies; in the remaining 10%, the presence of bacteria was documented in subsequent biopsies before the initiation of the treatment and infection was scored as mild in most cases.

The patients were classified as responders or non-responders to treatment (Table 1). All patients classified as responders showed lymphoma regression, and the median time for follow-up after remission was 29 mo (range: 6-39 mo). There were 33 (66%) patients considered as responders to treatment and 17 (34%) patients considered non-responders who did not reach remission after *H. pylori* eradication. The median time to CR after the completion of antibiotic therapy was 3.2 mo (range: 1-18 mo). The bacteria were successfully eradicated in all patients treated with the standard triple therapy applied, including all non-responders.

Non-responder cases were defined as tissues with

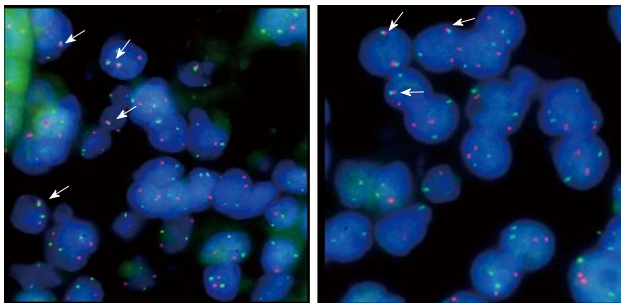

Figure 1 Fluorescence *in situ* hybridization to detect the translocation (11;18)(q21;121) using the probe LSI API2-MALT1 Dual Color (Vysis, Downers Grove, IL, United States). Arrows show translocation-positive mucosa-associated lymphoid tissue lymphomas.

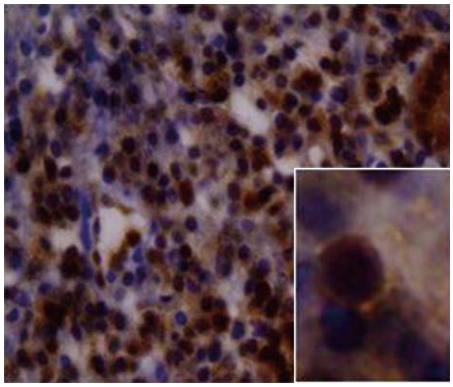

Figure 2 Aberrant expression of B-cell lymphoma 10 protein in gastric mucosa-associated lymphoid tissue lymphoma. Original magnification, × 400; inset in panel, × 1000.

histological persistence of gastric MALT lymphoma infiltrate despite successful *H. pylori* eradication as determined by histological and immunohistochemical examination. Within this group, seven cases, representing 14% of all cases analyzed, were considered positive for translocation. Cases were considered positive when more than 6% of tumor cells demonstrated the presence of fusion protein for a minimum count of 100 cells, which was the parameter set as the cut-off point for translocation-positive status for the technique. There was a statistically significant association between treatment response and the presence of the t(11;18)(q21;q21) translocation (Yales $\chi^2 = 12.57$, $P < 0.05$) because there were 7 patients within the group of non-responders who displayed the chromosomal arrangement of interest. Figure 1 shows the fluorescence patterns in positive and negative cases in the translocation study. When the response to treatment and the BCL10 staining pattern were analyzed, it was observed that all non-responders had nuclear expression of the BCL10 protein in a moderately nuclear pattern, as is shown in Figure 2. In addition, these cases showed evidence of translocation t(11;18)(q21;q21). However, in the responder group, BCL10 expression was confined to the cytoplasm. This finding confirms results reported elsewhere. Based on these results, the authors divided the cases into three groups (Table 1). Group A: patients who responded to the eradication and were negative for the studied translocation (66%); group B: patients who did not respond to eradication and were negative for t(11;18)(q21;q21) (20%); and group C: cases that did not respond to eradication of *H. pylori* and were positive for t(11;18)(q21;q21) (14%).

Table 2 Histological and immunohistochemical characteristics of 50 cases of gastric mucosa-associated lymphoid tissue lymphoma *n* (%)

Features	Group			*P* value		
	A	B	C	A *vs* B	A *vs* C	B *vs* C
n (%)	33 (66)	10 (20)	7 (14)			
Large cells						
Positive	8 (23.2)	0	0	> 0.05	> 0.05	NA
Negative	25 (75.8)	10 (100)	7 (100)			
Centrocyte-like/small lymphocytes						
Positive	16 (48.5)	6 (60)	7 (100)	> 0.05	< 0.05[a]	> 0.05
Negative	17 (51.5)	4 (40)	0			
Monocytoid cells						
Positive	8 (24.2)	3 (30)	0	> 0.05	> 0.05	> 0.05
Negative	25 (75.8)	7 (70)	7 (100)			
Plasma cell differentiation						
Positive	18 (54.5)	1(10)	1 (14.3)	< 0.05[a]	> 0.05	> 0.05
Negative	15 (45.5)	9 (90)	6 (85.7)			
Residual follicles						
Positive	5 (15.2)	2 (20)	0	> 0.05	> 0.05	> 0.05
Negative	28 (84.8)	8 (80)	7 (100)			
Colonization follicles						
Positive	6 (18.2)	3 (30)	1 (14.3)	> 0.05	> 0.05	> 0.05
Negative	27 (81.8)	7 (70)	6 (85.7)			
Dutcher bodies						
Positive	6 (18.2)	0	0	> 0.05	> 0.05	NA
Negative	27 (81.8)	10 (100)	7 (100)			
B-cell lymphoma 10						
Positive	0	10 (100)	7 (100)	< 0.05[a]	< 0.05[a]	NA
Negative	32 (100)	0	0			

[a]$P < 0.05$ is significant. NA: Not applicable.

The results of the comparisons between groups A and B (responders *vs* non-responders with fusion), show statistically significant differences in plasma cell differentiation variables with a higher percentage in group A. These groups also differed in the nuclear expression of BCL10 protein, which was observed more frequently in group B. The other variables showed no significant associations. Comparisons between groups A and C (responders *vs* non-responders without fusion) showed statistically significant differences in the presence of centrocytes/small lymphocytes, which appeared at a higher rate in group C, and nuclear expression of BCL10, which also occurred at a higher percentage in group C. Other variables did not show any significant associations. Comparisons between groups B and C did not show any significant associations with the variables assessed (Table 2). Characteristic examples of the morphology described above are presented in Figure 3.

DISCUSSION

Known predictive factors for assessing the outcome of MALT lymphoma include the evaluation of trans-

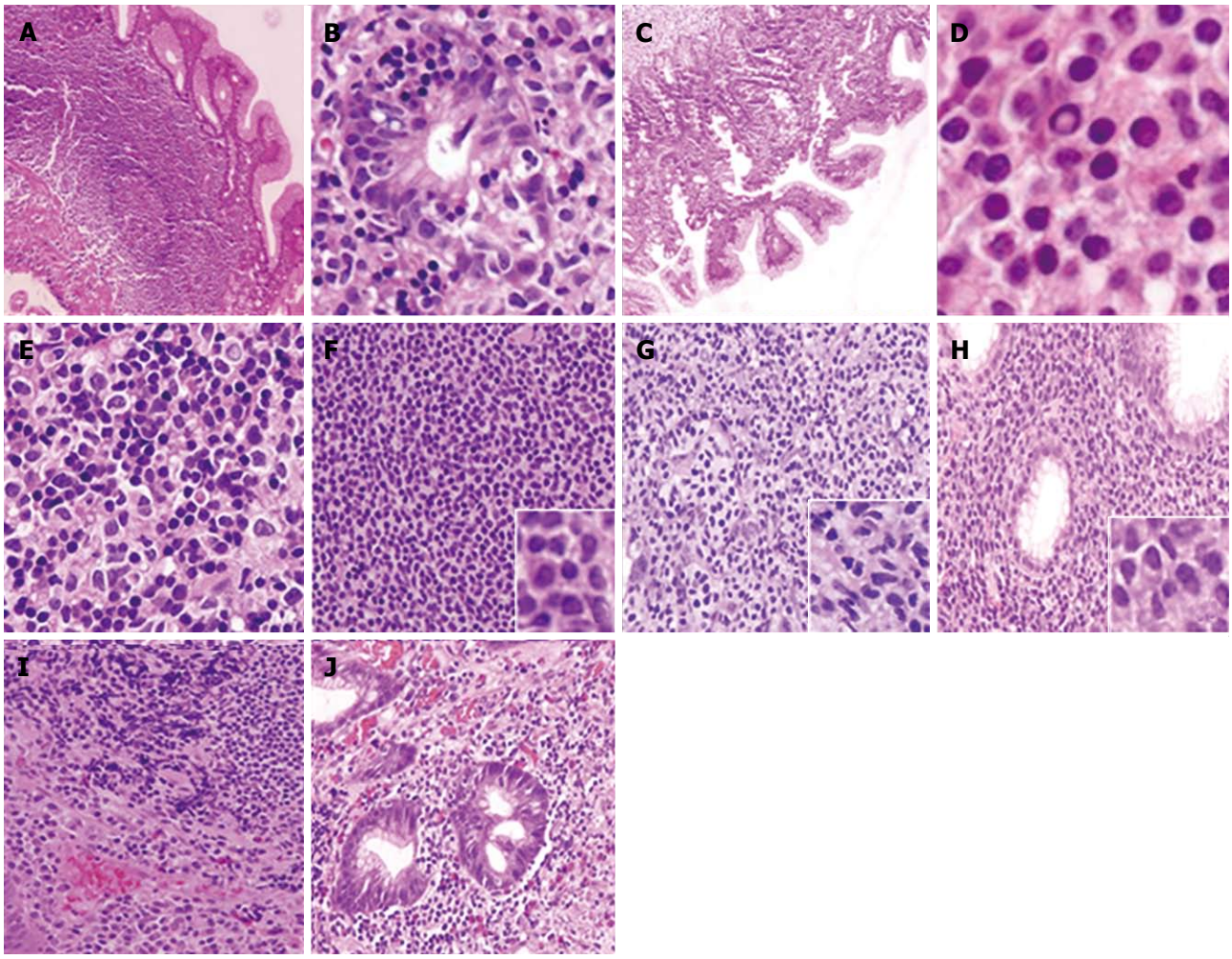

Figure 3 Stain showing tumor morphology (hematoxylin and eosin). A: Gastric mucosa-associated lymphoid tissue (MALT) lymphoma overview; B: Lymphoepithelial lesion; C: Accompanying intestinal metaplasia in the epithelial tissue surrounding the lymphoma; D: Dutcher's bodies; E: MALT lymphoma with clusters of large cells; F: Tumor cells of the centrocyte-like/small lymphocyte type; G: Monocytoid tumor cell type; H: Plasma cell differentiation; I: Mixed phenotype composed of centrocytes and plasma cell type; J: Presence of eosinophils. Original magnifications of panels A-J and inset in panel F-H are × 400 and × 1000, respectively.

formation to a high-grade B-cell lymphoma, clinical stage of the disease and molecular markers of response to treatment. It is now known that the translocation t(11;18)(q21;q21) occurs frequently in gastric MALT lymphomas in advanced stages of the disease showing resistance to treatment. However, new evidence has recently demonstrated that tumor regression might also be achieved in patients with t(11;18)(q21;q21) after *H. pylori* eradication[11]. In addition, it has been recognized that the presence of t(11;18)(q21;q21) in gastric MALT lymphomas does not exclude progression to DLBCL[4,12]. Further research is necessary to understand the molecular mechanisms involved in these latter situations.

Complete clinical staging for this study could not be obtained for all patients because cases studied were referred for pathological evaluation from different institutions. Also, this work was focused on the histological characteristics, response to bacterial eradication treatment and presence of molecular markers such as t(11;18)(q21;q21) and BCL10 protein expression. Outside of these factors, there are other variables that may also help predict the course of disease.

These results have demonstrated that BCL10 nuclear expression and the determination of the t(11;18)(q21;q21) translocation involved in neoplastic signaling processes mediated by NF-κB are two useful markers for predicting the independent status of *H. pylori* in gastric MALT lymphomas; its presence, therefore, allows for the identification of patients who will not respond to bacterial eradication therapy as a means of promoting tumor regression. The resistance of the tumor to therapy observed in translocation-positive cases is explained by the fact that the fusion protein generates a bypass of the NF-κB signaling pathway thereby allowing constitutive activation of the transcription factor, self-generating growth and independent antigenic stimulation. On the other hand, the association of BCL10 with tumor resistance can be explained by the presence of nuclear export signals of the gene *MALT1* that allows the export of BCL10 from the nucleus to the cytoplasm. Nakagawa *et al*[21] argued that IAP2 usually mediates BCL10 degradation through its ubiquitin ligase activity and inhibits antigen-receptor-

mediated signaling. This normal function is absent in the fusion protein, resulting in the stability of BCL10 in lymphomas with t(11;18)(q21;q21) and the activation of the NF-κB signal transduction pathway. Consequently, the translocation generates a gain-of-function activity in MALT1 and a loss of API2 function.

Thirty-four percent of patients treated with antibiotic therapy in the sample group showed resistance to tumor regression; within this group, there were cases in which the fusion signal was detected by FISH, a finding that may explain the mechanism of autonomous growth and independence of antigenic stimulation as previously discussed. This study therefore confirms that patients with extranodal marginal zone lymphoma carrying t(11;18)(q21;q21) do not respond to treatment for *H. pylori* as a therapeutic measure and should be treated by other methods, such as radiotherapy and chemotherapy. These results also endorse the recommendation that every patient with a diagnosis of gastric MALT lymphoma should be evaluated for the presence of the aforementioned translocation by molecular methods.

Treatment resistance in cases that do not have the t(11;18)(q21;q21) translocation could possibly be due to the carrying of other molecular aberrations, such as t(1;14)(p22;q32). However, such cases would have shown evidence of a strong nuclear BCL10 staining pattern and/or genetic evidence of the translocation.

Patients in whom the aforementioned translocations are not detected frequently have trisomy of chromosomes 3, 7, 12 or 18; these cases are still considered dependent on antigenic stimulation. Additionally, it is known that the trisomy of these chromosomes is seen in cases where transformation to high-grade tumors is present after the inactivation of the *P53* gene[21]. t(11;18)(q21;q21) and different aneuploidies, such as trisomy of chromosome 3, 7, 12 or 18, are usually considered mutually exclusive events, suggesting that at least two different routes lead to the development of the lymphoma. In patients with aneuploidy events, the oncogenesis mechanism is unclear; it is proposed that cell growth may be induced as a result of high copy numbers of genes involved in proliferation. Several genes located on chromosome 3 have been suggested as possibly responsible for these changes, including *BCL6* and *FOXP1*[21].

The authors found that cases classified as non-responders to therapy and positive for translocation are histologically characterized by the presence of more homogeneous tumor cells. In most cases, these cells were predominantly centrocyte-like/small lymphocytes, and this cell homogeneity could possibly result from a process of clonal selection. Additionally, in cases not responsive to therapy, fewer tumor cells appeared to differentiate into plasma cells, suggesting a decrease in the immune response by memory cells of the marginal zone. This is unlike the prominent cellular heterogeneity seen in responder patients, although this result has not been extensively studied and would require larger sample sizes to avoid possible statistical bias.

We applied Wotherspoon's criteria for histological evaluation of response to treatment given the current widespread use of this system adopted by the WHO in 2001 for the histological differential diagnosis of gastric MALT lymphoma and extensive use by pathologists worldwide for the evaluation of therapeutic response. However, there are other useful systems proposed for the evaluation of therapeutic response to treatment, such as the Groupe d'Etude des Lymphomes de l'Adult (GELA) system that can be used for the same objective[22]. There are no comparative studies that convincingly prove the superiority of any system, even though the GELA system has the advantage of assessment of stromal changes in the lamina propria[23]. The GELA system may become a useful tool if its reproducibility can be confirmed by further testing in a large series[24]. Therefore, the utilization of the WHO/Wotherspoon score accompanied by the assessment of stromal changes for the post-treatment evaluation in MALT lymphoma is an interesting topic for future studies.

ACKNOWLEDGMENTS

The authors thank Dr. Margarita Baldión for choosing tumor material, Dr. Adriana Plata for her assistance in the FISH procedure and Yubeli Abello for her technical collaboration.

COMMENTS

Background

Gastric mucosa-associated lymphoid tissue (MALT) lymphoma is caused by infection with *Helicobacter pylori* (*H. pylori*), which induces genetic abnormalities and malignant transformations. *H. pylori* eradication therapy leads to complete lymphoma regression in some patients in the early stages of the disease.

Research frontiers

There is considerable individual variability in the extent of the disease, response to treatment, relapse and event-free survival. Consequently, optimal management should be established and there is a need for improved understanding of the genetic abnormalities underlying gastric MALT lymphoma.

Innovations and breakthroughs

This is the first study in Colombia concerning the assessment and significance of the gastric MALT lymphoma-associated t(11;18)(q21;q21) chromosomal translocation and its relationship with *H. pylori* infection in a country with a high incidence of cancer associated with virulent *H. pylori* strains, a significant cause of mortality and a major public health problem.

Applications

This study confirms, in a previously unexplored population, the importance of molecular markers in the selection of different treatment regimens for individual patients to increase the probability of survival.

Terminology

Gastric MALT lymphoma is a low-grade malignant lymphoma of the stomach originating from B cells and is associated with chronic infection by *H. pylori*, a gram-negative bacterium strongly linked to the development of inflammation and stomach cancer. The disease carries chromosomal aberrations such as t(11;18)(q21;q21), useful for the evaluation of therapeutic response.

Peer review

It was shown that the presence of t(11;18)(q21;q21) and nuclear expression of BCL10 are associated with the treatment resistance. These findings have been documented in patients of other ethnic origins and are thus confirmatory; the results are of relevance to disease management in patients.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.692

World J Gastroenterol 2012 February 21; 18(7): 692-697
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Clinical impact of multidetector computed tomography before double-balloon enteroscopy for obscure gastrointestinal bleeding

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Received: January 30, 2011 Revised: April 26, 2011
Accepted: May 2, 2011
Published online: February 21, 2012

Abstract

AIM: To evaluate the clinical impact of multidetector computed tomography (MDCT) before double-balloon endoscopy (DBE) for patients with obscure gastrointestinal bleeding (OGIB).

METHODS: A retrospective analysis of prospectively collected cases with DBE and MDCT for overt OGIB was conducted from April 2004 to April 2010 at Changhua Christian Hospital. We evaluated the clinical impact of MDCT on the subsequent DBE examinations and the diagnostic yields of both MDCT and DBE respectively.

RESULTS: From April 2004 to April 2010, a total of 75 patients underwent DBE for overt OGIB. Thirty one cases received MDCT followed by DBE for OGIB. The overall diagnostic yields of DBE and MDCT was 93.5% and 45.2%. The MDCT had a high diagnostic yield of tumor *vs* non-tumor etiology of OGIB (85.7% *vs* 33.3%, P = 0.014). Additionally, the choice of initial route of DBE was correct in those with a positive MDCT *vs* negative MDCT (100% *vs* 52.9%, P = 0.003).

CONCLUSION: This study suggests MDCT as a triage tool may identify patients who will benefit from DBE and aid the endoscopist in choosing the most efficient route.

Key words: Multidetector computed tomography; Capsule endoscopy; Double-balloon endoscopy; Obscure gastrointestinal bleeding

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Yen HH, Chen YY, Yang CW, Liu CK, Soon MS. Clinical impact of multidetector computed tomography before double-balloon enteroscopy for obscure gastrointestinal bleeding. *World J Gastroenterol* 2012; 18(7): 692-697 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/692.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.692

INTRODUCTION

Obscure gastrointestinal bleeding (OGIB) is defined by the American Gastroenterological Association (AGA)[1] as bleeding of unknown cause after upper or lower endoscopy. OGIB constitutes approximately 5% of patients evaluated for gastrointestinal hemorrhage[2]. OGIB can be further classified into two clinical forms: (1) obscure-occult bleeding; and (2) obscure-overt type bleeding (those with hematochezia or melena). The management of overt OGIB is clinically challenging for the gastroenterologist. With the introduction of high yield diagnostic tools such

as capsule endoscopy (CE) and double-balloon endoscopy (DBE) since the year 2000, the AGA recommended the use of CE followed by DBE as the first line diagnostic tool in 2007[2]. CE has exhibited some limitations, such as capsule retention, incomplete examination, inability to provide therapy, and high cost[3]. Compared with CE, DBE offered a therapeutic ability which is more useful in the setting of overt OGIB and is cost-effective compared with CE[4]. Multidetector computed tomography (MDCT) is a non-invasive tool and some recent reports have used it to investigate OGIB[3,5-7]. The clinical impact of MDCT prior to DBE for OGIB has not been evaluated. Thus, we performed this study to evaluate, retrospectively, the role of MDCT and DBE in patients with OGIB.

MATERIALS AND METHODS

Patients

A retrospective review of the medical records of Changhua Christian hospital from April 2004 to April 2010 identified 75 patients who underwent DBE for OGIB. A retrospective analysis of this prospectively collected database of DBE identified 31 patients who received MDCT within one month prior to DBE for obscure overt gastrointestinal (GI) bleeding and were included for this analysis. OGIB was defined as gastrointestinal bleeding after a non-diagnostic upper and lower endoscopy. All patients provided written consent to undergo MDCT and endoscopy, including endoscopic treatments, such as hemoclip and argon plasma coagulation. All patients were informed that endoscopic examination and treatment are involved in the current standard therapeutic approach used in the evaluation of OGIB. The primary end point of the study was to evaluate the clinical impact of MDCT on the subsequent DBE examinations. The secondary end point of the study was the diagnostic yield for MDCT and DBE for the diagnosis of OGIB.

MDCT procedure

All the patients received MDCT with non-enhanced and triphasic helical computed tomography (CT) scanning[8]. No oral contrast material was given before the examination. First, patients were imaged with a MDCT scanner (LightSpeed Ultra 16, GE Medical Systems, Milwaukee, WI) in a craniocaudal direction beginning at the dome of the liver. A nonionic contrast medium (Optiray 350, Tyco Healthcare, Mansfield, MA) was then administered at a total dose of 100 to 120 mL with an injection rate of 3 mL/s through an antecubital vein. For triphasic acquisitions, scanning was started with a 10 s scan delay for the hepatic arterial phase after the attenuation value of the aorta reached 120 HU. Fifteen seconds after the end point of the hepatic arterial phase, the scans for the portal venous phase were acquired. Delayed-phase images were acquired 80 s after the end of the acquisition of the portal venous phase. Whole scanning was completed in 4 to 8 s with the patients holding their breath.

The MDCT was reviewed by one radiologist (Dr. Liu) with 10 years of experience with abdominal imaging. The finding of the presence of small intestinal neoplasm (Figure 1), active contrast extravasation (Figure 2) or hyperdense fluid accumulation was considered to be diagnostic of GI bleeding[9].

DBE procedure

The double-balloon method was developed as described by Yamamoto *et al*[10]. Briefly, the double-balloon endoscope (EN-450P5 or EN-450T5, Fujinon Co., Japan) had two balloons, one attached to the distal end of the scope and the other attached to a transparent overtube (length 140 cm). Through a technique of inflating and deflating the balloons, the 7 m long small intestine can be shrunk to less than 2 m. Therefore, the entire small intestine could be theoretically examined. Fluoroscopy was required to guide the insertion or withdrawal of the endoscope. An oral route or anal route was chosen depending on clinical suspicion of the lesion. The endoscopic examination was stopped when (1) a lesion was found; (2) the endoscope was unable to be inserted or (3) due to patient intolerance. All the patients were admitted and received conscious sedation with intravenous midazolam and meperidine. During the procedure, the patients were monitored with an oximeter, EKG, and blood pressure monitor. Oxygen *via* nasal cannula was provided, as necessary.

Statistical analysis

All data were analyzed with SPSS 16.0. All quantitative data were expressed as mean ± SD. The χ^2 test was used to compare two categorical data with $P < 0.05$ being considered statistically significant.

RESULTS

Clinical features

From 2004 to 2010, a total of 75 patients underwent DBE for OGIB and, of these, 31 patients were included in this analysis. These patients had a mean age of 68.6 years. Their mean hemoglobin levels were of 7.95 g/dL. Fourteen patients exhibited shock (defined as systolic blood pressure < 90 mmHg or drop of systolic pressure > 40 mmHg) during the presentation. The median time between the MDCT and DBE procedures was 2 d (range from 0 d to 12 d). The source attributed to OGIB was found in 93.5% of these 31 patients. Two patients had no diagnosis made after both DBE and CT examination and had no further bleeding during the follow-up period (Table 1).

Diagnostic yield of DBE and MDCT

The overall diagnostic yield of DBE was 93.5% and CT was 45.2%. The diagnostic yield of CT depends on the etiology: 100% (2/2) of bleeding angiodysplasia, 85.7% (6/7) of those had bleeding from tumors, 41.7% (5/12) of those from diverticulosis, 14.3% (1/7) of those from ulcers and 0% of those from lymphangiectasia (0/1). One patient had early colon cancer diagnosed by DBE, though missed by CT. The MDCT diagnostic yields were

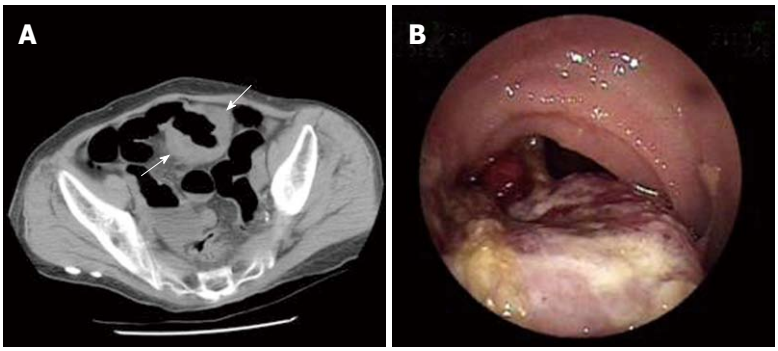

Figure 1 Multidetector computed tomography finding of small bowel neoplasm. A: Abdominal multidetector computed tomography performed after colonoscopy. Abundant bowel gas was observed as a contrast to distend the small intestinal lumen. A focal bowel thickening was discovered (arrows); B: Anal route double-balloon endoscopy disclosed ileal lymphoma accounting for the bowel wall thickening.

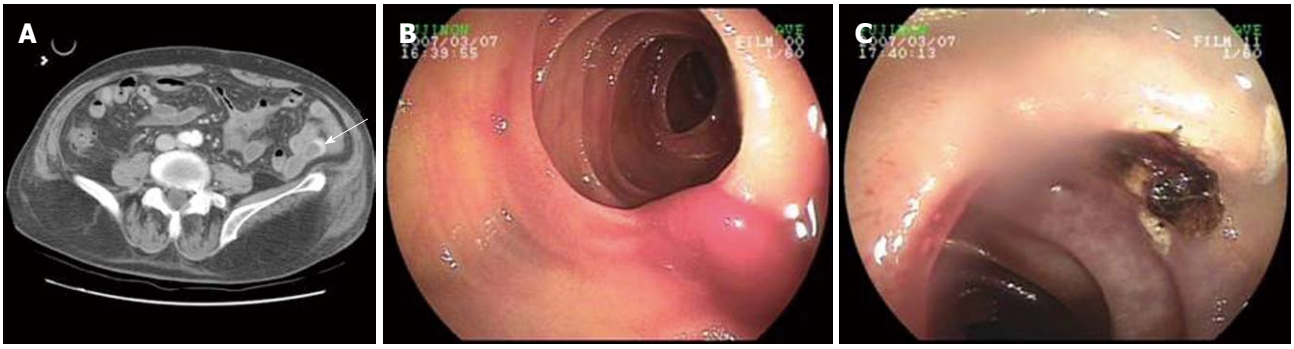

Figure 2 Multidetector computed tomography finding of small bowel bleeding. A: Multidetector computed tomography disclosed active contrast extravasations in the jejunum (arrow); B: Oral route double-balloon endoscopy disclosed active bleeding angiodysplasia; C: Endoscopic view after control of bleeding.

Table 1 Clinical features of patients (mean ± SD) *n* (%)

Item	Value
Sex (M/F)	13/18
Age (yr)	68.6 ± 11.3
Underlying diseases	
Hypertension	23 (74.2)
DM	4 (12.9)
Chronic renal failure	5 (16.1)
Cirrhosis	2 (6.5)
Abdominal operation	1 (3.2)
NSAID/aspirin use	9 (29.0)
Hemoglobin level (g/dL)	7.95 ± 1.62
Hospital stay (d)	12.8 ± 10.8
Shock at presentation	14 (45.2)
Median time between DBE and MDCT (d)	2 (0-12)
Final diagnosis	
Ulcers	7
Tumors	7
Angiodysplasia	2
Diverticulosis	10
Lymphangiectasia	1
Undiagnosed	2

DM: Design management; NSAID: Nonsteroidal antiinflammatory drugs; DBE: Double-balloon endoscopy; MDCT: Multidetector computed tomography.

Table 2 Diagnostic yield of double-balloon endoscopy *vs* multidetector computed tomography according to final diagnosis *n* (%)

Final diagnosis	DBE positive	MDCT positive	Total
Ulcers	7	1	7
Tumors	7	6	7
Angiodysplasia	2	2	2
Diverticulosis	12	5	12
Lymphangiectasia	1	0	1
Undiagnosed	0	0	2
Diagnostic yield	29 (93.5)	14 (45.2)	31

DBE: Double-balloon endoscopy; MDCT: Multidetector computed tomography.

not different according to the hospital stay, shock status, hemoglobin levels and timeframe between presentations to MDCT. The diagnostic yield of MDCT is high compared to the bleeders from tumor *vs* non-tumor (ulcerative or angiogenic cause) origin (85.7% *vs* 33.3%, $P = 0.014$) (Table 2).

Clinical impact of MDCT on subsequent DBE

The choice of initial route of DBE examination (for example, oral route or anal route) depended on the clinical suspicion of the lesion by the endoscopist. If previous imaging studies suggested the location of bleeding, the nearest route was chosen; if the patient had a history of hematemesis or tarry stool passage, the oral route was chosen; if the patient had bloody stool passage, the anal route was chosen. Our patients received DBE

Table 3 Impact of multidetector computed tomography on choice of enteroscopy insertion route

	Route choice right	Route choice wrong
Positive CT	14	0
Negative CT	9	8
Pearson's $\chi^2 = 8.88$, $P = 0.003$		

CT: Computed tomography.

via oral route only ($n = 18$), anal route only ($n = 4$), and both routes ($n = 9$). Among the 14 cases with a positive CT, the initial route of DBE was correct in all 14 cases. Among those 17 patients with a negative CT, the initial route of DBE was correct in 9 cases. A positive CT led to a correct choice of the route of DBE compared with a negative CT (100% *vs* 52.9%, $P = 0.003$) (Table 3).

DISCUSSION

The current suggested investigation into OGIB includes CE and DBE by the AGA in 2007[2]. Both methods have a variable diagnostic yield from 36% to 80%[2]. CE is non-invasive but has some limitations, such as capsule retention and incomplete examination[3]. Compared with CE, DBE is more invasive but offers therapeutic ability[4]. Unlike routine endoscopy, performing DBE is technically demanding and usually requires a two-physician team[11]. An initial approach with CE followed by DBE is ideal for patients with OGIB, but is not cost-effective[12]. Both procedures are expensive and not reimbursed by insurance in Taiwan. By contrast, MDCT is a non-invasive tool and had been used to evaluate OGIB[3,5-7]. In our previous reports[9,13,14], we determined that MDCT is particularly useful to localize the bleeding for subsequent endoscopy in the setting of active GI bleeding. MDCT is reimbursed by our insurance and is more readily available; therefore, approaching OGIB patients with initial MDCT followed by DBE was suggested as an alternative to the current AGA recommendation[2] in our institution since the introduction of DBE[15]. There are few studies that compare CE and MDCT in the management of OGIB[6,16]. In Zhang's report[16], the combination of MDCT with CE was not superior to CE alone in the diagnosis of OGIB. However, data regarding the role of MDCT before DBE in the setting of OGIB is lacking. Only one study from Chen *et al*[17] involving 70 patients suggested the usefulness of MDCT combined with DBE in the assessment of small bowel diseases. Thus, we performed this study to evaluate the role of MDCT prior to DBE in our institution during a 6-year-period.

In our study, we found DBE had higher diagnostic yield compared with MDCT (93.5% *vs* 45.2%) for OGIB. Both the diagnostic yield of DBE[2] and MDCT[3,5-7] are comparable to previous literature. Most of our patients exhibited severe GI bleeding (45.2% of shock at presentation), accounting for our high diagnostic yield. In this study, we discovered that MDCT is useful in two ways. Firstly, MDCT has a high diagnostic yield of small intestinal neoplasm compared with other non-tumor origins (ulcerative or angiogenic cause). Among seven patients with a tumor origin accounting for their OGIB, six patients were finally diagnosed to have small bowel tumors and all were diagnosed by MDCT prior to DBE procedure. The failure to diagnose one case of early colon cancer in the study can be explained by the fact that our MDCT protocol did not involve bowel preparation or optimal for colonic examination. The reported diagnostic rate of MDCT for small bowel neoplasm ranges from 67.4%[16] to 100%[5]. This is particularly useful in the management of patients with OGIB. In two recent large DBE series[18,19], patients with diagnosed neoplasm mostly benefited from the DBE due to the ability for long-term control of bleeding either endoscopically or surgically. By contrast, while a diagnosis of ulcer or vascular lesion was identified, only 40%[18,19] of the patients remained free of recurrent bleeding. Thus, the high diagnostic yield of MDCT for small bowel neoplasm allowed the clinician to identify patients that would most benefit from subsequent DBE procedures.

Secondly, in patients with positive MDCT, the choice of initial route of DBE examination was more likely to be correct compared to a negative MDCT (100% *vs* 52.9%, $P = 0.003$). As DBE usually requires examination *via* either the oral or anal route, the choice of correct insertion route of DBE is critical for allowing rapid approaches to the bleeding[20]. No recommended standard for selection of the insertion route of DBE currently exists. Although clinical presentation such as hematesis and stool color are useful for the endoscopist to choose the route of insertion, the initial route of DBE is not reliable, especially in the setting of massive bleeding[20]. Few studies[20,21] have utilized the finding of CE to guide the insertion route of subsequent DBE. In our study, we demonstrated that a positive MDCT is useful for guiding the insertion route of subsequent DBE.

Several methods of MDCT are described in the literature, including MDCT with oral saline as a contrast[16,22] or multi-phase CT-enterography[3,5,7]. The reported diagnostic yield for OGIB ranges from 30%[16] to 83%[22] with different MDCT protocols. No comparative study comparing the diagnostic yield of different CT protocols exists. In our institution, the triphasic MDCT protocol without oral contrast[8] utilized in our institution demonstrates a similar diagnostic yield as compared with previous reports. Although the use of fluid contrast can distend the intestinal lumen effectively and increase the diagnostic ability, especially for mucosal lesions[3,16,22], this may delay the subsequent DBE examination. In our institution, an emergent MDCT can be arranged as quickly as 2 h after a non-diagnostic endoscopy. If previous endoscopic studies suggested bleeding in the small bowel, the endoscopist would withdraw the endoscope without air suction, which leaves air in the bowel as a contrast (Figure 1). This approach is simple and similar to the recently described virtual enteroscopy[23]. *Via* this approach, once MDCT is positive, a DBE can be arranged early without the need to wait for

gastric emptying due to the use of an oral fluid contrast.

This study has some limitations. The first is in the retrospective nature of the study. Although CE or DBE are recommended for the diagnosis of OGIB[24], both of these procedures are expensive and neither is covered by health insurance providers in our region. In addition, MDCT is more readily available compared with CE and DBE and less invasive than conventional angiography. Thus, MDCT is used as the diagnostic and triage tool of choice for OGIB at our institution[9]. Therefore, our findings of the high diagnostic yields of both DBE and MDCT may be an overestimation because some patients with small intestinal tumors or those who have self-limited bleeding may not undergo subsequent DBE and were not included in this analysis. Secondly, the use of MDCT still has some disadvantages[4]. The procedure results in substantial radiation exposure and carries the risk of contrast nephrotoxicity that may limit its use in elderly patients, who constitute the main population of OGIB patients.

Our study demonstrates that MDCT provides a reasonable diagnostic yield for OGIB in comparison with previous reports[3,7,16,22]. The use of MDCT as a triage tool prior to DBE may aid the endoscopist in identifying patients who will benefit from the examination, and may aid the endoscopist in choosing the most efficient route of DBE examination. However, our study is limited to a retrospective nature and only a small number of patients were enrolled in this study. Further prospective studies with more patients are required to confirm our observation.

COMMENTS

Background

Capsule endoscopy followed by double-balloon endoscopy is the current standard approach for obscure gastrointestinal bleeding. Multidetector computed tomography (MDCT) has been used recently to investigate obscure gastrointestinal bleeding, but its role in current standard approach to obscure gastrointestinal bleeding (OGIB) has not been evaluated.

Research frontiers

This study analyzed the clinical impact of MDCT in a subset of patients with OGIB in a single medical center.

Innovations and breakthroughs

This study is novel in that the researchers analyzed the clinical utility of MDCT before double-balloon enteroscopy (DBE) for the indication of OGIB.

Applications

MDCT provides a reasonable diagnostic yield in this study. The use of MDCT as a triage tool prior to DBE may aid the endoscopist in identifying patients who will benefit from the examination and may aid the endoscopist in choosing the most efficient route of DBE examination.

Peer review

The authors demonstrated that MDCT may provide a reasonable diagnostic yield in a select group of patients with OGIB by a retrospective study.

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S- Editor Sun H **L- Editor** Rutherford A **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.698

World J Gastroenterol 2012 February 21; 18(7): 698-703
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Efficacy of imatinib dose escalation in Chinese gastrointestinal stromal tumor patients

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Supported by Beijing Cancer Hospital Foundation, No. 08-17 and Novartis Oncology
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Received: April 4, 2011 Revised: June 24, 2011
Accepted: July 1, 2011
Published online: February 21, 2012

Abstract

AIM: To investigate the efficacy and safety of imatinib dose escalation in Chinese patients with advanced gastrointestinal stromal tumor (GIST).

METHODS: Advanced GIST patients previously failing 400 mg imatinib treatment were enrolled in this study. Patients received imatinib with dose escalation to 600 mg/d, and further dose escalation to 800 mg/d if imatinib 600 mg/d failed. Progression-free survival, overall survival, clinical efficacy, c-kit/PDGFRA genotype and safety were evaluated.

RESULTS: 52 patients were enrolled in this study. For the 47 evaluable patients receiving imatinib (600 mg/d), the disease control rate was 40.4%, and the median progression-free survival for all patients was 17 wk (95% CI: 3.9-30.1). The median overall survival after dose escalation was 81 wk (95% CI: 36.2-125.8). Adverse events, mainly edema, fatigue, granulocytopenia and skin rash were tolerable. However, further dose escalation (800 mg/d) in 14 cases was ineffective, with disease progression and severe adverse events. Among 30 cases examined for gene mutations, patients with exon 9 mutations experienced a better progression-free survival of 47 wk.

CONCLUSION: Imatinib dose escalation to 600 mg/d is more appropriate for Chinese patients and may achieve further survival benefit.

Key words: Gene mutation; Gastrointestinal stromal tumor; Imatinib; Increased dose

Peer reviewer: Robert Jensen, MD, Digestive Disease Branch, National Institutes of Health, Building 10, Rm 9C-103, Bethesda, MD 20892, United States

Li J, Gong JF, Li J, Gao J, Sun NP, Shen L. Efficacy of imatinib dose escalation in Chinese gastrointestinal stromal tumor patients. *World J Gastroenterol* 2012; 18(7): 698-703 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/698.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.698

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are tumors that may occur throughout the gastrointestinal tract and in the omentum and mesentery, and account for about 2% of gastrointestinal tract tumors[1]. Various types of *KIT* gene mutations are present in the majority of GISTs[2]. Imatinib mesylate, a molecular tyrosine kinase inhibitor targeting *KIT*, platelet-derived growth factor receptor (PDGFR) and/or *bcr-abl* has remarkable clinical efficacy in the treatment of advanced GIST. Imatinib mesylate 400 mg/d is recommended in the first-line treatment of advanced GIST[3,4]. However, about 5% of patients show

primary resistance to imatinib and about 50% of patients develop secondary resistance within a median treatment duration of 2 years[4,5].

The results of two studies proved that after the failure of first-line treatment with imatinib 400 mg/d an escalated dose (800 mg/d) can still achieve tumor control and confer further survival benefits in some patients[6,7]. However, these studies were conducted in Western countries, thus whether Chinese patients with advanced GIST could benefit from an escalated dose of imatinib is unclear. It is also unknown whether increased dose increments are tolerated in these patients, due to differences in body weight and race between Chinese patients and Western patients.

KIT gene mutations can predict the objective efficacy of imatinib in the first-line treatment of advanced GIST[8], however, the relevance of these mutations to the objective response of imatinib dose escalation has not yet been reported. In this study, Chinese patients with advanced GIST, previously treated with 400 mg/d imatinib and who received dose escalation therapy, were examined to evaluate the median progression-free survival (PFS) and overall survival (OS) after imatinib dose escalation, objective efficacy, relationships between gene mutation types and efficacy, and the safety of imatinib dose escalation.

MATERIALS AND METHODS

Study design

This was a single arm, open-label, prospective study designed to evaluate the objective response and safety of imatinib dose escalation in Chinese GIST patients with resistance to imatinib 400 mg/d. The primary end point was PFS and the secondary end points were OS, disease control rate (DCR) defined as a combination of complete response (CR) + partial response (PR) + stable disease (SD), relationships between the gene mutation types and objective responses, and safety of imatinib dose escalation.

Adult patients (age $\geq$ 18 years) with a histologically confirmed recurrent or metastatic GIST which was CD117 positive, and who failed prior imatinib 400 mg/d treatment were eligible for the trial. The pathological and CD117 diagnosis was made immunohistochemically. All patients were Eastern Cooperative Oncology Group (ECOG) performance status 2 or lower and provided informed consent. Patients were assigned to receive imatinib 600 mg/d orally, taken once daily with food, in the form of 100 mg capsules. If the disease progressed after imatinib 600 mg therapy, the dose of imatinib was further increased to 800 mg/d until the next instance of tumor progression or tolerance failure. CT or MRI examination was conducted every 8 wk and the objective response was based on the Response Evaluation Criteria In Solid Tumors (RECIST) guidelines. The PFS was defined as the time from the first dose of imatinib 600 mg/d to the occurrence of progression, death from any cause, or withdrawal from the trial. OS was defined as the time from the first dose of imatinib 600 mg/d to the occurrence of death from any cause. Adverse events were graded according to the National Cancer Institute Common Toxicity Criteria version 2.0.

Detection of KIT and PDGFR gene mutations

Formalin-fixed, paraffin-embedded tumor tissue samples, taken prior to imatinib treatment, were collected for *KIT* and *PDGFR* gene mutation analyses. Genomic DNA was extracted from the tumor sample using the e.Z.N.A. FFPE DNA Kit (OMEGA Bio-Tek Inc., Norcross, GA, United States). Initially, polymerase chain reaction (PCR) amplification and mutational analyses of *KIT* exon 11 were performed. Patient samples negative for *KIT* exon 11 mutations were subsequently amplified with primers specific for *KIT* exons 9, 13, and 17. When *KIT* mutations were not identified, additional mutational analyses were conducted for exons 12 and 18 of the *PDGFR* gene.

Statistical analysis

All statistical analyses were based on the SPSS 15.0 platform (SPSS Inc., Chicago, IL, United States). PFS and OS curves were constructed according to the Kaplan-Meier method and the log-rank test was used to compare differences between PFS curves. Frequency and percentage descriptions were used for categorical variables and the chi-square test was conducted to compare the incidence of different events. If the theoretical frequency was lower than 1, Fisher's exact test was conducted. Continuous variables were expressed as mean ± SD and mean differences between two groups were compared using Student's *t*-test.

RESULTS

Patient characteristics following disease progression on imatinib 400 mg/d treatment

Between April 2004 and March 2009, 168 patients with advanced GIST received imatinib 400 mg/d. Of these patients, 52 were eligible for imatinib dose escalation therapy. Most of the patients had secondary resistance (96%) and two had primary resistance. The duration of imatinib 400 mg/d treatment ranged from 3.0 to 56.0 mo, with a median PFS of 20.0 mo (95% CI: 15.3-24.7). Before the initiation of imatinib dose escalation, three patients with progression in liver metastasis and one patient with new-onset pelvic metastasis received complete tumor resection, while one patient with liver metastasis received local radiofrequency ablation therapy and four patients received primary palliative surgical resection. The 52 eligible patients received imatinib dose escalation therapy of 600 mg/d orally. The clinical features of these patients are shown in Table 1.

Toxicities and dose reductions

Dose escalation therapy of imatinib 600 mg/d was well tolerated. All 52 patients had Grade 1 or 2 adverse events and only 7 patients had Grade 3 adverse events. Adverse events consisted of edema, fatigue, granulocytopenia and skin rash. Adverse events more severe than those found

Table 1 Clinical characteristics of the 52 patients *n* (%)

Characteristics	Patients
Sex	
Male	34 (65.4)
Female	18 (34.6)
Age (yr) (mean ± SD)	53.8 ± 14.0
Primary site	
Stomach	18 (34.6)
Small intestine	20 (38.5)
Abdominal cavity	9 (17.3)
Colon	2 (3.8)
Rectum	2 (3.8)
Pelvis	1 (1.9)
Prior surgical resection	
Yes	47 (90.4)
No	5 (9.6)
Site of metastasis	
Liver	29 (55.8)
Abdominal cavity	31 (59.6)
Pelvis	6 (11.5)
Lung	1 (1.9)
Bone	1 (1.9)
Subcutaneous	1 (1.9)
Prior response to imatinib 400 mg/d	
Complete remission	5 (9.6)
Partial remission	27 (51.9)
Stable disease	18 (34.6)
Progressive disease	2 (3.8)
Receiving regional treatment prior to dose escalation	
Complete tumor resection	4 (7.7)
Palliative tumor resection	4 (7.7)
Local Radiofrequency ablation treatment	1 (1.9)
No regional treatment	43 (82.7)

in the previous 400 mg/d treatment were experienced by 73.1% of patients, particularly edema and fatigue. Imatinib dose was reduced to 500 mg/d in one patient due to fatigue and abdominal pain. In other patients, 600 mg/d was well tolerated and discontinuation of imatinib was not required due to adverse events.

The dose of imatinib was further escalated to 800 mg/d in 14 patients due to disease progression during 600 mg/d treatment. All 14 patients had Grade 1 or 2 adverse events and showed worsening adverse events; predominantly edema, fatigue, abdominal pain, nausea, and loss of appetite. Among these patients, 6 had Grade 3 adverse events, and imatinib was discontinued in three and dose reduction to 600 mg/d in two. Hematologic and non-hematologic toxicities are summarized in Table 2.

Response rate

Of 52 patients, four were excluded due to complete tumor resection and one patient received local radiofrequency ablation therapy prior to dose escalation therapy. The remaining 47 patients all had measurable disease according to RECIST criteria and tumor assessment was performed at least once. Among these patients, three (6.4%) achieved PR, 16 (34.0%) had SD, and 28 (59.6%) showed disease progression (PD) after imatinib dose escalation to 600 mg/d. The overall DCR was 40.4%.

Fourteen patients with disease progression on 600 mg/d imatinib received further dose escalation to 800 mg/d, of which three patients discontinued therapy due to adverse reactions. Eleven patients with measurable disease all showed PD.

Table 2 Hematologic and non-hematologic toxicities of imatinib dose escalation *n* (%)

	Imatinib 600 mg/d		Imatinib 800 mg/d	
	Grade 1-2 (%)	Grade 3-4 (%)	Grade 1-2 (%)	Grade 3-4 (%)
Edema	42 (80.8)	0 (0)	14 (100)	0 (0)
Fatigue	32 (61.5)	1 (1.9)	9 (64.3)	5 (35.7)
Granulocytopenia	19 (36.5)	3 (5.8)	5 (35.7)	1 (7.1)
Skin rash	12 (23.1)	2 (3.8)	7 (50.0)	1 (7.1)
Anemia	7 (13.5)	2 (3.8)	5 (35.7)	0 (0)
Thrombocytopenia	2 (3.8)	0 (0)	1 (7.1)	0 (0)
Anorexia	8 (15.4)	1 (1.9)	6 (42.9)	0 (0)
Nausea	11 (21.2)	1 (1.9)	6 (42.9)	0 (0)
Alopecia	0 (0)	0 (0)	2 (14.3)	0 (0)
Abdominal pain	11 (21.2)	0 (0)	7 (50.0)	0 (0)
Diarrhea	5 (9.6)	0 (0)	1 (7.1)	0 (0)
Epiphora	2 (3.8)	0 (0)	2 (14.3)	0 (0)

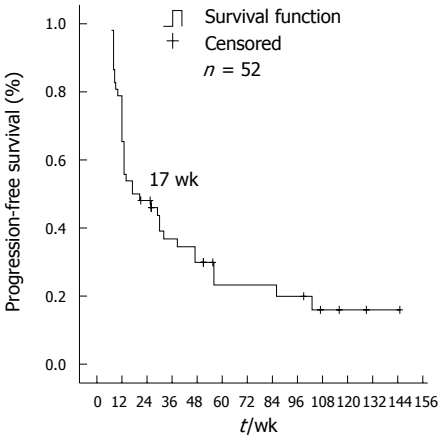

Figure 1 Progression-free survival for all patients. Forty-eight patients (92.3%) had disease progression after imatinib 600 mg/d treatment, and the median PFS of all the 52 patients was 17 wk (95% CI: 3.9-30.1). PFS: Progression-free survival; CI: Confidence interval.

PFS and OS

As of February 2011, 48 patients (92.3%) had progression after imatinib 600 mg/d treatment. The median PFS for all 52 patients was 17 wk (95% CI: 3.9-30.1) (Figure 1). Patients with PR or SD had a significantly longer PFS than patients who progressed on 600 mg/d dose escalation therapy. The median PFS for patients with PR or SD was 51 wk (95% CI: 26.8-75.2) and was 12 wk for patients with PD (95% CI: 10.6-13.4) ($P < 0.001$) (Figure 2).

As of February 2011, 13 patients were alive, 38 patients had died due to tumor progression and 1 patient had died due to other reasons. The median OS following dose escalation in all patients, starting from the first prescription of 600 mg/d imatinib, was 81 wk (95% CI: 36.2-125.8) (Figure 3). The 1-year survival rate for all patients was 63.5%.

Of the four patients who received complete resection of tumor metastasis before dose escalation, two experi-

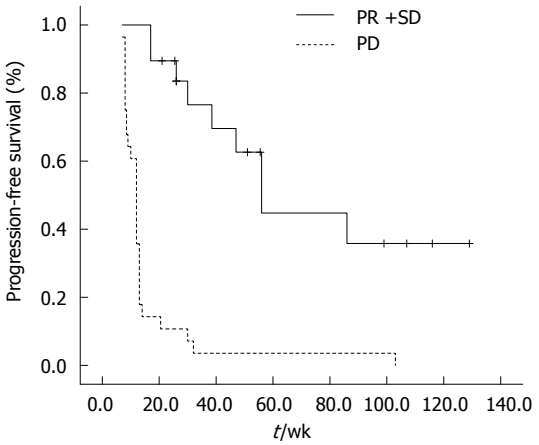

Figure 2 The median progression-free survival for partial response or stable disease patients was 51 wk (95% CI: 26.8-75.2) and that of patients with progressive disease was 12 wk (95% CI: 10.6-13.4) (*P* < 0.001). PR: Partial response; SD: Stable disease; CI: Confidence interval.

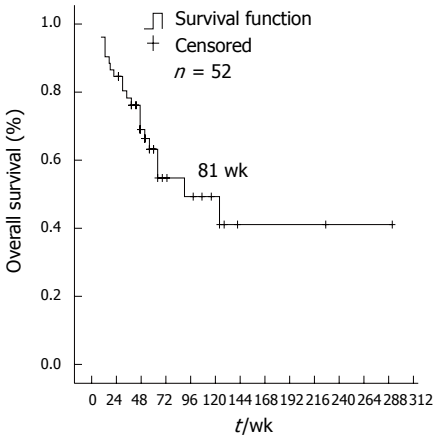

Figure 3 Median overall survival of all patients starting from the first prescription of 600 mg/d imatinib. Median OS was 81 wk (95% CI: 36.2-125.8). OS: Overall survival; CI: Confidence interval.

enced tumor recurrence and died of tumor progression. PFS in these two cases was 48 wk and 29 wk, respectively, and OS was 90 wk and 51 wk, respectively. The other two patients, and one patient receiving microwave treatment of local liver metastasis, had no recurrence; PFS was 135 wk, 151 wk, and 145 wk, respectively.

Gene mutation analyses

A total of 30 patients underwent gene mutation analysis before first-line imatinib 400 mg/d treatment, of which 19 (63.3%) had a mutation in *KIT* exon 11, 6 (20.0%) in *KIT* exon 9, and two (6.7%) in *KIT* exon 13. The remaining five (10.0%) patients showed wild-type GIST. No *PDGFR* gene mutations were found.

Among the 19 patients with *KIT* exon 11 mutations, 17 were evaluable. One patient achieved a PR and 7 patients remained stable. The remaining 9 patients progressively worsened. The overall DCR was 47.1%. Among the 4 evaluable *KIT* exon 9 patients, 1 achieved PR and 3 displayed SD. In the 3 evaluable wild-type GIST patients, 1 stabilized and the remaining 2 had PD. In patients with *KIT* exon 13 mutations, one was stable and the other had PD.

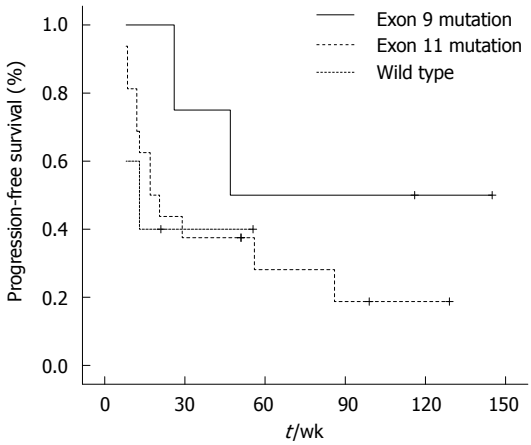

Figure 4 Progression-free survival of patients by gene mutation. PFS of the patients with exon 9 mutation, exon 11 mutation, and wild type were 47 wk, 21 wk, and 8 wk, respectively, but the difference was not significant (*P* = 0.083). PFS: Progression-free survival.

The median PFS of patients with *KIT* exon 11 mutations, *KIT* exon 9 mutations, and *KIT*/PDGFR wild-type patients was 21 wk (95% CI: 3.4-37.6), 47 wk (95% CI: 11.6-82.4), and 8 wk, respectively, as shown in Figure 4.

Although the difference in PFS between the three categories was not significant (*P* = 0.083), patients with *KIT* exon 9 mutations tended to have longer PFS than those with exon 11 mutations and wild-type patients.

Follow-up after imatinib treatment

Up to February 2011, of 52 patients, 4 are progression-free and continue to receive imatinib 600 mg/d treatment. All patients receiving further dose escalation to imatinib 800 mg/d have discontinued treatment. 21 patients received sunitinib treatment. 39 patients died due to tumor progression with the exception of 1 patient.

DISCUSSION

The efficacy of imatinib mesylate in advanced gastrointestinal stromal tumors was confirmed and patients with advanced disease can achieve prolonged OS. Imatinib 400 mg/d is recommended as the first-line treatment for advanced GIST patients. However, a proportion of GIST patients experience secondary resistance to imatinib after tumor remission, and tumor progression occurs. Results of the phase Ⅲ trials, EORTC62005 and US0033, confirmed that after resistance to 400 mg/d imatinib, dose escalation therapy to 800 mg/d can offer further disease control in 30%-40% of patients[6,7]. The results of the analyses in these two studies showed that patients obtained a PFS benefit of 11.3 and 21.4 wk after cross-over to 800 mg/d imatinib, which represents a further survival benefit for a number of GIST patients[9].

The conclusions in the present study are consistent with those of the above-mentioned studies: imatinib 600 mg/d dose escalation treatment improved PFS and OS of patients after progression on imatinib 400 mg/d. The

PFS of patients achieving PR and SD was superior to that of patients with PD, suggesting that disease remission or control may bring about a benefit in PFS. In addition, late follow-up revealed that about 40% of patients received sunitinib after failure of imatinib escalation treatment. Sunitinib, a multi-target tyrosine kinase inhibitor, provided prolonged OS for imatinib-resistant patients in a phase Ⅲ trial[10]. Thus, due to an unclear mechanism, sunitinib also contributed to the median OS of 81 wk.

In this study, of the 11 patients with evaluable disease receiving imatinib 800 mg/d dose escalation therapy, none of these patients benefited from this escalation, and adverse events were severe and intolerable. The results of the EORTC62005 and US0033 studies showed that Western patients were tolerant to imatinib 800 mg/d. However, because Chinese patients tend to have a smaller body surface area than Western patients, it is unclear whether Chinese patients can tolerate imatinib 800 mg/d dose escalation therapy. In this study, the median PFS of Chinese patients receiving 600 mg/d dose escalation therapy was similar to the PFS of Western groups in the two phase Ⅲ trials, and imatinib 600 mg/d dose escalation was generally well-tolerated. In addition, since none of the patients benefited from imatinib dose escalation therapy to 800 mg/d and adverse events were severe, imatinib dose escalation to 800 mg/d is not recommended when imatinib 600 mg/d is ineffective for the treatment of Chinese patients with advanced GIST.

Some gene mutations can predict the objective efficacy of imatinib for advanced GIST[8]. In addition, results from the EORTC62005 study have shown that, during the initial treatment, imatinib 800 mg/d may significantly prolong PFS in patients with GISTs harboring exon 9 mutations, although no further improvement in PFS was seen in patients with *KIT* exon 11 mutations[9]. However, the relationship between gene mutations and the efficacy of imatinib dose escalation therapy following secondary imatinib resistance is unclear. Previous studies have shown that secondary resistance to imatinib might be related to a secondary *c-kit*/*PDGFRA* mutation, gene amplification, or imatinib-related drug resistance due to changes in drug metabolism[11-13]. A recent, small sample size study[14] in Korea reported that dose escalation of imatinib to 600 mg/d or 800 mg/d following 400 mg/d treatment failure can produce better outcomes in patients with exon 9 mutations than with other mutation types. Due to the difficulty in obtaining tissue samples following drug resistance, the analysis of secondary gene mutations was not possible. Thus, the gene mutation analyses were conducted on tissue samples taken prior to the start of imatinib treatment. Similar to the results of the Korean study, patients with exon 9 mutations had a relatively long PFS after receiving imatinib dose escalation therapy. Although the sample size, especially the number of patients with exon 9 mutations, was comparatively small, and the results of the analysis of PFS with different mutation types showed no statistical difference, patients with exon 9 mutations showed a trend towards longer PFS following imatinib dose escalation therapy. Moreover, although the results of the EORTC62005 study showed that first-line treatment with imatinib 800 mg/d may not improve the objective efficacy and PFS in *KIT* exon 11 mutation patients compared with standard treatment of imatinib 400 mg/d, a proportion of *KIT* exon 11 mutation patients can still achieve disease control with dose escalation therapy after progression on previous treatment with imatinib 400 mg/d. As for *KIT* exon 11 mutation patients, dose escalation therapy is still one of the available treatment strategies following failure of imatinib standard treatment.

The NCCN guidelines recommend that GISTs with exon 9 mutations should receive high-dose imatinib treatment. Whether these patients can receive standard imatinib treatment followed by high-dose imatinib treatment following disease progression is still not known. Since the number of patients with exon 9 mutations in the present study was very small, no conclusion can be made. It is hoped that the EORTC62005 and S0033 studies will provide further insight.

A variety of molecular targeted compounds are available or under development to overcome imatinib resistance. These include the new generation tyrosine kinase inhibitor, nilotinib, the multiple kinase inhibitor sorafenib, and the molecular target of rapamycin inhibitor, everolimus, all of which have been shown to be effective in overcoming imatinib resistance in phase Ⅰ or Ⅱ studies of advanced GIST patients[15-17]. As a multi-target tyrosine kinase inhibitor, sunitinib has been approved by the United States Food and Drug Administration for the treatment of patients with advanced GIST whose disease has progressed on imatinib treatment. For patients who failed prior standard dose imatinib treatment, there has been no direct evidence to suggest whether it is preferable to use imatinib dose escalation therapy or switch to second-line treatment with sunitinib. An expanded, global, retrospective analysis of sunitinib showed that the OS of patients who received previous imatinib treatment of $\leqslant$ 400 mg/d was 93 wk, which was superior to the 70 wk observed in patients previously receiving > 400 mg/d imatinib, suggesting that patients previously receiving comparatively small doses of imatinib may obtain greater survival benefit after sunitinib treatment[18]. However, this suggestion still does not answer the above question, and further evidence is needed to select between the two secondary treatment strategies.

Dose escalation therapy to 600 mg/d after treatment failure with imatinib 400 mg/d is more appropriate for Chinese patients with advanced GIST due to good tolerability and survival benefits. Regarding the comparatively poor objective efficacy and severe adverse events, further escalation of imatinib dose to 800 mg/d following imatinib 600 mg/d failure is not recommended.

COMMENTS

Background

Imatinib 400 mg/d is recommended as the standard first-line treatment for advanced gastrointestinal stromal tumor (GIST). An escalated dose (800 mg/d) can still achieve tumor control and confer further survival benefits after failure

with imatinib 400 mg/d in some patients. However, whether Chinese patients with advanced GIST could benefit from an escalated dose of imatinib is unclear. It is also unknown whether increased dose increments are tolerated in these patients, due to differences in body weight and race between Chinese patients and Western patients. *KIT* gene mutation can predict the objective efficacy of imatinib in the first-line treatment of advanced GIST, however, its relevance to the objective response of imatinib dose escalation has not yet been reported.

Research frontiers

In this study, the authors demonstrate that imatinib dose escalation was effective in some Chinese GIST patients after failure of imatinib standard dose, and KIT genotype could predict the efficacy of imatinib dose escalation treatment.

Innovations and breakthroughs

This is the first report of imatinib dose escalation treatment for GIST in Chinese patients and the results showed imatinib escalation to 600 mg/d was more appropriate for Chinese patients than imatinib 800 mg/d. There are few reports on the relationship between KIT genotype and the efficacy of imatinib dose escalation. This study demonstrated that patients with KIT exon 9 mutations benefited more from imatinib dose escalation treatment.

Applications

The results of this study help to clarify the efficacy and safety of imatinib dose escalation treatment in Chinese GIST patients. More importantly, the appropriate treatment dose between Western patients and Chinese patients may be different. Genotype may be helpful in predicting the efficacy of imatinib dose escalation treatment.

Peer review

Generally, this is an interesting and well-done study that even though its patient size limits the conclusions of the genetic studies, it provides important clinical information for the management of these patients with higher drug doses.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.704

World J Gastroenterol 2012 February 21; 18(7): 704-711
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Apoptosis of human cholangiocarcinoma cells induced by ESC-3 from *Crocodylus siamensis* bile

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Supported by The National Natural Science Foundation of China, No. 81072014; the National Foundation for Fostering Talents in Basic Sciences, No. J1030626; the Thailand Sriracha Tiger Zoo Co. Ltd., Sriracha, Thailand
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Received: May 6, 2011 Revised: July 10, 2011
Accepted: July 17, 2011
Published online: February 21, 2012

Abstract

AIM: To investigate the effects of ESC-3 isolated from crocodile bile on the growth and apoptosis induction of human cholangiocarcinoma cells.

METHODS: ESC-3 was isolated from crocodile bile by Sephadex LH-20 and RP-18 reversed-phase column. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay was conducted to determine the effects of ESC-3 on the proliferation of human cholangiocarcinoma cell lines (QBC939, Sk-ChA-1 and MZ-ChA-1). Giemsa staining, Hoechst 33258 and acridine orange/ethidium bromide staining showed the morphological changes of Mz-ChA-1 cells exposed to ESC-3 at different concentrations. Flow cytometry with regular propidium iodide (PI) staining was performed to analyze the cell cycle distribution of Mz-ChA-1 cells and to assess apoptosis by annexin v-fluorescein isothiocyanate (V-FITC)/PI staining. Rh123 staining was used to detect the alteration of mitochondrial membrane potential ($\Delta\Psi m$). The protein levels of Bax, Bcl-2, Cdk2, cytochrome c and caspase-3 were further confirmed by Western blotting.

RESULTS: ESC-3 significantly inhibited the growth of three human cholangiocarcinoma cell lines and arrested Mz-ChA-1 cell cycle at G0/G1 phase. Mz-ChA-1 cells showed typical apoptotic morphological changes after treated with ESC-3 (10 μg/mL) for 48 h. Cell death assay indicated that Mz-ChA-1 cells underwent apoptosis in a dose-dependent manner induced by ESC-3. In addition, ESC-3 treatment could downregulate the protein level of Bcl-2 and upregulate the Bax, leading to the increase in the ratio of Bax to Bcl-2 in Mz-ChA-1 cells. Meanwhile, cytochrome c was released from the mitochondria into the cytosol, which subsequently initiated the activation of caspase-3. All these events were associated with the collapse of the mitochondrial membrane potential.

CONCLUSION: ESC-3, the active ingredient of crocodile bile, induced apoptosis in Mz-ChA-1 cells through the mitochondria-dependent pathway and may be a potential chemotherapeutic drug for the treatment of cholangiocarcinoma.

Key words: *Crocodylus siamensis* bile; Cholangiocarcinoma; Antiproliferation; Apoptosis; Mitochondria

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Song W, Shen DY, Kang JH, Li SS, Zhan HW, Shi Y, Xiong YX, Liang G, Chen QX. Apoptosis of human cholangiocarcinoma cells induced by ESC-3 from *Crocodylus siamensis* bile. *World J Gastroenterol* 2012; 18(7): 704-711 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/704.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.704

INTRODUCTION

Cholangiocarcinoma is the second most common primary hepatic tumor and currently accounts for 3% of all gastrointestinal cancers; its incidence has recently been increasing globally[1-3]. Clinically, cholangiocarcinoma patients are treated with surgical resection, radiation and chemotherapy. Although progress has been made in the diagnosis and treatment of cholangiocarcinoma in recent years, the prognosis is still unsatisfactory due to the lack of efficient anticancer drugs[4]. Currently, more than 30 compounds of natural origin are in various phases of clinical study for the treatment of several types of cancer[5]. Hence, it is a potential strategy to discover effective compounds from natural products for cancer treatment[6].

With the progress in research on traditional Chinese medicine (TCM), more natural products have been used in medical treatment, especially in the treatment of cancers. Bile is a fluid that consists of several acids, including cholic acid (CA) and chenodeoxycholic acid (CDCA) and has potential anticancer effects; CA and CDCA are the predominant active components in snakes[7]. Previous reports showed that bile acid played a key role in regulating cholangiocyte growth and secretion[8,9]. In addition, deoxycholic acid (DCA) has been shown to rapidly induce apoptosis in HCT116 cells, a colon tumor cell line[10]. The effects of synthetic derivatives of ursodeoxycholic acid (UDCA), such as HS-1183, and CDCA, such as HS-1199 and HS-1200, on the proliferation of human prostate carcinoma PC-3 cells have been investigated[11]. Based on these previous reports, it was suggested that components of crocodile bile could inhibit cell proliferation and induce apoptosis and may be a source of potential anti-tumor agent. In this study, we isolated anti-tumor component ESC-3 from crocodile bile, demonstrated the effects of ESC-3 on Mz-ChA-1 cells and elucidated the mechanism by which ESC-3 induces apoptosis.

MATERIALS AND METHODS

Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), bisbenzimide (Hoechst 33258), acridine orange (AO), ethidium bromide (EB), propidium iodide (PI) and proteinase K were purchased from Sigma-Aldrich Co. (St. Louis, MO, United States). RPMI-1640 medium and fetal calf serum were purchased from Gibco (Grand Island, NY, United States). Mouse monoclonal antibodies against human p53, Bax, Bcl-2, cytochrome c, CDK2 and caspase-3 were obtained from Santa Cruz Biotechnology, Inc. (CA, United States). *Crocodylus siamensis* bile was obtained from the Sriracha Tiger Zoo Thailand Co. Ltd.

Isolation of Crocodylus siamensis bile

The gallbladder containing bile was directly deposited after dried. The whole gallbladder was homogenized and extracted with phosphate buffered saline (PBS) for 4 h at 4 ℃ in triplicate and then centrifuged at 20 000 × *g* for 30 min at 4 ℃. The supernatant was pooled and lyophilized by vacuum freeze-drying, and the powder was stored at -20 ℃ until purification. The powder was redissolved in methanol and loaded onto a Sephadex LH-20 column (Pharmacia, Sweden) with a flow rate of 2 mL/min, and 1 mL per tube was collected. The anti-cancer compound-containing fraction was then loaded onto a RP-18 reversed-phase column (25 cm × 0.3 cm). The solvent system was methanol-0.01 M NaH_2PO_4 (70:30, v/v), which was adjusted to pH 4.0 by the addition of H_3PO_4. The solvent was filtered through a 0.22 μm filter (Millipore) prior to use. The detection wavelength was 215 nm.

Cell culture and treatment

The QBC939, Sk-ChA-1 and MZ-ChA-1 cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 ℃ in a humidified atmosphere containing 50 mL/L CO_2. Twenty-four hours after seeding, the cells were treated with culture medium containing various concentrations of ESC-3.

Cell viability assay

Cell viability was determined using the MTT assay. Briefly, cells were seeded in a 96-well plate at a density of 1 × 10^5/mL. After overnight growth, the cells were treated with various concentrations of ESC-3 for 12 h, 24 h, 36 h, 48 h or 72 h. After the ESC-3 treatment, 20 mL MTT (5 mg/mL) was added to each well, and the cells were cultured for another 4 h at 37 ℃. The medium was then removed, and 150 mL dimethyl sulfoxide (DMSO) was added to each well. The absorbance of each well was recorded at 490 nm.

Giemsa staining

Mz-ChA-1 cells from the control group and the group treated with 10 μg/mL ESC-3 for 48 h were seeded onto coverslips and grown for 24 h. After washing with PBS three times, the cells were stained with Giemsa staining solution for 10 min and observed under a light microscope (Olympus BH-2).

Hoechst 33258 and AO/EB staining

Cells treated with 10 μg/mL ESC-3 for 48 h were harvested and fixed with a mixture of glacial acetic acid and methanol (1:3, v/v) for 5 min and then washed twice with PBS. The cells were resuspended in Hoechst 33258 solution (5 μg/mL) and incubated at room temperature for 10 min. After three washes with PBS, the cells were dried thoroughly and observed under a fluorescence mi-

croscope. For AO/EB staining, the cells were harvested and washed twice with PBS. The cells were then incubated with 100 μL PBS plus 4 μL AO/EB solution (100 μg/mL AO and 100 μg/mL EB in PBS) for 3 min at room temperature in the dark and immediately observed under a fluorescence microscope.

Flow cytometry

After treatment with ESC-3 at different concentrations (0, 5, 10 and 15 μg/mL) for 72 h, the Mz-ChA-1 cells were harvested, washed twice with PBS and fixed with 70% ethanol at 4 ℃ overnight. After centrifugation, the cells were resuspended in 100 μg/mL RNase A at 37 ℃ for 30 min and subsequently stained with 50 μg/mL propidium iodide at 4 ℃ for 30 min in the dark. The cells were analyzed by flow cytometry at 488 nm, and the data were analyzed with the CellFit software.

For quantifying cell apoptosis, an annexin v-fluorescein isothiocyanate (V-FITC)/PI double staining assay was performed according to the manufacturer's instructions. Briefly, the cells were harvested and stained with annexin V-FITC and PI for 20 min at room temperature. The cells were then washed twice with PBS, and the fluorescence of the cells was measured by flow cytometry.

Analysis of mitochondrial transmembrane potential (ΔΨm)

After treatment with different concentrations of ESC-3 (0 μg/mL, 5 μg/mL, 10 μg/mL and 15 μg/mL) for 48 h, the Mz-ChA-1 cells were incubated with Rh123 (1 mg/mL in dimethyl sulfoxide) at 37 ℃ for 30 min and washed three times with PBS. The cells were harvested and analyzed by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Western blotting analysis

Western blotting analysis was performed as previously described. Briefly, cell lysates were prepared, separated on 12% sodium dodecyl sulfate polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ). Non-specific reactivity was blocked by incubating the membranes for 1 h in 5% nonfat milk at room temperature. The membranes were incubated with primary antibody overnight at 4 ℃. After three washes for 10 min with phosphate buffered saline tween-20 (PBST), the membranes were incubated at 37 ℃ for 1 h with the appropriate secondary antibody (1:5000 dilution, Sigma) and washed three times with PBST. Reactive proteins were detected with the enhanced chemiluminescence (ECL) detection system (Pierce). β-actin was used as an internal control.

Caspase-3 activation assay

To investigate caspase-3 activation after treatment with ESC-3, a caspase-3 colorimetric assay kit (Kaiji Bio Co., Nanjing, China) was used according to the manufacturer's instructions. Briefly, cells were harvested and lysed by incubation with cell lysis buffer on ice for 1 h, followed by centrifugation at 10 000 × *g* for 1 min. Enzymatic reactions were performed in a 96-well microplate, and 50 μL cell lysate was incubated with the substrate for 4 h at 37 ℃. The absorbance was measured at 405 nm. The total protein concentration was determined using the Coomassie brilliant blue method.

Statistical analysis

All results obtained from ESC-3-treated Mz-ChA-1 cells were analyzed using the SPSS software. Data were expressed as the means ± SD error of the mean (SEM) of separate experiments ($n \geqslant 3$). Differences between two treatments were considered significant at $P < 0.05$.

RESULTS

Purification of bile acid from Crocodylus siamensis gall bladder

The crude extract was subjected to separation on a Sephade × LH-20 column. As shown in Figure 1A, four peaks were obtained. The effects of each pooled peak on Mz-ChA-1 cells were tested. Peak 3 had the strongest anticancer activity (data not shown). Peak 3 was further analyzed by high performance liquid chromatography (HPLC) with methanol elution. Figure 1B shows that the elution profile had only one main peak, which was pooled and named ESC-3.

Effect of ESC-3 on proliferation of human cholangiocarcinoma cells

To evaluate the cytotoxicity of ESC-3 on the proliferation of human cholangiocarcinoma cells, QBC939, Sk-ChA-1 and MZ-ChA-1 cells were treated with 5 μg/mL, 10 μg/mL and 15 μg/mL ESC-3 for 48 h, as shown in Figure 1C, the cell survivals of the three cholangiocarcinoma cell lines were all sensitive to ESC-3 in a dose-dependent manner and MZ-ChA-1 cells are more sensitive to ESC-3 than are the other human cholangiocarcinoma cell lines.

With ESC-3 concentrations of 5 μg/mL, 7.5 μg/mL, 10 μg/mL, 12.5 μg/mL, 15 μg/mL and 17.5 μg/mL, the proliferation of Mz-ChA-1 cells was inhibited significantly. After treatment with various concentrations of ESC-3 for 72 h, the survival rates were 64.3%, 53.2%, 31.3%, 19%, 11.2% and 5.8%, respectively (Figure 1D).

Morphological changes of Mz-ChA-1 cells after exposure to ESC-3

Giemsa staining was used to visualize morphological changes under an optical inverted microscope. After exposure to 10 μg/mL ESC-3 for 48 h, the Mz-ChA-1 cells showed typical apoptotic morphology. When compared with the untreated cells, the chromatin of the ESC-3-treated cells was condensed, and the size of the cells decreased. As shown in Figure 2, after AO/EB staining, the untreated Mz-ChA-1 cells displayed green fluorescence, while the ESC-3-treated cells emitted orange and red fluorescence and were smaller in size. The results of the Hoechst 33258 staining showed that the nuclei of un-

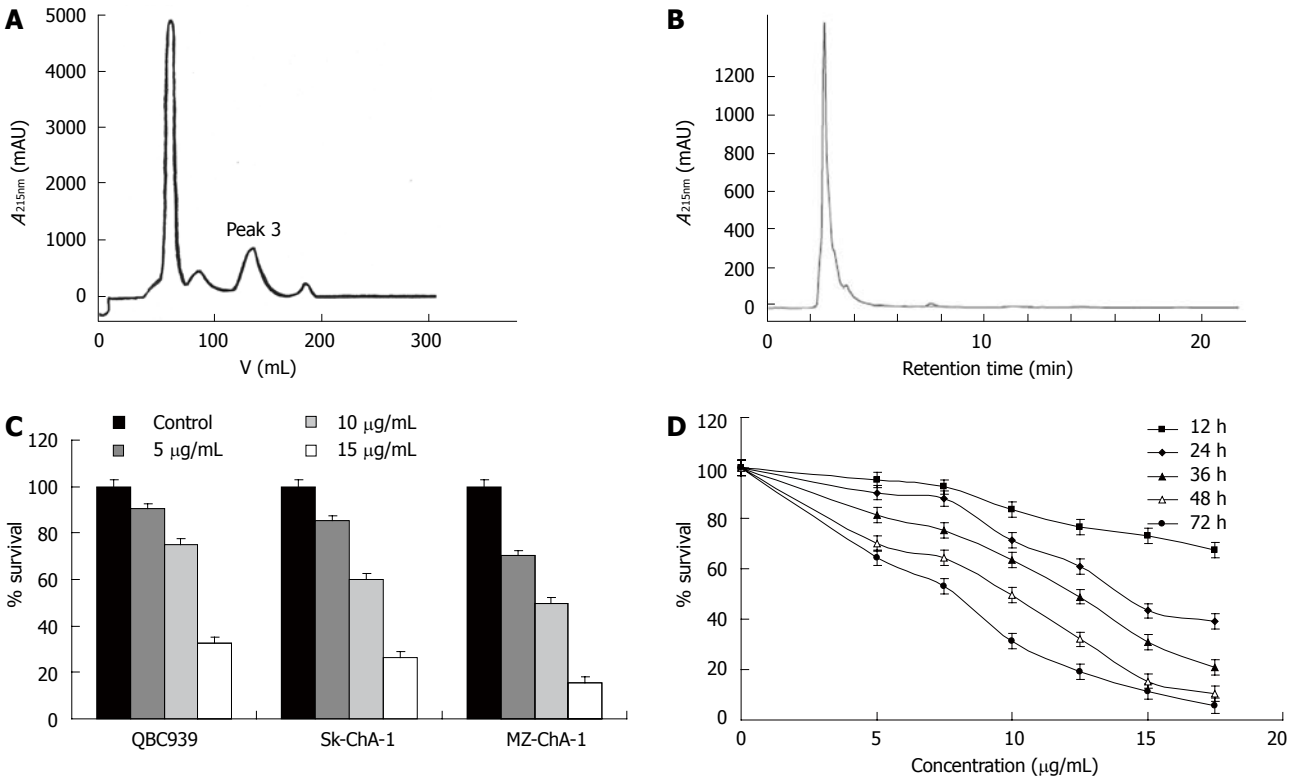

Figure 1 Isolation of ESC-3 from the gallbladder of *Crocodylus siamensis*. A: Using Sephadex LH-20; B: Using RP-18 reversed-phase columns; C: QBC939, Sk-ChA-1 and MZ-ChA-1 were treated continuously with different concentrations of ESC-3 for 48 h. Cell growth inhibition was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay; D: Growth inhibitory effects of ESC-3 on Mz-ChA-1 cells. Exponentially growing Mz-ChA-1 cells were treated with different concentrations of ESC-3 for different periods of time. Cell growth inhibition was analyzed by the MTT assay.

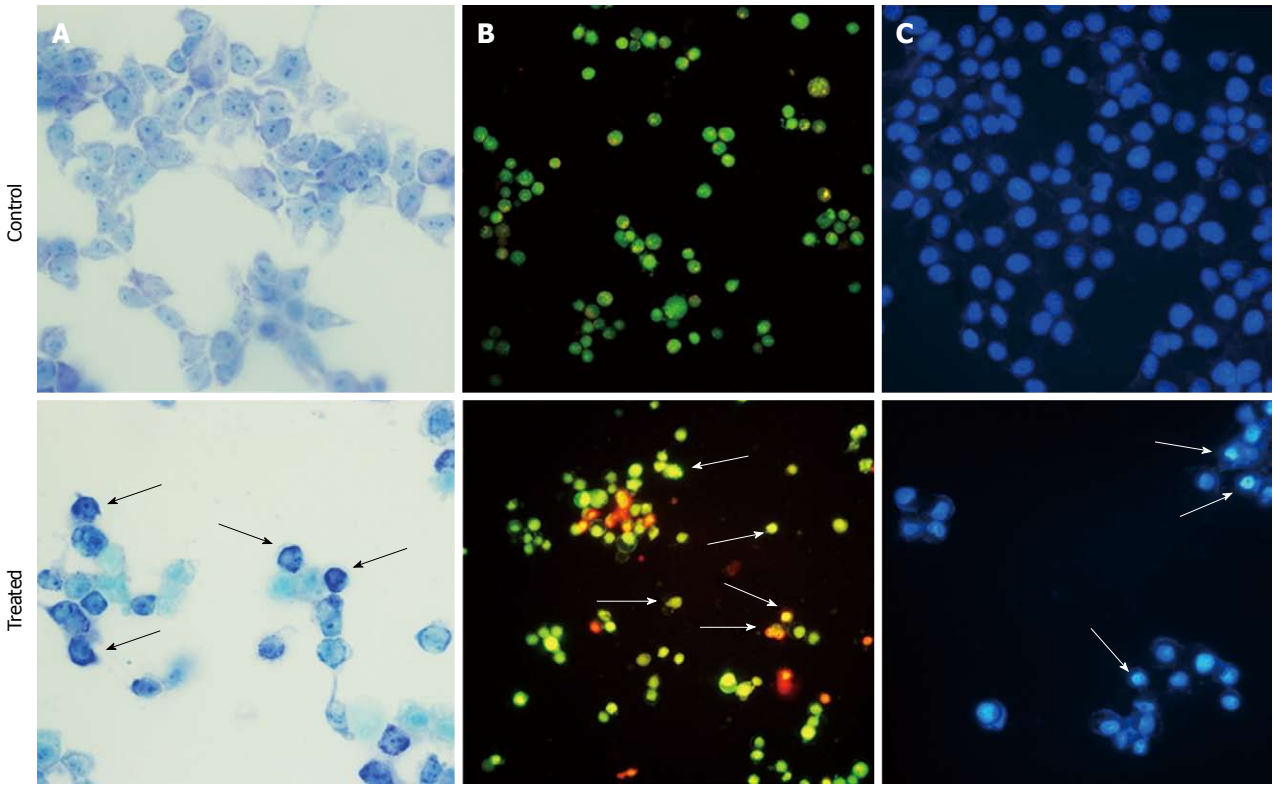

Figure 2 Morphological changes in the Mz-ChA-1 cells after exposure to different concentrations (0 μg/mL and 10 μg/mL) of ESC-3 for 48 h. A: Morphological changes visualized under an ordinary inverted phase-contrast microscope with Giemsa staining (magnification 400 ×); B: Morphological changes visualized under fluorescence microscope with AO/EB staining (magnification 200 ×); C: Morphological changes visualized under fluorescence microscope with Hoechst 33258 staining (magnification 400 ×). The arrows indicate the cells undergoing apoptosis. AO/EB: Acridine orange/ethidium bromide.

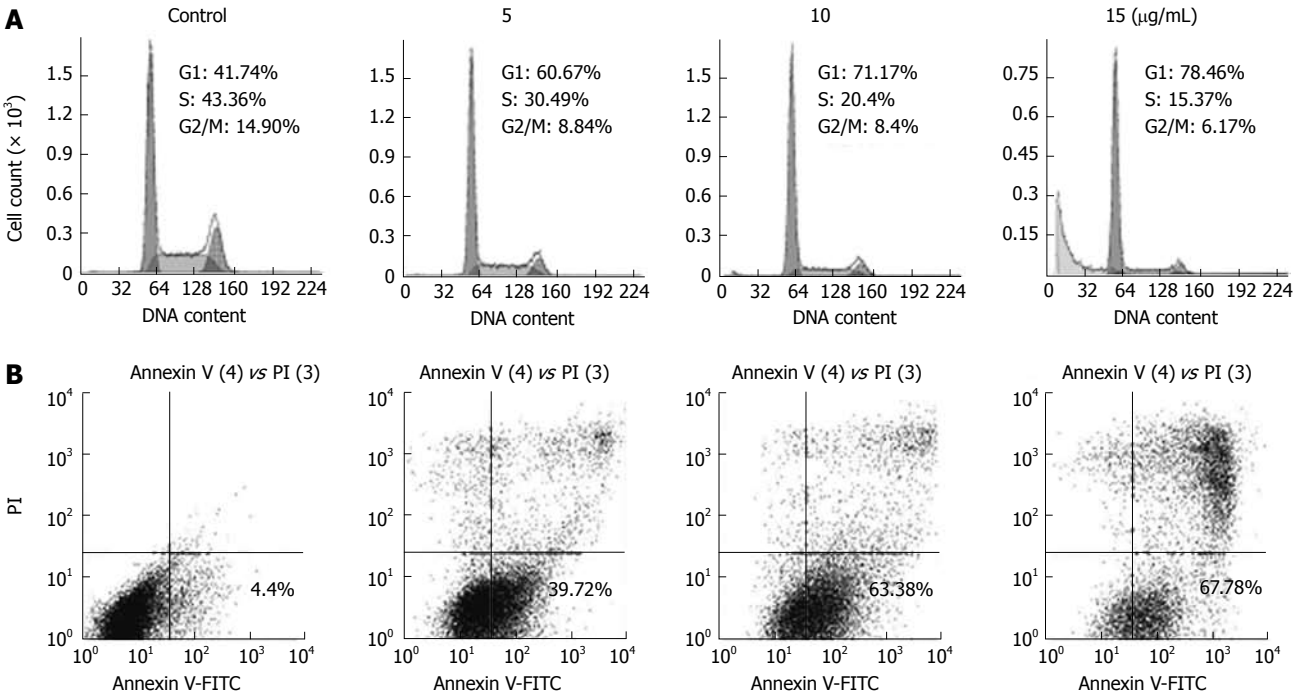

Figure 3 Effects of ESC-3 on cell cycle distribution and apoptosis. A: Cell cycle analysis of Mz-ChA-1 cells using flow cytometry with propidium iodide (PI) staining and the DNA histograms; B: Assessment of apoptosis using flow cytometry with annexin v-fluorescein isothiocyanate (V-FITC)/PI staining and the dot-plot graph of Mz-ChA-1 cells.

treated Mz-ChA-1 cells emitted a low fluorescence intensity in a homogeneous manner, and the nuclear structure was intact. Our data indicated that the ESC-3-treated Mz-ChA-1 cells displayed typical morphological features of apoptosis: condensed chromatin, gradual disintegration of the nuclear membrane, and pyknotic (shrunken and dark) nuclei.

Cell cycle analysis

To confirm that ESC-3 inhibited cellular proliferation by affecting the cell cycle distribution of the cells, we performed a cell cycle analysis after treatment with various concentrations of ESC-3 (0 μg/mL, 5 μg/mL, 10 μg/mL and 15 μg/mL). Forty-eight h after ESC-3 treatment, the cell cycle distribution of Mz-ChA-1 cells was altered in a dose-dependent manner. The percentage of cells in G0/G1 phase was 41.74% in the control group and increased to 78.46% after treatment with 15 μg/mL ESC-3, while the percentage of cells in G2/M phase decreased from 14.9% to 6.17% (Figure 3A). These results suggested that ESC-3 arrested Mz-ChA-1 cells at G0/G1 phase and suppressed cellular proliferation.

ESC-3-induced apoptosis

To further verify the apoptotic effect of ESC-3 on Mz-ChA-1 cells, flow cytometric analysis was conducted using dual staining with annexin V and PI, which was used to distinguish between viable, early apoptotic, late apoptotic or necrotic cells. As shown in Figure 3B, the population of early apoptotic cells increased with treatment with increasing ESC-3 concentrations (4.4%, 39.72%, 63.38% and 67.78% for 0 μg/mL, 5 μg/mL, 10 μg/mL and 15 μg/mL ESC-3, respectively). We, therefore, demonstrated that ESC-3 induced apoptosis.

Apoptosis via mitochondria-dependent pathway

Rhodamine 123 staining was used to measure the change in the mean fluorescence intensity as a measure of the mitochondrial membrane potential (ΔΨm), which drives the uptake and accumulation of Rh123 in the mitochondria. The hypofluorescence peak observed was indicative of a collapse in the ΔΨm and depolarization of the mitochondrial membrane. As shown in Figure 4A, significant changes in the mean fluorescent intensity of the cells and a collapse of the ΔΨm were observed in the Mz-ChA-1 cells 48 h after treatment with ESC-3 in a dose-dependent manner.

To investigate how ESC-3 induced apoptosis in Mz-ChA-1 cells, we examined the expression levels of apoptosis-related proteins, including Bax, Bcl-2, CDK2 and caspase-3, which are generally activated by the caspase-dependent apoptotic signal transduction pathways[12]. As shown in Figure 4B and C, after exposure to ESC-3 for 48 h, the procaspase-3 expression level was lowered, while the activity of caspase-3 increased significantly in dose- and time-dependent manners in Mz-ChA-1 cells. Moreover, the expression of Bax was increased, while the levels of the Bcl-2 and Cdk2 proteins significantly decreased; therefore, the ratio of Bax to Bcl-2 also increased in a time-dependent manner (Figure 4B). Furthermore, we observed that the levels of cytochrome c in the cytosol of ESC-3-treated Mz-ChA-1 cells increased in a dose-dependent manner. This suggests that the mitochondrial release of cytochrome c into the cytosol may

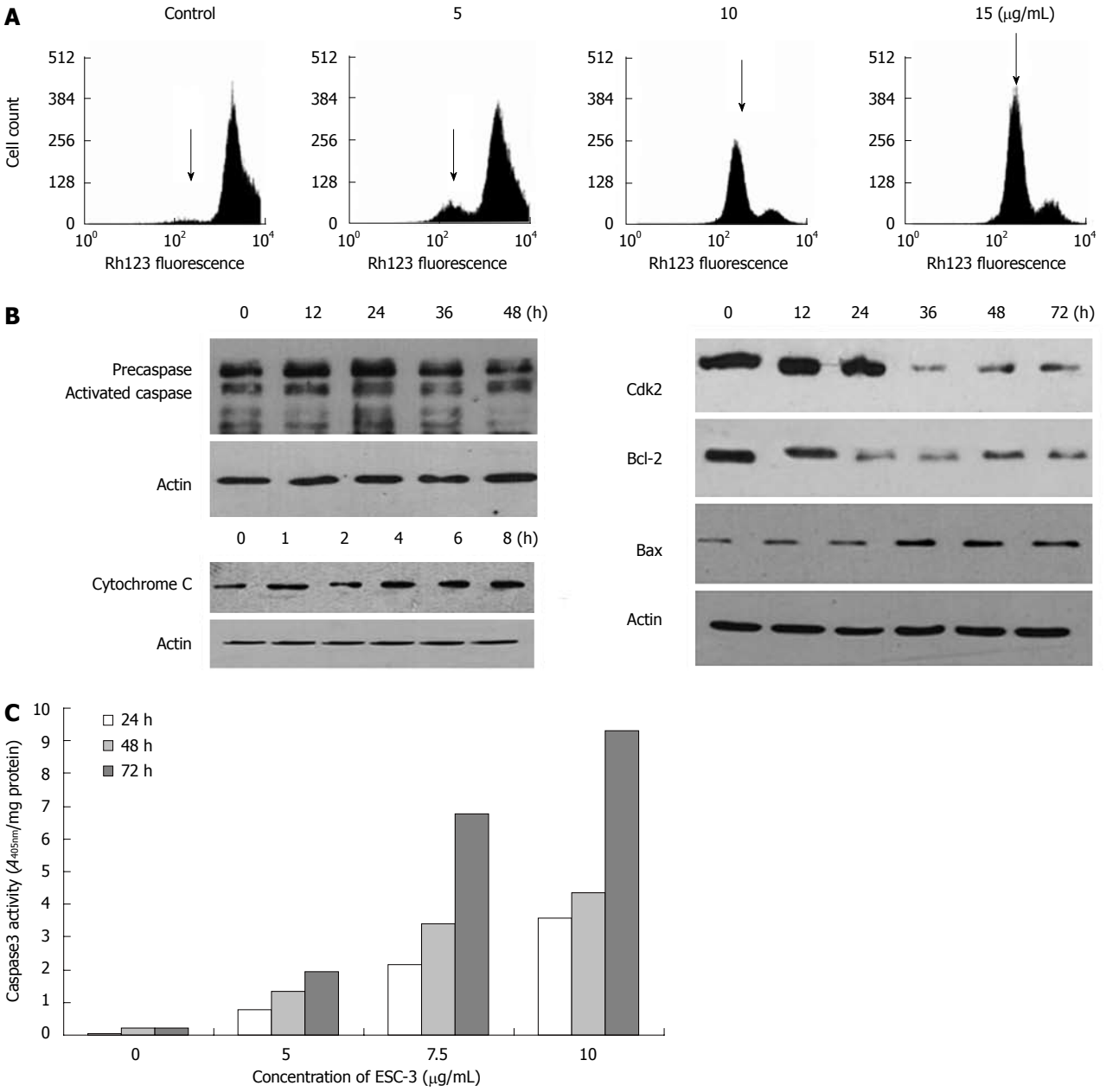

Figure 4 Apoptosis induced by ESC-3 through the mitochondria-dependent pathway. A: Effect of ESC-3 on the ΔΨm of cholangiocarcinoma cells. The increase in Rh123 hypofluorescence indicates a reduction in ΔΨm, which is shown with arrows; B: Expression of cytochrome C, caspase-3, CDK2, Bax, and Bcl-2 in Mz-ChA-1 cells treated with 10 μg/mL ESC-3 for different periods of time; C: Effect of ESC-3 on the activation of caspase-3 activity in Mz-ChA-1 cells. The cells were treated with 0 μg/mL, 5 μg/mL, 7.5 μg/mL and 12.5 μg/mL ESC-3, and caspase-3 activity was analyzed after 24 h, 48 h or 72 h.

play a role in induction of cell apoptosis by ESC-3.

DISCUSSION

Apoptosis is a process by which cells undergo programmed cell death under certain physiological or pathological conditions[13]. Apoptosis and its related signaling pathways have a profound effect on the progression of cancer[14]; therefore, the induction of apoptosis is a desirable goal for the prevention of cancer[15]. Recently, researchers have focused on screening novel anticancer drugs from organisms to identify compounds that could induce apoptosis.

In the present study, we isolated an anticancer compound, ESC-3, from the components of the *Crocodylus siamensis* bile. Our results demonstrated that ESC-3 significantly inhibited the proliferation of QBC939, Sk-ChA-1 and MZ-ChA-1 cells in a dose-dependent manner. Cell cycle arrest is one of the typical responses displayed by proliferating eukaryotic cells after exposure to DNA damaging agents, such as UV light and inhibitors[16]. To determine whether ESC-3 arrested the cell cycle in Mz-ChA-1 cells, we examined the cell cycle distribution of ESC-3-treated cells using flow cytometry. Our data indicated that ESC-3 induced a cell cycle arrest at G0/G1 phase.

Changes in cell morphology are the primary indicators of apoptosis[17-19]. There were significant morphological changes in Mz-ChA-1 cells after exposure to ESC-3,

including cell shrinkage, chromatin agglutination, marginalization, nuclear fragmentation, and apoptotic body formation. Early apoptotic cells exhibited bright annexin V-FITC fluorescence (annexin V-FITC positive only), and the proportion of apoptotic cells increased in a dose-dependent manner. It is reported that CDCA and DCA presented significant cytotoxic activities in ovarian cancer cells *via* inducing apoptosis[20], which is consistent with our results, suggesting that ESC-3 is capable of inducing apoptosis in Mz-ChA-1 cells.

The mechanisms of apoptosis mainly involve two signaling pathways: the mitochondrial pathway and the cell death receptor pathway[21]. Upregulation of Bax is often associated with the mitochondrial release of cytochrome c, which is a key element of the mitochondrial pathway[22,23]. Cytochrome c is regulated by the Bcl-2 family and has been shown to initiate the activation of caspase-3[24]. The Bcl-2 family consists of many important regulators of apoptosis, including Bcl-2, which prevents cells from entering apoptosis, and Bax and Bak, which induce cell death[25]. The increase in the ratio of Bax to Bcl-2 usually triggers cell death[22]. In the present study, we found that ESC-3 downregulated Bcl-2 expression while upregulating Bax expression, which resulted in an elevation of the ratio of Bax to Bcl-2 in Mz-ChA-1 cells. Hydrophobic bile acids have been reported to cause oxidative stress, DNA damage, and mitochondrial membrane instability in several cancer cells[26,27]. Mitochondrial dysfunction is correlated with DNA damage and oxidative stress[28]. ESC-3 also induced a loss of the mitochondrial membrane potential and the release of cytochrome c into the cytosol. Depolarization of the mitochondrial membrane was also detected. All these data suggest that DNA damage or oxidative stress may induce mitochondrial damage and dysfunction which finally triggers apoptosis in cancer cells. Thus, our data indicates that ESC-3-induced apoptosis of Mz-ChA-1 cells occurs through the mitochondrial pathway.

All of the typical signs of apoptosis result from a complex biochemical cascade of events[21]. A family of cysteine-dependent aspartate-directed proteases propagates death signaling by cleaving key cellular proteins. Caspase-3 is a well-known key executioner of apoptosis. Our results showed that ESC-3 activated caspase-3 and enhanced the levels of cleaved caspase-3 in a dose-dependent manner.

In summary, ESC-3 is a novel cytotoxic compound that blocked the proliferation of three cholangiocarcinoma cell lines and arrested the Mz-ChA-1 cell cycle at G0/G1 phase. ESC-3 induced apoptosis in Mz-ChA-1 cells in a dose-dependent manner *via* the mitochondria-dependent pathway. Understanding how ESC-3 regulates cell death would provide insight into the potential anticancer mechanisms and help screen novel active natural compounds for cancer treatment.

COMMENTS

Background

Cholangiocarcinoma is the second most common primary hepatic tumor and currently accounts for 3% of all gastrointestinal cancers; its incidence has recently been increasing globally. Progress in the diagnosis and treatment of cholangiocarcinoma has been achieved in recent years, but the prognosis is still unsatisfactory due to the lack of efficient anticancer drugs.

Research frontiers

Anti-tumor component ESC-3 isolated from crocodile bile could inhibit cell proliferation and induce apoptosis, and it may be a source of potential anti-tumor agent.

Innovations and breakthroughs

ESC-3 induced apoptosis in Mz-ChA-1 cells in a dose-dependent manner *via* the mitochondria-dependent pathway. Understanding how ESC-3 regulates cell death would provide insight into potential anticancer mechanisms and help screen novel active natural compounds for cancer therapy.

Applications

ESC-3, the active ingredient of crocodile bile may be a potential chemotherapeutic drug for the treatment of cholangiocarcinoma.

Peer review

This paper reports the effects of some extracts from crocodile's bile (ESC-3) on cholangiocarcinoma cells. In particular, these authors showed that ESC-3 inhibits cell proliferation, induces apoptosis and cell death. In addition, they provided evidences of Bax/Bcl-2 ratio increase and changes of the mitochondrial membrane potential in these cell lines. It is of interest to identify the natural products with anti-cancer activities in traditional Chinese medicine. Indeed, one component of the bear bile, i.e., the ursodeoxycholic acid, was found to have anti-cholestatic effect and it is now the only accepted therapy for many cholestatic disorders.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.712

World J Gastroenterol 2012 February 21; 18(7): 712-719
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Expression of OCT4 in human esophageal squamous cell carcinoma is significantly associated with poorer prognosis

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Received: May 20, 2011 Revised: July 14, 2011
Accepted: July 21, 2011
Published online: February 21, 2012

Abstract

AIM: To explore the expression pattern of OCT4 in human esophageal squamous cell carcinoma and its significance in diagnosis and prognosis.

METHODS: Using real-time polymerase chain reaction (PCR), Western blotting, immunocytochemistry and immunohistochemistry, the expression of OCT4 in three esophageal squamous cancer cell lines, KYSE70, KYSE140 and KYSE450, was characterized. OCT4 expression was investigated in a series of 153 esophageal squamous cell carcinoma samples using immunohistochemistry and explored its association with clinicopathological features.

RESULTS: Immunohistochemically, OCT4 positive immunostaining was observed in cancer cell nuclei. OCT4 was variably expressed in three esophageal squamous cancer cell lines. Among 153 specimens, 105 (68.7%) were negative or weakly positive for OCT4 staining; 21 (13.7%) were moderately positive and 27 (17.6%) were strongly positive. Higher expression level of OCT4 was significantly associated with higher histological grade ($P < 0.001$) and poor clinic outcome ($P < 0.001$).

CONCLUSION: The expression of OCT4 enables the tumor to have a higher degree of stemness, which in turn results in a poorer clinical outcome for patients with esophageal squamous cell carcinoma.

Key words: Esophageal squamous cell carcinoma; Immunohistochemistry; OCT4; Real-time polymerase chain reaction; Western blotting

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He W, Li K, Wang F, Qin YR, Fan QX. Expression of OCT4 in human esophageal squamous cell carcinoma is significantly associated with poorer prognosis. *World J Gastroenterol* 2012; 18(7): 712-719 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/712.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.712

INTRODUCTION

OCT4, also known as OCT3, belongs to the POU (Pit-Oct-Unc) transcription factor family[1]. The POU family of transcription factors can activate the expression of their target genes through binding the octameric sequence motif with an AGTCAAAT consensus sequence[2,3]. The expression of this gene is necessary for the maintenance of pluripotentiality in embryonic stem cells (ESCs) and primordial germ cells and is down-regulated in all differentiated cells *in vitro* as well as *in vivo*[2].

Previous studies have demonstrated that many cancers express OCT4 and that its expression appears to be important for cancer cell survival. Hattab *et al*[4] reported OCT4 immunoreactivity in all of 25 primary intracranial germinomas. Jin *et al*[5] discovered that the human breast

cancer cell line, MCF7, expressed at least four POU gene products including OCT4. Sung *et al*[6] proposed that with its superior sensitivity and easy interpretation compared with other markers, OCT4 immunostaining is a powerful tool for confirming the diagnosis of retroperitoneal seminoma. In addition, OCT4 has been shown to be expressed in human tumors including pancreatic and gastric carcinomas[7,8].

Recent studies have argued that the transcription factor, OCT4, exhibited the hallmark of global regulators during mammalian embryogenesis. OCT4 works together with SOX2, another type of transcription factor, which plays important roles in the regulation of organ development and cell type specification[9,10] during embryogenesis to co-ordinate their own transcriptions[11,12], *via* the OCT4/SOX2 complex in ESCs[13]. OCT4 constitutes part of an important gene regulatory network and is essential for embryogenesis and/or the pluripotency and self-renewal of ESCs[14].

Cancer cells, especially in poorly differentiated or undifferentiated tumors, have been characterized by many phenotypic traits similar to undifferentiated embryonic cells[15-17]. These similarities suggest the expression of genes determining cell renewal and stemness.

Esophageal cancer is one of the leading causes of cancer-related death worldwide, especially in some high risk populations in China, such as in Linxian (Western Anyang, Henan Province). In the present study, we first attempted to characterize the expression status of OCT4 in three esophageal cell lines, and then explore their clinicopathological and survival correlations in a series of 153 esophageal cancers in patients from Anyang, Henan, China. It was found that OCT4 was expressed in all three esophageal squamous cancer cell lines (KYSE70, KYSE140 and KYSE450). Furthermore, the expression of OCT4 was significantly associated with higher histological grade and poorer clinical survival, indicating that this embryonic self-renewal factor may exert a positive influence on tumor cell stemness, which in turn results in a negative clinical course for the tumors.

MATERIALS AND METHODS

Patients and cell lines

In this retrospective study, 153 consecutive esophageal cancer patients who underwent potentially curative surgery without preoperative chemotherapy or radiotherapy during the period of 1990-1994 at the Anyang Tumor Hospital, Henan, China were randomly selected. Among them, 93 were men and 60 were women, ranging from 33-73 years of age with a mean age of 56.4 years. According to the International Union Against Cancer (UICC) 1997 standard, 100 were classified as stage I or II and 53 cases as stage III or IV. The patients' information and tumor parameters are listed in Table 1. All the patients were followed at the Anyang Tumor Hospital until May 2004 supported by an international collaboration project between Anyang Tumor Hospital and the Norwegian Radium Hospital[18]. Among these patients, 97 (63.4%) patients died during the follow-up period. Surgically removed specimens were routinely fixed in buffered formalin and embedded in paraffin blocks for clinical diagnosis and reclassification for this study.

Table 1 Correlation of OCT4 expression with clinicopathological features of esophageal squamous cell carcinoma

Parameters	Cases	OCT4 Staining scores			*P* value
		0	1	2	
Age					0.493
< 51	48	30	6	12	
51-60	52	39	6	7	
> 60	53	36	9	8	
Gender					0.088
Male	93	70	10	13	
Female	60	35	11	14	
Location					0.716
Upper	14	10	2	2	
Middle	101	71	11	19	
Lower	35	23	7	5	
Missing	3				
Size					0.175
< 31 mm	25	22	2	1	
31-60 mm	105	67	17	21	
> 60 mm	14	10	1	3	
Missing	9	6	1	2	
Lymph node metastasis					0.296
-	99	72	11	16	
+	54	33	10	11	
UICC stage					0.276
Ⅰ + Ⅱ	100	73	12	15	
Ⅲ + Ⅳ	53	32	9	12	
T stage					0.407
Ⅰ	5	2	1	2	
Ⅱ	39	29	6	4	
Ⅲ	100	68	14	18	
Ⅳ	9	6	0	3	
Histological grade					< 0.001[a]
Well	53	48	5	0	
Moderate	60	48	3	9	
Poor	40	9	13	18	
Patients at follow-up					0.033[a]
Alive	56	45	3	8	
Dead	97	60	18	19	

UICC: International Union Against Cancer. [a]$P < 0.05$.

The human esophageal squamous cell carcinoma cell lines, KYSE70, KYSE140 and KYSE450 (DSMZ, Germany), were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 ℃ under 5% CO_2 and saturated moisture. The sample cells were collected and counted by trypan blue exclusion using a hemocytometer.

Immunocytochemistry

Immunocytochemistry was performed on all three cell lines. Simply, the cultured cells were detached and the cell suspension was centrifuged at 2000 rpm for 10 min. The supernatant was removed before the sediment was mixed with 2-3 drops of plasma and 2 drops of thrombin for 1 min, followed by the addition of 4% buffered formalin

to the coagulated cell mass, which was then fixed for 30 min and placed in linen paper for further conventional paraffin cytoblock preparation. Sections (4 μm thick) were cut from the paraffin-embedded cytoblocks and immunostained according to the same procedure as the tissue blocks described below.

Tissue arrays

Multitissue array blocks were made with the MTA-1 manual tissue arrayer (Beecher Instruments Inc., Sun Prairie, WI, United States). Briefly, 4 μm sections from the routinely made paraffin blocks were stained with H and E and reevaluated to confirm the diagnosis and to identify two representative tumor areas and one stromal area. Then, the related paraffin blocks were oriented and marked. From these blocks, tissue cores with a diameter of 0.6 mm were punched and arrayed in triplicate on a recipient paraffin block. When the block construction was complete, the block was placed into a 40 ℃ oven overnight to tighten the cylinders by slightly melting the paraffin. Then, 5 μm sections of these tissue array blocks were cut and placed on charged Super-Frost Plus glass slides and dried in a 60 ℃ oven for 2-4 h. These sections were used for immunohistochemical analysis. For those samples whose tissue array materials were not representative or not available, the paraffin-embedded conventional sections were also used for additional immunohistochemistry analyses.

Immunohistochemistry

Immunohistochemical analysis of OCT4 was performed on 5 μm sections which were prepared from the tissue microarray blocks. The Envision Plus detection system (Dako, Carpinteria, CA, United States) was used for immunostaining. The sections were deparaffinized in xylene and microwaved in 10 mmol/L citrate buffer (pH 6.0) to unmask the epitopes. Endogenous peroxidase activity was blocked by incubation with 0.03% hydrogen peroxide in methanol for 5 min. For the detection of OCT4, the sections were incubated with the polyclonal goat anti-human OCT4 antibody (microwaving retrieval in citrate buffer in 1:40 concentration, catalog no. AF1759, R and D Systems) for 30 min at room temperature. Then, mouse anti-goat IgG (sc-2489, Santa Cruz Biotechnology Inc, Santa Cruz, CA, United States) diluted at 1:100 was added for incubation for another 30 min followed by a gentle rinse with washing buffer three times. Thereafter, the sections were incubated with a peroxidase labeled polymer conjugated to goat anti-mouse IgG (Dako, Carpinteria, CA, United States) for 30 min before staining for 5 min with 3'3-diaminobenzidine tetrahydrochloride (DAB), counterstained by hematoxylin, dehydrated and mounted in Diatex. Known OCT4 positive seminoma was used as a positive control, while the same concentration of non-immune goat IgG was applied as a negative control for OCT4.

Evaluation of staining for OCT4

Only nuclear staining was considered as OCT4 positive. Both intensity and percentage of immunostained carcinoma cells were taken into consideration according to a previously published method with modification[19]. Briefly, the extent of positivity was scored as 0 when no positive cells were observed; 1 when the percentage of positive cells was < 10%; 2 when it was 10%-50%; and 3 when it was > 50%. The intensity was scored as 0 when no positive cells were identified; 1, weak; 2, moderate; and 3, strong staining. Multiplying the extent by intensity gave the following immunohistochemical staining grades as 0, 1, 2, 3, 4, 6 and 9. For statistical analyses, grades 0, 1 and 2 were considered as not or weakly stained and scored as 0, grades 3 and 4 were considered as moderately stained and scored as 1, and grades 6 and 9 were considered as strongly stained and scored as 2.

Total RNA and protein isolation

Total RNA and protein isolation was performed using a Total RNA and Protein Isolation kit (Macherey-Nagel, Düren, Germany) according to the user manual. About 5×10^6 cultured cells were collected and lysed. Through the NucleoSpin RNA/Protein column, RNA and DNA were bound to the column and protein was contained in the flow-through. After digestion of DNA, total RNA was isolated by washing the column. Protein was isolated from the flow-through and incubated for 3 min at 98 ℃ for dissolving and denaturation and stored at -20 ℃ until used. All of the preparations and handling steps of RNA took place in a laminar flow hood, under RNase-free conditions.

cDNA synthesis

RNA quality and quantity were determined by absorbance readings at 260 nm and 280 nm with the Nano Drop (ND-1000) spectrophotometer (Wilmington, DE, United States). RNA integrity was tested by polymerase chain reaction (PCR) amplification of the *gapdh*. Reverse transcription of RNA was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). cDNA was synthesized from 5 μg of total RNA isolated from the cell lines according to the manufacturer's handbook.

Primer and probe design

The primer pairs and hydrolysis probes for quantitative real-time PCR of *oct4* and *gapdh* were designed by Universal ProbeLibrary Assay Design Center (Roche, Mannheim, Germany). For *oct4*, the sense primer sequence was 5'-AGCAAAACCCGGAGGAGT-3' and the antisense primer sequence was 5'-CCACATCGGCCTGTGTATATC-3', giving a product of 114pb. For *gapdh*, the sense primer sequence was 5'-AGCCACATCGCTCAGACA-3' and the antisense primer sequence was 5'-GCCCAATACGACCAAATCC-3', giving a product of 66bp. The hydrolysis probes (#35, cat No. 04687680001 for *oct4*, and #60, cat No. 04688589001, for *gapdh*) were bought from Roche Diagnostics (Mannheim, Germany). In addition to the primer pairs for real-time PCR, the specific primer pair

for *oct4* which had been verified avoiding pseudogenes amplification by Suo *et al*[20] was also applied in this study for conventional PCR analyses.

Quantitative real-time PCR and conventional PCR

Quantitative real-time PCR was performed with the LightCycler 2.0 Real-Time PCR System (Roche, Mannheim, Germany) in a total volume of 20 μL in glass-capillaries containing 2 μL of cDNA, 0.5 μmol/L of each primer, 0.1 μmol/L of hydrolysis probe and 4 μL of LightCycler TaqMan Master Mix (Roche, Mannheim, Germany). Quantification was carried out according to a published method with some modification[21]. After the expression ratio value for target gene (*oct4*) *vs* house keeping gene (*gapdh*) was obtained, the ratio of KYSE450 was set as 1, and all other ratio values were normalized by this value as relative quantities. The PCR was initiated with a 12 min denaturation at 95 ℃ and terminated with a 30 s cooling step at 40 ℃. The cycling protocol consisted of denaturation at 95 ℃ for 10 s, annealing at 54 ℃ for 10 s and extension at 72 ℃ for 10 s and was cycled 45 times. Fluorescence detection was performed at the end of each extension step. The housekeeping gene *gapdh* and DEPC-H_2O were set as the internal control and negative control, respectively.

The specific conventional PCR for *oct4* was initiated with a 5 min denaturation at 95 ℃. Amplification was carried out for 30 cycles consisting of 30 s at 95 ℃, 50 s at 55 ℃ and 50 s at 72 ℃. An additional extension step of 5 min at 72 ℃ was added at the end of the cycles. *gapdh* was used as an internal control to confirm the success of the reverse-transcription action and the identical quantity of different cDNA templates. PCR products were analyzed by electrophoresis on 1.5% agarose gel.

Western Blot analysis

20 μL of protein samples were separated on a 10% SDS-acrylamide gel (Bio-Rad) for 1 h at 150V and the proteins were transferred to a nitrocellulose membrane (Whatman, Kent, United Kingdom). After blocking in 5% fat-free milk, the membrane was treated with the dilution of the primary antibody overnight at 4 ℃ and the dilution of the secondary IgG-horseradish peroxidase (HRP) conjugated antibody for 1 h at room temperature. The antibody used for immunohistochemistry was applied for Western blotting. The antibody was diluted in phosphate buffered saline containing 5% Blotto and 0.1% Tween-20. The stained membranes were visualized by an enhanced chemiluminescence reaction using the ECL Plus (GE Healthcare, Fairfield, CT, United States). Western blotting experiments were repeated at least three times on every sample with similar results.

Statistical analysis

Bivariate association between ordinal variables was assessed using Spearman's correlation (exact version). For categorical data, Pearson's χ^2 test was used. All tests of statistical significance were two-sided. Overall survival was calculated from the date of diagnosis to the date of death or May 1st, 2004. Survival curves were plotted according to the Kaplan-Meier method, and the log-rank test was used to determine significant differences among groups. Multivariate analysis according to Cox's proportional hazards regression model adjusted for clinicopathological factors (age, gender, tumor location, tumor size, lymph node metastasis, histological grade, and T stage) was performed to assess which tumor variables were independently correlated with overall survival. Statistical analyses were performed using the SPSS 16.0 package and $P < 0.05$ was considered as statistically significant.

RESULTS

Characterization of OCT4 in esophageal cancer cell lines

Quantitative real-time PCR was employed to analyze *oct4* mRNA level in three human esophageal squamous cell carcinoma cell lines (KYSE70, KYSE140 and KYSE450). Comparatively, the ratios of *oct4 vs gapdh* in KYSE70 and KYSE140 were 2.7 and 1.73 times that in KYSE450, respectively (Figure 1A). To further examine the protein level of OCT4, Western blotting analysis was performed on these three cell lines. Figure 1B shows 38KD OCT4 bands, which are in agreement with the NP_002692 OCT4 characterization on NCBI. The band of OCT4 in KYSE450 was weak, but in KYSE140 and KYSE70, it was strong and stronger, respectively. Immunocytochemistry of the cell lines cytoblock paraffin sections gave similar results as shown by RT-PCR and Western blotting (Figure 1D).

Six *oct4* pseudogenes have been proposed to exist using a bioinformatics approach to analyze the genomic nucleotide sequences[22]. To confirm the specific transcription of *oct4*, a specific primer pair for *oct4* conventional PCR examination was designed and the specificity of this pair was verified by Suo *et al*[20]. This primer pair was also applied in the present study for additional verification of OCT4 expression. As shown in Figure 1C, the predicted PCR products (408 bp) with the specific primer pair were detected in these cell lines, highly in KYSE70 and KYSE140 and weakly in KYSE450. This verified the immunocytochemistry and Western blotting results that OCT4 was variably expressed in the human esophageal squamous cell carcinoma cell lines.

OCT4 expression in tumor samples

Immunohistochemically, OCT4 positive immunostaining was observed in cancer cell nuclei (Figure 2). Among the 153 tumors, 100 were negative for OCT4 staining, and 5 were scored as 1 or 2. These tumors were again classified as either negative or weakly positive for OCT4 immunostaining (105 in total, 68.7%). Twenty-one (13.7%) were scored as 3 or 4 and classified as moderately positive. Twenty-seven (17.6%) were scored as 6 or 9 and classified as strongly positive (Table 2).

Clinicopathological correlations

The correlations between the clinicopathological features

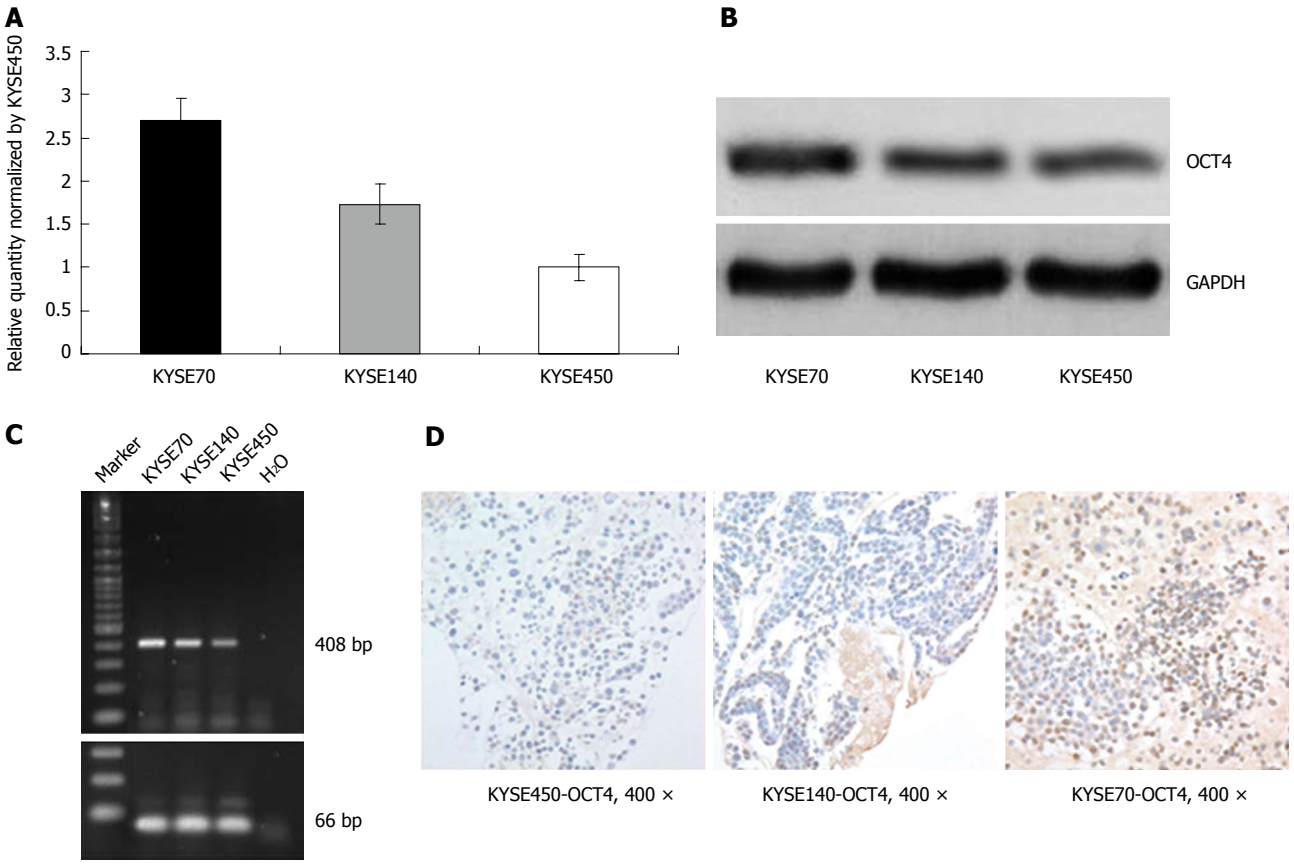

Figure 1 OCT4 expression in esophageal squamous cancer cell lines. A: The results of real-time polymerase chain reaction (PCR); B: The results of Western blotting; C: The results of conventional PCR; D: The results of immunocytochemistry. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

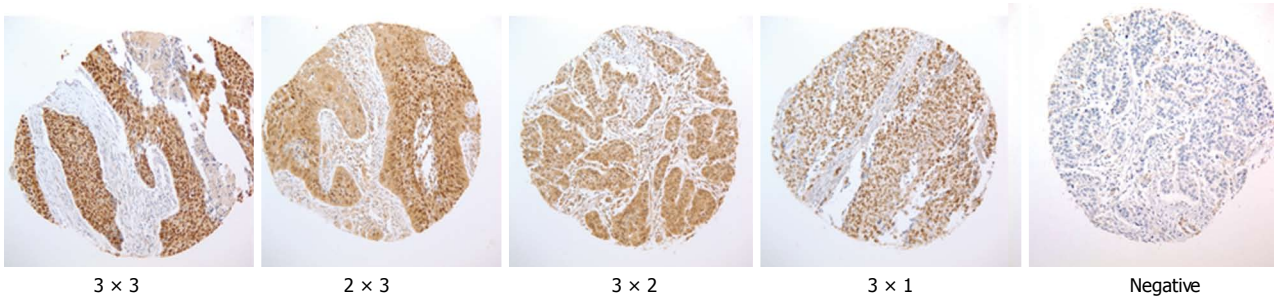

Figure 2 Evaluation of immunohistochemistry staining for OCT4 in tissue array materials of esophageal squamous cell carcinomas (multiplying the extent by intensity, × 400).

Table 2 Immunohistochemical staining results for OCT4

Score	OCT4	
	n	%
0	100	65.4
1	2	1.3
2	3	2
3	15	9.8
4	6	3.9
6	17	11.1
9	10	6.5
Total	153	100

and OCT4 expression in the primary tumors are summarized in Table 1. The expression level of OCT4 was not correlated with age, sex, tumor location, tumor size or clinical stage. However, a higher level of OCT4 expression was significantly associated with higher histological grade ($P < 0.001$), indicating its correlation with dedifferentiation of these tumors.

Relation to survival

Follow-up information was available for 153 patients for a minimum period of 10 years. The median follow-up time for the 56 patients still alive was 124 mo (range 118-155 mo) and for the remaining 97 patients who died during the follow-up period was 61 mo (range 1-139 mo). In univariate analysis, patients with low OCT4 expression

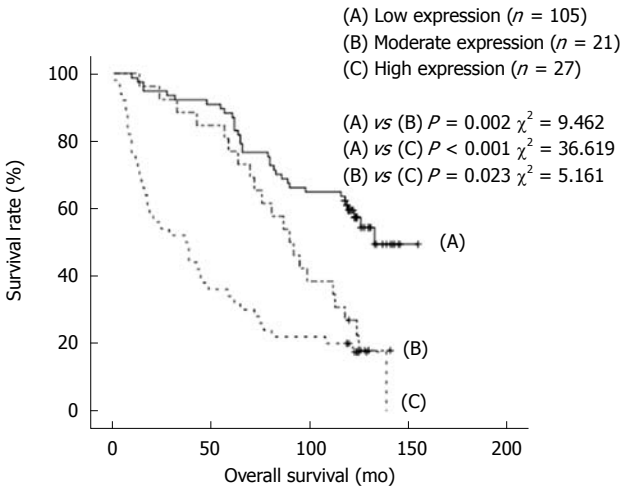

Figure 3 Kaplan-Meier survival curves for esophageal squamous cell carcinoma patients with regard to OCT4 protein expression.

level in tumors had a better overall survival than patients with tumor showing moderate or high OCT4 expression level ($P = 0.002$ and $P < 0.001$, respectively, Figure 3).

In multivariate Cox's regression analysis, we detected a 2.625-fold increased risk of tumor-related death for patients ($P < 0.001$) whose tumors showed high OCT4 expression level compared to patients with low expression level (Table 3).

Table 3 Multivariate analysis of death events related to esophageal squamous cancer

Parameters	Ratio of risk (RR)	95% CI	*P* value
Age	1.057	0.806-1.385	0.689
Gender	1.119	0.709-1.767	0.628
Location	1.170	0.785-1.745	0.441
Size	0.925	0.585-1.462	0.739
Lymph node metastasis	2.280	1.429-3.636	0.001[a]
T stage	1.885	1.204-2.951	0.006[a]
Histological grade	3.085	2.242-4.247	< 0.001[a]
OCT4	2.625	1.528-3.972	< 0.001[a]

CI: Confidence interval; [a]$P < 0.05$.

DISCUSSION

The expression of OCT4 was first discovered in human esophageal squamous cancer cell lines with the antibody AF1759 from R and D System for immunocytochemistry in the authors' lab. Since controversial results concerning the expression of OCT4 in tumors exist[23], the expression status of this factor in these cell lines was investigated. The expressions were repeatedly demonstrated immunocytochemically, with different positive and negative controls. To verify these findings, real-time PCR with hydrolysis probes was carried out, and repeatedly demonstrated similar results in these cell lines. Considering the fact that *oct4* pseudogenes are also transcribed[20], the *oct4* specific PCR primer pair (poct4a, 5'-TCCCTTCGCAAGCCCTCAT-3' and poct4b, 5'-TGACGGTGCAGGGCTCCGGGGAGGCCCCATC-3') were applied in conventional PCR for these three cell lines, obtaining similar results. All these experiments were repeated at least twice. The additional Western blotting analysis also disclosed similar results as shown by immunocytochemistry and PCR. Therefore, we can conclude that OCT4 is variably expressed in these esophageal squamous cancer cell lines.

Based on the findings on cell lines, this work was extended to clinical esophageal cancer samples for which official follow-up data were obtained during the international collaboration. To date, this study is the first to report the expression patterns of the embryonic stem cell factor OCT4 in esophageal squamous carcinomas.

Esophageal cancer is one of the most aggressive neoplasms and the overall prognosis for esophageal cancer patients is poor[24]. One of the reasons for the low survival rate is the tumor's intrinsic resistance to many clinical therapies, especially chemotherapy. Chemotherapy often removes the bulk of a tumor mass without preventing tumor recurrence, suggesting the survival of a subset of cancer stem cells. Recent studies have provided experimental evidence for the concept that human tumor growth may depend on a small portion of cancer stem cells[25].

The present investigation on esophageal squamous carcinomas revealed several novel observations. Firstly, it was discovered that 17.6% of these esophageal cancer samples had strong positive expression of OCT4. Secondly, although the expression level of OCT4 did not correlate with age, sex, tumor size, location, histological type or UICC stage, it was significantly associated with higher histological grade of the tumors and poorer clinical outcome of the patients.

It has been proposed that OCT4 is a key regulator of stem cell pluripotency and differentiation, indicating that it is the primary factor determining the fate of ESCs by controlling cell self-renewal and differentiation[26]. Phenotypically, human preimplantation embryonic cells resemble cancer cells in many ways, especially in their ability to grow indefinitely. Both types of cells undergo deprogramming to a proliferating state and become immortal, self-renewable and invasive. It has been reported that OCT4 is expressed in human tumors but not in normal somatic tissues[27], in agreement with the hypothesis that embryonic genes are re-activated in tumor cells.

In this series, OCT4 expression was strongly associated with the histological grade of the tumors and survival of the patients, which supports the findings of previous reports[28-30]. Given the previously reported functions of OCT4, the results suggest that it may play a role in conferring a less differentiated phenotype or inactivating the ability to differentiate in esophageal carcinomas.

It has been demonstrated that human somatic cells can be reprogrammed into pluripotent stem cells by either a combination of OCT4, SOX2, Nanog and Lin28 factors[31] or a combination of OCT4, SOX2, Klf4 and c-Myc factors[32]. A common feature of these studies is the contribution of OCT4 and SOX2 for pluripotency,

indicating that tumor cells with OCT4 expression may behave as or close to tumor stem cells. It is also known that SP positive tumor cells, which most probably harvest tumor stem cells, are resistant to chemotherapy and radiotherapy[33,34]. Thus, the present investigation on esophageal squamous cancer samples may indicate that OCT4 conveys a stemness feature in tumor cells, so that these tumors could easily develop and relapse, resulting in poorer clinical outcome.

The current results demonstrate that the ESC marker, OCT4, could be detected in human esophageal squamous cancer cell lines and related cancer tissues. The poorly differentiated esophageal squamous cancer cell lines expressed higher levels of OCT4. The higher expression level of OCT4 in esophageal carcinomas was correlated with higher histological grade and poorer overall survival of esophageal squamous cell cancer patients. Since the function of pluripotency and self-renewal of this factor has been characterized in adult human cells[31,32], the role of OCT4 in esophageal carcinogenesis, especially in consideration of tumor cell stemness, merits further studies.

COMMENTS

Background

The embryonic stem cell factor, OCT4, is essential for pluripotency and self-renewal of embryonic stem cells. Cancer cells, especially in poorly differentiated or undifferentiated tumors, have been characterized by many phenotypic traits similar to undifferentiated embryonic cells, indicating that OCT4 may be expressed in solid tumors.

Research frontiers

Previous studies have demonstrated that many cancers express OCT4 and that its expression appears to be important for cancer cell survival, such as in germinoma, breast cancer, seminoma, pancreatic and gastric carcinomas. However, the expression pattern of OCT4 in human esophageal squamous cell carcinoma and its significance in diagnosis and prognosis remain unclear. In this study, the authors demonstrated that OCT4 positive immunostaining was observed in cancer cell nuclei, and the expression of OCT4 enabled the tumor to have a higher degree of stemness, which in turn resulted in poorer clinical outcome for the patients with esophageal squamous cell carcinomas.

Innovations and breakthroughs

To date, this study is the first to report the expression patterns of the embryonic stem cell factor, OCT4, in esophageal squamous carcinomas. The present investigation revealed several novel observations. Firstly, it was discovered that 17.6% of these esophageal cancer samples had strong positive expression for OCT4. Secondly, although the expression level of OCT4 did not correlate with age, sex, tumor size, location, histological type or International Union Against Cancer stage, it was significantly associated with higher histological grade of the tumors and poorer clinical outcome of the patients.

Applications

By knowing that the embryonic stem cell (ESC) marker, OCT4, can be detected in human esophageal squamous cancer cell lines and related cancer tissues, and the higher expression level of OCT4 in esophageal squamous carcinomas was correlated with higher histological grade and poorer overall survival of esophageal squamous cell cancer patients, this study may represent a future strategy for therapeutic intervention in the treatment of patients with esophageal squamous carcinoma.

Terminology

OCT4 belongs to the POU (Pit-Oct-Unc) transcription factor family. The expression of this gene is necessary for the maintenance of pluripotentiality in ESCs and primordial germ cells and is down-regulated in all differentiated cells *in vitro* as well as *in vivo*.

Peer review

This study considers the investigation of the expression of the embryonic stem cell factor OCT4 in three esophageal cell lines and additionally in esophageal squamous carcinoma patients. Important finding of this study was the observation that OCT4 expression was strongly associated with the histological grade of the tumors and survival of the patients. The poorly differentiated esophageal squamous cancer cell lines expressed higher level of OCT4. This study make a great contribution to studies of OCT4 as one of the transcription factors and its role in cancer cell's survival. The study support the hypothesis that the expression of OCT4 as embryonic self-renewal factor may exert a positive influence on tumor cell stemness, which in turn results in a negative clinical course for the tumors.

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S- Editor Sun H **L- Editor** Webster JR **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i7.720

World J Gastroenterol 2012 February 21; 18(7): 720-726
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Pancreatic tuberculosis with acquired immunodeficiency syndrome: A case report and systematic review

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Received: October 13, 2011 Revised: December 23, 2011
Accepted: December 31, 2011
Published online: February 21, 2012

Abstract

Pancreatic tuberculosis (TB) is a relatively rare disease that can mimic carcinoma, lymphoma, cystic neoplasia, retroperitoneal tumors, pancreatitis or pseudocysts. Here, I report the case of a 31-year-old immigrant Burmese woman who exhibited epigastralgia, fever, weight loss and an epigastric mass. The patient was diagnosed with pancreatic TB and acquired immunodeficiency syndrome, and was treated with antituberculous drugs and percutaneous catheter drainage without a laparotomy. The clinical presentation, radiographic investigation and management of pancreatic TB are summarized in this paper to emphasize the importance of considering this rare disease in the differential diagnosis of pancreatic masses concomitant with human immunodeficiency virus infection. I also emphasize the need for both histopathological and microbiological diagnosis *via* fine-needle aspiration.

Key words: Pancreas; Tuberculosis; Abscess; Antituberculous drugs; Human immunodeficiency virus; Fine-needle aspiration

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Meesiri S. Pancreatic tuberculosis with acquired immunodeficiency syndrome: A case report and systematic review. *World J Gastroenterol* 2012; 18(7): 720-726 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i7/720.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i7.720

INTRODUCTION

Tuberculosis (TB) is an extremely common opportunistic infection in human immunodeficiency virus (HIV)-positive patients and is considered to be an acquired immunodeficiency syndrome (AIDS)-defining illness[1]. Because of the virulence of TB, its symptoms tend to manifest at an early stage of HIV infection. The most overt feature of TB in HIV-seropositive patients is their substantially greater likelihood of extrapulmonary involvement and dissemination[2]. Intraabdominal involvement is frequently observed in the abdominal lymph nodes (LNs), the spleen, the peritoneum, the liver, and the gastrointestinal tract[3]. However, even in HIV-infected patients, TB of the pancreas is relatively rare with an incidence of 0.46% based on ultrasonographic findings[4], and isolated primary pancreatic TB is particularly rare. Because of its rarity, the natural course of the disease is currently unknown. Several case reports have included a detailed review of this subject; however, we still lack a complete clinical picture of the disease[5-7]. Here, I report a case of pancreatic TB with AIDS that initially followed a classic pattern, but in which the patient subsequently exhibited an uncommon natural history of disease. I review the current literature regarding HIV-associated pancreatic TB. To the best of my knowledge, the present systematic review includes the largest number of published case reports on this subject, and is the first to describe the natural history of disease in a patient with pancreatic TB and AIDS.

CASE REPORT

In April 2010, a 31-year-old immigrant Burmese woman was admitted at Mae Sot General Hospital for approximately 1 wk due to epigastralgia. Two day before admission, she experienced constant severe abdominal pain and fever. She also reported weight loss of 5 kg over the preceding month. Upon admission, her temperature was 39.8 ℃, her pulse was 128 bpm, her respiratory rate was 18 breaths/min, and her blood pressure was 116/81 mmHg. Her weight and body mass index were 39 kg and 15.82 kg/m^2, respectively. She presented with oral thrush on her tongue and a pruritic papular eruption on her extremities and trunk. Her abdomen was bulging and contained a well-defined, round, smooth-surface, tender mass measuring 9 cm × 9 cm in the epigastrium. No hepatosplenomegaly or significant lymphadenopathy was observed.

A serological test for HIV was positive, and the patient's lymphocyte subset profile showed a CD4-positive cell count of 120 cells/mm^3 (range, 410-1 264/mm^3). A sputum examination for acid-fast bacilli (AFB) was negative, and a chest radiograph was normal. An ultrasound (US) of the upper abdomen revealed a lobulated, heterogeneous, hypoechoic cystic mass with septation and an irregular wall at the head of the pancreas (5.6 cm × 5.3 cm × 5.7 cm in size) (Figure 1). Multiple small LNs that ranged from 1.0 cm to 1.9 cm in diameter were observed in the paraaortic, peripancreatic, and porta hepatis regions.

US-guided fine-needle aspiration (FNA) of the mass was performed, and 50 mL of purulent, turbid fluid was harvested. The level of amylase in the cyst fluid was 33 801 U/L. An examination of a specimen using the Ziehl-Neelsen stain revealed AFB in the proteinaceous fluid and abundant acute inflammatory cells. An analysis of the cultures identified pure growth of *Mycobacterium tuberculosis* that was susceptible to isoniazid, rifampicin, ethambutol, and streptomycin. Consistent with the patient's clinical features, she was diagnosed with AIDS with a tuberculous pancreatic abscess. Note that a pancreatic pseudocyst typically contains an amylase-rich fluid, and when pus is present instead of sterile pancreatic juice, an infected pancreatic pseudocyst is referred to as a pancreatic abscess. Antituberculous drugs (ATDs) including isoniazid, rifampicin, pyrazinamide, and ethambutol were administered on the 6th d of admission. The treatment plan included quadruple ATDs for 2 mo followed by isoniazid and rifampicin for the next 7 mo. The patient was discharged after 12 d of hospitalization in an improved and stable condition, but she was still febrile at that time. Antiretroviral treatment was not planned due to a lack of monetary support.

The patient was readmitted 4 mo later due to epigastric fullness and shortness of breath. The epigastric mass was found to be enlarged. A physical examination revealed that the epigastric mass measured 15 cm × 15 cm in size. On admission, the patient had a temperature of 38.9 ℃, a pulse of 100 bpm, and a respiratory rate of 24 breaths/min; her blood pressure was 123/87 mmHg.

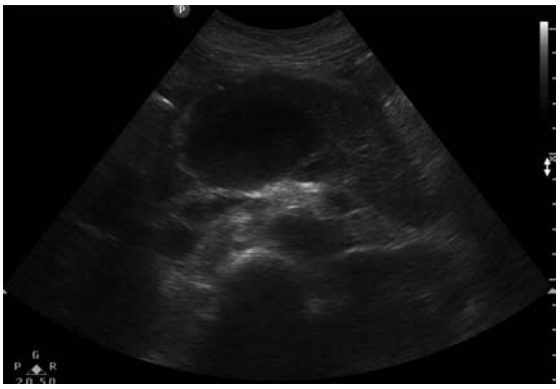

Figure 1 Ultrasound demonstrated a well-defined, lobulated cystic lesion with fluid debris at the pancreatic head. The size of the lesion is approximately 5.6 cm × 5.3 cm × 5.7 cm.

The patient's body weight was 39 kg. The patient's septic workups were unremarkable. An abdominal US revealed that the pancreatic cystic lesion had increased in size (11.8 cm × 8.7 cm × 11.6 cm) and contained a clear fluid (Figure 2A). Encasement of the celiac trunk was noted. Main portal vein compression and varices in the porta hepatis were observed (Figure 2B). A clear, serosanguinous fluid with an intracystic amylase level of 55 708 U/L was obtained from the pancreatic pseudocyst. Aerobic and mycobacterial cultures were negative. Although the patient's abdominal symptoms had improved, she still experienced an intermittent fever, cachexia, malnutrition (with a body weight of 34 kg and a serum albumin level of 21.0 g/L), and weakness within the month following admission. She continuously complied with the ATD regimen for 5 mo and 1 wk prior to discharge. Unfortunately, the patient was lost to follow-up after discharge and died 3 wk later (i.e., 6 mo after being diagnosed with pancreatic TB) in Myanmar from a suspected AIDS-related complication.

DISCUSSION

The PubMed, EMBASE databases, and Google were searched in July 2011 using the following terms in various combinations: tuberculosis, tuberculous, tubercular, mycobacterial infection and pancreas, pancreatitis, pancreatic, peripancreatic and HIV, AIDS, immunocompromised. The reference lists of the retrieved articles or of articles dealing with literature review were searched for additional studies. Only HIV-positive patients with informative clinical characteristics were reviewed.

A total of 43 cases of HIV-positive patients with pancreatic TB (including the present case) were reviewed[2,5-38]. Of these cases, 40 were primarily analyzed[2,5-23,25-38]. Table 1 illustrates the clinical characteristics of the pancreatic TB patients with AIDS. Most of the patients appeared to be affected between the age of 30 and 40 (this was the case in 53.7% of cases); the median age was 33 years with a range of 19-61 years, n = 41, and most of the patients (75.6%) were ≤ 43 years old. Five of the 40 patients (12.5%) had

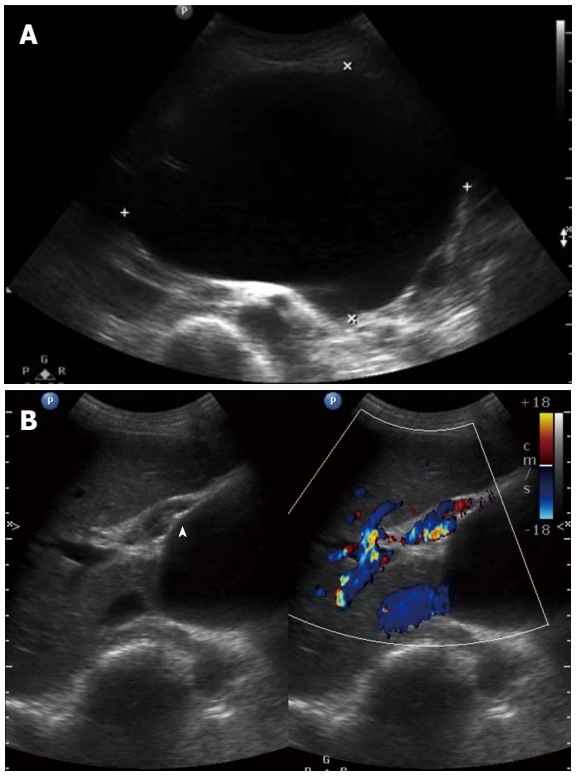

Figure 2 An abdominal ultrasound was performed 4 mo after discharge and revealed an increase in the size of the pancreatic cystic lesion (to 11.8 cm × 8.7 cm × 11.6 cm) with a clear fluid content (A). Portal vein compression (arrowhead) and varices in the porta hepatis were observed (B).

Table 1 Clinical characteristics, diagnostic studies, investigations yielding definitive diagnosis, preoperative diagnoses and definitive diagnosis of patients with pancreatic tuberculosis and acquired immunodeficiency syndrome

Clinical characteristics	*n* (%)
Gender[2,5-20,22-28,30-32,34-38]	
Male	28/39 (71.8)
Female	11/39 (28.2)
Known HIV-infected cases[2,5-23,25-38]	11/38 (28.9)
Positive tuberculin skin tests[2,5,6,9,12,13,16,29-31,33,34,38]	7/14 (50.0)
Symptoms[2,5-23,25-38]	
Abdominal pain	38/40 (95.0)
Fever	36/40 (90.0)
Weight loss	21/40 (52.5)
Anorexia	12/40 (30.0)
Night sweats	10/40 (25.0)
Nausea or vomiting	9/40 (22.5)
Diarrhea	7/40 (17.5)
Dysphagia	2/40 (5.0)
Cough	2/40 (5.0)
Dysuria, polyuria, and confusion	1/40 for each (2.5)
Signs[2,5-23,25-38]	
Epigastric mass	8/40 (20.0)
Significant superficial lymphadenopathy	7/40 (17.5)
Hepatomegaly	7/40 (17.5)
Oral thrush	6/40 (15.0)
Jaundice	6/40 (15.0)
Splenomegaly	2/40 (5.0)
Oral hairy leukoplakia, pruritic papular eruption, ascites, and disorientation	1/40 for each (2.5)
Diagnostic studies	*n* (%)
Evidence of TB on chest X-rays[2,5,6,8-10,12-15,17-20,23-25,27-32,34-37]	10/31 (32.3)
Abnormal CT scans[2,5-10,12-16,19-38]	
Pancreatic/peripancreatic mass with or without diffuse pancreatic enlargement	34/36 (94.4)
Only diffuse pancreatic enlargement	1/36 (2.8)
Small nodular lesions	1/36 (2.8)
Topographic lesions of the pancreatic mass from all imaging studies[2,5-10,12,13,15,16,18-25,27,29-38]	
Head of the pancreas	31/36 (86.1)
Body of the pancreas	8/36 (22.2)
Neck of the pancreas	3/36 (8.3)
Tail of the pancreas	2/36 (5.6)
Diagnostic clues of a simultaneous duodenal fistula	
Abnormal upper gastrointestinal study[13]	1/1 (100.0)
Abnormal gastroscopy[14,17,27,33,38]	2/5 (40.0)
Investigations yielding definitive diagnosis	(*n* = 40)[2,5-23,25-38] (%)
FNA with AFB staining and/or mycobacterial culture[a]	23 (57.5)
Laparotomy	14 (35.0)
Therapeutic diagnosis with evidence of miliary TB[b]	1 (2.5)
Discharge from pancreaticoduodenal fistula	1 (2.5)
Autopsy	1 (2.5)
Preoperative diagnoses	(*n* = 15)[2,5,7-13,16-17,19,22,25] (%)
Pancreatic mass	4 (26.7)
Pancreatic cancer	3 (20.0)
Pancreatic abscess of unknown pathogen	3 (20.0)
Acute pancreatitis	2 (13.3)
Tuberculous pancreatic abscess[c]	2 (13.3)
Lymphoma or retroperitoneal tumor	1 (6.7)
Definitive diagnosis	(*n* = 40)[2,5-23,25-38] (%)
Tuberculous pancreatic abscess without duodenal fistula	26 (65.0)
Tuberculous pancreatic abscess with duodenal fistula	2 (5.0)
Acute tuberculous pancreatitis without duodenal fistula	2 (5.0)
Acute tuberculous pancreatitis with duodenal fistula	1 (2.5)
Focal acute tuberculous pancreatitis with chronic pancreatitis	1 (2.5)
Pancreatic TB	8 (20.0)

TB: Tuberculosis; AIDS: Acquired Immunodeficiency Syndrome; HIV: Human Immunodeficiency Virus; CT: Computed tomography. [a]One was performed from the left supraclavicular lymph node instead of the pancreatic mass, and one patient underwent both fine needle aspiration and laparotomy due to clinical deterioration. [b]No histopathological specimens. [c]Diagnosis was based on a computed tomography scan only in one patient.

a confirmed history of TB; in 3 cases, the patient had had a positive purified protein derivative skin test, and there were 2 cases of pulmonary TB. The median CD4 count (n = 21) was 54/mm^3 (range, 0-718/mm^3); 76.2% and 90.5% of the patients had a CD4 count ⩽ 120 or ⩽ 190/mm^3, respectively. The median duration of abdominal pain (n = 31) before presentation was 30 d (range: 3 d-1 year).

The results of the diagnostic studies conducted in cases of pancreatic TB with AIDS are summarized in Table 1. The types of abdominal imaging studies (n = 43) used included computed tomography (CT) scanning (n = 36), US (n = 27), endoscopic US (n = 1), and magnetic resonance imaging (n = 1). In at least 20 cases, both US and CT scans were performed. A CT scan usually confirmed or completed the US findings and revealed a pancreatic/peripancreatic mass with or without diffuse pancreatic enlargement; this was true in all but 2 cases.

The masses ranged from cystic or hypodense masses to complex soft tissue masses or mixed solid/cystic masses. The CT data for the remaining cases indicated only diffuse pancreatic enlargement or small nodular lesions (< 1 cm) in the pancreas[24]. The pancreatic masses were 2-8 cm in diameter. Abdominal lymphadenopathy was detected as follows: peripancreatic LN (n = 9), retroperitoneal LN (n = 7), paraaortic/aortocaval LN (n = 4), porta hepatic/hepatic hilum LN (n = 4), mesenteric LN (n = 2), periportal LN (n = 2), unspecified abdominal LN (n = 2), celiac LN (n = 1), and splenic hilum LN (n = 1). Hepatomegaly and splenomegaly were noted in 10 and 9 patients, respectively. Concomitant hepatic and splenic granulomas and concomitant splenic and kidney granulomas were each observed in one patient. Other associated conditions included ascites (n = 3), extrahepatic bile duct obstruction and dilatation (n = 2), pancreatic duct dilatation (n = 1) or compression (n = 1), and ileocecal thickening (n = 1).

The diagnoses for the pancreatic TB cases are summarized in Table 1. FNA was attempted in 29 cases, but was successful in only 25 (86.2%), 2 of which did not include a documented AFB smear or mycobacterial culture. Only one specimen was successfully obtained using endoscopic US-guided aspiration[38], whereas the other specimens were obtained percutaneously[6,10,15,18,21,25-32,34-37]. In the cases in which AFB smears and/or mycobacterial cultures of the FNA specimens were conducted, 20 of 20 (100.0%) and 18 of 19 (94.7%), respectively, were positive. In the sole culture-negative FNA sample, *Mycobacterium tuberculosis* (*M. tuberculosis*) DNA was identified *via* the polymerase chain reaction (PCR) method. ATD susceptibility was studied in 9 samples, and only one was resistant to streptomycin; the others were sensitive to the prescribed first-line ATDs. PCR was performed in 4 cases, and all 4 were positive for *M. tuberculosis* DNA. The probability of positive results from at least one mycobacterial culture or AFB smear for body fluid (excluding the pancreatic mass) was 32.5% (13/40). In these cases, mycobacterial cultures of sputum (n = 8), blood (n = 3), bronchoalveolar lavage (n = 2), urine, ascites fluid, pleural effusion, stool, or preauricular LN (n = 1 for each) were taken, as were ABF smears of urine, bone marrow, or supraclavicular LN (n = 1 for each). Active pulmonary TB was documented in 9 cases.

The preoperative diagnoses made in all 15 cases in which laparotomies were conducted are summarized in Table 1. A provisional diagnosis of acute pancreatitis with a pseudocyst was made in one autopsy case[14]. Pancreatic TB was suspected in each of the cases in which FNA was performed; an AFB smear and/or mycobacterial culture was performed in each of these cases. The definitive diagnoses of pancreatic TB are listed in Table 1.

A laparotomy and/or FNA was performed in every case except for one that was diagnosed after autopsy and another in which a presumptive diagnosis was made based on an imaging study and a positive mycobacterial sputum culture[23]. There were 13 cases in which an exploratory laparotomy for a biopsy and/or open drainage was considered due to an uncertain diagnosis (n = 11) or a deteriorating tuberculous pancreatic abscess (n = 2). A distal pancreatectomy/ splenectomy was performed in one case for an unknown reason. In a second case, a Whipple's operation was performed to treat suspected pancreatic cancer.

Thirty-four patients received ATD treatment. The most commonly used treatment was a quadruple ATD regimen that consisted of isoniazid, rifampicin, pyrazinamide, and ethambutol (n = 15). The initial response to the ATDs usually occurred rapidly, manifesting between 72 h and 2 wk later; however, fever and cachexia occasionally persisted for years. The duration of ATD treatment ranged from 3 wk to 2 years. Of the 37 documented patients, 33 (89.2%) survived and were dischargeable after their admission. The remaining 4 patients deteriorated and died during their initial hospitalization (yielding a 10.8% in-hospital mortality rate) due to upper gastrointestinal bleeding[12], severe sepsis[14], viral meningoencephalitis[30], or an unreported cause (n = 1 for each)[31]. Twenty-four patients were followed from 3 wk to 32 mo (median, 9.50 mo), and 9 died during the follow-up period from AIDS-related causes, including full-blown AIDS (n = 5)[25,26], disseminated cryptococcosis[9], non-Hodgkin's lymphoma[2], pyogenic chest infection[18], and *Pneumocystis jirovecii* pneumonia (n = 1 for each)[7]. The probability of survival after discharge was determined *via* Kaplan-Meier analysis. A 35.9% long-term survival rate emerged with a median survival period of 18 mo. No recurrence of TB was found in the pancreas or in other organs. In 15 cases, follow-up abdominal imaging data were available. In all but one of these cases (the present case), the size of the pancreatic mass decreased. Antiretroviral drugs were prescribed in only 6 cases; most of the reports did not mention any antiretroviral treatment.

Pancreatic TB is extremely rare, even in countries in which TB is highly prevalent. Pancreatic TB most often occurs as a complication of miliary TB[9]. The low frequency of pancreatic TB may be partly due to the biological resistance of the pancreas to tubercular infection. Pancreatic enzymes, including lipases and deoxyribonucleases, have antimycobacterial effects[28]. However, the incidence of pancreatic TB has recently increased. In India, Bhansali did not discover a single case of pancreatic TB in a review of 300 cases of abdominal TB in 1977[39]; however, a recent study of collective data from 1999-2004 from the same endemic area detected pancreatic TB in 8.3% of the 384 patients who were diagnosed with abdominal TB[40]. Globalization, the HIV pandemic, and the worldwide resurrection of *M. tuberculosis* are all responsible for this increasing incidence[41]. Pancreatic TB typically presents in the following patient types: in patients who reside in endemic tuberculous zones, sporadically in no-risk healthy patients, and in patients who are immuno-compromised[26]. In AIDS cases, tuberculous pancreatic abscesses are most common, accounting for 70.0% of cases. In addition,

71.1% of cases include no previous serological evidence of HIV infection, and 76.2% of patients are severely immunocompromised hosts with a CD4 cell count of $\leqslant 190/mm^3$. If the diagnosis is delayed, pancreatic TB can be fatal; the disease has a 10.8% mortality rate (which is comparable to the mortality rate of 9.1% in immunocompetent patients)[42]. However, pancreatic TB responds well to standard ATDs.

Diagnosing pancreatic TB is challenging. From 1989-1998, 13 of 21 cases were diagnosed postoperatively or postmortem, although this was true for only 2 of 19 cases in subsequent years. This trend indicates increasing surgeon awareness of cases in HIV-infected patients. Pancreatic TB can be classified radiologically as follows: the most common form is mass-forming (with or without diffuse pancreatic enlargement) and accounts for 94.4% of cases, but there is also a diffuse form and a small, nodular form[24]. The masses can be radiographically similar to pancreatic tumors, abscesses, lymphomas, or pseudocysts. US scans of the abdomen are simple, non-invasive, cost-effective, and readily available; thus, they are usually used as an initial diagnostic tool and exhibit excellent sensitivity. Furthermore, US scans can reveal focal hypoechoic lesions[12,13,18,20,21,32,34,35,38] and heterogeneously hypo-isoechoic lesions[5,12,14,18] (primarily in the head of the pancreas), diffuse enlargement of the pancreas[14,20,23] and enlarged peripancreatic[12,21,25] and other abdominal LNs[2,11,18,20,21,31]. Based on the 90.6% sensitivity of association (half of all pancreatic TB patients are HIV-positive), Nagar *et al*[40] recommended that TB of the pancreas be a differential diagnosis for pancreatic masses associated with peripancreatic lymphadenopathy. Occasionally, biliary dilatation results from the obstruction of the common bile duct[18,35], pancreatic duct dilatation[12], air bubble(s) within the mass[12], encasement of the celiac artery, or compression of the portal vein with collaterals (as in the present case). Tubercular etiology should be suspected in the case of certain ancillary findings, including characteristic lesions in other solid viscera such as the liver, kidney, or spleen; hepatomegaly[12,31]; splenomegaly[12,31]; ascites[12,20]; peritoneal nodularity; mural thickening in the ileocecal area[20]; pulmonary TB[2,11,12,14,18,19,23,28,32]; or pleural effusion[14]. Because of its high sensitivity, a CT scan should be used to rule out associated pathology and to plan for further disease management. However, radiographic signs of pancreatic TB are neither specific nor pathognomonic, and most radiographic findings can be observed in pancreatitis of any cause or pancreatic carcinoma[20].

A definitive diagnosis is usually based on a histopathological or microbiological examination of a specimen that is obtained from the pancreas or based on peripancreatic LNs exhibiting chronic granulomatous inflammation with caseous necrosis and multinucleated giant cells. In addition, an AFB smear using Ziehl-Neelsen or auramine staining can also be employed for this purpose. Nevertheless, in pancreatic TB cases with advanced HIV-associated immunosuppression and AIDS, there is a striking paucity of granuloma formation with little cellular recruitment, and the AFB findings are copious[43]. In non-HIV-infected patients, the success rate of FNA in diagnosing pancreatic TB is 50.0% for specimens that are obtained percutaneously[42]. This rate is much lower than the 85.7% rate in HIV-infected cases in the present study, which reflects the operator-dependent nature of the procedure. Therefore, it is reasonable to attempt a second percutaneous image-guided FNA by a more experienced operator or an endoscopic US-guided FNA if the first attempt fails. The extremely high identification rate (100.0%) of acid-fast smears of FNA specimens from HIV-positive individuals, compared with the 23%-38% detection rate associated with mixed specimens from HIV-negative individuals[28,44], indicates the much higher pathogen burden and lower immunological response in these immunocompromised hosts. As a result, and given the simplicity and availability of the test and the rapid results that it produces, FNA with an acid-fast smear should be a required test for pancreatic TB with immunodeficiency. A PCR assay, when used to detect mycobacterial DNA, yields highly specific same-day results. Although its sensitivity to TB in FNA specimens has not yet been determined, the PCR assay is increasingly used as an adjunct to special staining techniques and mycobacterial cultures. It may yield positive results even when specimen cultures are negative[31,32].

In cases of AIDS with suspected pancreatic TB, mycobacterial smears and cultures from the sputum, bronchoalveolar lavage, blood, urine, stool, bone marrow, superficial LNs, ascites, or pleural effusion specimens should be performed due to the augmented yield of these sites. The more aggressive the investigation, the more likely it is that a primary infection will be discovered. In a study of abdominal TB in HIV infection, all of the cases exhibited at least one extraabdominal TB site (with 3.9 sample sites/case). The incidence of positive mycobacterial cultures from various sites was as follows; extraabdominal LNs (87%), sputum (82%), blood (74%), stool (63%), ascites (67%), bone marrow (56%), and urine (35%). Disseminated TB was present in 93% of cases[3]. Therefore, the total number of case reports of primary or isolated pancreatic TB may be overestimated, and it may thus be exceedingly rare.

In most cases of pancreatic TB, medication is the preferred treatment; surgery and the drainage of fluid are not preferred. A standard multiple ATD regimen with directly observed therapy for 6-12 mo is usually effective[5]. In the present case, the fluid was clear and sterile even though the pancreatic mass was subsequently further enlarged. However, if the tuberculous pancreatic mass is enlarged or causes symptoms even after the ATDs have been employed for a reasonable period, minimally invasive procedures should be considered. These might include percutaneous catheter drainage (preferably in patients with no pancreatic duct-pseudocyst communications and pancreatic duct strictures, those with immature

or infected pseudocysts, those at high surgical risk, or those who exhibit malnourishment)[45] or endoscopic internal drainage. Although the prognosis for this disease is good in immunocompetent patients (only 1 out of 58 cases that were reported in the Chinese-language literature resulted in death[41]), the prognosis is grave in AIDS patients due to the underlying disease[25], particularly in settings where antiretroviral therapy is unavailable[46].

Pancreatic TB is extremely rare, has various clinical presentations, and tends to masquerade as a pancreatic malignancy, cystic tumor, or pseudocyst. In HIV-infected patients, a differential diagnosis of a pancreatic mass that is combined with a histopathological and microbiological diagnosis *via* FNA can often prevent unnecessary surgery.

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S- Editor Gou SX **L- Editor** Cant MR **E- Editor** Zhang DN

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World J Gastroenterol 2012 February 21; 18(7): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2012 February 21; 18(7): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

November 9-13, 2012
American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel Diseases
Hollywood, FL 33028, United States

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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2012 February 21; 18(7): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1352 experts in gastroenterology and hepatology from 64 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

Maximization of personal benefits

The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJG* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJG* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJG* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion; and (4) Maximization of the benefits of employees: It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality articles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

Aims and scope

The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

Columns

The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

Name of journal

World Journal of Gastroenterology

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

Editorial office
World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
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Indexed and abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifer, and Directory of Open Access Journals. ISI, Thomson Reuters, 2010 Impact Factor: 2.240 (35/71 Gastroenterology and Hepatology).

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SPECIAL STATEMENT

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Biostatistical editing
Statistical review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc*. The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

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In the interests of transparency and to help reviewers assess any potential bias, *WJG* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

Sample wording: [Name of individual] has received fees for serving as a speaker, a consultant and an advisory board member for [names of organizations], and has received research funding from [names of organization]. [Name of individual] is an employee of [name of organization]. [Name of individual] owns stocks and shares in [name of organization]. [Name of individual] owns patent [patent identification and brief description].

Statement of informed consent
Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics committee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. Authors should also draw attention to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964, as revised in 2004).

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When reporting the results from experiments, authors should follow the highest standards and the trial should conform to Good Clinical Practice (for example, US Food and Drug Administration Good Clinical Practice in FDA-Regulated Clinical Trials; UK Medicines Research Council Guidelines for Good Clinical Practice in Clinical Trials) and/or the World Medical Association Declaration of Helsinki. Generally, we suggest authors follow the lead investigator's national standard. If doubt exists whether the research was conducted in accordance with the above standards, the authors must explain the rationale for their approach and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study.

Before submitting, authors should make their study approved by the relevant research ethics committee or institutional review board. If human participants were involved, manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and appropriate informed consent of each. Any personal item or information will not be published without explicit consents from the involved patients. If experimental animals were used, the materials and methods (experimental procedures) section must clearly indicate that appropriate measures were taken to minimize pain or discomfort, and details of animal care should be provided.

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Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of Baishideng Publishing Group Co., Limited, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the ICMJE to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www.clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

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Title: Title should be less than 12 words.

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Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

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Abstract

There are unstructured abstracts (no less than 256 words) and structured abstracts (no less than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no less than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections.

AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/…"; MATERIALS AND METHODS (no less than 140 words); RESULTS (no less than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$; CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:...; B:...; C:...; D:...; E:...; F:...; G: …*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of *P* values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of *P* values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunolog-

ic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

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Organization as author

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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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World Journal of Gastroenterology®

Volume 18 Number 8
February 28, 2012

Published by Baishideng Publishing Group Co., Limited
Room 1701, 17/F, Henan Building,
No. 90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-31158812
Telephone: +852-58042046
E-mail: bpg@baishideng.com
http://www.wjgnet.com

ISSN 1007-9327 CN 14-1219/R Local Post Offices Code No. 82-261

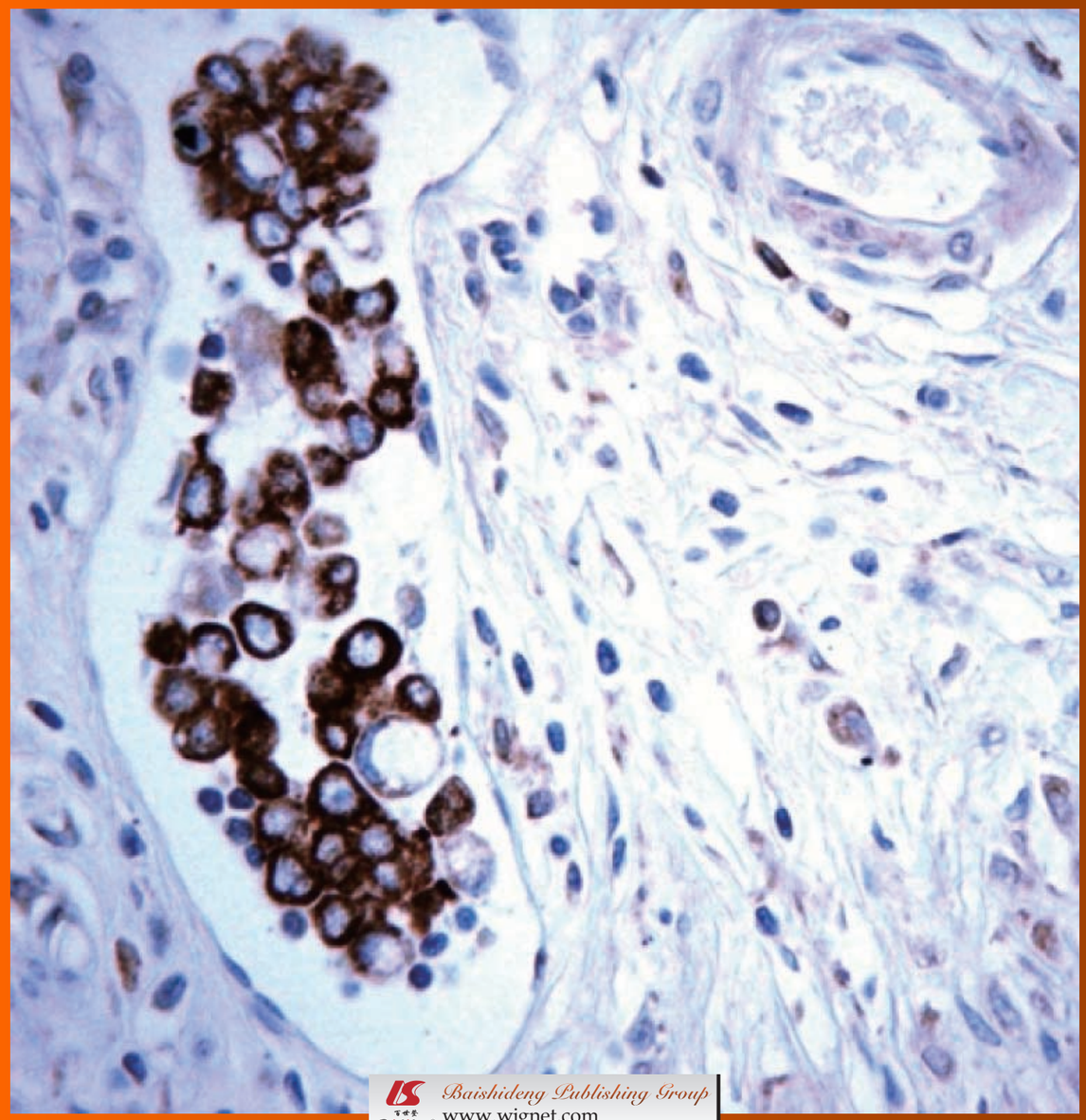
ISSN 1007-9327

World Journal of Gastroenterology
www.wjgnet.com
Volume 18 Number 8 Feb 28 2012

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

World Journal of *Gastroenterology*

World J Gastroenterol 2012 February 28; 18(8): 727-864



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World Journal of Gastroenterology

Contents

Weekly Volume 18 Number 8 February 28, 2012

World Journal of Gastroenterology
Volume 18 Number 8 February 28, 2012

Contents

NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

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PUBLISHER
Baishideng Publishing Group Co., Limited
Room 1701, 17/F, Henan Building,
No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-31158812
Telephone: +852-58042046
E-mail: bpg@baishideng.com
http://www.wjgnet.com

PRINT SUBSCRIPTION
RMB 300 Yuan for each issue, RMB 14400 Yuan for one year.

PUBLICATION DATE
February 28, 2012

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

ONLINE SUBMISSION
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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.727

World J Gastroenterol 2012 February 28; 18(8): 727-735
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Role of cytokines and chemokines in non-alcoholic fatty liver disease

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Supported by The Swiss National Science Foundation, No. 32003B-134963/1 and "Sir Jules Thorn Trust Reg" Foundation, Gustave and Simone Prévot Foundation to Montecucco F; EU FP7 AtheroRemo, No. 201668 and Swiss National Science Foundation, No. 310030B-133127 and Novartis Foundation and Swiss Heart Foundation to Mach F
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Received: June 7, 2011 Revised: July 27, 2011
Accepted: August 4, 2011
Published online: February 28, 2012

Abstract

Non-alcoholic fatty liver disease (NAFLD) includes a variety of histological conditions (ranging from liver steatosis and steatohepatitis, to fibrosis and hepatocarcinoma) that are characterized by an increased fat content within the liver. The accumulation/deposition of fat within the liver is essential for diagnosis of NAFLD and might be associated with alterations in the hepatic and systemic inflammatory state. Although it is still unclear if each histological entity represents a different disease or rather steps of the same disease, inflammatory processes in NAFLD might influence its pathophysiology and prognosis. In particular, non-alcoholic steatohepatitis (the most inflamed condition in NAFLDs, which more frequently evolves towards chronic and serious liver diseases) is characterized by a marked activation of inflammatory cells and the up-regulation of several soluble inflammatory mediators. Among several mediators, cytokines and chemokines might play a pivotal active role in NAFLD and are considered as potential therapeutic targets. In this review, we will update evidence from both basic research and clinical studies on the potential role of cytokines and chemokines in the pathophysiology of NAFLD.

Key words: Inflammation; Non-alcoholic fatty liver disease; Cytokine; Chemokine

Peer reviewers: Maria Concepción Gutiérrez-Ruiz, PhD, Department of Health Sciences, Universidad Autónoma Metropolitana-Iztapalapa, DCBS, Av San Rafael Atlixco 186, Colonia Vicentina, México, DF 09340, Mexico; Astrid van der Velde, PhD, AMC Liver Center, Meibergdreef 69-71, 1105 BK Amsterdam, The Netherlands

Braunersreuther V, Viviani GL, Mach F, Montecucco F. Role of cytokines and chemokines in non-alcoholic fatty liver disease. *World J Gastroenterol* 2012; 18(8): 727-735 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/727.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.727

INTRODUCTION

In the last decade, there has been a remarkable scientific effort to improve our understanding of the pathogenesis, diagnosis, and treatment of non-alcoholic fatty liver disease (NAFLD). Clinical studies revealed dramatically high prevalence of NAFLD worldwide[1,2]. Worrying data on the prevalence of NAFLD in children and adolescents was also revealed[3]. Importantly, in American adolescents followed in the National Health and Nutri-

tion Examination Survey between 1999 and 2004, serum elevation of hepatic enzymes [i.e., alanine aminotransferase (ALT)] was observed in 6% to 11% of subjects (depending of ethnicity)[4]. Furthermore, serum ALT increase was positively associated with waist circumference and insulin resistance, suggesting that NAFLD might be considered as the hepatic manifestation of other epidemic diseases, such as metabolic syndrome and obesity[1,2]. In fact, in obese children and adolescents, NAFLD affects about 20% to 74%, indicating that this disease might start early during life, providing more time for its deleterious evolution[5-7]. However, we believe that NAFLD is limited to patients suffering from obesity, metabolic syndrome, or other fat-related diseases. Although NAFLD has been described as an increased hepatic accumulation of fat (steatosis), a recent scientific consensus defined it as a complex spectrum of diseases, ranging from asymptomatic steatosis with possible aminotransferase alterations to non-alcoholic steato-hepatitis (NASH), cirrhosis, and also hepatocellular carcinoma[8-10]. Whether these conditions are different stages of a common progressive disease or should be considered as different entities, is still an open question. Thus, additional pathophysiological studies on improved animal models are needed to clarify this issue. Indeed, NAFLD is often underestimated, under diagnosed, and not treated in the current medical practice; therefore, its pathophysiological history is at risk of remaining a mystery for several years.

At present, the most suitable area for improving our knowledge of the pathophysiology of NAFLD is represented by the chronic inflammation that underlies all NAFLD entities/stages[11]. Soluble cytokines and chemokines, regulating inflammatory cell function and survival, could be considered as very promising candidates. On the other hand, hormonal axes, adipocytokines, and growth factors have also received attention from NAFLD scientists. In the following paragraphs, we focus on cytokines and chemokines, updating evidence of their role in NAFLD pathophysiology, both in human (Table 1) and animal studies.

CYTOKINES

Cytokines are soluble molecules that are involved in intercellular communication and are produced by a wide variety of cells in the body, including most types of liver cells[12]. They comprise several subfamilies, including interferons, interleukins, tumor necrosis factors (TNF), transforming growth factors (TGF), colony-stimulating factors, and chemokines. Cytokines mediate several fundamental biological processes, including body growth, adiposity, lactation, hematopoiesis, as well as inflammation and immunity. However, they are also implicated in various pathologies, such as atherosclerosis, rheumatoid arthritis, systemic lupus erythematosus, psoriasis, as well as NAFLD[13-16].

Under physiological conditions, constitutive cytokine generation is absent or minimal in the liver. Nevertheless, pathological stimuli like lipid accumulation induces hepatic cells to produce these inflammatory molecules. Cytokines might play an active role in the development and the potential progression of NAFLD through stimulation of hepatic inflammation, cell necrosis and apoptosis, and induction of fibrosis. Nevertheless, they are also essential for liver regeneration following injury[16]. Evidence on the pathophysiological role of cytokine in NAFLD is reported and discussed below.

TNF-α

TNF-α is an inflammatory mediator secreted by several inflammatory cell types, including monocyte/macrophages, neutrophils, and T-cells, but also by many other tissues, such as the endothelium, adipose tissue, or neuronal tissue. In the liver, TNF-α is secreted directly by hepatocytes and Kupffer cells or indirectly by abdominal fat[17]. Several studies have shown that TNF-α is a key factor in the development of NAFLD and NASH in both humans and animals. Hotamisligil *et al*[18] showed for the first time a relationship between TNF-α expression and insulin resistance in NASH. The authors stated that adipose tissue represents an important source of obesity-induced inflammation, notably by the expression of TNF-α, which can induce inflammation and insulin resistance. Indeed, in several rodent models of obesity, TNF-α expression in adipose tissue was upregulated as compared to controls[18]. Accordingly with these study, obese mice lacking TNF-α showed an improved insulin sensibility[19]. Recently, mice treated with the anti-TNF-α drug thalidomide showed some improvements in the hepatic alterations mediated by a high-fat diet[20]. Moreover, the use of anti-TNF-α antibodies in an experimental model of NASH decreased inflammation, necrosis, and fibrosis in rats[21].

Although TNF-α inhibition in animal models of NAFLD presents encouraging therapeutic perspectives, in humans, the role of this cytokine remains controversial. In patients, TNF-α levels were shown to be higher in obese than in lean individuals, and were correlated with insulin resistance[22,23]. Moreover, a positive correlation was observed between the degree of liver fibrosis and circulating TNF-α levels in patients with NASH[24]. Another study showed increased TNF-α expression in the liver and adipose tissue in NASH patients with significant fibrosis in comparison with those with a slight or nonexistent fibrosis[25]. More recently, Hui *et al*[26] strengthened these results, showing increased TNF-α levels in subjects with steatohepatitis as compared to controls. The potential involvement of TNF-α in NAFLD pathophysiology was recently suggested by genetic studies on its polymorphisms[27,28]. Moreover, treatment with pentoxifylline (a molecule inhibiting TNF-α) decreased the serum levels of aminotransferases and displayed hepatic beneficial effects in patients with NASH[29].

Nevertheless, the involvement of TNF-α in insulin resistance and NAFLD is questionable. Some studies did

Table 1 Summary of human studies concerning the role of cytokines and chemokines in non-alcoholic fatty liver disease

Cytokine/chemokine	References	Findings	Approach/sample size	Treatment
TNF-α	Hotamisligil *et al*[22]	Increase of TNF-α in fat tissue of obese subjects Correlation between TNF-α and IR	18 control and 19 obese pre-menopausal women	-
	Dandona *et al*[23]	Higher TNF-α levels in obese subjects that contribute to IR Decrease of TNF-α levels and IR with weight loss	30 control and 38 obese women	-
	Lesmana *et al*[24]	Correlation between TNF-α serum levels and liver fibrosis	30 patients with NASH	-
	Crespo *et al*[25]	Overexpression of TNF-α in liver and adipose tissue in patient with NASH Increase of p55 TNF-α receptor expression in fibrotic liver Enhancement of TNF-α expression with advanced liver fibrosis	52 obese patients	-
	Hui *et al*[26]	Increase of TNF-α and TNFR2 in patients with NASH	109 patients with NAFLD	-
	Valenti *et al*[27]	Higher prevalence of 238 TNF-α polymorphism in patients with NAFLD	99 subjects with NAFLD	-
	Zhou *et al*[28]	238 TNF-α polymorphism association with NAFLD susceptibility	117 subjects with NAFLD	-
	Lee *et al*[29]	Reduction of aminotransferase in patients treated with Pentoxifylline	20 patients with NASH	Pentoxifylline 400 mg three time per day
	Müller *et al*[30]	No significant increase of TNF-α or its receptor levels in patients with IGT	80 subjects with IGT, 152 subjects with type II diabetes and 77 control subjects	-
	Bruun *et al*[31]	No correlation between IR and TNF-α levels	19 obese and 10 lean men	-
	Ofei *et al*[32]	No effect on insulin sensitivity	21 obese NIDDM patients	Single injection of CDP571 (anti-TNF-α antibody)
	Bernstein *et al*[33]	No effect on insulin sensitivity	56 subjects with metabolic syndrome	Etanercept (TNF-α antagonist) 50 mg 1 time per week, for 4 wk
TGF-β1	Annoni *et al*[40]	Enhancement of TGF-β1 expression in man with active liver disease	16 patients with active liver disease	-
	Castilla *et al*[44]	Association of TGF-β1 levels and fibrosis in chronic liver disease	46 patients with elevated serum ALT	-
	Milani *et al*[45]	High TGF-β1 mRNA expression in fibrotic liver	2 subjects control, 1 subject with cirrhosis, and 9 subjects with hepatitis B viral liver disease	-
	Hasegawa *et al*[47]	TGF-β1 levels are useful to differentiate between NAFLD and NASH Benefits of α-tocopherol to treat NASH	12 patients with non-alcoholic steatohepatitis and 10 patients with non-alcoholic fatty liver	α-tocopherol 300 mg/d during 1 yr
	Dixon *et al*[48]	Association of polymorphism inducing angiotensinogen and TGF-β1 and advanced hepatic fibrosis	105 obese patients	-
IL-6	Kopp *et al*[58]	Correlation between IL-6 and IR	37 obese patients	-
	Kugelmas *et al*[60]	Elevated IL-6 concentration in serum of patients with NASH Decrease of IL-6 with the treatment	16 patients with NASH	Vitamin E 800 IU/d
	Haukeland *et al*[61]	Higher levels of IL-6 in patients with NAFLD	47 patients (22 simple steatosis, 25 NASH) and 30 controls	-
	Wieckowska *et al*[63]	Higher hepatic IL-6 expression in patients with NASH Association with IL-6 levels and the disease severity Correlation between hepatic IL-6 expression and IR	50 patients with suspected NALFD	-
IL-10	Esposito *et al*[69]	Association between low levels of IL-10 and metabolic syndrome	50 obese and 50 normal-weight women	-
	Calcaterra *et al*[70]	No association between metabolic syndrome and low levels of IL-10	70 severely obese and 30 non-obese children and adolescents	-
CCL2/MCP-1	Haukeland *et al*[61]	Elevated levels of CCL2 in patients with NAFLD and NASH	47 patients (22 simple steatosis, 25 NASH) and 30 controls	-
	Westerbacka *et al*[92]	Increase of CCL2 in steatotic liver of patients with NAFLD	24 subjects (8 controls, 16 with NAFLD)	-
	Greco *et al*[93]	Correlation between CCL2 gene expression and liver fat content in patients with NAFLD	10 subjects with low and high extremes of fat liver	-
CCL5/RANTES	Wu *et al*[99]	Higher CCL5 expression in adipose tissue of obese patients than in lean controls	21 morbidly obese patients, 10 obese patients with metabolic syndrome, and 3 lean controls	-

	Kirovski *et al*[100]	Upregulation of hepatic and circulating CCL5 levels in patients with NAFLD	45 patients with NAFLD and 61 controls with normal liver	-
CXCL8/IL-8	Bahcecioglu *et al*[103]	Higher serum levels of IL-8 in patients with NASH and cirrhosis compared to control group	28 patients with NASH, 14 patients with cirrhosis, and 15 controls	-
	Torer *et al*[104]	Higher IL-8 serum levels in the patients with NASH than in patients with hepatosteatosis	57 patients with NASH and 35 patients with NALFD	-
	Jarrar *et al*[105]	Higher levels of IL-8 in NAFLD patients compared to obese and non-obese subjects Independent association of IL-8 levels with NASH	26 patients with NASH, 19 patients with simple steatosis, 38 obese controls, and 12 non-obese controls	-
	Abiru *et al*[106]	No differences of IL-8 between NASH, simple steatosis, and control groups	23 patients with NASH, 21 patients with simple steatosis, and 18 healthy controls	-
CXCL9/Mig	Wasmuth *et al*[109]	Association CXCL9 serum levels and CXCL9 liver expression with liver fibrosis due to NASH	441 individuals with HCV	

NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steato-hepatitis; TNF: Tumor necrosis factor; TGF: Transforming growth factor; IL: Interleukin; MCP: Monocyte chemotactic protein; RANTES: Regulated on activation normal T-cell expressed and secreted; Mig: Monokine induced by interferon-γ; IR: Insulin resistance; IGT: Impaired glucose tolerance; NIDDM: Noninsulin-dependent diabetes mellitus; ALT: Alanine aminotransferase; HCV: Hepatitis C virus.

not show any correlation between insulin resistance and TNF-α levels[30,31], whereas two clinical studies, using an antagonist and an anti-TNF-α antibody, did not show any improvements in insulin sensitivity[32,33]. Moreover, in a recent study, Lucero *et al*[34] did not observe any difference in circulating levels of TNF-α between patients with NAFLD as compared to controls without NAFLD.

TGF-β

TGF-β is a cytokine/growth factor with immunosuppressive, anti-inflammatory, and pro-fibrotic properties[35]. In the liver, TGF-β1 is the most abundant isoform, and it is secreted by immune cells, stellate cells, and epithelial cells[36]. TGF-β1 plays a pivotal role in hepatic fibrosis by mediating the activation of stellate cells and their production of extracellular matrix proteins[37-39]. Indeed, Kupffer and stellate cells produce TGF-β1, which induces the transformation of resting stellate cells to myofibroblasts[40]. In experimental models of hepatic fibrosis induced by CCl_4 or schistosomiasis, expression of TGF-β1 is upregulated[41-43]. Moreover, in patients with liver fibrosis, the expression of TGF-β1 mRNA is increased[40,44,45]. Stärkel *et al*[46] showed that the upregulation of TGF-β1 is an early molecular step in the progressive fibrotic steatohepatitis. A study by Hasegawa and co-workers showed that TGF-β1 levels were increased in patients with NASH as compared to hepatic steatosis. Thus, the measurement of serum levels of TGF-β1 might be useful to distinguish NASH patients in the spectrum of NAFLD[47]. Moreover, polymorphisms that induce high angiotensinogen and TGF-β1 are associated with advanced hepatic fibrosis in obese patients with NAFLD[48].

Interleukin-6

The role of interleukin-6 (IL-6) in liver pathology is very complex, and its participation in the development of NAFLD remains unclear. IL-6 activates several cells, such as immune cells, hepatocytes, hematopoietic stem cells, and osteoclasts[49]. Furthermore, IL-6 has a wide range of biological functions, including induction of inflammation and oncogenesis, regulation of immune response, and support of hematopoiesis[49]. IL-6 was initially considered as a hepatoprotector in liver steatosis, capable of reducing oxidative stress and preventing mitochondrial dysfunction[50,51]. Furthermore, this potential hepatoprotective effect of IL-6 was confirmed in other models of liver disease, such as ischemic preconditioning models and in liver regeneration after partial hepatectomy in mice[52-55].

Nevertheless, IL-6 is a key element in the acute phase response, mediating the synthesis of several acute phase proteins (such as C-reactive protein and serum amyloid A)[56]. Thus, we cannot exclude the possibility that IL-6 might also play an indirect deleterious role in NAFLD pathogenesis. In diet-induced obese mice, treatment with IL-6 antibodies improved sensitivity to insulin[57]. Furthermore, IL-6 is considered as a predictor marker of insulin resistance and cardiovascular diseases. In patients undergoing bariatric surgery, decreased IL-6 concentrations were associated with weight loss and insulin resistance improvement[58]. Serum IL-6 levels are higher in animal models and patients with NAFLD[59-61]. Recently, Mas and co-workers showed that diet-induced NASH was reduced in IL-6 knockout mice as compared to controls[62]. In humans with NASH, a positive correlation between IL-6 expression in hepatocytes and the severity of NAFLD was observed [63].

Thus, although IL-6 could improve hepatic regeneration and repair, it could also sensitize the liver to injury, stimulate hepatocyte apoptosis, induce insulin resistance, and participate in NASH development. Recent studies from Yamaguchi illustrated this paradoxical role of IL-6 in NAFLD. Indeed, IL-6 pathway neutralization with tocilizumab, a specific antibody against the IL-6 receptor, enhanced hepatic steatosis, but improved liver damage in mice with methionine choline deficient (MCD) diet-induced NASH[64]. Furthermore, Yamaguchi *et al*[65], in

a second study, showed that not only upregulation of IL-6, but also severe suppression of hepatic IL-6/signal transducer and activator of transcription 3 signaling may lead to the progression of NASH.

IL-10

IL-10 is considered as an anti-inflammatory cytokine that regulates the inflammation in several organs and tissues in physiological or pathological situations[66]. It inhibits T cell-, monocyte-, and macrophage-mediated functions. In the liver, IL-10 has been detected in several cells, including hepatocytes, stellate cells, and Knuppfer cells, but only few studies have been performed to investigate the role of endogenous IL-10 in the progression of NAFLD. A study using IL-10-deficient mice fed on high fat diet, suggested that endogenous IL-10 was protective for hepatic steatosis, but not for concomitant insulin resistance[67]. In another study, Cintra and co-workers observed that the inhibition of IL-10 (either using an anti-IL-10 antibody or an IL-10 antisense oligonucleotide) led to increased expression of pro-inflammatory markers (TNF-α, IL-6, IL-1β, and F4/80) and impaired insulin signal transduction and steatosis[68]. In humans, Esposito and co-workers showed an inverse correlation between IL-10 levels and metabolic syndrome in obese woman, suggesting a potential IL-10-mediated benefit in metabolic syndrome patients also affected by NAFLD[69]. However, Calcaterra *et al*[70] did not confirm this association in obese children and adolescents.

CHEMOKINES

Chemokines (chemotactic cytokines) are small heparin-binding proteins known to induce mainly leukocyte trafficking, growth, and activation in inflammatory sites[71,72]. Many cell types, including endothelial cells, smooth muscle cells, leukocytes, hepatocytes, and stellate cells, can secrete them. Approximately 50 currently identified chemokines are classified in four subfamilies (C, CC, CXC, CX_3C) according to their structural arrangement of N-terminal conserved cysteine residues. Chemokines need to bind to their coupled seven transmembrane protein G coupled receptors on target cells to induce cellular changes. Chemokines and their receptors have been implicated in multiple inflammatory diseases, such as atherosclerosis, multiple sclerosis, psoriasis, and insulin resistance[73]. Expression of several chemokines and chemokine receptors has been shown to be upregulated in the livers of obese patients with severe steatosis and NASH[74]. Inflammatory processes are crucial in the potential progression of NAFLD; therefore, chemokines might also play a pivotal role in NAFLD pathophysiology[75].

CCL2/monocyte chemotactic protein-1

CCL2 is a potent chemoattractant that is principally secreted by macrophages and, to a lesser extent, by activated endothelial cells, smooth muscle cells, and hepatic stellate cells[76-79]. It activates target cells (mainly macrophages) through binding with its receptor, CCR2[76-79]. It is widely secreted in adipose tissue and plasma of obese mice[80]. Monocyte/macrophage infiltration in adipose tissue has been observed in animal models and humans[81]. Monocyte/macrophage accumulation in the steatotic liver was reduced in mice fed on high-fat diet and who were deficient for *CCL2* or *CCR2* genes[82]. However, the role of CCL2 is actually more complex, extending far beyond the monocyte/macrophage chemoattractant effect. For instance, low density lipoprotein receptor and CCL2 double knockout mice showed alterations in glucose and lipid metabolism induced by high-fat diet[83]. When fed with a normal chow diet, these mice are characterized by lower alterations in the lipid and glucose profile. However, obesity is also reduced under normal diet, suggesting that different food intake might regulate CCL2-mediated inflammation in mice prone to develop obesity and atherosclerosis. Importantly, CCL2 deficiency in mice fed on a high-fat diet decreases insulin resistance and hepatic steatosis[84]. On the other hand, mice overexpressing CCL2 in adipose tissue presented increased insulin resistance and hepatic triglyceride levels[84].

Interestingly, CCL2 was also upregulated in the livers of animals with high-fat diet-induced NASH[85]. This pathophysiological aspect of CCL2 directly contributed to the lipid accumulation in hepatocytes *via* the activation of peroxisome proliferator-activated receptor α gene expression[86]. More recently, Obstfeld and co-workers showed that hepatic myeloid cells might play a crucial role in the promotion of obesity-induced hepatic steatosis. Indeed, they observed that obesity upregulates CCL2 expression in hepatocytes, leading to the recruitment of CCR2-positive myeloid cells and thus, promoting hepatosteatosis[87]. Pharmacological treatments inhibiting the CCL2/CCR2 pathway in several mouse models of metabolic diseases significantly improved obesity, insulin resistance, hepatic steatosis, and inflammation in the adipose tissue[88-90]. These benefits were not confirmed by other studies. For example, CCL2 deletion in an experimental model of MCD diet-induced steatosis did not improve liver fat accumulation and associated inflammation[91].

In humans, only few studies have been performed to investigate the role of CCL2 in NAFLD pathology. Haukeland *et al*[61] showed that patients with NAFLD had low-grade systemic inflammation and presented with higher serum levels of CCL2 compared to controls. Moreover, CCL2 has been confirmed to also be elevated in the steatotic livers of NAFLD patients[92]. More recently, another study showed that CCL2 expression was positively correlated with liver fat content in patients with NAFLD[93]. These studies suggest an important participation of CCL2 in the potential progression of simple steatosis to NASH. Therefore, although the role of CCL2 in metabolic diseases requires further investigation, these studies suggest a potential direct role of CCL2 in NAFLD and, in particular, in NASH.

CCL5/regulated on activation normal T-cell expressed and secreted

CCL5 is involved in several chronic immune-inflammatory diseases, such as atherosclerosis, acute myocardial infarction, myocarditis, rheumatoid arthritis, and multiple sclerosis[94,95]. It is secreted by various cells, such as endothelial cells, smooth muscle cells, macrophages, or hepatic stellate cells. This chemokine is mainly involved in migration of T cells, monocytes, neutrophils, and dendritic cells through binding to its cognate transmembrane receptors, CCR1, 3 and 5. The receptor CCR5 has been identified on isolated hepatic stellate cells, suggesting that these hepatic cells are both the target and source of CCL5[96,97]. The association of CCL5 with NAFLD was shown recently in humans and mice. Indeed, two studies showed that obesity increased hepatic expression of CCL5 in a murine model of NASH and in obese patients[98,99]. Hepatocytes are the major source of serum and hepatic CCL5 in NAFLD[100]. CCL5 release in the liver is mediated by hepatocellular lipid accumulation, suggesting that hepatic steatosis *per se* has pathophysiological relevance[100]. CCL5 is also involved in the progression of hepatic fibrosis in mice *via* CCR1 and CCR5 triggering[97]. More recently, Berres and co-workers defined CCL5 as a critical mediator of experimental liver fibrosis. Indeed, antagonism of CCL5 on receptor CCR5 improved experimental liver fibrosis in mice, indicating that CCL5 is a promising therapeutic target to reduce NAFLD[101].

CXCL8/IL-8

CXCL8/IL-8 is a CXC chemokine produced by several cell types, including inflammatory and endothelial cells[102]. The major role of this chemokine is to orchestrate neutrophil recruitment within inflamed tissues. There is little data documenting its potential role in NAFLD. Serum levels of CXCL8 were significantly higher in subjects with NASH as compared to hepatosteatosis or healthy control group[103,104]. More recently, Jarrar and co-workers showed that serum levels of CXCL8 were higher in NAFLD patients as compared to obese and non-obese patients[105]. In addition, CXCL8 serum levels were independently associated with NASH[105]. Conversely, the study from Abiru and co-workers did not confirm this association or any significant differences in serum CXCL8[106].

Other CXC chemokines

Chemokines CXCL9/monokine induced by interferon-γ, and CXCL10/interferon inducible protein-10, which bind the common receptor CXCR3, are generally not detectable in most non-lymphoid tissues under physiological conditions. However, in some inflammatory conditions, interferon gamma might increase their release. CXCR3 is found at high levels on activated T cells, memory T cells, and natural killer cells[107]. CXCL9 and CXCL10 mainly induce the migration of these cell types. In the liver, endothelial cells highly express CXCL9 leading to the transmigration of the CXCR3-expressing lymphocytes[108]. Recently, high levels of CXCL9 were found in the livers of patients with NASH[109]. In this study, Wasmuth and co-workers identified the CXCL9-CXCR3 axis as a potential anti-fibrotic pathway in the liver in both humans and animals.

CONCLUSION

The take-home message of the present update on the inflammatory pathophysiology of NAFLD do not recommend any optimistic insights for the near future. Much research remains to be done to clarify the pathophysiology of NAFLD and to identify selective targets for treatment. The involvement of cytokines and chemokines and their receptors in the pathogenesis of NAFLD is only partially understood. Although the first studies attempting therapeutic strategies targeting the chemokine system have been recently published, we believe that scientific interest in NAFLD should be increased. In particular, effort is required to improve the consideration of NAFLD as a dangerous condition that should not be underestimated or by-passed. Another crucial aspect is represented by the identification of common cytokines and chemokines between NAFLD and metabolic or cardiovascular diseases. The most promising mediators (such as TNF-α, CCL2 and CCL5) also require more selective inhibitory drugs to safely improve NAFLD, limiting the potential risk of deleterious immune-suppression.

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S- Editor Tian L **L- Editor** Stewart GJ **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.736

World J Gastroenterol 2012 February 28; 18(8): 736-745
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Developments in metastatic pancreatic cancer: Is gemcitabine still the standard?

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Received: May 29, 2011 Revised: August 26, 2011
Accepted: September 3, 2011
Published online: February 28, 2012

Abstract

In the past 15 years, we have seen few therapeutic advances for patients with pancreatic cancer, which is the fourth leading cause of cancer-related death in the United States. Currently, only about 6% of patients with advanced disease respond to standard gemcitabine therapy, and median survival is only about 6 mo. Moreover, phase Ⅲ trials have shown that adding various cytotoxic and targeted chemotherapeutic agents to gemcitabine has failed to improve overall survival, except in cases in which gemcitabine combined with erlotinib show minimal survival benefit. Several meta-analyses have shown that the combination of gemcitabine with either a platinum analog or capecitabine may lead to clinically relevant survival prolongation, especially for patients with good performance status. Meanwhile, many studies have focused on the pharmacokinetic modulation of gemcitabine by fixed-dose administration, and metabolic or transport enzymes related to the response and toxicity of gemcitabine. Strikingly, a phase Ⅲ trial in 2010 showed that, in comparison to gemcitabine alone, the FOLFIRINOX regimen in patients with advanced disease and good performance status, produced better median overall survival, median progression-free survival, and objective response rates. This regimen also resulted in greater, albeit manageable toxicity.

Key words: Chemotherapy; Palliative therapy; Metastasis; Biomarkers; Pancreatic neoplasms

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Ying JE, Zhu LM, Liu BX. Developments in metastatic pancreatic cancer: Is gemcitabine still the standard? *World J Gastroenterol* 2012; 18(8): 736-745 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/736.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.736

INTRODUCTION

In 2010, there were an estimated 43 140 new cases and 36 800 deaths from pancreatic cancer in the United States[1]. This represents the 10th most common new cause of cancer and the fourth most common cause of cancer-related death in 2010, highlighting the disproportionate mortality associated with this disease. Additionally, unlike most of the more frequent causes of cancer mortality (lung, colon, prostate, and breast), whose death rates are declining, the death rate for pancreatic cancer has remained relatively stable, which also indicates the limited progress in this field.

Data from the 2000-2007 Surveillance, Epidemiology and End Results registry indicate that at diagnosis, the majority of pancreatic cancer is advanced (50.5% metastatic, 8% localized, 25.9% regional spread, and 15.5% unstaged)[2]. Only 15%-20% of patients are eligible for

surgery at diagnosis[3]. Standard surgical procedures include pancreaticoduodenectomy (stomach-or pylorus-preserving) for tumors of the pancreatic head, and distal pancreatectomy with splenectomy for tumors arising in the body or tail of the pancreas[4]. Although radical resection alone, or in combination with other therapy, is the only way to eradicate the disease, only 10%-20% of patients who have radical resection survive beyond 5 years, underscoring the need for better preoperative staging and more effective systemic therapy.

This article reviews a variety of clinical trials for locally advanced or metastatic pancreatic cancer, including those using cytotoxic drugs and targeted agents, to evaluate if gemcitabine (Gem) should still remain the standard chemotherapy for advanced or metastatic pancreatic cancer after 15 years of developments in this field. We also briefly discuss the pharmacokinetic modulation by fixed-dose administration and metabolic or transport enzymes, related to the response and toxicity of Gem.

PALLIATIVE CHEMOTHERAPY IN PANCREATIC THERAPY

5-Fluorouracil

5-Fluorouracil (5-FU) was considered the only chemotherapeutic option for about 20 years until the development of Gem. A systematic review and meta-analysis of nine trials from the American Society of Clinical Oncology (ASCO) 2003, which compared 5-FU-based combination to best support care, showed positive results in overall survival (OS) (6.38 mo *vs* 3.87 mo, $P < 0.0001$)[5].

Gem

Single agent Gem: Gem became the standard regimen for advanced pancreatic cancer after a randomized trial in 1997 that presented significant improvements in median OS compared to 5-FU (5.6 mo *vs* 4.4 mo, $P = 0.002$). One hundred and twenty-six untreated pancreatic cancer patients randomly received either 5-FU (600 mg/m^2 once weekly) or Gem (1000 mg/m^2 weekly × 7 with 1 wk off, followed by weekly × 3 wk every 4 wk). The clinical benefit response (CBR) also significantly improved in Gem-treated patients (23.8% *vs* 4.8%, $P = 0.0022$)[6].

Fixed-dose Gem: There has been some evidence suggesting that maintaining a constant dose rate of Gem with a fixed dose rate regimen (FDR) may improve outcomes[7-9]. An initial trial was done by Tempero and colleagues to test this hypothesis. Patients were administered either 2200 mg/m^2 Gem over 30 min (standard arm) or 1500 mg/m^2 Gem over 150 min (FDR arm) on days 1, 8 and 15 of every 4-wk cycle. Patients treated with FDR had a trend towards improved survival (8 mo *vs* 5 mo, $P = 0.013$) but more severe adverse events, namely hematological toxicity[10]. However, a subsequent phase Ⅲ Eastern Cooperative Oncology Group (ECOG) trial failed to demonstrate a statistically significant improvement in OS of Gem FDR regimen over the standard administration (6.2 mo *vs* 4.9 mo)[11].

Biomarkers of Gem: Gem is a specific analog of the pyrimidine nucleotide deoxycytidine, and a prodrug that requires cellular uptake and intracellular phosphorylation[12]. Gem intracellular uptake is mainly mediated by the human equilibrative nucleoside transporter (hENT1) and to a lesser extent, by human concentrative nucleoside transporters (hCNTs), hCNT1 and hCNT3. Gem is phosphorylated to its monophosphate form by deoxycytidine kinase (dCK), and this step is essential for further phosphorylation to its active triphosphate form. The active diphosphate metabolite of Gem is also active and inhibits DNA synthesis indirectly by inhibiting ribonucleotide reductase (RRM1). Gem is inactivated by deoxycytidine deaminase (CDA) and deoxycytidylate deaminase into 2',2'-difluorodeoxyuridine[13]. Many studies have demonstrated the relationship between Gem metabolic or transport enzymes and clinical outcome.

In clinical studies of pancreatic cancer, the high expression of hENT1 in tumors has been associated with improved survival in patients treated with Gem[14-16]. In the RTOG 9704 trial, 538 patients were randomly assigned to groups that were given either Gem or 5-FU after surgical resection. Immunohistochemistry analysis showed that hENT1 expression was associated with OS and disease-free survival (DFS) in the group given Gem ($P = 0.004$ and $P = 0.003$, respectively), whereas hENT1 expression was not associated with survival in the group given 5-FU.

The relationship between the expression of dCK, RRM1, and efficacy of Gem is inconsistent[17-24]. A study reported in ASCO 2011 evaluated the association of hENT1, dCK and RRM1 with efficacy of Gem in 434 patients with resected pancreatic cancer. In multivariate models, Gem was associated with better OS in hENT1 high tumors [$n = 163$; hazard ratio (HR): 0.44, 95% CI: 0.28-0.69, $P < 0.001$] and in dCK high tumors ($n = 302$; HR: 0.57, 95% CI: 0.41-0.78, $P = 0.001$). In contrast, in dCK low tumors ($n = 114$, $P = 0.73$) and in hENT1 low tumors ($n = 249$, $P = 0.66$), patients derived no benefit from Gem[25]. Fujita *et al*[17] also showed high dCK expression groups had a significantly longer DFS in the Gem-treated group ($P = 0.0067$).

Several reports have demonstrated that the genotype of degradation enzymes such as CDA, is related to Gem-mediated severe adverse reactions[26-30]. Major contributing factors for Gem clearance are genetic polymorphisms of CDA. The CDA 208AA homozygote allele and its related haplotype, is associated with severe drug toxicity in Japanese cancer patients after Gem-based chemotherapy. However, Tanaka *et al*[30] from MD Anderson Cancer Center have shown that the CDA A-76C genotypes were significantly associated with grade 3/4 neutropenia ($P = 0.020$).

S-1

S-1 is an oral fluorinated pyrimidine, which contains tegafur, gimeracil, and oteracil in a molar ratio of 1:0.4:1[31-32].

Tegafur, a prodrug of 5-FU, is gradually converted to 5-FU by hepatic microsomal enzymes. Gimeracil inhibits the degradation of 5-FU by inhibiting dihydropyrimidine dehydrogenase; oteracil is a competitive inhibitor of orotate phosphoribosyltransferase that inhibits phosphorylation of 5-FU in the gastrointestinal tract to reduce the gastrointestinal toxicity associated with 5-FU.

Two phase Ⅱ trials of S-1 in untreated patients with metastatic pancreatic cancer have shown that this appears to be an active and well-tolerated drug[33,34]. A randomized, open-label, three-arm, phase Ⅲ study (GEST) was reported in ASCO 2011, in which 834 chemotherapy-naive unresectable advanced pancreatic cancer patients with an ECOG performance status (PS) of 0-1 were randomly assigned to receive Gem (1000 mg/m^2, days 1-8 and 15, q4w), S-1 (80/100/120 mg/d based on BSA, days 1-28, q6w), or GS (Gem 1000 mg/m^2, days 1 and 8 plus S-1 60/80/100 mg/d based on BSA, days 1-14, q3w)[35]. S-1 was confirmed to be non-inferior to Gem with respect to OS (9.7 mo *vs* 8.8 mo, $P < 0.001$). Response rate (RR) was 13.3% and 21.0%, respectively. Grade 3/4 hematological toxicities were more common in the Gem arm (neutropenia 41.0% *vs* 8.8%, thrombocytopenia 11.0% *vs* 1.5%), while digestive toxicities were more common in the S-1 arm (anorexia 7.3% *vs* 11.4%, diarrhea 1.1% *vs* 5.5%). This is the first trial demonstrating that oral S-1 has similar efficacy and tolerable toxicity to Gem. Therefore, its potential as a first-line treatment for pancreatic cancer warrants further study.

Gem-based combination chemotherapy

Due to the dissatisfactory results of single-agent Gem for advanced pancreatic cancer, a variety of phase Ⅲ trials have been initiated to investigate the efficacy and toxicity of Gem-based combination therapy (Table 1).

Gem and FU: Two phase Ⅲ trials which compared Gem plus FU with single-agent Gem in patients with advanced disease did not show any benefit in terms of survival[36,37]. In a phase Ⅲ ECOG trial, 322 patients with advanced pancreatic cancer were randomized into groups treated with Gem alone or Gem combined with 5-FU. OS was 5.4 mo for Gem and 6.7 mo for Gem plus FU ($P = 0.09$). Progression-free survival (PFS) was 2.2 mo *vs* 3.4 mo ($P = 0.022$).

Gem and capecitabine: Capecitabine is an orally administered fluorouracil pro-drug, which is activated by a three-step targeted process (carboxylesterases, cytidine deaminase, and thymidine phosphorylase). This drug mimics the continuous infusion of 5-FU, and its intratumoral activation improves the therapeutic index and reduces toxicity in normal tissue[38-40].

A phase Ⅲ trial conducted by Herrmann and colleagues randomized 319 patients into a Gem (1000 mg/m^2 on days 1 and 8) plus capecitabine (1300 mg/m^2 on days 1-14 every 21 d) (GemCap) arm, or a standard Gem arm. OS was not statistically significant different between the two arms (8.4 mo *vs* 7.2 mo, $P = 0.23$). Only subgroup analysis of patients with good PS (Karnofsky performance status 90-100) showed significant prolongation of median OS in the GemCap arm compared to the Gem arm (10.1 mo *vs* 7.4 mo, respectively, $P = 0.014$)[41]. In addition, a more recent analysis of the CBR and quality of life (QOL) did not disclose differences between the two treatment arms[42]. In the more recently reported United Kingdom Phase Ⅲ trial, a higher dose intensity of Gem (12% higher) and capecitabine (44% higher) was used in 533 patients. GemCap significantly improved objective response rate (ORR) (19.1% *vs* 12.4%; $P = 0.034$) and PFS (5.3 mo *vs* 3.8 mo, $P = 0.004$), and was associated with a trend towards improved OS (7.1 mo *vs* 6.2 mo, $P = 0.08$) compared with Gem alone[43]. A meta-analysis of these two studies revealed a survival benefit for GemCap combination (HR: 0.86; 95% CI: 0.75-0.98).

Gem and S-1: In the GEST trial, 454 patients were enrolled in the Gem arm or GS arm[35]. There was no significant difference in OS between these two treatment groups (8.8 mo *vs* 10.1 mo, $P = 0.15$), but PFS differed significantly (4.1 mo *vs* 5.7 mo, $P < 0.0001$). RR was 13.3% and 29.3% for the Gem and GS arms, respectively. The EQ-5D QOL score in the GS arm was significantly better than that in the Gem arm ($P = 0.003$). This trial suggests that GS contributes to a better QOL. Further clinical investigations are needed to ensure its efficacy.

Gem and platinum derivatives: Platinum derivatives are frequently used in combination schedules to treat pancreatic cancer[44-47]. Encouraging results have been obtained in several phase Ⅱ trials using Gem combined with cisplatin, with a RR ranging from 11.5% to 26.0%, and median OS ranging from 7.1 mo to 8.2 mo[44-46]. The following phase Ⅲ trial conducted by Colucci and colleagues did not present a benefit in survival for combination treatment (7.5 mo *vs* 6.0 mo, $P = 0.43$), despite a marked improvement in RR (26.4% *vs* 9.2%, $P = 0.02$) and time to progression (TTP) (4.6 mo *vs* 1.8 mo, $P = 0.048$)[48]. Recently, Colucci and colleagues reported another phase Ⅲ trial with the same regimen. There, the improvement in PFS and RR disappeared when the number of patients increased from 107 to 400[49]. Another randomized phase Ⅲ trial conducted by Heinemann *et al*[50] also did not demonstrate statistically significant differences in OS (7.5 mo *vs* 6.0 mo , $P = 0.15$), PFS (5.3 mo *vs* 3.1 mo , $P = 0.053$) and RR (10.2% *vs* 8.2%).

The French Multidisciplinary Clinical Research Group in Oncology (GERCOR) conducted a phase Ⅱ study of Gem and oxaliplatin (GemOx) in 64 patients with advanced or metastatic pancreatic cancer[51]. The encouraging results (PFS 5.3 mo, OS 9.2 mo) in this study prompted the initiation of a phase Ⅲ trial, conducted by both GERCOR and the Italian Group for the Study of Gastrointestinal Tract Cancer. In this phase Ⅲ study, GemOx was superior in terms of PFS (5.8 mo *vs* 3.7 mo, $P = 0.04$), RR (26.8% *vs* 17.3%, $P = 0.04$), and clinical

Table 1 Gem combined chemotherapy regimens

Phase Ⅲ trial	Combination	No. of patient	OS (mo)	*P* value	PFS (mo)	*P* value	RR (%)	*P* value
Berlin *et al*[36]	GemFU *vs* Gem	322	6.7 *vs* 5.4	0.09	3.4 *vs* 2.2	0.022	6.9 *vs* 5.6	NR
Riess *et al*[37]	GemFU *vs* Gem	473	6.2 *vs* 5.85	0.68	NR	0.44	NR	NR
Cunningham *et al*[43]	GemCap *vs* Gem	533	7.1 *vs* 6 .2	0.08	5.3 *vs* 3.8	0.004	19.1 *vs* 12.4	0.034
Herrmann *et al*[41]	GemCap *vs* Gem	319	8.4 *vs* 7.2	0.234	4.3 *vs* 3.9	0.103	10 *vs* 7.8	NS
Colucci *et al*[48]	GemCIS *vs* Gem	107	7.5 *vs* 5	0.43	4.6 *vs* 1.8	0.048	26.4 *vs* 9.2	0.02
Heinemann *et al*[50]	GemCIS *vs* Gem	195	7.5 *vs* 6	0.15	5.3 *vs* 3.1	0.053	10.2 *vs* 8.2	NS
Colucci *et al*[49]	GemCIS *vs* Gem	400	8.3 *vs* 7.2	0.38	3.9 *vs* 3.8	0.8	10.1 *vs* 12.9	0.37
Louvet *et al*[52]	GemOX *vs* Gem	313	9.0 *vs* 7.1	0.13	5.8 *vs* 3.7	0.04	26.8 *vs* 17.3	0.04
Poplin *et al*[11]	GemOX *vs* Gem	832	5.7 *vs* 4.9	0.09	2.7 *vs* 2.6	0.1	6 *vs* 9	0.11
Stathopoulos *et al*[59]	GemIRI *vs* Gem	145	6.4 *vs* 6.5	0.97	2.8 *vs* 2.9	0.795	15 *vs* 10	0.387
O'Reilly *et al*[61]	Gem-EXE *vs* Gem	349	6.7 *vs* 6.2	0.52	3.7 *vs* 3.8	0.22	6.3 *vs* 4.6	NR
Oettle *et al*[55]	Gem-PEM *vs* Gem	565	6.2 *vs* 6.3	0.85	3.9 *vs* 3.3	0.11	14.8 *vs* 7.1	0.004
Ioka *et al*[35]	GS *vs* Gem	454	10.1 *vs* 8.8	0.15	5.7 *vs* 4.1	0.0001	29.3 *vs* 13.3	NR

OS: Overall survival; PFS: Progression-free survival; RR: Response rate; Gem: Gemcitabine; NR: Not reported; NS: Not significant.

benefit (38.2% *vs* 26.9%, $P = 0.03$) in both the metastatic and locally advanced population[52]. However, median OS did not significantly improved (9.0 mo *vs* 7.1 mo, $P = 0.13$). The largest trial, the ECOG 6201 trial which was mentioned earlier, showed that the median OS was 4.9 mo for the standard Gem arm, and 5.7 mo for the GemOx arm. These differences were not statistically significant, and GemOx caused higher rates of neuropathy, nausea and vomiting[11].

Gem and pemetrexed: Pemetrexed is a multi-targeted antifolate that has synergistic activity with Gem[53]. Miller *et al*[54] reported an OS of 6.5 mo, and a median time to treatment failure (TTF) of 4 mo in a phase Ⅱ trial of pemetrexed as a single agent therapy for advanced pancreatic cancer. A phase Ⅲ trial was carried out in 565 advanced pancreatic patients who were randomly assigned to either Gem plus pemetrexed (PG) or Gem alone. No significant differences between the two treatment arms were observed in terms of OS (6.2 mo *vs* 6.3 mo, $P = 0.85$) and PFS (3.9 mo *vs* 3.3 mo, $P = 0.11$), although RR was significantly better in the PG arm (14.8% *vs* 7.1%, $P = 0.004$), although hematologic toxicity was significantly more common in this arm as well[55].

Gem and topoisomerase inhibitors: Irinotecan (CPT-11) and exatecan are the most widely used topoisomerase inhibitors[56,57]. The first phase Ⅱ trial to evaluate the combination regimen of irinotecan with Gem showed a median survival of 5.7 mo and a median TTP of 2.8 mo[58]. A subsequent phase Ⅲ study failed to improve OS (6.4 mo *vs* 6.5 mo, $P = 0.970$), TTP (2.8 mo *vs* 2.9 mo, $P = 0.795$) and tumor RR (15% *vs* 10%, $P = 0.387$). The incidence of grade 3 diarrhea was higher in the combination group, but grade 3/4 hematologic toxicity and QOL were similar[59]. Another topoisomerase inhibitor, exatecan (DX-8951f), was studied in a randomized phase Ⅲ trial and was shown to be inferior to Gem in respect to RR and improvement in QOL[60]. Furthermore, the combination of exatecan and Gem failed to show any significant survival benefit over Gem alone in a phase Ⅲ study (6.7 mo *vs* 6.2 mo, $P = 0.52$)[61]. Patients in the combination treatment arm experienced significantly more grade 3/4 toxicity, in particular neutropenia (30% *vs* 15%, $P = 0.001$), thrombocytopenia (17% *vs* 5%, $P = 0.004$), and vomiting (11% *vs* 5%, $P = 0.04$).

Meta-analysis

To overcome the statistical limitation of the individual trials, a meta-analysis was performed to investigate the treatment effects of combination regimens.

In 2007, Sultana and colleagues reported a systematic review and meta-analysis of 4060 patients, comparing Gem-based combination chemotherapy with Gem alone in patients with locally advanced and metastatic pancreatic cancer[62]. OS was significantly better for combination chemotherapy (HR: 0.91; 95% CI: 0.85-0.97), and results from subgroup analysis suggested a survival advantage for Gem combined with either a platinum agent (HR: 0.85; 95% CI: 0.74-0.96) or capecitabine (HR: 0.83; 95% CI: 0.72-0.96), although there was insufficient evidence to support combinations of Gem with either 5-FU (HR: 0.98; 95% CI: 0.86-1.11) or irinotecan (HR: 1.01; 95% CI: 0.84-1.22). Another meta-analysis conducted by Banu and colleagues, which included 5886 patients with advanced pancreatic cancer, showed a small but significant improvement in OS for patients receiving Gem-based doublets compared to Gem alone[63]. In addition, a meta-analysis conducted by Heinemann and colleagues, which included 4465 patients, also revealed a significant survival benefit for Gem combination chemotherapy, with a pooled HR of 0.91 ($P = 0.004$)[64]. The analysis of platinum-based combinations indicated a HR of 0.85 ($P = 0.010$), whereas for fluoropyrimidine-based combinations, the HR was 0.90 ($P = 0.030$). No risk reduction was observed when combining Gem with irinotecan, exatecan or pemetrexed. For the 1682 patients with adequate information on baseline PS, analysis indicated that patients with a good PS had a marked survival benefit when receiving combination chemotherapy (HR: 0.76, $P < 0.0001$). In contrast, application of combination chemotherapy to patients with an initially poor PS

Table 2 Gem in combination with targeted therapy

Phase Ⅲ trial	Combination	No. of patient	OS (mo)	*P* value	PFS	*P* value	ORR (%)	*P* value
Moore *et al*[76]	Gem ± Erlotinib	569	6.24 *vs* 5.91	0.038	3.75 *vs* 3.55	0.004	8.6 *vs* 8.0	NS
Philip *et al*[79]	Gem ± Cetuximab	745	6.3 *vs* 5.9	0.23	3.4 *vs* 3.0	0.18	12 *vs* 14	0.59
Kindler *et al*[83]	Gem ± BEV	602	5.8 *vs* 5.9	0.95	3.8 *vs* 2.9	0.07	13 *vs* 10	NS
Van Cutsem *et al*[84]	Gem + Erlotinib ± BEV	607	7.1 *vs* 6.0	0.2087	4.6 *vs* 3.6	0.0002	13.5 *vs* 8.6	0.057
Goncalves *et al*[87]	Gem ± Sorafenib	104	8.5 *vs* 9.2	0.146	3.8 *vs* 5.6	0.601	NR	NR

OS: Overall survival; PFS: Progression-free survival; ORR: Objective response rate rate; Gem: Gemcitabine; BEV: Bevacizumab; NR: Not reported; NS: Not significant.

appeared to be ineffective (HR: 1.08, $P = 0.40$). However, a meta-analysis conducted by Bria and colleagues, involving 6296 patients, did not show significant differences in the primary endpoint OS, although a significant advantage was evident with regard to both PFS and the ORR, especially in platinum combination therapy[65].

Taken together, it appears that patients taking Gem in combination with either a platinum analog or capecitabine, may have a clinically relevant survival prolongation, especially if they have good PS.

Targeted therapy

Several pathways have been targeted in an attempt to treat pancreatic cancer, but the results from most of these have been disappointing[66-68] (Table 2).

Epidermal growth factor receptor inhibitors

The overexpression of epidermal growth factor receptor inhibitors (EGFR) has been reported in pancreatic cancer, thus EGFR inhibitors has emerged as a new therapeutic approach for this disease. EGFR can be inhibited by small molecular tyrosine kinase inhibitors that block the intrinsic tyrosine kinase activity pathway and monoclonal antibodies directed against the extracellular ligand binding domain[69-75].

Gem and erlotinib: The original approval of erlotinib to be used in the treatment of pancreatic cancer was obtained in 2005 after a double-blind international Phase Ⅲ trial in which a total of 569 patients were randomly assigned to receive standard Gem plus erlotinib at 100 mg or 150 mg daily, or Gem alone plus placebo[76]. The study showed statistically significant improvements in OS (6.24 mo in the erlotinib arm and 5.91 mo in the control arm, $P = 0.038$) and PFS (3.75 mo *vs* 3.55 mo , $P = 0.004$). However, ORR were not significantly different (8.6% *vs* 8.0%), and most patients had disease stabilization. Although this study was statistically significant, major concerns still remained about using erlotinib in light of the minimal median survival duration benefit and high costs. In addition, patients receiving erlotinib had higher frequencies of rash, diarrhea, infection and stomatitis, although they were generally well-tolerated and were notably of grade 1 or 2[77]. As in studies of anti-EGFR agents in colorectal cancer, the presence of rash was associated with a higher likelihood of achieving disease control ($P = 0.05$).

Gem and cetuximab: A phase Ⅱ trial of cetuximab combined with Gem in pathologically confirmed EGFR-expressing pancreatic cancer patients showed moderate activity (TTP 3.8 mo, OS 7.1 mo)[78]. However, a phase Ⅲ study (SWOG S0205) that assigned 745 patients to Gem plus cetuximab or Gem alone did not have any survival benefits[79]. There were also no significant differences in terms of OS (6.3 mo *vs* 5.9 mo, $P = 0.23$), PFS (3.4 mo *vs* 3.0 mo, $P = 0.18$) and RR (12% *vs* 14%, $P = 0.59$).

Antiangiogenesis

Vascular endothelial growth factor receptor (VEGF) is known to stimulate cell growth, survival, and proliferation. VEGF inhibitors effectively reduce neovascularization. They inhibit new and recurrent tumor vessel growth and improve the capacity of the tumor vasculature for effective delivery of antitumor growth compounds[80,81].

Gem and bevacizumab: A phase Ⅱ study evaluated the combination of bevacizumab plus Gem in 52 advanced pancreatic patients; the median OS and PFS were 8.8 and 5.4 mo, respectively[82]. These interesting results led to a phase Ⅲ study that failed to confirm the previous findings. It showed median OS was 5.8 mo *vs* 5.9 mo ($P = 0.95$), and PFS was 3.8 mo *vs* 2.9 mo ($P = 0.07$) in the bevacizumab arm and the control arm, respectively[83]. Subgroup analysis demonstrated statistically significant differences in survival by PS. Patients with a PS of 0 survived a median of 7.9 mo compared to 4.8 mo, and 2.4 mo for PS 1 and PS 2 patients, respectively ($P = 0.001$). Another Roche sponsored trial, in which 607 patients with metastatic pancreatic cancer were randomized to Gem and erlotinib with or without bevacizumab, showed no significant prolongation of survival with the addition of bevacizumab (7.1 mo *vs* 6.0 mo, $P = 0.20$), although the PFS was statistically significantly improved (4.6 mo *vs* 3.6 mo, $P = 0.0002$)[84]. Bevacizumab was reported to be safe in this combination, despite an increase in the incidence of epistaxis, hypertension, and proteinuria.

Gem and sorafenib: With its inhibitory effects against Raf-1 kinase and VEGF-2, sorafenib is an oral anticancer agent that targets Ras-dependent signal transduction as well as angiogenic pathways[85]. A phase Ⅰ trial demonstrated that Gem in combination with sorafenib was well tolerated and had activity in advanced pancreatic cancer patients[86]. A multicenter, randomized, double-blind, pla-

cebo-controlled phase Ⅲ trial (BAYPAN study), which was reported in ASCO 2011, compared Gem combined with sorafenib to Gem combined with placebo in 104 untreated patients with locally advanced or metastatic pancreatic adenocarcinoma[87]. There was no significant differences in median PFS between the two groups (5.6 mo *vs* 3.8 mo, $P = 0.601$). Median OS were also similar (9.2 mo *vs* 8.5 mo, $P = 0.146$).

Other targeted agents: Recently there have been many phase Ⅰ or Ⅱ trials assessing the efficacy and safety of other novel targeted agents, including the hypoxia-activated prodrug (TH-302), the PARP inhibitor (ABT-888), the anti-IGF-1R antibody (MK-0646, GAN), and the fully human monoclonal antibody directed against prostate stem cell antigen. Some of these have shown promising results[88-93].

Non-Gem containing regimen

In 2005, Conroy *et al*[94] evaluated the RR and toxicity of FOLFIRINOX in 46 chemotherapy-naive advanced pancreatic adenocarcinoma patients. The FOLFIRINOX regimen comprised oxaliplatin 85 mg/m^2 and irinotecan 180 mg/m^2 plus leucovorin 400 mg/m^2 followed by bolus FU 400 mg/m^2 on day 1, then FU 2400 mg/m^2 as a 46-h continuous infusion. This report showed promising results with an RR of 26%, a TTP 8.2 mo, and an OS of 10.2 mo. Despite the fact that grade 3/4 neutropenia occurred in 52% of patients, patients had improvement in all functional scales of the EORTC QLQ-C30. Based on these data, Conroy and colleagues conducted a phase Ⅲ trial comparing FOLFIRINOX with Gem as first-line treatment for metastatic pancreatic adenocarcinoma in 342 patients with good PS (0-1)[95]. The median OS was 11.1 mo in the FOLFIRINOX group compared to 6.8 mo in the Gem group (HR: 0.57; 95% CI: 0.45-0.73; $P < 0.001$). Median PFS was 6.4 mo *vs* 3.3 mo (HR: 0.47; 95% CI: 0.37-0.59, $P < 0.001$). The ORR was 31.6% *vs* 9.4% ($P < 0.001$). At 6 mo, 31% of the patients in the FOLFIRINOX group had a definitive degradation of the QOL, *vs* 66% in the Gem group (HR: 0.47; 95% CI: 0.30-0.70; $P < 0.001$). Grade 3/4 toxicities were more common in the FOLFIRINOX, diarrhea 12.7% *vs* 1.8%, nausea 15.6% *vs* 6.3%, vomiting 14.5% *vs* 8.3%, fatigue 23.6% *vs* 17.8%, neutropenia 45.7% *vs* 21%, and febrile neutropenia 5.4% *vs* 1.2%. In the FOLFIRINOX arm, 42% of patients received support with granulocyte colony-stimulating factor. Two patients died from treatment-related causes: one from febrile neutropenia in the FOLFIRINOX group and one from cardiac decompensation in the Gem group. This trial was highly selective: only 39% of patients had a primary tumor in the head of the pancreas; whereas in clinical practice, about two-thirds of patients present with a primary tumor in the pancreas, possibly requiring biliary stents. Therefore, for patients with a good PS, normal bilirubin, and a good supportive care system, FOLFIRINOX could be a viable option.

CONCLUSION

As a first-line therapy, Gem has been the standard treatment for pancreatic cancer since 1997, despite its low RR and short OS. Trials for Gem-based combination regimens have failed to improve survival. Striking, FOLFIRINOX regimen increased median OS from 6.8 mo to 11.1 mo ($P < 0.0001$), and RR was 31.6% for patients with a good PS. Thus the question that arises is whether FOLFIRINOX should be considered standard therapy for advanced pancreatic cancer instead of Gem. Does this trial represent the start of a change for the management of advanced pancreatic cancer? The FOLFIRINOX regimen was quite toxic because nearly half of the patients suffered grade 3/4 toxicity. Considering the efficacy and toxicity, FOLFIRINOX could be a viable option in selected patients (good PS, normal bilirubin, and a good supportive care system). This trial provided more evidence that Gem does not have to be the backbone of chemotherapy in pancreatic cancer[96-98]. The GEST trial was the first to show that oral S-1 had similar efficacy and tolerable toxicity to Gem in pancreatic cancer patients with good PS. Considering the convenience of oral S-1, it may be worthwhile to initiate further studies. Based on its high RR, further studies are needed to explore the value of a FOLFIRINOX regimen in borderline or unresectable pancreatic tumors, together with radiation for a possible downstaging effect. FOLFIRINOX should also be studied in adjuvant settings where patients can potentially accept more toxic effects. In the future, better tolerated doublet regimens such as FOLFOX and FOLFIRI should be compared to Gem. It will also be necessary to screen the dominant group based on the expression and genotype of metabolic or transport enzymes of Gem.

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S- Editor Lv S **L- Editor** Kerr C **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.746

World J Gastroenterol 2012 February 28; 18(8): 746-753
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Routine blood tests to predict liver fibrosis in chronic hepatitis C

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Supported by Clinical Study Project XMRP, No. CMRPG 690081, from Chiayi Chang Gung Memorial Hospital
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Received: March 23, 2011 Revised: May 26, 2011
Accepted: May 30, 2011
Published online: February 28, 2012

Abstract

AIM: To verify the usefulness of FibroQ for predicting fibrosis in patients with chronic hepatitis C, compared with other noninvasive tests.

METHODS: This retrospective cohort study included 237 consecutive patients with chronic hepatitis C who had undergone percutaneous liver biopsy before treatment. FibroQ, aspartate aminotransferase (AST)/alanine aminotransferase ratio (AAR), AST to platelet ratio index, cirrhosis discriminant score, age-platelet index (API), Pohl score, FIB-4 index, and Lok's model were calculated and compared.

RESULTS: FibroQ, FIB-4, AAR, API and Lok's model results increased significantly as fibrosis advanced (analysis of variance test: $P < 0.001$). FibroQ trended to be superior in predicting significant fibrosis score in chronic hepatitis C compared with other noninvasive tests.

CONCLUSION: FibroQ is a simple and useful test for predicting significant fibrosis in patients with chronic hepatitis C.

Key words: Liver fibrosis; Noninvasive test; FibroQ; Aspartate aminotransferase; Alanine aminotransferase; FIB-4 index; Aspartate aminotransferase to platelet ratio index; Lok's model; Cirrhosis discriminant score; Pohl score

Peer reviewer: Luigi Muratori, MD, PhD, Assistant Professor, Department of Clinical Medicine, University of Bologna, via Massarenti, 9, Bologna 40138, Italy

Hsieh YY, Tung SY, Lee K, Wu CS, Wei KL, Shen CH, Chang TS, Lin YH. Routine blood tests to predict liver fibrosis in chronic hepatitis C. *World J Gastroenterol* 2012; 18(8): 746-753 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/746.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.746

INTRODUCTION

Viral hepatitis C is one of the most common liver diseases in the world, affecting an estimated 200 million individuals[1], with a particularly high prevalence in Southern Taiwan[2]. Approximately 60%-80% of infected individu-

als develop chronic hepatitis[2], and patients with higher degrees of fibrosis may progress rapidly to cirrhosis and hepatocellular carcinoma. Approximately 20% of patients with chronic hepatitis C advance to cirrhosis, and 5% of them develop hepatocellular carcinoma[3,4].

Knowledge of the extent of liver fibrosis is important for the clinical management of chronic hepatitis C. Patients with no fibrosis or with only portal fibrosis at the time of diagnosis have more favorable outcomes and a lower chance of reaching end-stage liver disease than patients with severe fibrosis or cirrhosis[5-8]. The probability of developing cirrhosis and/or other unfavorable outcomes is closely related to fibrosis stage, therefore, liver biopsy is recommended prior to antiviral treatment. However, liver biopsy adds expense, requires an experienced clinician, and may cause complications, including mortality in 0.018% of patients[9]. In addition, sampling errors and inter- and intraobserver variations may lead to understaging of cirrhosis, particularly macronodular cirrhosis[10-13]. Hence, several noninvasive tests have been proposed to assess the severity of hepatic fibrosis as an alternative to liver biopsy. As reported by Akkaya *et al*[14], alanine aminotransferase (ALT) levels in patients with hepatitis C virus (HCV) infection correlate with periportal bridging/necrosis, and Lu *et al*[15] have reported that thrombocytopenia is a surrogate for cirrhosis. Furthermore, aspartate aminotransferase (AST)-to-platelet ratio index (APRI)[16] and AST/ALT ratio (AAR)[17], cirrhosis discriminant score (CDS)[18], age-platelet index (API)[19], Pohl score[20], FIB-4 index[21], and Lok's model[22] are well-known parameters that are based on routine laboratory data, and are therefore readily available in clinical practice (Table 1). These parameters have been reported to predict the presence of significant fibrosis and extensive fibrosis in some patients[14-23].

Recently, we proposed a novel index, FibroQ[23], which is calculated from common laboratory test results that include prothrombin time international normalized ratio (PT INR), platelet count, AST, ALT, and age, as 10 × (age × AST × PT INR)/(ALT × platelet count) to predict significant fibrosis. In a previous study, we enrolled 140 patients with hepatitis B virus (HBV) and HCV infection. To focus on HCV, 113 of these patients were included in the 237 patients in the present study. The aims of the present study were to assess the value of the FibroQ index and to determine its threshold values to differentiate significant fibrosis. We also compared the discriminatory performance of FibroQ to that of AAR, APRI, CDS, API, Pohl score, FIB-4 index, and Lok's model.

Table 1 Fibrosis tests composed of laboratory parameters

Fibrosis test	Calculation
AAR	AST/ALT
APRI	[(AST/ULN)/platelet count (10^9/L)] × 100
FibroQ	(10 × age × AST × PT INR)/(PLT × ALT)
CDS	Platelet count (10^9/L): > 340 = 0; 280-339 = 1; 220-279 = 2; 160-219 = 3; 100-159 = 4; 40-99 = 5; < 40 = 6 ALT/AST ratio: > 1.7 = 0; 1.2-1.7 = 1; 0.6-1.19 = 2; < 0.6 = 3
API	INR: < 1.1 = 0; 1.1-1.4 = 1; > 1.4 = 2 CDS is the sum of the above (possible value, 0-11) Age (yr): < 30 = 0; 30-39 = 1; 40-49 = 2; 50-59= 3; 60-69 = 4; > 70 = 5 Platelet count (10^9/L): ≥ 225 = 0; 200-224 = 1; 175-199 = 2; 150-174 = 3; 125-149 = 4; ≤ 125 = 5 API is the sum of the above (possible value, 0-10)
Pohl score	Positive: AAR ≥ 1 and platelet count < 150 × 10^9/L
FIB-4 index	[Age (yr) × AST (U/L)]/[platelet count (10^9/L) × ALT (U/L) 1/2]
Lok's model	Log odds (predicting cirrhosis) = -5.56 to 0.0089 × platelet (× 10^3/mm^3) + 1.26 × AST/ALT ratio + 5.27 × PT INR Predicted probability = exp (log odds)/[1 + exp (log odds)]

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AAR: AST/ALT ratio; APRI: AST-to-platelet ratio index; API: Age-platelet index; CDS: Cirrhosis discriminant score; ULN: Upper limit of normal; PLT: Posterolateral thoracotomy; PT-INR: Prothrombin time international normalized ratio.

MATERIALS AND METHODS

We retrospectively studied 250 consecutive treatment-naïve patients with chronic HCV infection that was confirmed by the presence of anti-HCV antibody by enzyme immunoassay methods (Abbott Architect I 2000; Abbott, Champaign, IL, United States) as recorded in the departmental files of the Department of Gastroenterology, Chang Gung Memorial Hospital, Chiayi, between May 2005 and December 2008. All patients were Taiwanese. The research study was approved by the Clinical Research Sub-committee of the hospital. Patients with the following conditions were excluded from the study: those co-infected with human immunodeficiency virus or HBV, and those with alcohol consumption in excess of 20 g/d, hepatocellular carcinoma, liver transplantation, antiviral or immunosuppressive therapy, metabolic liver disease, insufficient liver tissue for fibrosis staging, or recent warfarin or other anticoagulant usage. Thirteen patients were excluded due to incomplete data on liver function tests or platelet count within 1 mo before the date of biopsy. The clinical data were reviewed, and the following parameters were recorded: sex, age, AST, ALT, platelet count, PT INR, hemoglobin, white blood cell count, serum creatinine, free thyroxine, thyroid-stimulating hormone, and bilirubin. Liver biopsies were performed by hepatologists and were interpreted by a single pathologist using the Metavir fibrosis score[10]. Significant liver fibrosis and extensive liver fibrosis were defined as Metavir fibrosis scores of ≥ 2 (F2, F3 and F4) and ≥ 3 (F3 and F4), respectively.

Statistical analysis was performed using SPSS software version 12.0 (SPSS Inc., Chicago, IL, United States). Patient characteristics were represented as the mean ± SD. Bivariate Spearman's rank correlation coefficient (r_s) was calculated to measure the relationship between the clinical variables and degree of fibrosis. Receiver operating characteristic (ROC) curves were constructed for each test. To evaluate the diagnostic accuracies of the simple fibrosis prediction tests, their sensitivity, specific-

Table 2 Clinical characteristics of 237 patients with chronic hepatitis C

Male/female	135/102
Age (yr)	54.3 ± 11.6 (19-76)
AST (U/L)	101.2 ± 56.0 (21-348)
ALT (U/L)	156.3 ± 92.5 (24-637)
PT INR	1.049 ± 0.080 (0.88-1.34)
PLT (× 10^3/μL)	170.9 ± 55.1 (60-373)
Hemoglobin (g/L)	14.4 ± 1.37 (9.0-18.0)
WBC count (x 10^9/L)	5.85 ± 1.73 (2.8-16.7)
Creatinine (mg/dL)	0.94 ± 0.23 (1.0-2.0)
FT4 (μg/dL)	1.12 ± 0.19 (1.0-2.0)
TSH (mU/mL)	2.06 ± 2.13 (0.1-23.0)

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PLT: Posterolateral thoracotomy; PT-INR: Prothrombin time international normalized ratio; WBC: White blood cell; FT4: Free thyroxine; TSH: Thyroid stimulating hormone.

Table 3 Correlation of histological fibrosis severity with the variables

	Bivariate Spearman's rank correlation coefficient (95% CI)	*P* value
Age (yr)	0.232 (0.108-0.349)	< 0.001
AST (U/L)	0.188 (0.062-0.308)	0.004
ALT (U/L)	-0.028 (-0.155 to 0.100)	0.666
PT INR	0.337 (0.219-0.445)	< 0.001
PLT (× 10^3/μL)	-0.326 (-0.435 to -0.207)	< 0.001
Hb	-0.176 (-0.297 to -0.050)	0.005
WBC	-0.053 (-0.179 to 0.075)	0.404
Cr	-0.152 (-0.274 to -0.025)	0.02
FT4	-0.167 (-0.288 to -0.040)	0.014
TSH	0.065 (-0.063 to 0.191)	0.341
Bil(T)	0.135 (0.008-0.258)	0.04
AAR	0.341 (0.223-0.449)	< 0.001
APRI	0.322 (0.203-0.431)	< 0.001
FibroQ	0.444 (0.336-0.541)	< 0.001
FIB-4	0.429 (0.319-0.528)	< 0.001
CDS	0.185 (0.059-0.305)	0.004
API	0.360 (0.244-0.466)	< 0.001
Lok's model	0.430 (0.320-0.528)	< 0.001
Pohl score	0.144 (0.017-0.267)	0.027

We used Fisher's Z-transform to compute asymmetric confidence limits for the Spearman's rank correlation coefficients. Hb: Hemoglobin; Bil(T): Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PLT: Posterolateral thoracotomy; PT-INR: Prothrombin time international normalized ratio; WBC: White blood cell; FT4: Free thyroxine; TSH: Thyroid stimulating hormone; AAR: AST/ALT ratio; APRI: AST-to-platelet ratio index; API: Age-platelet index; CDS: Cirrhosis discriminant score.

ity, positive predictive value (PPV), negative predictive value (NPV), and the area under the ROC curve (AUC) were calculated. In tests of significance, two-sided $P < 0.05$ was considered significant.

RESULTS

Patient characteristics

The demographics of the 237 patients and standard laboratory tests around the time of liver biopsy are summarized in Table 2. The mean age of the 237 patients (135 male and 102 female) was 54.3 ± 11.6 years. The AST range was 21-348 U/L (mean value, 101.2 ± 56.0 U/L). The ALT range was 24-637 U/L (mean value, 156.3 ± 92.5 U/L). The platelet count range was 60-373 × 10^3/μL (mean value, 170.9 ± 55.1 × 10^3/μL).

Correlations between fibrosis stage and fibrosis-predicting models

Correlations between routine blood tests, fibrosis-predicting models, and histological fibrosis stage are summarized in Table 3. The highest correlation was observed for FibroQ ($r_s = 0.444$), Lok's model ($r_s = 0.430$), and FIB-4 ($r_s = 0.429$) ($P < 0.001$). Figure 1 shows the box-plots of fibrosis scores according to Metavir fibrosis stage. Weaker correlations were also found between other scores and histological fibrosis stage, especially for API ($r_s = 0.360$), AAR ($r_s = 0.341$), and APRI ($r_s = 0.322$) ($P < 0.001$).

There were 41 (17.3%) patients with Metavir stage F1 fibrosis, 85 (35.9%) with F2, 98 (41.4%) with F3, and 13 (5.5%) with F4. AAR, FibroQ, FIB-4, API, and Lok's model results increased significantly as the fibrosis advanced (Table 4, analysis of variance test: $P < 0.001$).

ROC curve analysis

ROC curves evaluating the diagnostic accuracies of FibroQ, AAR, APRI, CDS, API, Pohl score, FIB-4 index, and Lok's model were constructed and superimposed (Figure 2) to determine which score would have the most clinical utility to predict significant fibrosis (≥ F2). The AUC (95% CI) using the procedures described by Hanley and McNeil[24] was greatest for FibroQ (0.789, 0.720-0.857), then FIB-4 (0.785, 0.686-0.830), followed by Lok's model (0.768, 0.695-0.840), API (0.739, 0.660-0.818), AAR (0.709, 0.626-0.792), APRI (0.651, 0.566-0.736), CDS (0.580, 0.485-0.674), and Pohl score (0.523, 0.429-0.617) (Table 5). The AUC of FibroQ was significantly higher than those of AAR, APRI, CDS, and Pohl score ($P < 0.05$).

To predict extensive fibrosis (≥ F3), ROC curves for FibroQ, AAR, APRI, CDS, API, Pohl score, FIB-4 index, and Lok's model were also constructed and superimposed to determine which score would have the most clinical utility (Figure 3). The AUC curves (95% CI) using the procedures described by Hanley and McNeil[24] were greatest for FibroQ (0.728, 0.662-0.793), then FIB-4 (0.725, 0.659-0.791), followed by Lok's model (0.721, 0.656-0.786), API (0.696, 0.628-0.764), APRI (0.681, 0.613-0.749), AAR (0.675, 0.607-0.743), CDS (0.609, 0.537-0.680), and Pohl score (0.532, 0.458-0.606) (Table 5). The AUC of FibroQ was significantly higher than those of CDS or Pohl score ($P < 0.05$).

Sensitivity, specificity, positive predictive value, and negative predictive value

Table 6 shows the performance of FibroQ, AAR, APRI, CDS, API, Pohl score, FIB-4, and Lok's model at various cutoff levels for the prediction of significant fibrosis (F2,

Table 4 Correlation between fibrosis score and aspartate aminotransferase-to-platelet ratio index, aspartate aminotransferase/alanine aminotransferase ratio, and FibroQ, FIB-4, cirrhosis discriminant score, age-platelet index, Lok's model, Pohl score

Metavir fibrosis score Patient number (%)	F1 41 (17.3)	F2 85 (35.9)	F3 98 (41.4)	F4 13 (5.5)
AAR	0.566 ± 0.162[c,e]	0.673 ± 0.250	0.749 ± 0.222	0.776 ± 0.175
APRI	1.433 ± 1.040[a]	1.919 ± 1.904	2.320 ± 1.390	2.508 ± 1.102
FibroQ	1.485 ± 0.855[g,i]	2.532 ± 1.660[m]	3.563 ± 2.056	3.581 ± 1.812
FIB-4	1.79 ± 1.13[g,i,m]	2.79 ± 2.03	3.75 ± 1.97	3.82 ± 1.57
CDS	5.61 ± 1.16	5.69 ± 1.19	6.14 ± 1.12	6.15 ± 1.41
API	3.95 ± 2.06[g,i,m]	5.25 ± 2.24	6.40 ± 2.29	6.15 ± 1.63
Lok's model	0.22 ± 0.12[k,m,o]	0.32 ± 0.18	0.43 ± 0.19	0.46 ± 0.18
Pohl score	0	0.01 ± 0.11	0.08 ± 0.28	0.04 ± 0.19

[a]$P < 0.05$, F1 *vs* F3; [c]$P < 0.01$, F1 *vs* F3; [e]$P < 0.05$, F1 *vs* F4; [g]$P < 0.05$, F1 *vs* F2; [i]$P < 0.01$, F1 *vs* F3 and F4; [k]$P < 0.01$, F1 *vs* F2, F3 and F4; [m]$P < 0.01$, F2 *vs* F3; [o]$P < 0.05$, F2 *vs* F4. AAR: AST/ALT ratio; APRI: AST-to-platelet ratio index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; API: Age-platelet index; CDS: Cirrhosis discriminant score.

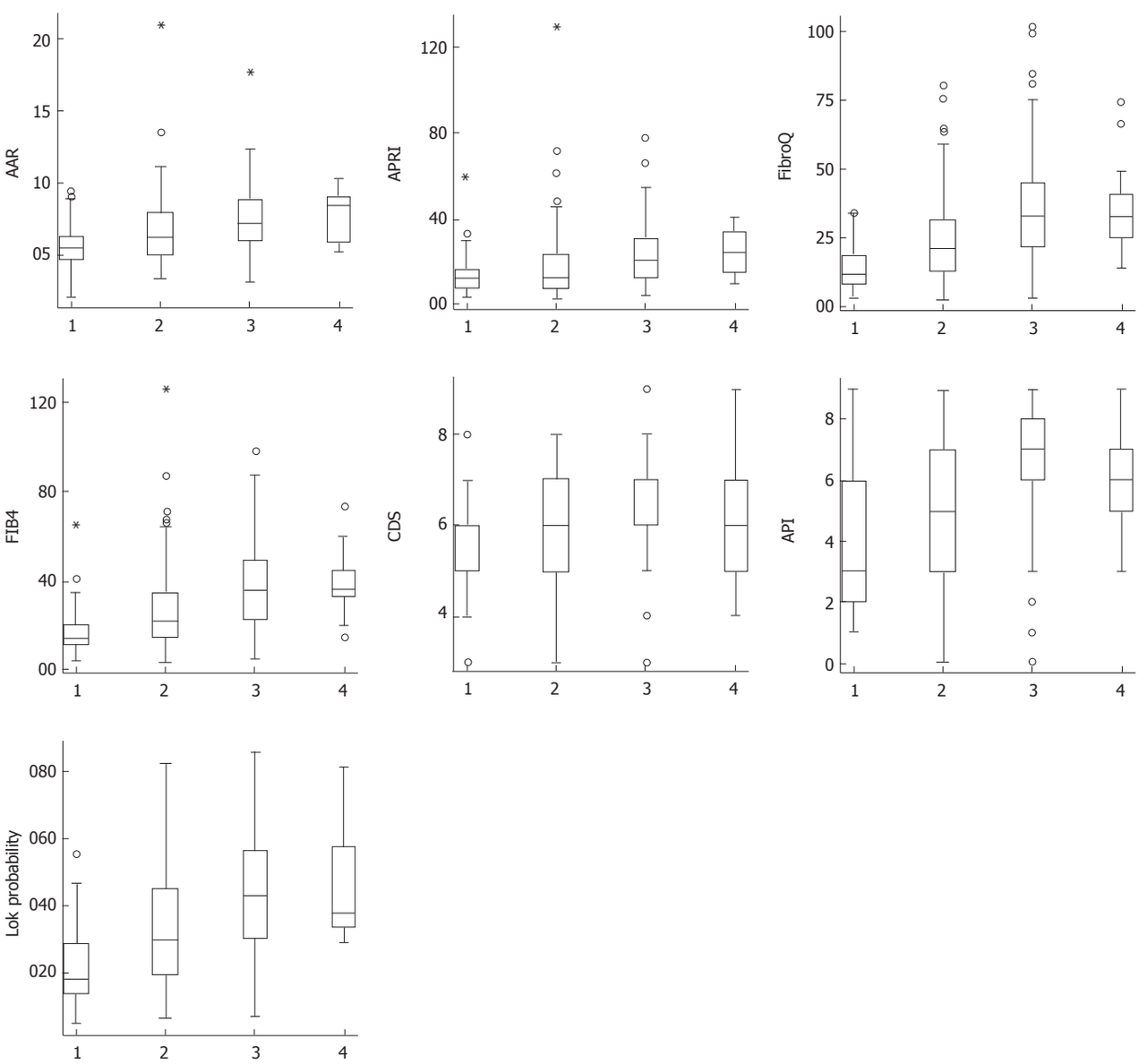

Figure 1 Score values according to Metavir fibrosis stages. Each outlier value is represented by a small circle symbol (o) in the Box Plot graph. If an outlier is more than 3 times the inter-quartile range away from Q1 or Q3, it is classified as an extreme outlier, asterisk sign (*). The top and bottom of each box are the 25th and the 75th percentiles. The line through the box is the median, and the errors bars are the 5th and 95th percentiles. AAR: AST/ALT ratio; APRI: AST-to-platelet ratio index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; API: Age-platelet index; CDS: Cirrhosis discriminant score.

Table 5 Performance of simple fibrosis prediction tests for significant fibrosis (F2, F3 and F4) and extensive fibrosis (F3 and F4)

Metavir fibrosis score *vs*	AUC (F2, F3, F4) (95% CI)	AUC (F3, F4) (95% CI)
AAR	0.709 (0.626-0.792)	0.675 (0.607-0.743)
APRI	0.651 (0.566-0.736)	0.681 (0.613-0.749)
FibroQ	0.789 (0.720-0.857)	0.728 (0.662-0.793)
FIB-4	0.785 (0.686-0.830)	0.725 (0.659-0.791)
CDS	0.580 (0.485-0.674)	0.609 (0.537-0.680)
API	0.739 (0.660-0.818)	0.696 (0.628-0.764)
Lok's model	0.768 (0.695-0.840)	0.721 (0.656-0.786)
Pohl score	0.523 (0.429-0.617)	0.532 (0.458-0.606)

AAR: AST/ALT ratio; APRI: AST-to-platelet ratio index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; API: Age-platelet index; CDS: Cirrhosis discriminant score; AUC: Area under the receiver operating characteristic curve.

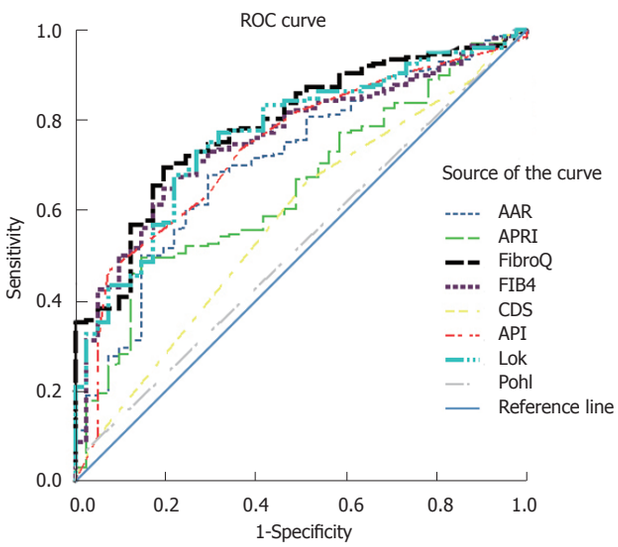

Figure 2 Receiver operating characteristic curves of simple noninvasive tests evaluated for prediction of significant fibrosis (F2, F3 and F4). AAR: AST/ALT ratio; APRI: AST-to-platelet ratio index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; API: Age-platelet index; CDS: Cirrhosis discriminant score; ROC: Receiver operating characteristic.

F3 and F4) and extensive fibrosis (F3 and F4). To compare our results with those of previous reports, the sensitivity, specificity, PPV, and NPV of the simple fibrosis prediction tests were calculated using cutoff values exactly as originally described[16,17,21-23]. Using a cutoff value of the FibroQ score of > 1.6 to predict the presence of significant fibrosis resulted in a sensitivity of 77.6%, specificity of 65.9%, PPV of 91.6%, and NPV of 38.0% in 166 (70%) of the 237 patients. With AAR, the cutoff levels to predict the presence (AAR > 1.0) of significant fibrosis had a sensitivity of 8.16%, specificity of 100%, PPV of 100%, and NPV of 18.5%. Using APRI, the cutoff values to predict the presence (APRI > 1.5) or absence (APRI < 0.5) of significant fibrosis had a sensitivity of 56.6% and 96.9%, specificity of 58.5% and 9.7%, PPV of 86.7% and 83.7%, and NPV of 22.0% and 40.0%, respectively.

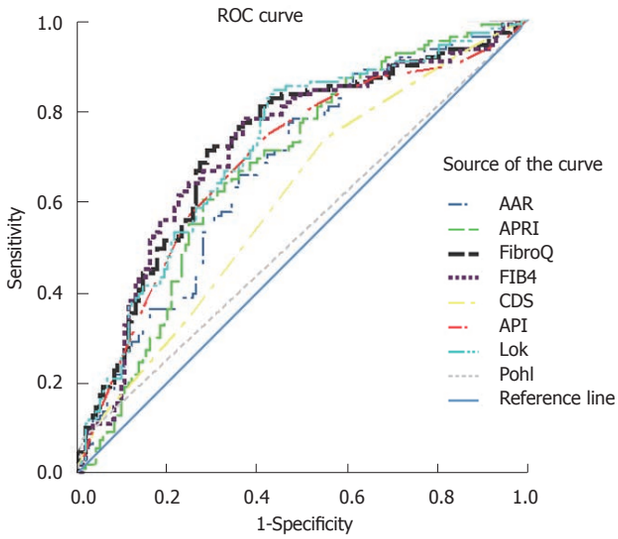

Figure 3 Receiver operating characteristic curves of simple noninvasive tests evaluated for prediction of extensive fibrosis (F3 and F4). AAR: AST/ALT ratio; APRI: AST-to-platelet ratio index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; API: Age-platelet index; CDS: Cirrhosis discriminant score; ROC: Receiver operating characteristic.

In Lok's model, the cutoff values to predict the presence (Lok's model > 0.5) or absence (Lok's model < 0.2) of extensive fibrosis had a sensitivity of 37.8% and 88.3%, specificity of 88.1% and 37.3%, PPV of 73.7% and 55.4%, and NPV of 61.7% and 78.3%, respectively. At a cutoff of FIB-4 < 1.45, the NPV to exclude extensive fibrosis (F3 and F4) was 75.9% with a sensitivity of 87.4%. A cutoff of FIB-4 > 3.25 had a PPV of 70.1% and a specificity of 77%.

Since lower cutoffs were originally described to exclude significant fibrosis, specific attention must be paid to NPVs that ranged from 18.0% to 41.7%. For the same cutoffs, NPVs to exclude extensive fibrosis showed superior performance compared with performance on significant fibrosis cases, ranging from 54.8% to 90.0%. For example, a FibroQ < 1.4, which was observed in 25.7% of patients, excluded significant fibrosis with 36.1% certainty and excluded extensive fibrosis with 75.4% certainty. The best predictive value was observed for positive Pohl score, but this cutoff selected only 3.8% of patients.

DISCUSSION

To assess the pathological grade and stage of chronic viral hepatitis, liver biopsy is necessary. However, liver biopsy is invasive, costly, and has its own limitations[11,25]. Hence, several noninvasive tests combining biological parameters have been proposed to attempt to predict the degree of fibrosis, with the objective of replacing liver biopsy[20,26-29]. There are also some noninvasive tests, such as PⅢP (N -terminal peptide of type Ⅲ procollagen)[29], fibrometer[26], Hepascore[30], FibroTest[31], and Forn's score[27]. The current study excludes these tests due to their expense (e.g., procollagen level), because they can only be checked in the laboratory (e.g., hyaluronic acid),

Table 6 Diagnostic accuracy of simple fibrosis prediction tests for significant and extensive fibrosis

Score	Cut-off value	(%)	Significant fibrosis (F2, F3 and F4)				Extensive fibrosis (F3 and F4)			
			Sen	Spe	PPV	NPV	Sen	Spe	PPV	NPV
FibroQ	> 0.6	94.9	96.4	12.2	84.0	41.7	97.3	7.14	48.0	75.0
	> 1.2	79.7	85.7	48.8	88.9	41.7	90.1	29.4	52.9	77.1
	> 1.4	74.3	80.1	53.7	89.2	36.1	86.5	36.5	54.5	75.4
	> 1.6	70.0	77.6	65.9	91.6	38.0	85.6	43.7	57.2	77.5
	> 1.8	66.2	74.0	70.7	92.4	36.3	83.8	49.2	59.2	77.5
	> 2.0	60.8	69.4	80.5	94.4	35.5	82.0	57.9	63.2	78.5
	> 2.6	44.3	51.0	87.8	95.2	27.3	64.9	73.8	68.6	70.5
AAR	> 0.4	97.0	95.4	12.2	83.9	35.7	96.4	7.1	47.8	69.2
	> 0.6	61.2	67.9	70.7	91.7	31.5	77.5	53.2	59.3	72.8
	> 0.8	28.7	31.6	85.4	91.2	20.7	36.9	78.6	60.3	58.6
	> 1.0	6.8	8.16	100	100	18.5	10.8	96.8	75.0	55.2
APRI	> 0.5	95.8	96.9	9.7	83.7	40.0	99.1	7.1	48.5	90.0
	> 1	72.6	75.5	41.5	86.0	26.2	87.4	40.5	56.4	78.5
	> 1.5	54.0	56.6	58.5	86.7	22.0	69.4	59.5	60.2	68.8
	> 2	39.7	45.4	87.8	94.7	25.2	55.0	73.8	64.9	65.0
CDS	> 5	88.6	88.8	12.2	82.9	18.5	92.8	15.1	49.0	70.4
	> 6	63.7	66.3	48.8	86.1	23.3	73.9	45.2	54.3	66.3
	> 7	29.1	30.6	78.0	87.0	19.0	34.2	75.4	55.1	56.5
	> 8	8.9	9.7	95.1	90.5	18.1	12.6	94.4	66.7	55.1
API	> 4	75.9	81.6	51.2	88.9	36.8	87.4	34.1	53.9	75.4
	> 5	67.1	73.5	63.4	90.6	33.3	82.0	46.0	57.2	74.4
	> 6	57.4	63.3	70.7	91.2	28.7	74.8	57.9	61.0	72.3
	> 7	39.7	46.4	92.7	96.8	26.6	56.8	75.4	67.0	66.4
Pohl score	Positive	3.8	4.59	100	100	18.0	7.2	99.2	88.9	54.8
FIB-4	> 1.45	75.5	81.6	53.7	89.4	37.9	87.4	34.9	54.2	75.9
	> 2	62.0	69.4	73.2	92.5	33.3	81.1	54.8	61.2	76.7
	> 2.5	53.6	61.2	82.9	94.5	30.9	74.8	65.1	65.4	74.5
	> 3	46.0	52.6	85.4	94.5	27.3	66.7	72.2	67.9	71.1
	> 3.25	40.9	47.4	90.2	95.9	26.4	61.3	77.0	70.1	69.3
Lok's model	> 0.2	74.7	81.6	58.5	90.4	40.4	88.3	37.3	55.4	78.3
	> 0.4	35.0	40.8	92.7	96.4	24.7	51.4	79.4	68.7	64.9
	> 0.5	24.1	28.6	97.6	98.2	22.2	37.8	88.1	73.7	61.7

AAR: AST/ALT ratio; APRI: AST-to-platelet ratio index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; API: Age-platelet index; CDS: Cirrhosis discriminant score; Sen: Sensitivity; Spe: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

or because they are not included in the routine monitoring and investigations of patients with chronic liver disease (e.g., haptoglobin or cholesterol).

We believe that an ideal noninvasive test for assessing liver fibrosis should be reliable, reproducible, and based on readily available tests and parameters. APRI, AAR, FibroQ, FIB-4, CDS, API, Lok's model, and Pohl score fulfilled these criteria, therefore, we compared these measures for evaluation of patients with chronic hepatitis C. The aim was to validate the usefulness of these simple tests in a community hospital in an area with hyperendemic HCV infection.

In addition, bias of the biopsy examination is also acknowledged. Regev *et al*[32] have reported discordances in fibrosis stage in one third of patients when right and left liver lobes were compared. As a liver biopsy specimen from a cirrhotic liver is often fragmented, the inadequate size of biopsy samples can lead to an underestimation of fibrosis as reported by Colloredo *et al*[33] and Bedossa *et al*[11]. Therefore, some cases diagnosed as F3 may have had cirrhosis (F4). Biopsy length and fragmentation were not recorded or compared in our study; however, a recent study has found that these variables did not affect the performance of their model[34]. Furthermore, we should notice that four different staging systems were used in the original studies for liver fibrosis tests. AAR results were based on the Scheuer system; APRI and FIB-4 were based on the Ishak system; FibroQ, API, and Pohl's score were based on the Metavir system; and CDS and Lok's model were based on a modified Knodell system. The different staging systems using either a 5-stage (F0-F4) or a 7-stage (F0-F6) scale prevented us from comparing fibrosis scores more precisely.

Although the current study was retrospective, it had some advantages over previous studies. First, the patients were all from a treatment-naïve population with HCV infection as the only problem. Second, we compared all fibrosis markers that combined routine blood tests rather than markers only available in the laboratory. Third, all histological assessment was performed blindly by one pathologist, thus preventing interobserver bias in fibrosis staging. Finally, using a 5-stage fibrosis scoring system in this study resulted in a lower tendency to induce interpretation error than would be the case with a 7-stage scoring system[22].

We divided the patients in two different ways to obviate liver biopsy. The first grouping was F1 *vs* F2, F3 and F4, the second was F1 and F2 *vs* F3 and F4. The ratio-

nale for the F1 *vs* F2, F3 and F4 grouping was that there was no need for treatment in patients with mild fibrosis. Patients with severe fibrosis or cirrhosis need screening for hepatocellular carcinoma, esophageal varices, and complications of portal hypertension, therefore, we made a second patient grouping of F1 and F2 *vs* F3 and F4. Applying the cutoff values, we were able to discriminate patients according to two relevant thresholds of fibrosis based on the needs for clinical treatment decision-making.

The result of our current study showed that Pohl score and CDS had high specificity but low sensitivity, and their AUCs were not statistically different from 0.5. In addition, FibroQ, FIB-4, and Lok's model showed the best performance characteristics. The AUCs for predicting significant fibrosis were 0.789, 0.785 and 0.768, respectively. The AUCs for predicting extensive fibrosis were 0.728, 0.725 and 0.721, respectively[21-23]. To evaluate the accuracy of the fibrosis index, we also checked the percentage of patients that were correctly classified according to the stage of fibrosis. Using FibroQ results below the lower cutoff value (0.6) and above the higher cutoff value (1.6), 157 of 178 patients (88.2%) were correctly identified as having or not having significant fibrosis, which was a better performance than in the original study on the use of FibroQ[23]. Using FIB-4, 72.3% of the 155 patients with FIB-4 values outside 1.45-3.25 would be correctly classified, and liver biopsy could be avoided in 65.4% of patients, which is slightly lower than the value of 71% reported by Sterling *et al*[21]. Using Lok's model with a cutoff of 0.2 to exclude extensive fibrosis, only 11.7% of those with extensive fibrosis were misclassified. Using a cutoff of 0.5, only 11.9% of those without extensive fibrosis had a score > 0.5. These results are compatible with those reported by Cheung *et al*[22]. Using the same cutoff values, 7.8% and 14.8% of patients were misclassified in their study.

In summary, the current study demonstrated that FibroQ, FIB-4, and Lok's model were simple methods that correlated well with the stages of fibrosis in patients with chronic hepatitis C. FibroQ showed a trend to be superior to the other modalities evaluated. Further prospective studies involving larger numbers of patients are warranted to validate the usefulness of FibroQ in clinical practice.

COMMENTS

Background

Viral hepatitis C is one of the most common liver diseases in the world, affecting an estimated 200 million individuals. Knowledge of the extent of liver fibrosis is important for the clinical management of chronic hepatitis C. Liver biopsy is recommended prior to antiviral treatment. However, liver biopsy may cause complications, including mortality in 0.018% of patients. Hence, several noninvasive tests have been proposed to assess the severity of hepatic fibrosis.

Research frontiers

Aspartate aminotransferase (AST)-to-platelet ratio index (APRI) and AST/alanine aminotransferase (ALT) ratio (AAR), cirrhosis discriminant score (CDS), age-platelet index (API), Pohl score, FIB-4 index, and Lok's model are well-known parameters that are based on routine laboratory data and are therefore readily available in clinical practice.

Innovations and breakthroughs

Recently, the authors proposed a novel index, FibroQ, which was calculated from common laboratory test results that included prothrombin time international normalized ratio (PT INR), platelet, AST, ALT, and age, calculated as 10 × (age × AST × PT INR)/(ALT × platelet count) to predict significant fibrosis. FibroQ trended to be superior in predicting significant fibrosis score in chronic hepatitis C compared with other noninvasive tests.

Applications

FibroQ is a simple and useful noninvasive test for predicting significant fibrosis in patients with chronic hepatitis C.

Terminology

FibroQ, APR, AAR, CDS, API, Pohl score, FIB-4 index, and Lok's model are well-known parameters that are based on routine laboratory data and are reported to predict the presence of liver significant fibrosis and extensive fibrosis.

Peer review

The study is of particular practical medical interest. The results provide sufficient evidence that the FibroQ correlates with significant liver fibrosis in patients with hepatitis C virus.

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S- Editor Sun H **L- Editor** Kerr C **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.754

World J Gastroenterol 2012 February 28; 18(8): 754-766
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Effects of resistin-like molecule β over-expression on gastric cancer cells *in vitro*

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Supported by The National Natural Science Foundation of China, No. 30200284, No. 30600278, No. 30772359, No. 81071997 and No. 81072073; Program for New Century Excellent Talents from Universities, No. NCET-06-0641; Scientific Research Fund for the Returned Overseas Chinese Scholars, No. 2008-889; Fundamental Research Funds for the Central Universities, No. 2010JC025
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Received: October 1, 2011 Revised: January 5, 2012
Accepted: January 18, 2012
Published online: February 28, 2012

Abstract

AIM: To investigate the effects of resistin-like molecule β (RELMβ) over-expression on the invasion, metastasis and angiogenesis of gastric cancer cells.

METHODS: Human RELMβ encoding expression vector was constructed and transfected into the RELMβ lowly-expressed gastric cancer cell lines SGC-7901 and MKN-45. Gene expression was measured by Western blotting, reverse transcription polymerase chain reaction (PCR) and real-time quantitative PCR. Cell proliferation was measured by 2-(4,5-dimethyltriazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetry, colony formation and 5-ethynyl-20-deoxyuridine incorporation assays. The *in vitro* migration, invasion and metastasis of cancer cells were measured by cell adhesion assay, scratch assay and matrigel invasion assay. The angiogenic capabilities of cancer cells were measured by tube formation of endothelial cells.

RESULTS: Transfection of RELMβ vector into SGC-7901 and MKN-45 cells resulted in over-expression of RELMβ, which did not influence the cellular proliferation. However, over-expression of RELMβ suppressed the *in vitro* adhesion, invasion and metastasis of cancer cells, accompanied by decreased expression of matrix metalloproteinase-2 (MMP-2) and MMP-9. Moreover, transfection of RELMβ attenuated the expression of vascular endothelial growth factor and *in vitro* angiogenic capabilities of cancer cells.

CONCLUSION: Over-expression of RELMβ abolishes the invasion, metastasis and angiogenesis of gastric cancer cells *in vitro*, suggesting its potentials as a novel therapeutic target for gastric cancer.

Key words: Resistin-like molecule β; Gastric cancer; Invasion; Metastasis; Angio-genesis

Peer reviewers: Henning Schulze-Bergkamen, National Centre for Tumor Diseases, University of Heidelberg, Im Neuenheimer Feld 350, Heidelberg 69120, Germany; Ki Baik Hahm, Professor, Gachon Graduate School of Medicine, Lee Gil Ya Cancer and Diabetes Institute, Incheon 406-840, South Korea

Zheng LD, Yang CL, Qi T, Qi M, Tong L, Tong QS. Effects of resistin-like molecule β over-expression on gastric cancer cells *in vitro*. *World J Gastroenterol* 2012; 18(8): 754-766 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/754.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.754

INTRODUCTION

Gastric cancer is one of the most common cancer types in the world[1]. In spite of the standardization of surgical techniques and multimodal therapy, the postoperative survival of patients with advanced gastric cancer still remains very low[1]. Recent evidence shows that goblet cell-derived proteins, such as intestinal trefoil factor (ITF) and mucin 2 (MUC2), participate in the pathogenesis of gastric cancer[2-6]. As a member of trefoil peptide family that is expressed exclusively in the goblet cells of small intestine and colon[7], ITF is over-expressed in several cancer tissues including gastric cancer[2,7], and promotes tumor cell invasion and angiogenesis[7-9]. Blocking ITF expression *via* an antisense strategy suppresses the *in vitro* growth and tumorigenicity of gastric cancer cells[3], suggesting that ITF may serve as a potential target in the control of gastrointestinal cancer progression. Similarly, MUC2 is expressed in the goblet cells of colon, small intestine and airways[10], and is aberrantly expressed in gastric cancer[4,5]. Measuring the MUC2 transcriptional levels is a sensitive and specific approach to detect lymph node micrometastasis in gastric cancer patients[6]. These results suggest that goblet cell-specific proteins may be involved in the progression of gastric cancer, which are potential targets for regulating the invasion, metastasis and angiogenesis of gastric cancer.

Resistin-like molecule β (RELMβ), also known as Found in Inflammatory Zone 2 (FIZZ2), belongs to a family of resistin-like cytokine molecules consisting of small and cysteine-rich secretory proteins[11]. As a novel goblet cell-specific protein that is abundantly expressed in proximal and distal colon[11,12], RELMβ is induced by intestinal microbial colonization, and plays a key role in epithelial barrier function and integrity[12,13]. In addition, RELMβ functions not only as a Th2 cytokine immune effector but also as an inhibitor of chemotaxis of parasites, through interfering with parasite nutrition by directly binding to the chemosensory components of parasites[13]. Recent evidence shows that RELMβ has the potentials to contribute to the airway remodeling in diseases such as asthma[14], and is involved in the pathogenesis of fibrotic lung diseases as a Th2-associated multifunctional mediator[15] and the development of scleroderma-associated pulmonary hypertension[16]. However, the role of RELMβ in cancer development still remains unclear.

Our previous studies have indicated that RELMβ is over-expressed in a majority of human colon cancer tissues[17], and in the metaplastic epithelium of Barrett's esophagus and associated dysplasia[18]. Moreover, RELMβ is aberrantly expressed in the goblet cells of intestinal metaplasia and cytoplasm of cancer cells in gastric cancer tissues, which is positively correlated with tumor differentiation and longer overall survival, and inversely correlated with tumor infiltration and lymph node metastasis, indicating the value of RELMβ in predicting the outcomes of gastric cancer patients[19]. In this study, to further elucidate the exact role of RELMβ in the progression of gastric cancer, we investigated the effects of RELMβ over-expression on the RELMβ lowly-expressed gastric cancer cells. We found that over-expression of RELMβ attenuated the invasion, metastasis and angiogenesis of cancer cells, suggesting the anti-tumor role of RELMβ in the progression of gastric cancer.

MATERIALS AND METHODS

Cell culture

Human gastric cancer cell lines SGC-7901 and MKN-45 were obtained from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Human endothelial cell line HUVEC (CRL-1730) was purchased from American Type Culture Collection (Rockville, MD, United States). The cells were grown in RPMI1640 medium (Life Technologies, Inc., Gaithersburg, MD, United States) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Inc., Gaithersburg, MD, United States), penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were maintained at 37 ℃ in a humidified atmosphere of 5% CO_2.

Vector construction and transfection

Full-length RELMβ cDNA was amplified from human colon tissues, subcloned between the restrictive sites Hind Ⅲ and Bam HI of pcDNA3.1/Zeo(+) (Invitrogen, Carlsbad, CA, United States), and validated by sequencing. The primers used for the RELMβ cDNA amplification were 5'-CGCCCAAGCTTATGGGGCCGTCCTCTTGC-3' (forward) and 5'-CGCGGATCCTCAGGTCAGGTGGCAGCA-3' (reverse). The recombinant pcDNA 3.1-RELMβ or empty vector (mock) was transfected into SGC-7901 and MKN-45 cells with Lipofectamine 2000 (Life Technologies, Inc., Gaithersburg, MD, United States), according to the manufacturer's instructions. To monitor the transfection efficiency, the cancer cells were co-transfected with pEGFP-N1 (Clontech, Mountair View, CA, United States).

Western blotting

Western blotting was performed as previously described[20], with antibodies specific for RELMβ (Abcam Inc, Cambridge, MA, United States), matrix metalloproteinase-2 (MMP-2), MMP-9, v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets1), vascular endothelial growth factor (VEGF) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States). ECL substrate kit (Amersham, Piscataway, NJ, United States) was used for the chemiluminscent detection of signals with autoradiography film (Amersham).

Reverse transcription polymerase chain reaction and real-time quantitative reverse transcription polymerase chain reaction

The reverse transcription reactions were conducted with Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN, United States). The polymerase chain reac-

Table 1 Primers sets used for reverse transcription polymerase chain reaction and real-time reverse transcription polymerase chain reaction

Primer set	Primers	Sequence	Product size (bp)
RELMβ	Forward	5′-ATGGGGCCGTCCTCTTGCCTCC-3′	336
	Reverse	5′-TCAGGTCAGGTGGCAGCAGCG-3′	
MMP-2	Forward	5′-CCAAAACGGACAAAGAGT-3′	275
	Reverse	5′-ATCAGGTGTGTAGCCAAT-3′	
MMP-9	Forward	5′-CAGAGATGCGTGGAGAGT-3′	220
	Reverse	5′-TCTTCCGAGTAGTTTTGG-3′	
Ets1	Forward	5′-TTCACTAAAGAACAGCAAC-3′	205
	Reverse	5′-TGTCCCCAACAAAGTCTG-3′	
VEGF	Forward	5′-GGCAGAATCATCACGAAG-3′	276
	Reverse	5′-TGTGCTGTAGGAAGCTCA-3′	
GAPDH	Forward	5′- AGAAGGCTGGGGCTCATTTG-3′	258
	Reverse	5′- AGGGGCCATCCACAGTCTTC-3′	

RT-PCR: Reverse transcription polymerase chain reaction; RELMβ: Resistin-like molecule β; MMP: Matrix metalloproteinase-2; Ets1: E26 oncogene homolog 1; VEGF: Vascular endothelial growth factor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

tion (PCR) amplification was performed with the primer sets indicated in Table 1. Real-time PCR with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, United States) was performed using ABI Prism 7700 Sequence Detector (Applied Biosystems). The fluorescent signals were collected during extension phase, Ct values of the sample were calculated, and the transcript levels were analyzed by $2^{-\Delta\Delta Ct}$ method.

MTT colorimetric assay

Cancer cells were cultured in 96-well plates at 5×10^3 cells per well and transfected with pcDNA3.1-RELMβ or empty vector (mock). After transfection for 24 h, 72 h and 120 h, cell viability was monitored by the 3-(4,5-dimethyltriazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, MO, United States) colorimetric assay[20]. All experiments were done with 6-8 wells per experiment and repeated at least three times.

Colony formation assay

Seventy-two hours after transfection, the cells were seeded at a density of 300/mL on 35-mm dishes. Colony formation assay was performed as previously described[20]. Positive colony formation (more than 50 cells/colony) was counted. The survival fraction of cells was expressed as the ratio of plating efficiency of treated cells to that of untreated control cells.

EdU incorporation assay

Cancer cells were cultured in 96-well plates at 5×10^3 cells per well, transfected with pcDNA3.1-RELMβ or empty vector (mock) for 72 h, then exposed to 50 μmol/L of 5-ethynyl-20-deoxyuridine (EdU, Ribobio, China) for additional 4 h at 37 ℃. The cells were fixed with 4% formaldehyde for 15 min and treated with 0.5% Triton X-100 for 20 min at room temperature. After washing with phosphate buffered saline for three times, the cells of each well were reacted with 100 μL of 1 × Apollo® reaction cocktail for 30 min. Subsequently, the DNA contents of cells in each well were stained with 100 μL of Hoechst 33342 (5 μg/mL) for 30 min and visualized under a fluorescent microscope.

Cell adhesion assay

Seventy-two hours after transfection, 2×10^4 cancer cells were inoculated into each well of 96-well plates that were precoated with 100 μL of 20 μg/mL matrigel (BD Biosciences, Franklin Lakes, NJ, United States), and incubated at 37 ℃ in serum-free complete medium (pH 7.2) for 2 h. Cell adhesion was measured as previously described[20]. And 0%, 20%, 50% and 100% of inoculated cells were directly fixed in 4% paraformaldehyde 2 h after inoculation.

Scratch migration assay

Cancer cells were cultured in 24-well plates and transfected with pcDNA3.1-RELMβ or empty vector (mock). Seventy-two hours after transfection, the cells were scraped with the fine end of 1-mL pipette tips (Time 0). Scratch migration assay was performed as previously described[20]. Remodeling was measured as diminishing distance across the induced injury and normalized to the 0 h control.

Matrigel invasion assay

The Boyden chamber technique (transwell analysis) was applied as previously described[20]. Briefly, 72 h after transfection, homogeneous single cell suspensions (1×10^5 cells/well) were added to the upper chambers and allowed to invade for 24 h at 37 ℃ in a CO_2 incubator. The migrated cells were counted according to the published criteria[21].

Tube formation assay

Fifty microliters of growth factor-reduced matrigel were polymerized on 96-well plates. HUVECs were serum starved in RPMI1640 medium for 24 h, suspended in RPMI1640 medium preconditioned with pcDNA3.1-RELM- or empty vector-transfected SGC-7901 or MKN-45 cells, added to the matrigel-coated wells at the density of 5×10^4 cells/well, and incubated at 37 ℃ for 18 h. Tube formation was visualized using a Leitz inverted microscope equipped with a Sony color digital DXC-S500 camera. Anti-angiogenic activity was detected by measuring the length of tube walls formed in the discrete endothelial cells in each well compared with the controls.

Statistical analysis

Unless otherwise stated, all data were shown as mean ± SE. Statistical significance ($P < 0.05$) was determined by *t* test or analysis of variance (ANOVA) followed by assessment of differences using SigmaStat 2.03 software (Jandel, Erkrath, Germany).

RESULTS

Transient transfection-mediated over-expression of RELMβ in gastric cancer cells

To examine the effects of RELMβ over-expression on

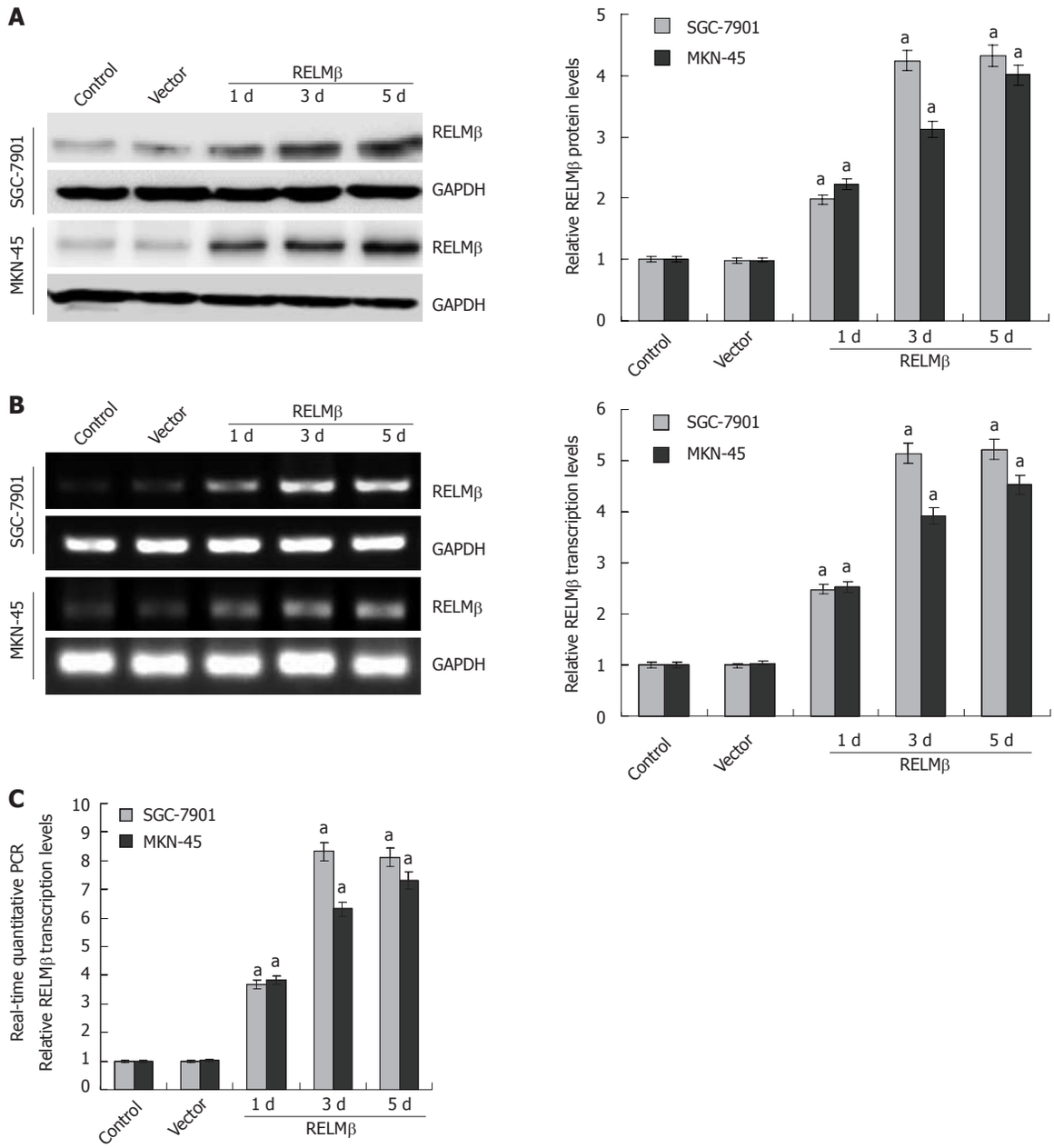

Figure 1 Transient transfection-mediated over-expression of resistin-like molecule β in gastric cancer cells. A: Western blotting indicated that low resistin-like molecule β (RELMβ) protein was detected in the parental SGC-7901 and MKN-45 cells, and transient transfection of the empty vector (mock) did not affect the expression levels of RELMβ. However, transient transfection of pcDNA3.1-RELMβ for 24 h, 72 h and 120 h resulted in increased RELMβ expression; **B: 24 h, 72 h and 120 h** after **transfection, reverse transcription polymerase chain reaction (RT-PCR) indicated the increased RELM**β transcription levels in pcDNA3.1-RELMβ transfected SGC-7901 and MKN-45 cells, but not in mock group; C: Real-time quantitative RT-PCR further demonstrated that transfection of pcDNA3.1-RELMβ for 24 h, 72 h and 120 h resulted in upregulation of RELMβ transcription levels in SGC-7901 and MKN-45 cells. The symbol (a) indicates a significant increase **compared with parental** cells ($P < 0.01$). Triplicate experiments were performed with essentially identical results.

human gastric cancer, the RELMβ cDNA was amplified from human colon tissues, subcloned into pcDNA3.1/Zeo(+) and validated by sequencing. Gastric cancer SGC-7901 and MKN-45 cells were transfected with pcDNA3.1-RELM or empty vector (mock). The transfection efficiency was monitored by co-transfection with the enhanced green fluorescent protein (EGFP) reporter vector pEGFP-N1. Seventy-two hours after transfection, EGFP expressed within the cytoplasm of cancer cells, with the transfection efficiency around 60% (data not shown). The protein and mRNA expression of RELMβ was examined by Western blotting, reverse transcription polymerase chain reaction (RT-PCR), and real-time quantitative RT-PCR. As shown in Figure 1A-C, low RELMβ protein and mRNA could be detected in the parental SGC-7901 and MKN-45 cells, and transient transfection of empty vector did not affect the expression levels of RELMβ ($P > 0.05$). However, RELMβ was significantly increased in the pcDNA3.1-RELMβ-transfected cells ($P < 0.01$). These results indicated that the eukaryotic vector

for RELMβ used in this study was efficient in up-regulating the expression of RELMβ in gastric cancer cells.

Over-expression of RELMβ did not affect the in vitro cell proliferation of gastric cancer cells

The effects of RELMβ over-expression on proliferation of SGC-7901 and MKN-45 cells were measured by MTT colorimetric assay. We found that transfection of pcDNA 3.1-RELMβ or empty vector (mock) did not affect the cell proliferation when compared with the parental cells ($P > 0.05$, Figure 2A). In addition, colony formation and EdU incorporation assays further revealed that over-expression of RELMβ did not influence the proliferation of cultured SGC-7901 and MKN-45 cells ($P > 0.05$, Figure 2B and C). These results indicated that over-expression of RELMβ did not affect the *in vitro* proliferation of gastric cancer cells.

Over-expression of RELMβ attenuated the adhesion, migration and invasion of gastric cancer cells in vitro

Since the adhesion, migration and invasion are three critical steps involved in metastasis, and RELMβ expression in gastric cancer is correlated with tumor infiltration and lymph node metastasis[19], we examined the effects of RELMβ over-expression on these characteristics in cultured gastric cancer cells. In the adhesion assay, SGC-7901 and MKN-45 cells transfected with pcDNA3.1-RELMβ exhibited markedly reduced ability in adhesion to the pre-coated matrigel, when compared with parental cells ($P < 0.01$, Figure 3A). However, the cells transfected with empty vector (mock) had similar adhesive abilities as parental cells (Figure 3A). In addition, transfection of pcDNA3.1-RELMβ into SGC-7901 and MKN-45 cells resulted in an impaired migration capacity ($P < 0.01$), when compared with the parental and mock cells as evidenced by scratch migration assay (Figure 3B). Moreover, over-expression of RELMβ abolished the invasion capabilities of SGC-7901 and MKN-45 cells, when compared with the parental and mock cells as evidenced by transwell analysis ($P < 0.01$, Figure 3C). These results suggested that over-expression of RELMβ suppressed the adhesion, invasion and metastasis of gastric cancer cells *in vitro*.

Over-expression of RELMβ decreased the expression of MMP-2 and MMP-9 in gastric cancer cells

To explore the mechanisms underlying RELMβ-mediated suppression on the adhesion, invasion and metastasis of gastric cancer cells, the protein and mRNA expression of MMP-2 and MMP-9 were examined by Western blotting, RT-PCR and real-time quantitative RT-PCR. As shown in Figure 4A, B and C, the expression of MMP-2 and MMP-9 was significantly decreased in the pcDNA3.1-RELMβ-transfected SGC-7901 and MKN-45 cells ($P < 0.01$) as compared with the parental cells. However, transient transfection of the empty vector (mock) did not affect the expression level of MMP-2 or MMP-9. These results indicated that over-expression of RELMβ attenuated the expression of MMP-2 and MMP-9 in gastric cancer cells.

Over-expression of RELMβ inhibited the in vitro angiogenesis of gastric cancer cells

We further investigated the effects of RELMβ over-expression on the *in vitro* angiogenic capabilities of SGC-7901 and MKN-45 cells. As shown in Figure 5, extensive tube formation of endothelial cells was observed in parental and mock cells. However, when the endothelial cells were treated with the medium preconditioned with pcDNA3.1-RELMβ-transfected SGC-7901 or MKN-45 cells, the tube formation was significantly suppressed ($P < 0.01$, Figure 5). These results indicated that over-expression of RELMβ remarkably decreased the angiogenesis of gastric cancer cells *in vitro*.

RELMβ attenuated the expression of VEGF, but not Ets1 in gastric cancer cells

Since Ets1 is one of the most important transcription factors to promote tumor angio-genesis[22], and based on the evidence that resistin, a member of the RELM family, influences the VEGF expression in cancer cells[23], we hypothesized that RELMβ might affect its expression in gastric cancer cells. The expression levels of Ets1 and VEGF were examined by Western blotting, RT-PCR and real-time quantitative RT-PCR. As shown in Figure 6A, B and C, the protein and mRNA levels of Ets1 and VEGF could be detected in the parental SGC-7901 and MKN-45 cells, and transient transfection of the empty vector (mock) did not affect their expression levels. However, VEGF, but not Ets1, was significantly decreased in the pcDNA3.1-RELMβ-transfected cells ($P < 0.01$). These results indicated that over-expression of RELMβ attenuated the expression of VEGF in gastric cancer cells.

DISCUSSION

Resistin-like molecules/found in inflammatory zone (RELM/FIZZ) gene family consists of four members, including resistin, RELMα, RELMβ and RELMγ, which exhibit unique distribution patterns in mammalian species[11]. Resistin, a small and cysteine-rich protein hormone secreted from adipose tissue, is named for its ability to induce insulin resistance[24]. RELMα is expressed in several tissues including white adipose tissue and lung, and participates in the regulation of inflammatory process[25,26]. RELMβ is highly conserved in all examined mammalian species, and its expression is tightly restricted to intestinal goblet cells, from where it is secreted apically into the intestinal lumen as a homodimer[11]. RELMγ is expressed in mouse spleen, bone marrow and intestine, and may play a role in promyelocytic differentiation[27,28]. Currently, although most studies have focused on the roles of RELMβ in intestinal defense against parasitic nematode infection and colonic inflammation[29], the functions of RELMβ remain to be further elucidated. Interestingly, recent evidences reveal the close relationship between resistin and prostate cancer[30], gastric cancer[31], colorectal cancer[32,33], breast cancer[34,35], and endometrial cancer[36]. It has been indicated that resistin induces cell

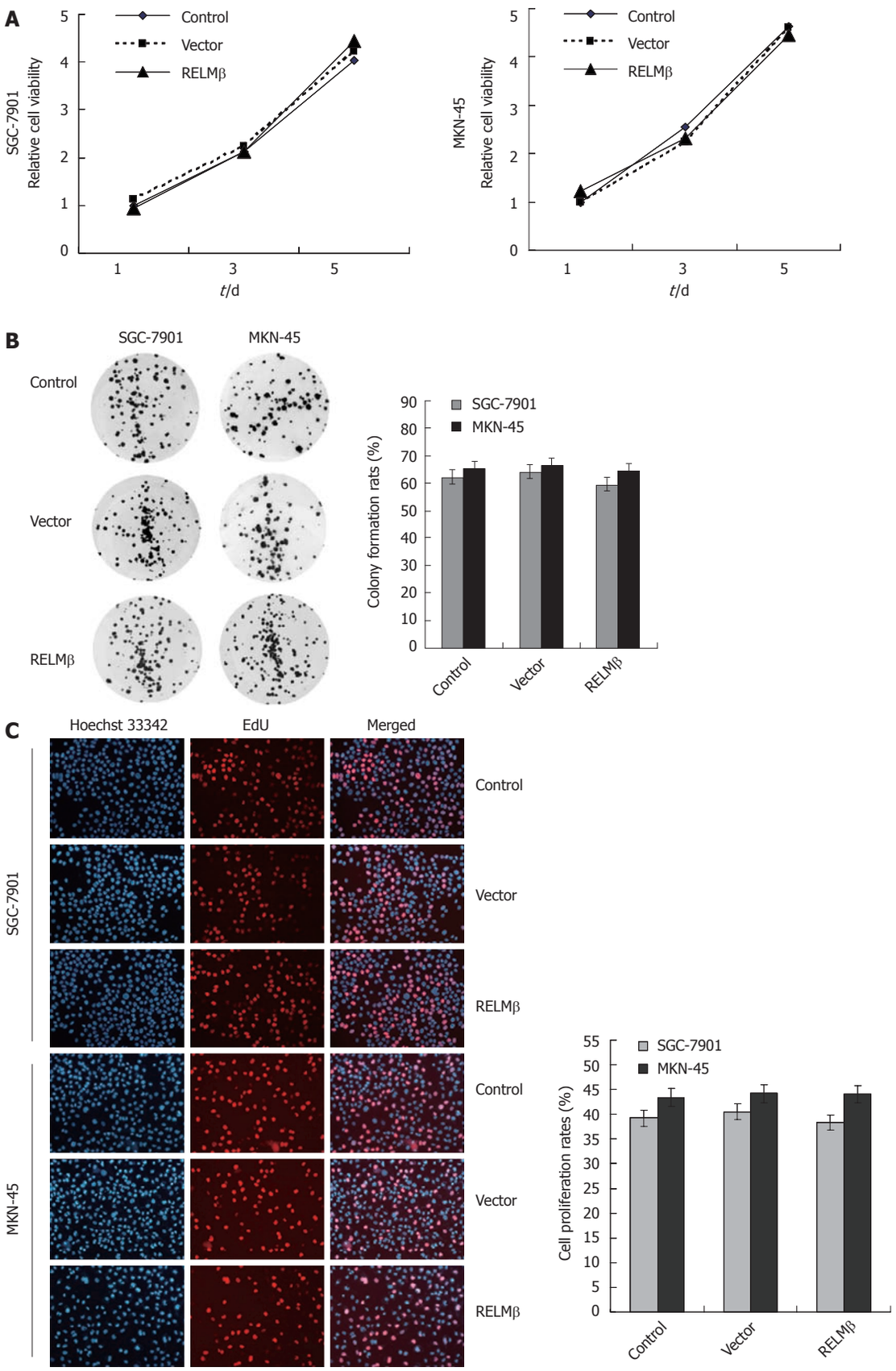

Figure 2 Upregulating resistin-like molecule β expression did not affect the *in vitro* proliferation of gastric cancer cells. A: In 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay, transfection of pcDNA3.1-resistin-like molecule β (RELMβ) or empty vector (mock) for 24 h, 72 h and 120 h, did not affect the cell proliferation, when compared with the parental SGC-7901 and MKN-45 cells ($P > 0.05$); B: Colony formation assay indicated that 72 h after transfection, over-expression of RELMβ did not affect the *in vitro* proliferation of SGC-7901 and MKN-45 cells ($P > 0.05$); C: 5-ethynyl-20-deoxyuridine incorporation assay revealed that 72 h after transfection, over-expression of RELMβ did not influence the proliferation of cultured SGC-7901 and MKN-45 cells ($P > 0.05$). Triplicate experiments were performed with essentially identical results.

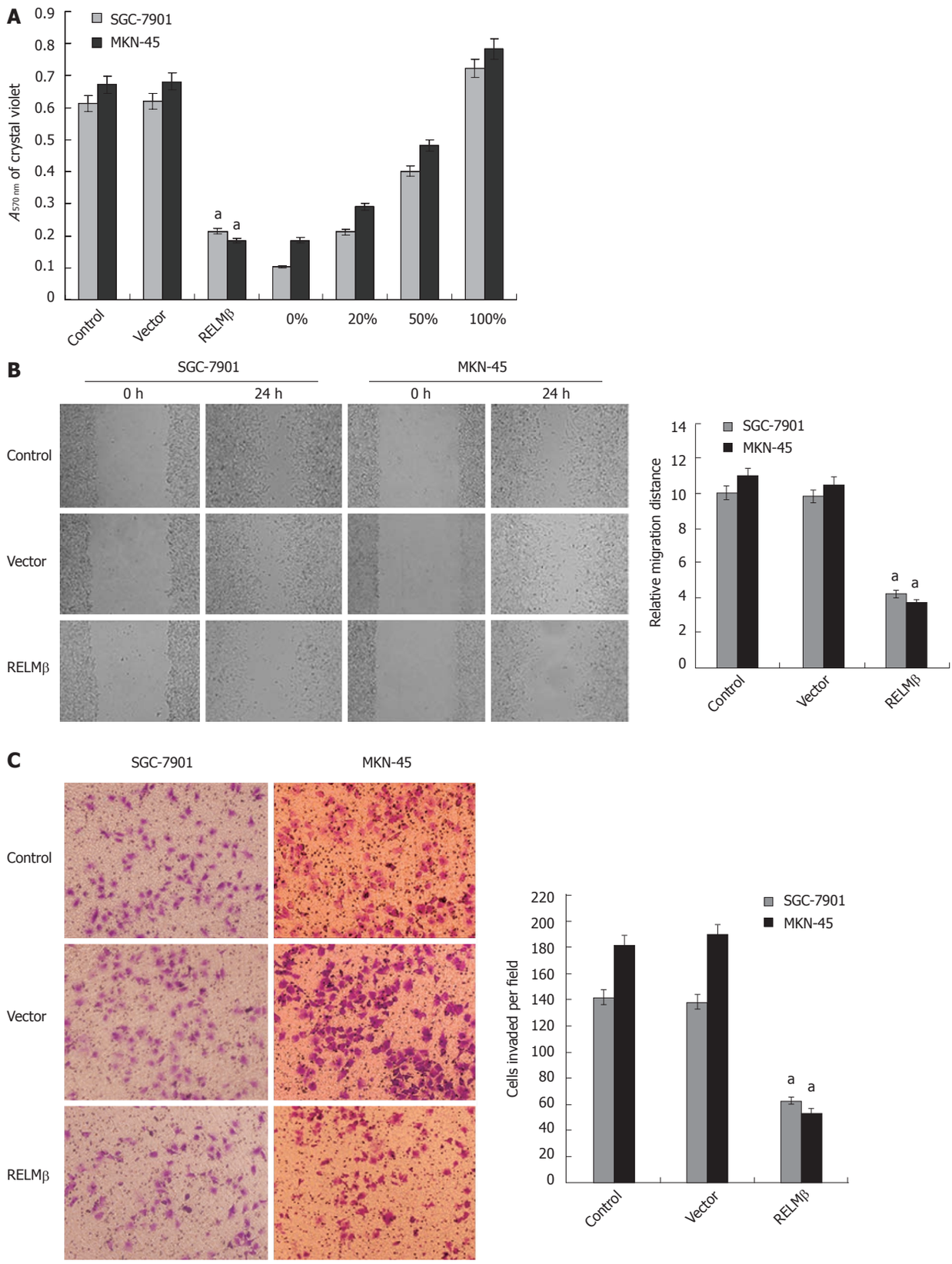

Figure 3 Over-expression of resistin-like molecule β attenuated the adhesion, migration and invasion of gastric cancer cells *in vitro*. A: In the adhesion assay, SGC-7901 and MKN-45 cells transfected with pcDNA3.1-resistin-like molecule β (RELMβ) for 72 h exhibited markedly reduced ability in adhesion to the precoated matrigel, when compared with parental cells. However, the cells transfected with empty vector (mock) had a similar adhesive ability as parental cells; B: Scratch migration assay indicated that transfection of pcDNA3.1-RELMβ into SGC-7901 and MKN-45 cells for 72 h resulted in an impaired migration capacity, when compared with the parental cells and mock group; C: Transwell analysis indicated that transfection of pcDNA3.1-RELMβ for 72 h abolished the invasive capabilities of SGC-7901 and MKN-45 cells, when compared with the parental and mock cells. The symbol (a) indicates a significant decrease compared with parental cells ($P < 0.01$). Triplicate experiments were performed with essentially identical results.

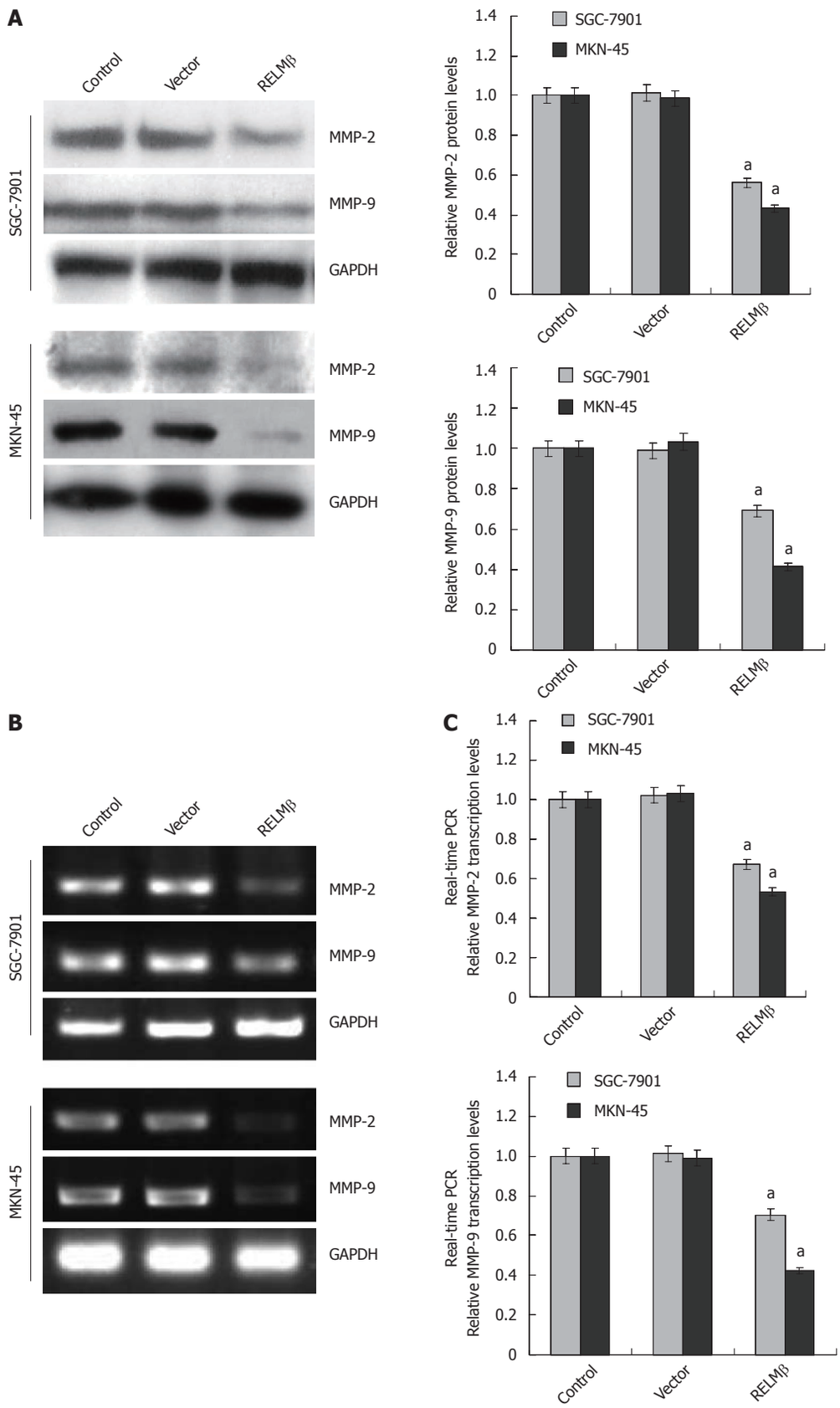

Figure 4 Over-expression of resistin-like molecule β decreased the expression of matrix metalloproteinase-2 and matrix metalloproteinase-9 in gastric cancer cells. A: Western blotting indicated that 72 h **after transfection, over-expression of resistin-like molecule** β (RELMβ) abolished the expression of matrix metalloproteinase (MMP)-2 and MMP-9 in SGC-7901 and MKN-45 cells. However, transfection of empty vector (mock) did not influence their expression; B: Reverse transcription polymerase chain reaction (RT-PCR) indicated the decreased MMP-2 and MMP-9 transcription levels in SGC-7901 and MKN-45 cells transfected with pcDNA3.1-RELMβ for 72 h, but not in mock group; C: Real-time quantitative RT-PCR further demonstrated that transfection of pcDNA3.1-RELMβ for 72 h resulted in decreased transcription levels of MMP-2 and MMP-9 in SGC-7901 and MKN-45 cells. The symbol (**a) indicates a significant decrease compared with parental cells (***P* < 0.01). Triplicate experiments were performed with essentially identical results. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

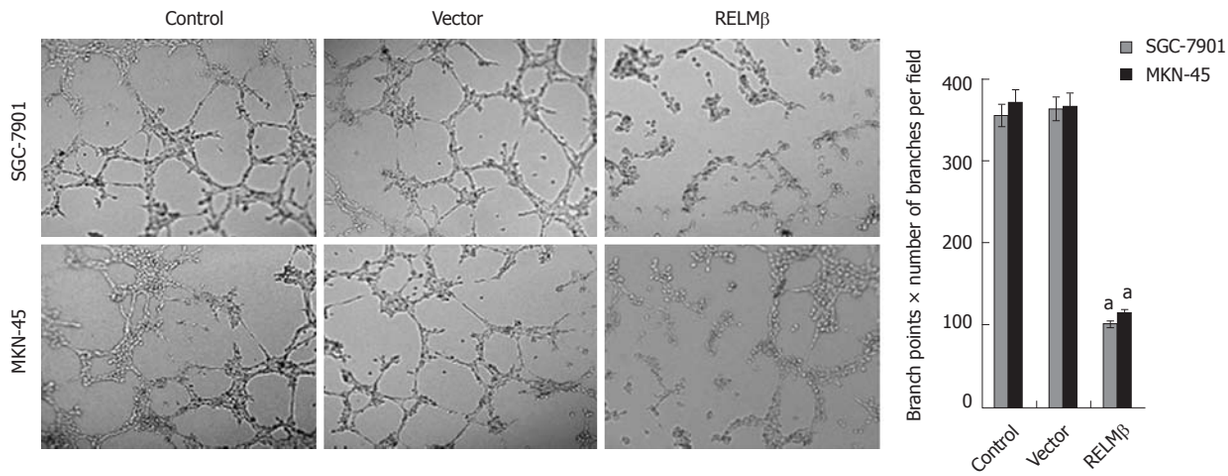

Figure 5 Over-expression of resistin-like molecule β inhibited the *in vitro* angiogenic capabilities of gastric cancer cells. Extensive tube formation of endothelial cells was observed in parental and empty vector (mock) groups. However, when the endothelial cells were treated by the medium preconditioned with pcDNA3.1-resistin-like molecule β (RELMβ)-transfected *SGC*-7901 or MKN-45 cells, the tube formation was suppressed. The symbol (**a) indicates a significant decrease compared with parental cells (***P*** < 0.01)**. Triplicate experiments were performed with essentially identical results.

proliferation of prostate cancer through phosphatidylinositol 3-kinase (PI-3K)/Akt signaling pathways[37]. In addition, transfection of RELMγ into promyelocytic HL60 cells resulted in increased proliferation rate and an altered response to retinoic acid-induced granulocytic differentiation[27]. Thus, these findings indicate the potential role of RELM/FIZZ gene family in the progression of cancer.

Our previous studies have revealed that RELMβ is virtually absent in normal gastric mucosa, whereas gastric cancer exhibits aberrant RELMβ expression[19]. Patients showing positive RELMβ expression have a significantly longer overall survival than those with negative expression, indicating the prognostic value of RELM-β in predicting the outcomes of gastric cancer[19]. Current literatures show conflicting results regarding the role of RELMβ in cell proliferation[38,39]. McVay *et al*[38] reported that RELMβ did not alter colonic epithelial proliferation or barrier function in the dextran sodium sulfate-induced model of murine colonic injury. In cultured lung adenocarcinoma A549 cells, transfection of a RELMβ encoding expression vector resulted in increased proliferation *via* the PI-3K pathway[39]. In this study, we found low expression levels of RELMβ in the poorly or moderately differentiated gastric cancer cell lines SGC-7901 and MKN-45. Unexpectedly, over-expression of RELMβ did not affect the proliferation of SGC-7901 and MKN-45 cells as evidenced by MTT colorimetry, colony formation and EdU incorporation assays. We believe that the effects of RELMβ on cell proliferation varied among different cancer types. In our previous studies, we have observed the correlation between the intensity of RELMβ and metastatic index heparanase, one of the key enzymes involved in the invasion and metastasis of gastric cancer[19]. We found that in primary gastric cancer tissues, lower RELMβ intensity was correlated with higher heparanase expression[19]. In this study, we chose the RELMβ lowly-expressed gastric cancer cell lines as models, and demonstrated that over-expression of RELMβ resulted in attenuated adhesion, migration and invasion, three important steps for cancer metastasis.

MMPs are a family of enzymes that proteolytically degrade various components of the extracellular matrix (ECM), and are closely correlated with tumor invasive and metastatic potentials[40]. MMP-2 and MMP-9 participate in the degradation of basement membrane and the remodeling of ECM[41], and appear to promote tumor initiation, invasion, and metastasis[42]. Tumor cells can synthesize and secret large amounts of MMP-2 and MMP-9 in a paracrine and/or autocrine manner to stimulate angiogenesis[41]. Previous studies show that high levels of MMP-2 and/or MMP-9 have a significant correlation with the invasion and metastasis of gastric cancer[43,44], and are associated with poor prognosis[44]. In this study, we found that over-expression of RELMβ inhibited the expression of MMP-2 and MMP-9 in gastric cancer cells, which at least in part, contributed to the RELM-mediated suppression of migration and invasion of cancer cells.

Angiogenesis, the process of new capillary formation from pre-existing vessels to provide oxygen and nutrients to tumor, plays an essential role in invasion and metastasis of malignancies[45]. Previous studies indicate that resistin increases *in vitro* angiogenesis in human coronary artery endothelial cells and umbilical vein endothelial cells[46]. As a mouse homolog of RELMβ, hypoxia-induced mitogenic factor (HIMF) is found to promote angiogenesis and participate in pulmonary vascular remodeling and fibrotic lung disease[47-49]. RELMβ is expressed in the lung tissue of patients with sclerodermaassociated pulmonary hypertension[16], and recombinant RELMβ induces the proliferation and activation of extracellular signal regulated kinase 1/2 (ERK1/2) in primary cultured human pulmonary endothelial and smooth muscle cells[16]. However, the influence of RELMβ on the angiogenic capabilities of cancer cells still remains exclusive.

In the current study, we demonstrated the anti-angiogenic properties of RELMβ in gastric cancer cells. It has

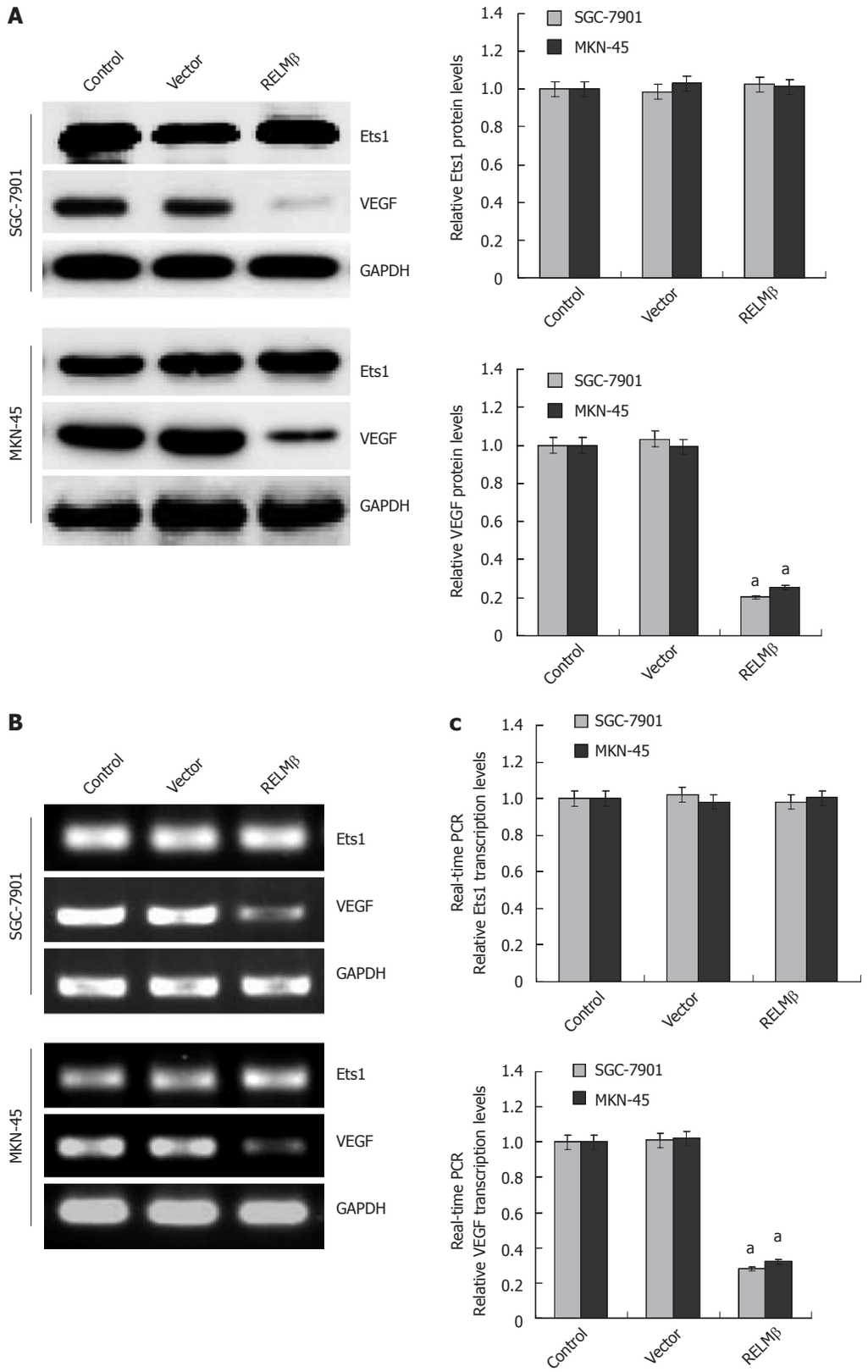

Figure 6 Over-expression of resistin-like molecule β decreased the expression of vascular endothelial growth factor, but not v-ets erythroblastosis virus E26 oncogene homolog 1, in gastric cancer cells. A: Western blotting indicated that 72 h after transfection, over-expression of resistin-like molecule β (RELMβ) abolished the expression of vascular endothelial growth factor (VEGF), but not v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets1), in SGC-7901 and MKN-45 cells. Moreover, transfection of empty vector (mock) did not influence the expression of VEGF and Ets1; B: Reverse transcription polymerase chain reaction (RT-PCR) indicated the decreased VEGF transcription levels in SGC-7901 and MKN-45 cells transfected with pcDNA3.1-RELMβ for 72 h, but not in mock group. Moreover, the Est1 transcription levels were not influenced by transfection of pcDNA3.1-RELMβ or empty vector (mock); C: Real-time quantitative RT-PCR further demonstrated that transfection of pcDNA3.1-RELMβ for 72 h resulted in decreased transcription levels of VEGF, but not of Ets1, in SGC-7901 and MKN-45 cells. The symbol (a) indicates a significant decrease from parental cells ($P < 0.01$). Triplicate experiments were performed with essentially identical results. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

been established that VEGF is secreted by most tumor cells, and plays a determinant role in regulating tumor angiogenesis through inducing cell proliferation, differentiation, and migration of vascular endothelial cells[50]. VEGF induces the formation of new vessels by targeting VEGF receptor 2 signaling pathway, and benefits primary tumor growth and metastasis[51]. Thus, targeting constitutive VEGF and/or its receptors has been an attractive approach for cancer therapy. Our results further showed that over-expression of RELMβ inhibited the expression of VEGF in gastric cancer cells. Based on our recent evidence that recombinant RELMβ protein possesses anti-angiogenic effects *via* decreasing the proliferation, migration, and tube formation of human umbilical vein endothelial HUVEC cells (data not shown), we believe that RELMβ is of potential values as a novel therapeutic target for human gastric cancer.

The mechanisms underlying RELMβ expression in gastric cancer still remains exclusive. Previous evidence indicates that a region between -418 and -588 in the human RELMβ promoter contains two potential caudal type homeobox (CDX) binding sites[52]. Moreover, CDX-2, but not CDX-1, binds to the human RELMβ promoter and thereby transactivates RELMβ expression in a goblet cell-specific fashion[52]. However, our preliminary findings indicate that CDX-2 does not transactivate the RELMβ expression in cultured gastric cancer cells (data not shown). The constitutive expression of RELMβ in gastric cancer tissues with or without intestinal metaplasia[19] suggests that other transcription factors are involved in the regulation of RELMβ expression in gastric cancer, which warrants further investigations.

In summary, for the first time, we have demonstrated that over-expression of RELMβ can efficiently inhibit the invasion, metastasis and angiogenesis of gastric cancer cells. It is likely that the RELMβ over-expression depresses the expression of MMP-2 and MMP-9, thus inhibiting the invasion and metastasis of gastric cancer. In addition, transfection of RELMβ suppresses the VEGF expression, which may result in decreased angiogenesis of gastric cancer cells. These results suggest a potential strategy for gastric cancer therapy *via* modulating or regulating the RELMβ expression. Further knocking down the RELMβ expression in RELMβ highly-expressed cell lines and *in vivo* studies are warranted to investigate the role of RELMβ in the development and progression of gastric cancer.

COMMENTS

Background

According to the previous studies of the authors, the aberrant expression of resistin-like molecule β (RELMβ), an intestinal goblet cell-specific protein, in gastric cancer tissues, is positively correlated with tumor differentiation and longer overall survival, and inversely correlated with tumor infiltration and lymph node metastasis. However, the exact roles and underlying mechanisms of RELMβ in the progression of gastric cancer still remain unknown.

Research frontiers

Although most studies of RELMβ have focused on its function in intestinal defense against parasitic nematode infection of the intestine and colonic inflammation, increasing attention has been paid to the role of RELMβ in tumor biology. The authors in their previous studies have demonstrated that RELMβ is a biomarker of intestinal metaplasia in Barrett's esophagus, and over-expressed in gastric cancer and colon cancer. However, no study has yet investigated the exact role of RELMβ in invasion, metastasis and angiogenesis of gastric cancer.

Innovations and breakthroughs

In this study, the authors demonstrate, for the first time, that over-expression of RELMβ can efficiently inhibit the invasion, metastasis and angiogenesis of gastric cancer cells. It is likely that the RELMβ over-expression depresses the expression of matrix metalloproteinase (MMP)-2 and MMP-9, thus inhibiting the invasion and metastasis of gastric cancer. In addition, transfection of RELMβ suppresses the vascular endothelial growth factor expression, which may result in decreased angiogenesis of gastric cancer cells.

Applications

RELMβ expression is a useful prognostic factor for predicting the outcomes of gastric cancer patients. The effects of RELMβ over-expression on the invasion, metastasis and angiogenesis of gastric cancer cells suggest a potential strategy for gastric cancer treatment *via* modulating or regulating the RELMβ expression. Further knocking down the RELMβ expression in RELMβ highly-expressed cell lines and *in vivo* studies are warranted to investigate the role of RELMβ in the development and progression of gastric cancer.

Terminology

RELMβ is a recently described goblet cell-specific protein that belongs to the resistin-like molecules, or found in inflammatory zone (*RELM/FIZZ*) gene family and functions as a critical immune-effector molecule in the expulsion of gastrointestinal tract nematodes.

Peer review

The authors reported a study elucidating the effects of RELMβ over-expression on the invasion, metastasis and angiogenesis of gastric cancer cell lines. It revealed that transient transfection of RELMβ into low-expressing gastric cancer cell lines resulted in attenuated adhesion, migration and invasion, three important steps for cancer metastasis. In addition, over-expression of RELMβ suppressed the angiogenic capabilities of cancer cells. The results are original and may represent a novel strategy for the treatment of gastric cancer.

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S- Editor Gou SX **L- Editor** Ma JY **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.767

World J Gastroenterol 2012 February 28; 18(8): 767-777
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Rifaximin *vs* conventional oral therapy for hepatic encephalopathy: A meta-analysis

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Received: April 29, 2011 Revised: June 16, 2011
Accepted: June 23, 2011
Published online: February 28, 2012

Abstract

AIM: To characterize the efficacy of rifaximin in the management of hepatic encephalopathy (HE) as several randomized controlled studies have shown contradictory results on its effectiveness in comparison to other oral agents.

METHODS: We performed a systematic review and random effects meta-analysis of all eligible trials identified through electronic and manual searches. Twelve randomized controlled trials met the inclusion criteria with a total of 565 patients.

RESULTS: The clinical effectiveness of rifaximin was equivalent to disaccharides or other oral antibiotics [odds ratio (OR) 0.96; 95% CI: 0.94-4.08] but with a better safety profile (OR 0.27; 95% CI: 0.12-0.59). At the completion of treatment protocols, patients receiving rifaximin showed lower serum ammonia levels [weighted mean difference (WMD) = -10.65; 95% CI: -23.4-2.1; *P* = 0.10], better mental status (WMD = -0.24; 95% CI: -0.57-0.08; *P* = 0.15) and less asterixis (WMD -0.1; 95% CI -0.26-0.07; *P* = 0.25) without reaching statistical significance. On the other hand, other psychometric outcomes such as electroencephalographic response and grades of portosystemic encephalopathy were superior in patients treated with rifaximin in comparison to the control group (WMD = 0.21, 95% CI: -0.33-0.09, *P* = 0.0004; and WMD = -2.33, 95% CI: -2.68-1.98, *P* = 0.00001, respectively). Subgroup and sensitivity analysis did not show any significant difference in the above findings.

CONCLUSION: Rifaximin appears to be at least as effective as other conventional oral agents for the treatment of HE with a better safety profile.

Key words: Hepatic encephalopathy; Lactulose; Neomycin; Non-absorbable disaccharides; Rifaximin

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Eltawil KM, Laryea M, Peltekian K, Molinari M. Rifaximin *vs* conventional oral therapy for hepatic encephalopathy: A meta-analysis. *World J Gastroenterol* 2012; 18(8): 767-777 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/767.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.767

INTRODUCTION

Hepatic encephalopathy (HE) is a reversible neuropsychiatric and functional syndrome occurring in 50%-70% of

patients with advanced liver disease[1]. The pathophysiology of HE is complex and it manifests with progressive deterioration of the superior neurological functions. HE occurs in the presence of insufficient hepatic clearance of toxins absorbed from the intestine resulting in neurochemical abnormalities across the blood brain barrier[2]. The clinical manifestations of HE range from altered mental status to deep coma[3]. Elevated serum ammonia level is the best described cause of HE and is detected in 60%-80% of affected patients[4,5]. Current treatment strategies[6] are aimed at reducing the serum level of ammonia. This is done by introducing agents that reduce or inhibit production of intestinal ammonia or minimize its absorption from the gastrointestinal tract as well as correcting precipitating factors such as gastrointestinal hemorrhage, electrolyte imbalances and constipation[7].

For both acute and chronic HE, the mainstay treatment has been the use of non-absorbable disaccharides[3] since they decrease the absorption of ammonia through cathartic effects and by altering the colonic pH[6]. Several oral antibiotics such as neomycin, paromomycin, metronidazole, vancomycin and rifaximin have shown some degree of effectiveness in lowering serum ammonia concentration by reducing the intestinal flora responsible for its production[8]. With the exception of rifaximin, all the other antibiotics have been associated with some side effects such as ototoxicity and nephrotoxicity (neomycin)[9,10] and neurotoxicity (metronidazole)[11,12]. Vancomycin may be a safer option, however, its use has been associated with the development of bacterial resistance[13]. On the other hand, rifaximin is a poorly-absorbed broad spectrum antibiotic with very few systemic side effects and at low risk of inducing bacterial resistance[14,15]. These properties make rifaximin an ideal antibiotic for the treatment of patients with HE as several studies have shown a significant decrease in plasma ammonia levels[16-18] with minimal impact on the normal gastrointestinal flora[13].

Several small randomized controlled trials (RCT) comparing rifaximin with oral disaccharides or with other antibiotics have found that rifaximin is effective and safe. Nevertheless, these trials were insufficiently powered. A meta-analysis of randomized controlled trials comparing disaccharides *vs* antibiotics for the treatment of HE has shown superior outcomes with the use of antibiotic therapy[19]. Sub-group analysis of five studies comparing rifaximin to disaccharides favored the use of rifaximin ($P = 0.04$)[19]. On the other hand, a more recent and larger meta-analysis including seven studies comparing rifaximin with non-absorbable disaccharides showed no significant difference between the two interventions, although rifaximin had fewer side effects[20]. In light of these limitations, we conducted a systematic review of the literature to identify, appraise and collectively analyze all RCTs comparing rifaximin with conventional oral therapies for the treatment of patients with HE.

MATERIALS AND METHODS

Data sources and study selection

Randomized controlled trials comparing oral rifaximin to non-absorbable disaccharides and other antibiotics used for the treatment of HE were searched in PubMed, Excerpta Medica Database, Scopus, Web of Science, Cochrane central register of controlled trials, and hepatobiliary group in the Cochrane library, EMBASE, CINAHL through December 4th 2010 without restriction on the publication status or language. Database specific search terms for rifaximin (rifaximin, rifamycins), disaccharides (disaccharides, lactulose, lactitol, sugar alcohols) and antibiotics (anti-bacterial-agents, antibiotics) were combined and all reference sections of eligible studies and review articles on the topic were hand-searched for additional potential studies. Two reviewers (Eltawil KM and Molinari M) independently assessed the eligibility of all potential abstracts and titles. When in disagreement or in the presence of insufficient information, the full text of the potential paper was reviewed for eligibility. Authors of all potential trials were also contacted by electronic mail for additional information if the published data were insufficiently described.

Inclusion and exclusion criteria

We included all RCTs that reported the effect of rifaximin *vs* non-adsorbable disaccharides or other antibiotics on the grade of HE according to Conn's modification of Parsons Smith classification[21], irrespective of language and publication status. Exclusion criteria were: studies conducted on pediatric patients, studies that compared the use of rifaximin *vs* placebo, non-controlled clinical trials, studies that assessed the efficacy of rifaximin in preventing HE, trials including patients with psychiatric illness, with undercurrent infections, with hypersensitivity to rifaximin and other antibiotics and/or intolerance to non-absorbable disaccharides, trials that included individuals affected by gastrointestinal bleeding and studies reporting results of the same population published more than once.

Outcomes

The primary outcomes of this study were the effectiveness and the safety of the use of rifaximin for the treatment of patients with at least one episode of HE. Secondary outcomes were reduction of serum ammonia levels and changes in psychometric parameters [mental status, asterixis, electroencephalographic characteristics and portosystemic encephalopathy (PSE) sum] measured at the end of the treatment.

Definitions

The study population was defined as patients older than 18 years of age with a diagnosis of reversible neurological decline secondary to end-stage liver disease. Effectiveness was calculated by the proportion of patients

who had resolution or clinical improvement of HE during the treatment. Partial neurological response was measured by mental status scores according to Conn's classification[21] as follows: 0, no personality or behavioral abnormality; 1, trivial lack of awareness, euphoria or anxiety, shortened attention span, or impairment of ability to add or subtract; 2, lethargy, disorientation with respect to time, obvious personality change, or inappropriate behavior; 3, somnolence or semi-stupor, responsiveness to stimuli, confusion, gross disorientation, or bizarre behavior; and 4, coma.

Side effects of rifaximin and other oral therapies assessed in this study were: severe diarrhea, episodes of intense abdominal pain and at least one of the following symptoms: nausea, anorexia and weight loss.

Serum ammonia levels were assessed at the end of the treatment and expressed in mg/dL.

The severity of asterixis was graded according to established criteria as follows: 0, no tremors; 1, few flapping motions; 2, occasional flapping motions; 3, frequent flapping motions; and 4, almost continuous flapping motions[22,23].

Electroencephalogram (EEG) abnormalities recorded in patients with HE were scored according to criteria previously published in the medical literature[24]: 0, well-structured EEG with stable and symmetrical posterior basic rhythm (8 Hz-13 Hz) dominant in the posterior regions medium amplitude without slow activities or epileptic pattern; 1, unstable or suppressed alpha rhythm frequently replaced by high prevalence of diffuse beta rhythm (normal-limit EEG); 2, low frequency alpha rhythm (8 Hz) disturbed by random waves in the theta range over both hemispheres (mild signs of encephalopathy); 3, background activity in the theta range, diffused over both hemispheres, random appearance of high waves in the delta range (distinctive features of encephalopathy); and 4, severe disorganization of EEG activity without any normal element (signs of severe encephalopathy).

Grades of PSE were calculated as the sum of the degree of mental status abnormality scores, the severity of asterixis, level of serum ammonia elevation and the degree of EEG abnormality[25].

Data extraction

Two independent reviewers extracted publication variables and clinical data for each study. The following variables were collected: the name of the primary author, journal and year of publication, country where the study was carried out, number of patients randomized in each arm, daily dosage of oral therapy, duration of the treatment, allocation sequence generation, allocation concealment, power calculation, study design, methods used to deal with missing data, appropriate description of attrition and drop-outs.

Clinical variables extracted were: the proportion of patients that experienced improvement of their neurological function (effectiveness), common side effects, serum ammonia level and psychometric parameters.

Assessment of study quality

The quality of included studies was scored using the Cochrane Collaboration risk assessment tool[26]. The methodological quality was essentially based on the attention that each study design paid to control potential bias based on the available description reported in the methodology of each paper. When in doubt, authors were contacted by digital letters. The randomization methods were classified as the primary way to control bias and the randomization process was evaluated by the allocation sequence generation and allocation concealment. The randomization methods were considered adequate if based on a table of random numbers, computer-generated random numbers or based on similar techniques. Allocation concealment was classified as adequate if based on central randomization, if using identically appearing coded drugs, if serially numbered opaque sealed envelopes were employed or when other equivalent methods were used. Blinding was extracted and appraised for caregivers, patients and assessors. Studies were classified as single-blinded if the patients did not have any opportunity to know the nature of their therapy and double-blinded if the authors described in their methods how they prevented patients and caregivers or assessors knowing the nature of the treatments. In addition, we appraised the risk of attrition bias by assessing the number and reason for dropouts and withdrawals and whether all patients were accounted for in the report and analysis of the study. Quality of the included studies also assessed the way the authors described sample size calculation to power the trial and if the sample size was achieved, whether there was a clear definition of primary outcomes and if they were reported and whether a crossover design was used.

Statistical analysis

All statistical analyses were performed using RevMan Version 5.0.5 software[27] (Nordic Cochrane Centre, Copenhagen, Denmark). The meta-analysis was performed using the random effects model of DerSirmonian and Laird[28] due to expected clinical heterogeneity. The results are reported as pooled odds ratios (OR) for binary and weighted mean differences (WMD) for continuous outcomes, both with 95% CI. Pooled OR and WMD were calculated using the general inverse variance (IV) with random effect model. Measure of the degree of inter-trial heterogeneity was explored with the I^2 test[29]. Heterogeneity was evaluated with a χ^2-based Q statistic of OR and defined at a P value less than 0.1 and potential reasons for heterogeneity were explored. Data on all patients randomized were extracted to allow intention-to-treat analysis. For patients with missing data, carry-forward of the last observed response was used. Only data from the first period of cross-over trials were included. For the primary outcome measure, we performed subgroup analyses of trials stratified by the treatment regimen and methodological quality. The preferred reporting items for systematic reviews and meta-analysis

Table 1 Characteristics of included studies

Authors	Country	Rifaximin (*n*)	Control (*n*)	Rifaximin dose (mg/d)	Comparative agent	Duration of treatment	Outcomes
Bucci *et al*[35]	Italy	30	28	1200	Lactulose 45 mL/d	15 d	Mental status, asterixis, cancellation test, reitan test, EEG, serum ammonia, degree and severity of HE
Di Piazza *et al*[33]	Italy	8	6	1200	Neomycin 4500 mg/d	21 d	Bradylalia, flapping tremor, performance, visual evoked potentials and the trial making test
Fera *et al*[37]	Italy	20	20	1200	Lactulose 120 mL/d	90 d	Mental status, asterixis, cancellation test, reitan test, EEG, PSE severity
Festi *et al*[17]	Italy	20	15	1200	Neomycin 3000 mg/d	21 d	Asterixis, EEG, blood ammonia
Festi *et al*[17]	Italy	9	12	1200	Lactulose 60 mL/d	21 d	Asterixis, EEG, blood ammonia
Loguercio *et al*[39]	Italy	14	13	1200	Lactulose 90 mL/d	3 mo	Mental status, asterixis, NCT, blood ammonia
Mas *et al*[36]	Spain	50	53	1200	Lactitol 60 g/d	5-10 d	HE grade, mental status, asterixis, NCT, EEG, PSE index, blood ammonia
Massa *et al*[18]	Italy	20	20	1200	Lactulose 90 mL/d	15 d	Mental status, asterixis, cancellation test, EEG, trail making test, PSE index, blood ammonia
Miglio *et al*[38]	Italy	25	24	1200	Neomycin 3000 mg/d	6 mo	HE grade, blood ammonia, neuropsychiatric signs
Paik *et al*[21]	South Korea	32	22	1200	Lactulose 90 mL/d	7 d	Mental status, flapping tremors, NCT, blood ammonia, HE index
Parini *et al*[34]	Italy	15	15	1200	Paromomycin 1500 mg/d	10 d	Blood ammonia, state of consciousness, behavior, intellectual functions, neurologic symptoms
Pedretti *et al*[25]	Italy	15	15	1200	Neomycin 3000 mg/d	21 d	PSE index, blood ammonia, EEG, NCT, asterixis, trail making test, mental status
Song *et al*[32]	South Korea	39	25	1200	Lactulose 90 mL/d	7 d	Blood ammonia, mental status, flapping tremors, NCT, HE index

EEG: Electroencephalogram; HE: Hepatic encephalopathy; PSE: Portosystemic encephalopathy; NCT: Number connection test.

recommendations were used for study reporting[30,31].

RESULTS

Ascertainment of the studies

After initial screening, a total of 220 potentially relevant trials were identified through the electronic searches as summarized in Figure 1. After subsequent evaluation for eligibility, we retained 12 published RCTs that assessed the effectiveness of rifaximin for the treatment of patients with HE. One study was available only in abstract form[32]. The remaining studies were excluded because they were prospective cohort studies or randomized controlled trials assessing the effectiveness of rifaximin for the prevention rather than for the treatment of HE.

Study characteristics

Table 1 lists the characteristics of the included studies. All trials were single-center studies except one which was multicentric[17] with the total number of patients per study ranging from 14 to 136 and with a minimum therapeutic interval of 7 d to a maximum of 6 mo. The majority of studies treated one arm of patients with rifaximin at a dose of 1200 mg/d divided in three doses, although some used 1100 mg in two divided doses. The comparison arm of patients received non-absorbable oral disaccharides (lactulose or lactitol) at doses ranging from 45 to 120 mL/d for lactulose and 60 g/d for lactitol or antibiotic therapy with neomycin or paromomycin. One study[33] was designed as a double cross study where patients received rifaximin in addition to oral disaccharides for one week and were switched to neomycin in

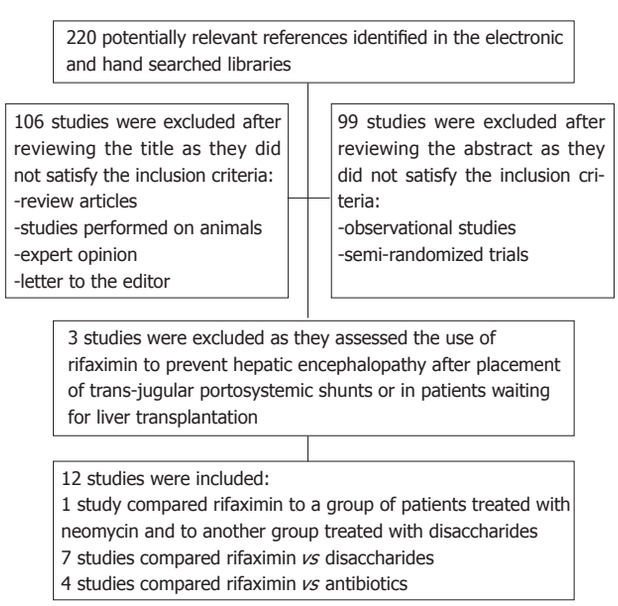

Figure 1 Flowchart of the included studies.

Table 2 Risk of bias in the published controlled trials

Author	Allocation system described	Allocation concealment	Patient	Blinding Personnel	Assessor	Handling of missing data	Power calculation for number of patients to be treated
Bucci *et al*[35]	Yes	Yes	Yes	Yes	NA	Unclear	No
Di Piazza *et al*[33]	No	No	Yes	Yes	NA	Unclear	No
Fera *et al*[37]	No	Yes	Yes	No	NA	Unclear	No
Festi *et al*[17]	No	No	No	No	NA	Unclear	No
Loguercio *et al*[39]	No	No	Yes	No	NA	Unclear	No
Mas *et al*[36]	Yes	Yes	Yes	Yes	NA	Unclear	No
Massa *et al*[18]	No	Yes	Yes	Yes	NA	Unclear	No
Miglio *et al*[38]	No	Yes	Yes	Yes	NA	Unclear	No
Paik *et al*[21]	Yes	Yes	Yes	No	NA	Unclear	No
Parini *et al*[34]	Yes	Yes	Yes	No	No	Unclear	No
Pedretti *et al*[25]	Yes	Yes	Yes	Yes	NA	Unclear	No
Song *et al*[32]	NA	NA	NA	NA	NA	NA	NA

NA: Not available.

combination with oral disaccharides on the third week of therapy. Festi *et al*[17] carried on a randomized controlled trial that included 4 arms of patients with HE: in one group the effect of rifaximin was compared to non-absorbable disaccharides and in the other group rifaximin was compared to neomycin.

The majority of control patients who received neomycin were treated with a total of 3000 mg/d divided in three doses except for participants in Di Piazza's study[33] who were treated with a total daily dose of 4500 mg divided in three administrations. Paromomycin was used in only one study[2] and administered at a total dose of 1500 mg/d divided in three doses of 500 mg each.

The majority of trials that used non-absorbable oral disaccharides aimed at inducing several soft bowel movements per day although none of the included studies reported how they monitored their participants.

Risk of bias

We assessed the risk of bias for all the included studies based on published reports and information provided by the authors (Table 2). The allocation system was described in five trials[21,25,34-36] and allocation concealment was clearly defined in eight trials[18,21,25,34-38]. The majority of trials were blinded to patients; however, blinding of observers was described only in six studies[18,25,33,36-38]. Methods for handling missing data, description of drop-outs and possible causes of attrition and power calculations were not adequately described in any of the included studies. One study was available only in abstract form and therefore the risk of bias appraisal was not satisfactory.

Primary outcomes

Effectiveness: First, the effectiveness of therapy was assessed by comparing rifaximin to non-adsorbable disaccharides (lactulose or lactitiol) and then to other oral antibiotics (neomycin or paromomycin). After that, the overall effectiveness of rifaximin *vs* other conventional oral therapies was assessed by combining the two subgroups.

Using the random-effect model, the pooled analysis of 7 studies[17,18,21,32,36,37,39] that investigated the efficacy of rifaximin (n = 184) *vs* non-absorbable disaccharides (n = 165) revealed that both groups experienced either full resolution of HE or clinical improvement that was considered significant by the primary investigators without reaching statistical significance (OR = 1.92, 95% CI: 0.79-4.68, P = 0.15).

Similar findings were observed when the data of all 5 RCTs comparing the efficacy of rifaximin *vs* other antibiotics[17,25,33,34,38] were pooled. This confirmed that rifaximin (n = 68) had similar effectiveness to neomycin or paromomycin (n = 60, neomycin or paromomycin) (OR = 2.77, 95% CI: 0.35-21.83, P = 0.21).

The results of the combined analysis with both groups of patients receiving antibiotics or disaccharides showed a trend that favored the use of rifaximin without statistical significance (OR = 1.96, 95% CI: 0.94-4.08, P = 0.07) (Figure 2).

Adverse events: The following were side effects used in our analysis: severe diarrhea beyond the expected cathartic effect of disaccharides, abdominal pain, and the combination of nausea, anorexia and weight loss. First, each of the adverse events experienced by patients was compared separately. Second, all the adverse events were pooled and compared between the group of patients who received rifaximin (n = 980) and the control group (n = 988).

Participants who received rifaximin had less risk of suffering from diarrhea (OR = 0.20, 95% CI: 0.04-0.92, P = 0.04) although the rate of abdominal pain nausea/anorexia/weight loss was similar between the two groups (P = 0.40, P = 0.06, respectively). Yet, combined analysis of all the adverse events favored the use of rifaximin as it was associated with fewer side effects (OR = 0.27, 95% CI: 0.12-0.59, P = 0.001) (Figure 3).

Secondary outcomes

Serum ammonia level: At the end of 7 RCTs[17,21,25,34-36,38], a significant reduction in serum ammonia level was observed in both treatment arms; rifaximin *vs* non-adsorbable disaccharides and rifaximin *vs* neomycin or

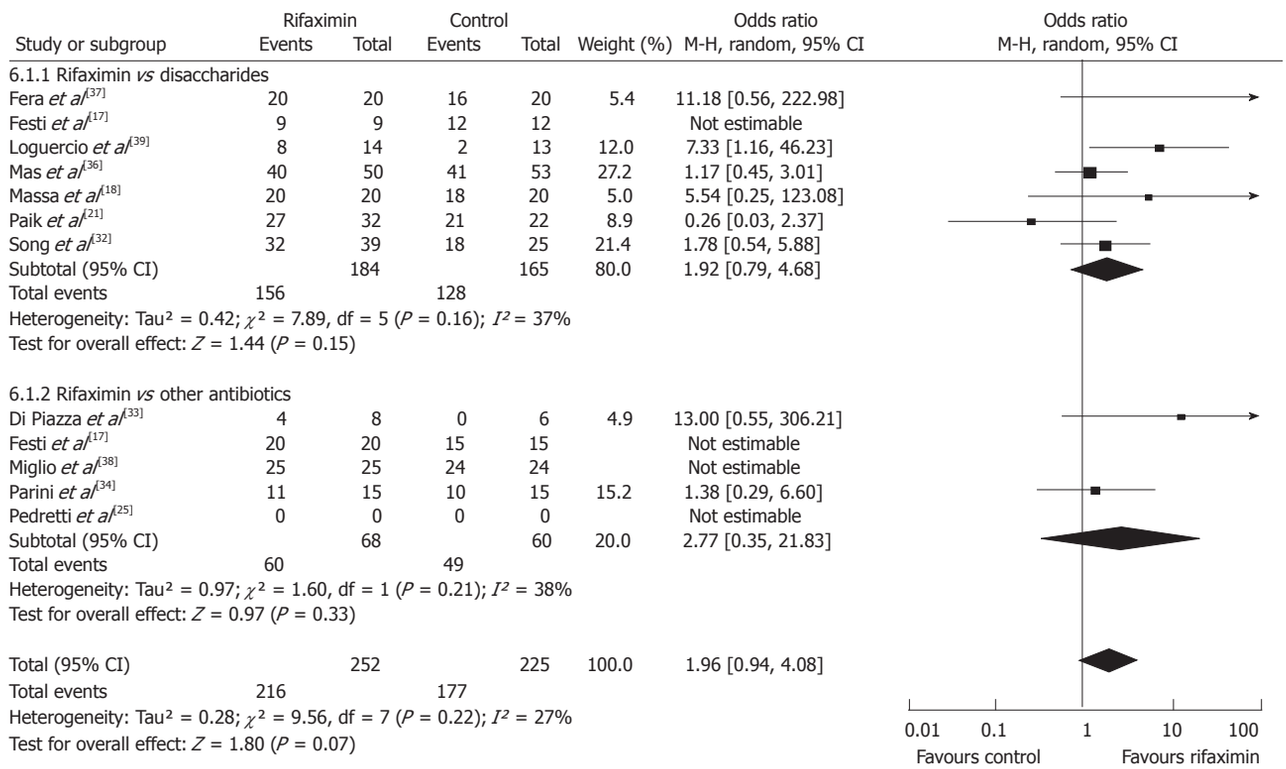

Figure 2 Efficacy of rifaximin *vs* oral disaccharides, rifaximin *vs* other oral antibiotics and rifaximin *vs* control therapy which included the combination of oral disaccharides and other antibiotics. M-H: Mantel Haenszel.

paromomycin. Participants who received rifaximin (n = 138) had lower serum ammonia levels in comparison to patients who received non-adsorbable disaccharides (n = 128) although the difference did not reach statistical significance (P = 0.30). Similar results were observed when comparing rifaximin (n = 60) *vs* other oral antibiotics (n = 60) (P = 0.33) although the differences were not statistically significant. When compared to all the controls (n = 176), patients treated with rifaximin (n = 181) had an overall lower mean serum ammonia level but this, too, was not statistically significant (WMD = -10.65, 95% CI: -23.46-2.17, P = 0.10) (Figure 4).

Psychometric parameters: Improvement in mental status and degree of asterixis after rifaximin therapy were compared to controls in seven trials with no statistically significant results (P = 0.15 and P = 0.25, respectively) (Table 3).

The changes in EEG patterns and PSE sum were studied in 3 trials (rifaximin *vs* control)[18,25,36]. For both parameters, the meta-analysis showed a statistically significant improvement favoring the use of rifaximin (WMD = 0.21, 95% CI: -0.33-0.09, P = 0.0004, and WMD = -2.33, 95% CI: -2.68-1.98, P = 0.00001, respectively) (Table 3). The overall improvement in psychometric parameters measured between the two drug groups was statistically significant favoring the use of rifaximin (P = 0.005) as summarized in Table 3.

Sensitivity analysis

A sensitivity analysis for both primary and secondary outcomes was conducted to explore heterogeneity on the basis of the quality of study design. By excluding studies considered at higher risk of bias according to the Cochrane Collaboration risk assessment tool, we identified consistency of findings and no statistically significant changes were noted for all the comparisons performed.

DISCUSSION

Hepatic encephalopathy represents a challenging clinical complication of liver insufficiency and presents with a wide spectrum of neuropsychiatric symptoms that range from mild disturbances in cognitive function to coma to even death[13,40]. The pathogenesis of this complex syndrome is thought to be multifactorial, but a key role is played by circulating gut-derived toxins such as ammonia[2,40]. With appropriate medical treatment most of the clinical manifestations of HE are reversible when precipitating factors are corrected[2]. The most common known conditions responsible for HE include: gastrointestinal bleeding, infections or systemic inflammation, renal and electrolyte abnormalities, dehydration, use of narcotics and other psychoactive medications, constipation and an excess protein intake[6].

Traditionally, non-absorbable disaccharides have been used as the first-line therapy for patients with HE[13] even if their effectiveness in comparison to placebo has not been proven[19]. Although safe, the need to adjust disaccharide doses to achieve two to three loose bowel movements per day often leads to frequent nausea, vomiting, and flatulence and affects compliance[3]. Poorly absorbed oral antibiotics such as neomycin, vancomycin or paro-

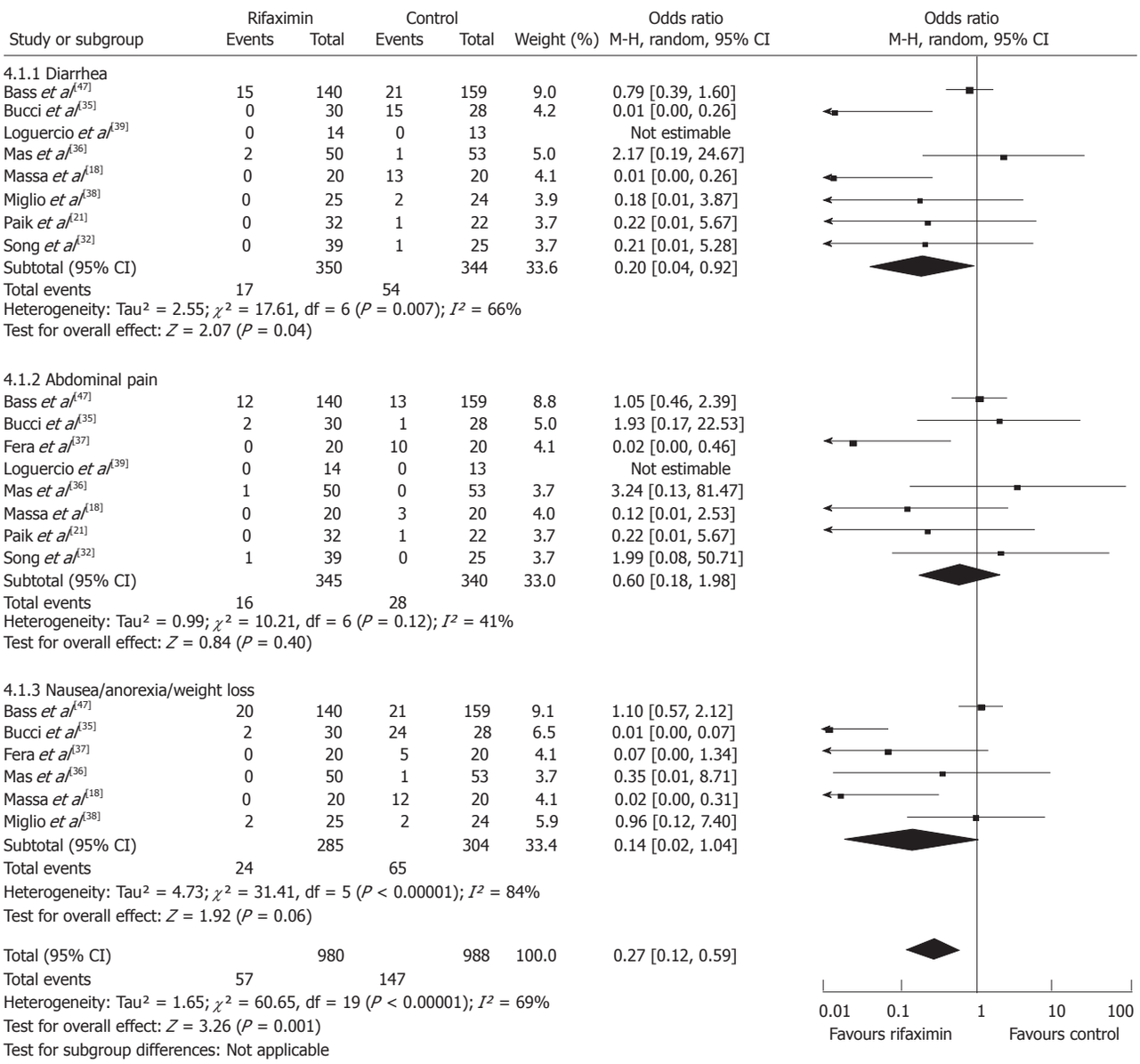

Figure 3 Adverse events experienced by patients treated with rifaximin *vs* control therapy. M-H: Mantel Haenszel.

momycin seem to be more effective than disaccharides[19] with fewer side effects, although ototoxicity[41], nephrotoxicity[42], neurotoxicity and bacterial resistance have been described[20,42]. This significant risk of severe toxicity is the reason why most of these agents are seldom used in modern practice.

On the other hand, rifaximin is an agent that appears to be effective in the treatment of HE without carrying the risk of severe side effects. It has the advantage of being well tolerated and has minimal risk of causing bacterial resistance[43]. It was initially introduced in Italy in 1987[44] and recently approved in the United States for the treatment[45] and prevention[46] of HE. The purported advantages of rifaximin over other oral agents make it a very attractive choice for treatment of HE, although at considerably greater expense[13]. A recent mathematical model has shown that initial disaccharide monotherapy followed by rifaximin as a second-line therapy would be the most cost-effective strategy[47]. So far, the evidence for use of rifaximin as the first-line therapy for HE has been supported only by underpowered randomized controlled trials with conflicting results. A previous meta-analysis of seven randomized controlled trials concluded that rifaximin was not superior to non-absorbable disaccharides, except that it was better tolerated[20]. One of the limitations of that study was that rifaximin was not compared to other established oral therapies and that psychometric functional outcomes were not included in the final analysis.

In this study, we incorporated 12 randomized controlled trials published in the last 20 years assessing the efficacy and adverse events of rifaximin *vs* other oral therapies such as disaccharides and antibiotics. In addition to the effectiveness and safety profile, we also analyzed the effects of these treatments on serum ammonia levels and several other psychometric outcomes.

Table 3 Summary of the meta-analysis on psychometric outcomes measured at the end of each randomized controlled trial

Variable	Rifaximin Mean	Rifaximin SD	Rifaximin Total	Control Mean	Control SD	Control Total	Mean difference IV, random, 95% CI
Mental status							
Bucci *et al*[35]	0.8	0.5	30	1.2	0.3	28	-0.40 [-0.61, -0.19]
Loguercio *et al*[39]	0.42	0.67	14	0.9	0.74	13	-0.48 [-1.01, 0.05]
Massa *et al*[18]	0.6	0.2	20	1.2	0.3	20	-0.60 [-0.76, -0.44]
Paik *et al*[21]	0.5	0.7	32	0.3	0.4	22	0.20 [-0.09, 0.49]
Parini *et al*[34]	0.22	0.39	15	0.16	0.34	15	0.06 [-0.20, 0.32]
Subtotal (95% CI)			111			98	-0.24 [-0.57, 0.08]
Heterogeneity: Tau² = 0.11; χ^2 = 32.85, df = 4 (P < 0.00001); I^2 = 88%							
Test for overall effect: Z = 1.45 (P = 0.15)							
Asterixis							
Bucci *et al*[35]	0.5	0.3	30	0.9	0.5	28	-0.40 [-0.61, -0.19]
Mas *et al*[36]	0	0.5	50	0	0.5	53	0.00 [-0.19, 0.19]
Massa *et al*[18]	0.1	0.2	20	0.1	0.2	20	0.00 [-0.12, 0.12]
Paik *et al*[21]	0.3	0.7	32	0.4	0.6	22	-0.10 [-0.45, 0.25]
Parini *et al*[34]	0.28	0.5	15	0.16	0.04	15	0.12 [-0.13, 0.37]
Pedretti *et al*[25]	1.6	0.7	15	2	0.8	15	-0.40 [-0.94, 0.14]
Subtotal (95% CI)			162			153	-0.10 [-0.26, 0.07]
Heterogeneity: Tau² = 0.02; χ^2 = 14.47, df = 5 (P = 0.01); I^2 = 65%							
Test for overall effect: Z = 1.15 (P = 0.25)							
EEG							
Bucci *et al*[35]	0.4	0.2	30	0.6	0.3	28	-0.20 [-0.33, -0.07]
Mas *et al*[36]	0.6	0.9	50	0.9	0.9	53	-0.30 [-0.65, 0.05]
Pedretti *et al*[25]	0.4	0.5	15	0.6	0.6	15	-0.20 [-0.60, 0.20]
Subtotal (95% CI)			95			96	-0.21 [-0.33, -0.09]
Heterogeneity: Tau² = 0.00; χ^2 = 0.28, df = 2 (P = 0.87); I^2 = 0%							
Test for overall effect: Z = 3.52 (P = 0.0004)							
PSE sum							
Mas *et al*[36]	4	0.1	50	6	2	53	-2.00 [-2.54, -1.46]
Massa *et al*[18]	3	0.5	20	5.5	0.5	20	-2.50 [-2.81, -2.19]
Pedretti *et al*[25]	7.1	2.4	15	9.3	2.7	15	-2.20 [-4.03, -0.37]
Subtotal (95% CI)			85			88	-2.33 [-2.68, -1.98]
Heterogeneity: Tau² = 0.02; χ^2 = 2.52, df = 2 (P = 0.28); I^2 = 21%							
Test for overall effect: Z = 13.11 (P < 0.00001)							

IV: Inverse variance; EEG: Electroencephalogram; PSE: Portosystemic encephalopathy.

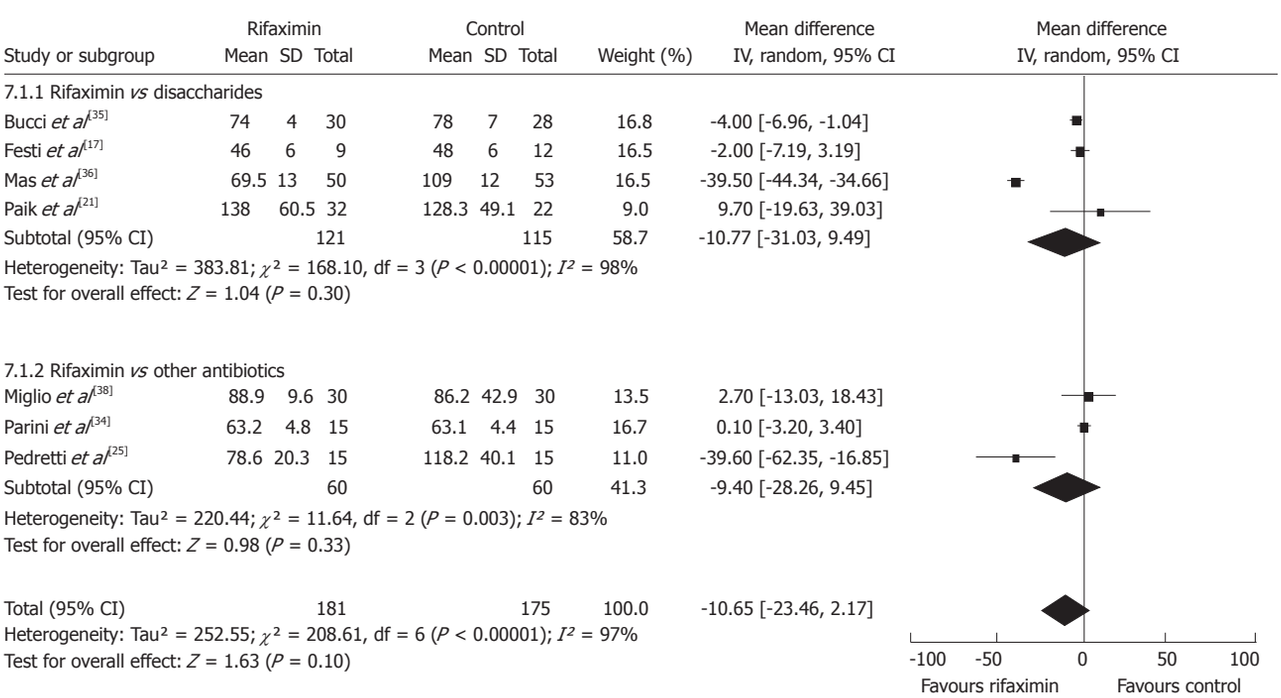

Figure 4 Serum ammonia levels at the end of the treatment protocols: rifaximin *vs* oral disaccharides, rifaximin *vs* other oral antibiotics and rifaximin *vs* control therapy which included the combination of oral disaccharides and other antibiotics. IV: Inverse variance.

Statistical assessment of patients' compliance was not possible as none of the included trials measured attrition or reported any drop-outs as all the included participants appeared to be able to complete the treatment protocols.

The results of this study confirm that rifaximin has similar effectiveness to other oral therapies but with fewer side effects. As such, it is the first new effective treatment for HE in a long time and its impact on patients' quality of life and survival has yet to be fully realized.

With regard to other secondary outcomes measured at completion of treatment protocols, patients receiving rifaximin had lower serum ammonia levels and superior mental status and asterixis profiles in comparison to the control group without reaching statistical significance. On the other hand, the grade of electroencephalographic abnormalities and PSE sums showed better profiles for participants treated with rifaximin when compared to their controls. These findings are of some importance as HE is a syndrome with a wide spectrum of neuropsychiatric abnormalities and it is important to be able to quantify subtle clinical changes during the course of therapy.

All trials in the present review excluded patients with uncorrected precipitating factors causing HE. Nevertheless, we could not obtain individual patient data to determine if response to the treatments varied according to the grade or etiology of liver disease.

One of the major limitations of this study was that the trials that satisfied the inclusion criteria had been performed during a relatively long period of time (1991-2005), the lengths of the treatment protocols varied significantly (5 d to 6 mo) and there was a lack of data on the severity of liver disease or other co-morbidities for each population. These important aspects were most likely responsible for the heterogeneity observed among the pooled studies. Minor imputations were required for some studies specially when considering standard deviations. In addition, pooling may not have been appropriate in all cases because of the heterogeneity between trials, but we attributed heterogeneity to statistical rather than clinical reasons.

On the other hand, this study also has several strengths. An extensive literature search was performed and provided the most up-to-date information on the effects of rifaximin in the treatment of HE. Using two reviewers, the inclusion or exclusion of studies and data extraction were performed independently and therefore more accurately. In addition, we did not exclude potential studies due to language, publication status or year of publication, and the fact that included trials were performed in several countries and in different settings increases the external validity of our results. Another strength of this study is that we assessed not only the efficacy and side effects of the treatments, but we also included other important clinical outcomes that could be measured objectively such as serum ammonia levels, asterixis and electroencephalographic features. Because we used a rigorous search strategy to reduce the introduction of potential publication bias, a funnel plot was not included as the number of trials was moderate and it would not have added any significant information.

In summary, this study has shown that rifaximin is comparable to other oral agents in regard to clinical efficacy for HE and is associated with fewer side effects. These results did not change during sensitivity analysis based on the quality of the trials, and the effect of rifaximin was more favorable in improving psychometric parameters and serum ammonia level measured at the end of the protocols. Given its safety profile, rifaximin should be considered as second-line in the treatment of HE patients who fail disaccharide therapy and as first-line in those intolerant of disaccharides.

COMMENTS

Background

Hepatic encephalopathy (HE) represents a debilitating and even potentially deadly complication occurring in patients with advanced liver disease. The clinical manifestations of encephalopathy range from altered mental status to deep coma. Rifaximin is an oral broad spectrum, non-absorbable antibiotic with very few systemic side effects and is used to treat or prevent hepatic encephalopathy in cirrhotic patients.

Research frontiers

The authors performed a systematic review of the literature and meta-analysis of the effectiveness and safety of rifaximin compared to other conventional oral agents such as non-absorbable disaccharides (lactulose) and other antibiotics (e.g., neomycin, metronidazole). Conventional therapies are known to cause local side effects such as diarrhea and abdominal pain in the case of Lactulose and systemic side effects such as nephrotoxicity and neurotoxicity with neomycin and metronidazole, respectively.

Innovations and breakthroughs

Several studies have shown contradictory results on the effectiveness of rifaximin in comparison to other oral agents.

Applications

The analysis has shown rifaximin to be as effective as non-absorbable disaccharides concerning improvement in the clinical symptoms of patients with hepatic encephalopathy with a lower incidence of side effects such as diarrhea, abdominal pain and nausea.

Terminology

HE: HE is a worsening of brain function that occurs when the liver is no longer able to remove toxic substances in the blood; Systematic review: A literature review focused on a research question that tries to identify, appraise, select and synthesize all high quality research evidence relevant to that question; Meta-analysis: A combination of the results of several studies that address a set of related research hypotheses.

Peer review

Rifaximin is a novel antimicrobial agent with a wide spectrum of activity that has shown promise as an alternative antimicrobial treatment option for HE. Rifaximin appears to be at least as effective as other conventional drug therapy and has been associated with fewer adverse events due to its limited systemic absorption.

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S- Editor Sun H **L- Editor** Webster JR **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.778

World J Gastroenterol 2012 February 28; 18(8): 778-784
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Immunoexpression of cyclooxygenase-2 in primary gastric carcinomas and lymph node metastases

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Supported by Institute of Molecular Pathology and Immunology of the University of Porto; Coordination for the Development of Post-Graduation Programs; and National Council for Scientific and Technological Development
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Received: January 27, 2011 Revised: March 24, 2011
Accepted: March 31, 2011
Published online: February 28, 2012

Abstract

AIM: To evaluate immunoexpression of cyclooxygenase-2 (COX-2) in primary gastric carcinomas and respective lymph node metastases.

METHODS: Immunohistochemistry to analyze COX-2 expression was performed on tissue microarray slices obtained from 36 specimens of gastrectomy and satellite lymph nodes from patients with gastric carcinoma.

RESULTS: Immunostaining was seen in most cases, and COX-2 expression was higher in lymph node metastases than in corresponding primary gastric tumors of intestinal, diffuse and mixed carcinomas, with a statistically significant difference in the diffuse histotype ($P = 0.0108$).

CONCLUSION: COX-2 immunoexpression occurs frequently in primary gastric carcinomas, but higher expression of this enzyme is observed in lymph node metastases of the diffuse histotype.

Key words: Cyclooxygenase-2; Gastric carcinoma; Immunoexpression; Lymph node metastases; Tissue microarray

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INTRODUCTION

Gastric carcinoma is one of the most common malignancies worldwide[1]. Despite improvements in the detection of early gastric cancer and more efficient surgical procedures, mortality remains high. Current challenges include studies on gastric carcinogenesis, tumor progression, and the investigation of possible molecular targets that could be utilized in diagnosis, prevention and therapeutic approaches.

Several recent studies have focused on gastric carcinogenesis. According to a known model, it is a multistep process beginning with chronic gastritis and proceeding to gastric atrophy, intestinal metaplasia, dysplasia and cancer[2]. *Helicobacter pylori* (*H. pylori*), a cause of chronic gastritis, is considered a carcinogenic agent and a link between inflammatory and neoplastic processes in the stomach[3,4].

Cyclooxygenase-2 (COX-2) is an important enzyme that catalyzes arachidonic acid to prostaglandins, which participate in inflammatory and neoplastic processes. *H. pylori*-associated with chronic gastritis induces COX-2 expression in gastric mucosa[5]. Following eradication of bacteria in gastritis, there is a tendency for COX-2 expression to reduce or disappear[5]. COX-2 expression is seen more frequently in intestinal metaplasia than in normal gastric mucosa[6] and is prominent in dysplasias[7].

COX-2 immunostaining levels correlate with the degree of dysplasia in the epithelia and stroma[8]. It is widely accepted that COX-2 immunoexpression occurs in the lamina propria and epithelia in gastritis, in intestinal metaplasia, in dysplasia, and more strongly in adenocarcinomas in a progressive manner according to the degree of the lesion[6,9-11].

In contrast to the many published reports on gastric carcinogenesis, we found few studies on the involvement of COX-2 in gastric cancer progression, all of them concerning local invasion. Results are controversial, with some authors finding a relationship between a higher degree of COX-2 expression and advanced local invasion[12-14] and others finding no correlation[15-17].

Several reports evaluated a possible association between COX-2 expression in primary gastric tumors and the presence or absence of lymph node metastasis, with mixed results. In some reports, COX-2 positivity[14,16,17] and COX-2 overexpression in primary carcinomas[18,19] were associated with the presence of lymph node metastases. Others found no correlation[13,15,20]. None of these studies evaluated COX-2 expression in both primary and metastatic gastric carcinomas. Here, we evaluated the immunoexpression of COX-2 in primary gastric carcinomas and respective lymph node metastases.

MATERIALS AND METHODS

Patients and tissue samples

Thirty-six gastric cancer specimens were obtained from patients surgically treated at the Cancer Institute of Ceará, Brazil. Formalin-fixed, paraffin-embedded tissue samples of primary gastric adenocarcinomas and their respective lymph node metastases were stained with hematoxylin and eosin and histologically analyzed. Gastric carcinomas were classified as intestinal (n = 10), diffuse (n = 12), mixed (n = 8), or unclassified (n = 6) (according to Lauren's classification system)[21].

Tissue microarray

Tissue microarrays (TMAs) were constructed from the primary samples and metastatic lymph node samples. One donor block was identified in each case, and a tissue core 2 mm in diameter representative of the neoplasm without necrotic or hemorrhagic areas was punched (TMA-builder LabVision™ catalog #-TMA 001), then placed into receptor blocks of TMAs (24 samples/block). Sections 2 μm in thickness were obtained from the TMA blocks to perform immunohistochemistry.

Immunohistochemistry

Paraffin sections were dewaxed and rehydrated. Endogenous peroxide was blocked with a 3% H_2O_2 solution in methanol for 10 min, and for unmasking antigens, a retrieval solution (Vector Co™) 1% in hot water (98 ℃) was added and incubated for 20 min. Ultra V block (TA-125-UB, LabVision Co™) was added to the sample and incubated for 10 min to inhibit nonspecific background staining. A rabbit monoclonal antibody against human COX-2 (Clone SP21, pm 70 kDa, LabVision Co™, 1:200) was applied for 60 min. After washing with phosphate buffered saline (PBS), the sections were incubated with a secondary anti-polyvalent biotinylated goat antibody (TP-125-BN, LabVision Co™) for 15 min, washed with PBS, incubated with a streptavidin-coupled peroxidase complex (TS-125-HR, LabVision Co™) for 15 min, then washed again with PBS. Reactions were processed at room temperature (approximately 20 ℃) in an automated immunostainer (Autostainer, LabVision Co™, model 480-2D) at the Institute of Molecular Pathology and Immunology of the University of Porto. A 3% H_2O_2 solution in methanol and 60 mg chromogen diaminobenzidine in PBS were applied for 10 min at 37 ℃ in the dark. After washing with distilled water for 3 min, sections were counterstained with hematoxylin, dehydrated, diaphanized with xylene, mounted and analyzed. Samples containing fewer than 100 cells were excluded from analysis.

Scoring and evaluation

The positivity criterion was cytoplasmic staining of COX-2 in malignant cells. Immunoexpression of COX-2 was evaluated regarding intensity and extension using a modified scoring table based on Rajnakova *et al*[12] (using intensity × extension, rather than intensity + extension). We used the following definitions: Intensity = degree of immunostaining which predominates in the TMA sample (0- absent; 1- mild; 2- moderate; 3- strong); extension = percentage of predominant staining intensity in the sample (0- negative or rare cells; 1- < 25%; 2- 25%-50%; 3- 50%-75%; 4- > 75% of immunoreactive neoplastic cells). A combined score of 0-12 was calculated by intensity × extension. Final COX-2 expression was classified as low (< 6) or high (≥ 6) based on median cut-off. Scores were established by two independent observers without knowledge of previous clinical findings, resulting in a high level of concordance (89%; $P < 0.05$). Cases out of concordance were defined by consensus.

Table 1 Expression of cyclooxygenase-2 in gastric cancer histotypes - stomach

Histotype	COX-2 (combined scores)								
	0	1	2	3	4	6	8	9	12
Intestinal	1	2	-	1	1	3	1	-	1
Diffuse	-	1	-	1	4	2	4	-	-
Mixed: Intestinal component	-	-	-	-	4	-	2	1	1
Diffuse component	-	-	-	-	4	-	3	-	1
Unclassified	1	-	-	1	1	1	1	-	1

COX-2: Cyclooxygenase-2.

Table 2 Expression of cyclooxygenase-2 in gastric cancer histotypes - lymph node

Histotype	COX-2 (combined scores)								
	0	1	2	3	4	6	8	9	12
Intestinal	1	1	-	-	2	1	4	-	1
Diffuse	-	1	-	-	1	-	4	-	6
Mixed	1	-	-	1	-	-	1	1	4
Unclassified	2	-	-	-	1	-	1	-	2

COX-2: Cyclooxygenase-2.

Controls

COX-2 positive controls were obtained from colonic adenocarcinoma sections[22]. Gastric non-tumoral mucosa and normal colonic mucosa distant from the tumor were used as negative controls. In known positive cases, there was no background staining when the primary antibody was not included. Stained inflammatory cells in the tumor stroma represented internal positive controls in cancer negative cases and fibroblasts were considered as an internal negative control in samples with positive neoplastic cells.

Statistical analysis

The non-parametric Mann-Whitney unpaired test (two-tailed) was used to compare median scores among different gastric carcinoma histotypes. The non-parametric Wilcoxon paired test (two-tailed) was utilized to test whether the median staining scores were significantly different between each primary gastric histotype sample of carcinoma and respective lymph node metastases. A P value of less than 0.05 was regarded as statistically significant.

Ethics

This study was approved by the Research Ethics Committee of the Cancer Institute of Ceará, Brazil (protocol number 32/2004) and conforms to The Code of Ethics of the World Medical Association (Declaration of Helsinki).

RESULTS

Table 1 shows the primary gastric cancer distribution by histological type and detailed scores. There is a marked concentration of scores at 4 and 8 and dispersion of other scores.

In lymph nodes, the highest score of 12 occurred at a much higher frequency (13 cases) than in primary tumors (only 4 cases), notably in diffuse and mixed carcinomas, while intestinal histotype was given a score of 8 more frequently than in primary carcinomas (Tables 1 and 2).

Analysis of low (< 6) and high (⩾ 6) COX-2 expression levels showed no differences among primary and metastatic respective histotypes (Table 3).

In lymph nodes (Table 4), high COX-2 expression (score ⩾ 6) was found in most cases (25/36 = 69%), mainly in diffuse (10/12 = 83%) and mixed histotypes (6/8 = 75%), but also in intestinal tumors (6/10 = 60%; Table 4). The highest lymph node expression difference was not statistically significant in the whole sample (P = 0.1488).

Nevertheless, in the diffuse histotype, COX-2 immunoexpression in lymph nodes was significantly higher than that in the stomach (P = 0.0108), as shown in Figure 1.

The highest COX-2 expression in metastatic diffuse carcinomas occurred not only in the whole sample of this histotype. Analysis of each case at primary and metastatic sites revealed increased expression in 75% of cases (9/12), similar expression levels in 1/12 cases (8%) and reduced expression levels in only two cases (17%). In five of nine cases with increased immunoexpression, there was a change from low to high COX-2 expression (Figure 2).

Figure 3 shows high COX-2 expression in metastatic tumors compared to respective primary carcinomas in every histological type (except unclassified).

An interesting isolated finding was observed in case 12 (diffuse histotype), shown in Figure 4. The neoplastic cells invading the gastric wall were poorly stained but the cells in the vessel lumina had strong immunoexpression, similar to metastatic lymph node implants. We will comment on these findings in the discussion.

Table 3 Expression of cyclooxygenase-2 (low and high) in gastric cancer histotypes - stomach

COX-2 scores	Histotype				
	Intestinal	Diffuse	Mixed	Unclassified	Total
< 6	5	6	4	3	18
⩾ 6	5	6	4	3	18
Total	10	12	8	6	36

COX-2: Cyclooxygenase-2.

Table 4 Expression of cyclooxygenase-2 (low and high) in gastric cancer histotypes - lymph node

COX-2 scores	Histotype				
	Intestinal	Diffuse	Mixed	Unclassified	Total
< 6	4	2	2	3	11
⩾ 6	6	10	6	3	25
Total	10	12	8	6	36

COX-2: Cyclooxygenase-2.

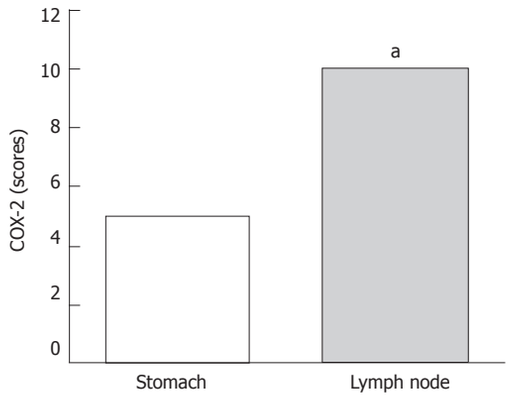

Figure 1 Cyclooxygenase-2 expression in gastric cancer, diffuse histotype. Lymph node expression is significantly higher than in the stomach (Median scores). [a]*P* = 0.0108, Mann-Whitney test. COX-2: Cyclooxygenase-2.

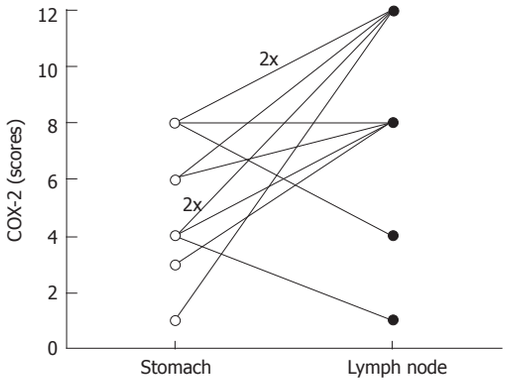

Figure 2 Cyclooxygenase-2 expression in primary diffuse gastric cancer and respective metastasis. Lymph node expression is higher than stomach expression levels in most cases (Individual case scores). *P* = 0.0137, Wilcoxon paired test. 2x means two overlaid cases. COX-2: Cyclooxygenase-2.

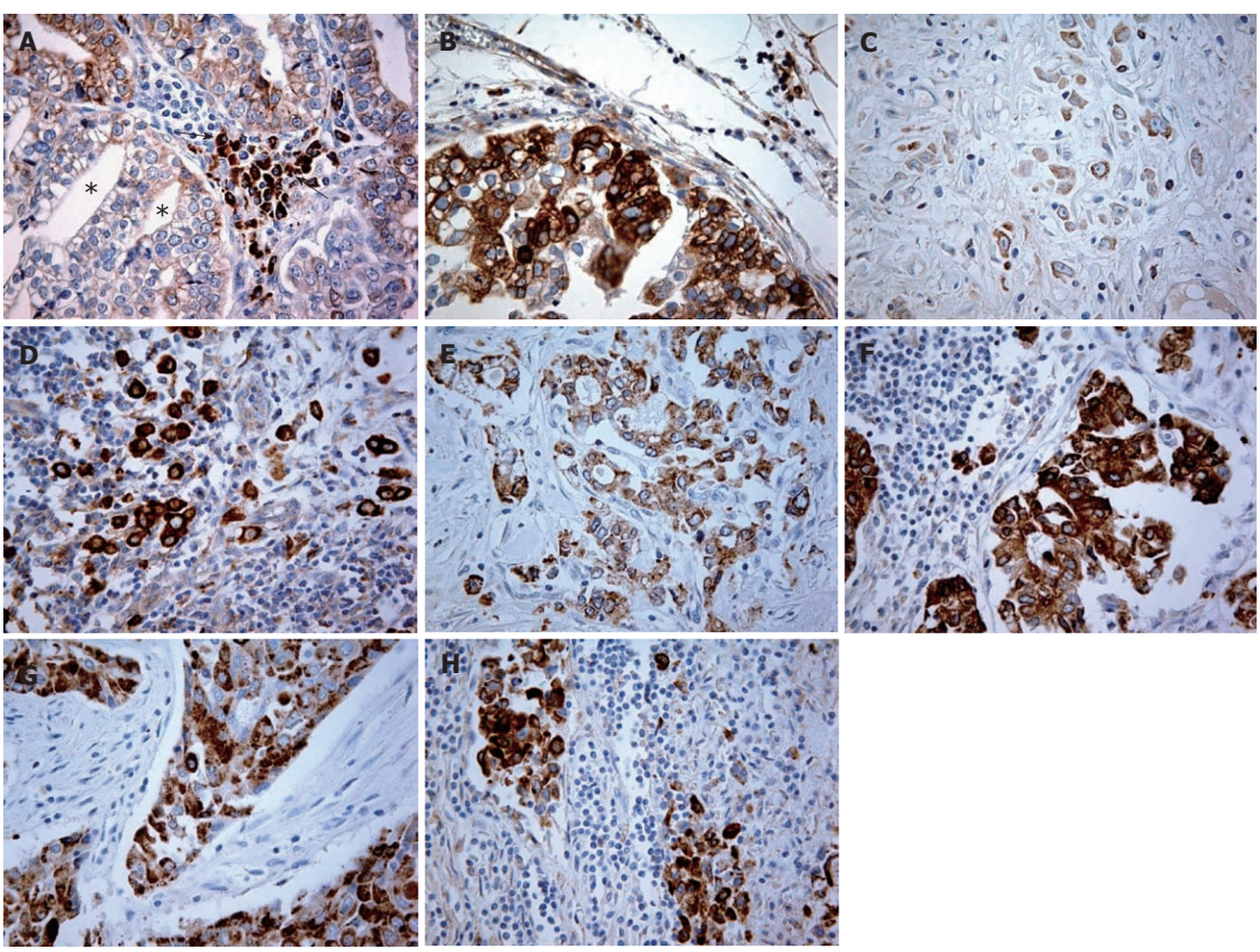

Figure 3 Cyclooxygenase-2 expression in gastric cancer. A, B: Intestinal type, case 12; A: Stomach = score 1, cancer cells were negative or scarcely stained (*). Strong cyclooxygenase-2 staining of inflammatory cells (mononuclear) into the stroma (arrows); B: Lymph node = score 8; C, D: Diffuse type, case 3; C: Stomach = score 4; D: Lymph node = score 12; E, F: Mixed type, case 7; E: Stomach = score 8 (both components); F: Lymph node = score 12; G, H: Unclassified, case 5; G: Stomach = score 6; H: Lymph node = score 8. All magnifications 400 ×.

DISCUSSION

The study of simultaneous COX-2 immunoexpression in the primary tumor and its metastasis is rarely seen in the current literature. In a case report recently published, Scheer *et al*[23] revealed a strong multifocal COX-2 expression in about 80% of cells in the primary hypopharyngeal carcinoma and 10% in the frontotemporal bone metastasis. In addition to that, Kasper *et al*[24] found various degrees of cytoplasmic COX-2 expression in

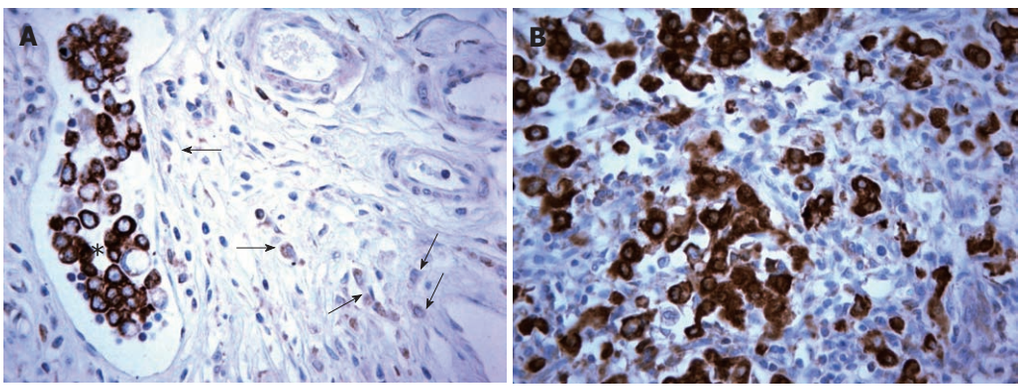

Figure 4 Cyclooxygenase-2 expression in gastric cancer and diffuse type: primary tumor, emboli and metastasis. A: Stomach: Low cyclooxygenase-2 (COX-2) expression in perivascular malignant cells (arrows). Tumor embolus with strongly stained neoplastic cells (*); B: Lymph node: High COX-2 expression at the same intensity seen in the embolus. Case 3, diffuse histotype, 400 ×.

57 colorectal carcinomas and in the corresponding liver metastases. As far as we know, there is no report regarding the simultaneous COX-2 expression in gastric cancer and in its respective lymph node metastasis.

In our study, we verified that COX-2 was expressed in 34/36 (94%) of the primary gastric carcinoma cases, similar to our results from a larger sampling (data not shown) and according to the frequency reported by other authors[14,20]. There is a broad variation in the frequency of COX-2 expression in gastric carcinomas, ranging from 43% to 100%[10,20]. In most reports, however, the frequency lies in the range of 60% to 80%[8,12,13,15,19,25].

Possible explanations for this variation are differences in sample characteristics, for instance, heterogeneity of sampling areas (inflammatory or ulcerated areas near cancer increase COX-2 expression) and variation in the usage of anti-inflammatory non-steroidal drugs, which reduce COX-2 expression. Other possibilities are methodological and technical procedures like time of fixation, sensitivity and specificity of monoclonal/polyclonal antibodies, and criteria for evaluation of immunoexpression. In many studies, including our own, separate scores were applied to intensity and extension and scores were then multiplied or added[8,10,12,19,20,25]. We used a scoring system ranging from 0 to 12 with a cut-off in the middle (low expression < 6; high expression ⩾ 6), based on median.

When scores are sufficiently simplified, defined for example as low and high expression, reproducible results must be obtained. In our sample, elevated COX-2 expression was found in 50% of primary gastric carcinoma cases, similar to the findings of Sun *et al*[19] (55%), both with a cut-off based on median. Other reports with similar score criteria showed high COX-2 expression in 61% and 62%[8,25], percentages not so different from ours.

This study showed high COX-2 expression in primary gastric carcinomas in half of the cases and without significant differences between histotypes, in agreement with other reports[9,20]. Nevertheless, other authors have found a significantly higher frequency of positive cases[6,25,26] or cases with higher expression of COX-2[10,26] in the intestinal carcinomas than in diffuse tumors.

The importance of COX-2 expression on cancer biology, in different malignant tumors including gastric carcinomas, must be emphasized. COX-2 influences neoplastic behavior in many ways, primarily through prostaglandin E_2 (PGE_2) synthesis promoting cellular proliferation[27-29], invasiveness[30-32], angiogenesis and inhibition of apoptosis[33,34]. These effects undoubtedly contribute to the process of tumor survival and progression.

In a larger series (intestinal and diffuse histotypes, results not shown), we found strong COX-2 immunostaining in 58% of tumors at stage T1 and in 48% of more advanced lesions (T2 to T4), in both histotypes (intestinal = 42%; diffuse = 55%). These percentages were higher in mixed carcinomas (data not shown). These findings reinforce the importance of COX-2 presence in local tumor progression of gastric carcinomas in all histological types.

In the lymph nodes, our results clearly showed higher COX-2 expression in metastatic tumors than in respective primary gastric carcinomas of the diffuse histotype. Diffuse carcinomas showed a strong increase in COX-2 lymph node immunostaining compared to primary gastric tumors, not only in the whole group but in most individual cases (Figure 2). To the best of our knowledge, this is the first report to evaluate COX-2 expression simultaneously in both sites and to demonstrate the strongest enzyme marking in a more advanced stage of gastric cancer compared to primary invasive tumors. Recently, Kim *et al*[35] compared COX-2 (and many other proteins) expression levels in primary gastric carcinomas and respective lymph node metastases and found no differences. Further investigation is needed to resolve this discrepancy.

We have no answer as to why, in the present study, the diffuse histotype showed the higher staining difference in metastatic lesions. Another question is "at what level of local invasion through the gastric wall does COX-2 expression increase?" There is an interesting finding in Figure 4 in which we show that neoplastic cells invading the gastric wall, near a vessel, were poorly stained while cells presented in the tumor embolus had a strong COX-2 immunoexpression, similar to the expression

found in the respective lymph node metastases.

This is an isolated finding and we cannot make definitive conclusions. However, this observation does raise further questions: do the neoplastic cells in the neighboring vessel first increase COX-2 expression and, as a consequence, invade the vessel? Or, alternatively, do the cells invade the vessel wall and then the vessel microenvironment would induce COX-2 expression? Perhaps the latter would be a suitable answer, since there are no strongly stained cells in tissue around the vessel. These questions are outside the scope of this study and must be investigated using a larger sample size with many histopathological sections for vessel immunohistochemical markers and even better addressed with experimental models.

In summary, we have shown that COX-2 immunoexpression occurs frequently in primary gastric carcinomas and higher expression is seen in lymph node metastases of the diffuse histotype. These findings emphasize the importance of COX-2 as a potential marker of tumor progression and its possible use in diagnosis, prognosis or development of therapeutic tools against gastric cancer. The role of COX-2 in neoplastic invasion through the gastric wall, in vessel tumor emboli formation and in establishing distant metastases requires further study.

ACKNOWLEDGMENTS

We thank Professor Manuel Sobrinho-Simões and Dr. Fátima Carneiro who made possible this international cooperation and Mrs. Dina Leitão for her excellent technical assistance. We are indebted to the Hospital of Cancer, Cancer Institute of Ceará (Brazil) for providing patient information and tissue blocks for the study.

COMMENTS

Background

Gastric carcinoma is one of the most common malignancies worldwide. It is widely accepted that cyclooxygenase-2 (COX-2) immunoexpression occurs in the lamina propria and epithelia in gastritis, in the intestinal metaplasia, in dysplasia, and more strongly in adenocarcinomas in a progressive manner according to the degree of the lesion. However, there is a lack of studies exploring the immunoexpression of COX-2 in primary gastric carcinomas and respective lymph node metastases.

Research frontiers

In some reports, COX-2 positivity and COX-2 overexpression in primary carcinomas were associated with the presence of lymph node metastases, but some others found no correlation. In this study, the authors evaluated the immunoexpression of COX-2 in primary gastric carcinomas and respective lymph node metastases.

Innovations and breakthroughs

This study reports that COX-2 immunoexpression occurs frequently in primary gastric carcinomas and higher expression is seen in lymph node metastases of the diffuse histotype.

Applications

The importance of COX-2 expression on cancer biology, including gastric carcinomas, must be emphasized. COX-2 influences neoplastic behavior by promoting cellular proliferation, invasiveness, angiogenesis and inhibition of apoptosis, which contribute to the process of tumor survival and progression. The differential expression of molecular markers among cancer histotypes is of high importance regarding a possible improved therapeutic approach.

Terminology

Gastric carcinomas are classified as intestinal, diffuse and mixed histotypes. The molecular characterization of these histotypes might provide a better knowledge concerning metastatic potential.

Peer review

The authors performed immunohistochemistry on COX2 in primary gastric cancer and respective lymph node metastases, and found a higher COX2 expression in metastatic tumors compared to respective primary tumors, especially in diffuse type gastric cancer. Since the study on COX2 expression in the primary and its metastases is rarely seen, this work is significant.

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S- Editor Sun H **L- Editor** Webster JR **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.785

World J Gastroenterol 2012 February 28; 18(8): 785-793
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Vaccination with dendritic cells pulsed with hepatitis C pseudo particles induces specific immune responses in mice

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Received: March 3, 2011 Revised: March 26, 2011
Accepted: June 13, 2011
Published online: February 28, 2012

Abstract

AIM: To explore dendritic cells (DCs) multiple functions in immune modulation.

METHODS: We used bone-marrow derived dendritic cells from BALB/c mice pulsed with pseudo particles from the hepatitis C virus to vaccinate naive BALB/c mice. Hepatitis C virus (HCV) pseudo particles consist of the genotype 1b derived envelope proteins E1 and E2, covering a non-HCV core structure. Thus, not a single epitope, but the whole "viral surface" induces immunogenicity. For vaccination, mature and activated DC were injected subcutaneously twice.

RESULTS: Humoral and cellular immune responses measured by enzyme-linked immunosorbent assay and interferon-gamma enzyme-linked immunosorbent spot test showed antibody production as well as T-cells directed against HCV. Furthermore, T-cell responses confirmed two highly immunogenic regions in E1 and E2 outside the hypervariable region 1.

CONCLUSION: Our results indicate dendritic cells as a promising vaccination model for HCV infection that should be evaluated further.

Key words: Dendritic cell; Hepatitis C; Pseudo particles; Immune responses; Vaccination

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Weigand K, Voigt F, Encke J, Hoyler B, Stremmel W, Eisenbach C. Vaccination with dendritic cells pulsed with hepatitis C pseudo particles induces specific immune responses in mice. *World J Gastroenterol* 2012; 18(8): 785-793 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/785.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.785

INTRODUCTION

Despite many developments and improving results in treating hepatitis C virus (HCV) infection, chronic hepatitis C stays a severe medical health problem world wide with over 170 million people infected[1,2]. Even though treatment of chronic infected patients with pegylated interferon and ribavirin currently results in a sustained virological response in 40%-80%[3,4], there remains a large number of HCV positive patients, non-responders and relapsers. Until development of an effective vaccine, chronic hepatitis C remains one of the most important infectious diseases.

The main problem in developing such a vaccine is the limited understanding of the type of immune response that is necessary for viral clearance and the occurrence of various genotypes and quasispecies of HCV, evolving rapidly under selection pressure by the immune response[5-7]. So most likely a vaccine must induce a broad immune response to clear HCV infection[5,8,9]. And indeed, humoral and cellular immune responses to several of the viral proteins have been shown to be associated with clearance of HCV infection in experimental settings[10-14]. Infected people develop variant-specific neutralizing antibodies[15]. A main target of these antibodies is considered to be the hypervariable region 1 (HVR1) of the envelope glycoprotein E2[7,11,16,17] but also other regions in the envelope protein[18,19]. E2 covalently linked to E1, the second envelope glycoprotein of HCV, forms the virus envelope[20]. Chimpanzees immunized by recombinant E1 and E2 protein, synthesized in mammalian cells, showed protection against HCV challenge[21,22]. Anti-HVR1 antibodies even have some cross-reactive activity to different HVR1 sequences[23] and may persist up to 7 years[24]. Still, re-infection and viral persistence occurs even in the presence of these antibodies[25,26]. Besides this humoral immune response, cellular immune responses appear to be critical for HCV clearance. Development of an early class 1 restricted $CD8^+$ cytotoxic T lymphocyte (CTL) response to HCV structural and non-structural proteins is associated with HCV clearance[27,28]. Human leukocyte antigen-A2, -A3 and -B7 restricted CTL responses have been identified to regions of HCV core, E1, nonstructural (NS)3, NS4 and NS5 proteins[29,30]. The additional inclusion of T helper epitopes has been shown to produce even stronger $CD8^+$ responses[10,12]. It seems likely that an effective vaccine against HCV should therefore be capable of inducing a T helper cell, CTL and neutralizing antibody response in multiple major histocompatibility complex (MHC) types.

Considering that, key players could be dendritic cells (DCs). DC are the most potent type of antigen presenting cells and induce immune antiviral responses[31-33]. Found within the peripheral tissues and lymphoid organs, DC are perfectly suited to detect and capture pathogens. Their antigen presenting capability is crucial for generation of $CD4^+$ T-cells priming B-cells for antibody production. By production of CD40 and interleukin-2 (IL-2), DC provide help to $CD8^+$ cells. To fulfill their function, DC have to mature, normally triggered by exposure to viruses or other pathogens[5]. Interestingly DC from HCV carriers show impaired maturation, determined by absence of cell surface molecules[34,35], as well as reduced IL-2 production[36]. Loss of DC function is probably a direct consequence of HCV infection[14]. Ex vivo generated and matured DC therefore might be the most potent candidate for a cell based HCV vaccine. In fact, there are some promising results published for immunization with DC against the human immunodeficiency virus and HCV[32,33,37,38].

In sera of patients with chronic HCV infection antibodies directed towards E1 and E2 can be found as mentioned above[39]. Chimpanzees immunized with recombinant HCV E1 and E2 showed protection against HCV infection[40]. Peptide immunizations have been successful in producing humoral and cellular immune responses[41,42]. But peptides do not deliver a great number of epitopes and are not folded in the native protein form. To overcome these limitations virus like particles have been created, consisting of both envelope proteins E1 and E2 on an HCV core or retroviral core protein, the latter termed HCV pseudo particles (HCVpp)[43-45]. These particles mimic HCV virions in the best possible way and are therefore an interesting stimulant. In this study, we investigated if HCVpp are able to activate mature murine DCs *ex vivo* and if so, if vaccination with these DC induces specific immune responses against HCV *in vivo*.

MATERIALS AND METHODS

Cells and culture conditions

293T cells and Huh-7 cells were maintained in Dulbecco's Modified Eagle Medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS) (Invitrogen, Karlsruhe, Germany), 2 mmol/L L-glutamine (Invitrogen, Karlsruhe, Germany), 100 units/mL penicillin and 100 units/mL streptomycin (Invitrogen, Karlsruhe, Germany). Bone marrow derived dendritic cells were maintained in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10 % FCS, 2 mmol/L L-glutamine, 100 U/mL Penicillin, 100 mg/mL streptomycin and 50 μmol/L 2-mercaptoethanol (Invitrogen, Karlsruhe, Germany).

Preparation of HCVpp

Pseudoparticles were generated as described previously[45]. Briefly, 293T cells were transfected using a calcium phosphate-based transfection kit with three expression vectors encoding an envelope glycoprotein, viral core components, and a viral genome containing a green fluorescent protein marker gene. The plasmids used in our laboratory are the following: Cytomegalovirus (CMV)-Gag-Pol murine leukemia virus (MLV) packaging construct, encoding the MLV gag and pol genes; the MLV-green fluorescent protein (GFP) plasmid, encoding an MLV-based transfer vector containing a CMV-GFP internal transcriptional unit and the plasmid phCMV-E1E2-HCV, encoding the HCV E1 and E2 glycoproteins of Con1-isolate, genotype 1b. After 40-46 h supernatants containing pseudoparticles were filtered through 0.45 μm pore size membrane and for further concentration a centrifugation with Amicon Ultra-15 centrifugal filter units (MWCO 100K) was done. Infectious titers of the concentrated supernatants were then determined by infection of Huh7 cells as previously described[45]. The infectious titers, expressed as transducing units per milliliter, were deduced from the transduction efficien-

cies, determined as the percentage of GFP-positive cells measured by fluorescence activated cell sorting (FACS) analysis[46].

Generation of bone-marrow derived dendritic cells

For preparation of bone-marrow derived DC, we used 6-10 wk old BALB/c mice (Charles River Breeding Laboratories, Sulzfeld, Germany). They were maintained under barrier-sustained conditions and handled according to international guidelines. After sacrificing the animals, the tibia and femur bones were used to prepare bone marrow cells. With minor adaptations, cultivation of bone marrow cells was done following the Inaba protocol[47]. On day 7 we pooled non-adherent and loosely adherent cells. The isolated cell suspensions were either taken for FACS analysis or plated into 12 well culture plates (Greiner Labortechnik, Kremsmuenster, Germany) at a density of 1.5×10^6 cells per well in one ml of complete bone marrow-derived dendritic cell (BMdDC) medium.

FACS analysis of BMdDC

Flow cytometry analysis for measuring the expression of different surface molecules was performed with a FACSCalibur® cytometer and data was analysed with Cell Quest Pro software (Beckton Dickinson, Franklin Lakes, United States). For staining, 2×10^5 cells were incubated in staining buffer [phosphate buffer solution (PBS) and 0.5% body surface area, Invitrogen, Paisley, United Kingdom] with either 1 μL of specific antibodies or the corresponding isotype control (APC anti-mouse CCR-7 (Biozol, Eching, Germany), R-PE anti-mouse CD 11c, FITC anti-mouse CD 86, FITC anti-mouse MHC Ⅱ (I-Ad) (all from BD Bioscience, Heidelberg, Germany) for 30 min on ice in the dark. Stained cells were pelleted for 3 min at 2000 r/mim (Biofuge pico; Kendro, Hanau, Germany) and were washed twice with staining buffer. A negative control with unstained cells was run first to determine the baseline fluorescence. Checking for unspecific binding, marker setting was done with isotype controls. For instrument settings and compensation of R-PE and FITC, samples stained with individual fluorescent probes were used.

Activation of BMdDC

On day 7, FACS analysis revealed 60%-70% mature DC, which were placed in a 12-well culture plate with a concentration of 1.5×10^6 cells per mL. Twenty-four hours later, the DC were activated by adding nothing (negative control), HCVpp (7.5×10^5), 1 μg/mL *E. coli* lipopolysaccharide (LPS) (Sigma, St. Louis, MO), or HCVpp together with LPS into the culture medium. LPS is a known co-stimulatory factor for DC[48]. Cells were harvested on day 9 (24 h after activation) and washed extensively. Activation of DC was measured by FACS analysis. For immunization, 1.0×10^6 DC were collected in 75 μL of 0.9% sodium chloride per mouse.

Table 1 Different vaccination groups with 8 mice each

No.	Vaccine	Purpose
1	Sodium chloride	Negative control
2	DC 293T-supernatant, concentrated with Amicon filtration as the HCVpp	Negative control to rule out immunogenic components of Amicon filter and 293T supernatant
3	HCVpp	Immunogenic impact of HCVpp
4	DC activated with HCVpp	Treatment group without adjuvant
5	DC activated with HCVpp + LPS	Treatment group with adjuvant

DC: Dendritic cell; HCVpp: Hepatitis C virus pseudo particles; LPS: Lipopolysaccharide.

Vaccination schedule of the mice

Different groups of 8 mice each were subcutaneously injected with vaccines on day 1 and day 15 (Table 1). In all experiments HCVpp were used at a concentration of 7.5×10^5/mL after amicon filtration. Two weeks after the second vaccination, sera and spleen cells were collected for immunological analysis. Sera were centrifuged at 13 000 r/min for 30 min and plasma was collected and stored at -80 ℃.

Isolation of splenocytes

Collected spleens were ground on ice in complete RPMI medium (Invitrogen, Paisley, United Kingdom), centrifuged (1000 r/min, 5 min) and resuspended in PBS twice. The purified cells were treated with erythrocyte lysis buffer, centrifuged, washed and resuspended in complete RPMI medium. The isolated cells were counted with a Neubauer chamber. Splenocytes were used for immunological analysis at a concentration of 3×10^6 cells per mL.

Immunological analysis

Interferon-gamma (IFN-γ) enzyme-linked immunosorbent spot test (ELISPOT) assay was performed using 3×10^5 splenocytes per well (96 well plates, Millipore, Bedford, United States) from each vaccination group precoated with anti-IFN-γ antibodies (5 μg/mL; Beckton Dickinson, Franklin Lakes, United States). HCV specific T-cell responses were examined after stimulation with either HCVpp or overlapping peptides from PepSet ™ (Mimotopes, Clayton Victoria, Australia) of the E1 and E2 Protein of the Con1 HCV sequence which was used for HCVpp production. Altogether, 69 peptides (24 covering the E1 protein and 45 covering the E2 protein) consisting of 20 amino acids each with an offset of 8, were pooled by 7 peptides for better handling as described earlier[49]. As a positive control, concanavalin A (1 μg/mL, Sigma, St. Louis, United States) was added. Following standard protocol IFN-γ spot-forming cells (SFC) were counted by a computer-based image analyser (Zeiss-Vision, Eching, Germany). All results were expressed as mean SFC/3×10^5 splenocytes of quadruplicate measurements.

Enzyme-linked immunosorbent assay (ELISA) was

Table 2 Amino acid sequence of the E1 and E2 protein of the hepatitis C virus Con1b isolate

E1	192 - 231	GYEVRNVSGVYHVTNDCSNASIVYEAAD-MIMHTPGCVPCV
aa 192-383	232 - 271	RENNSSRCWVALTPTLAARNASVPTTTIRRH-VDLLVGAAA
	272 - 311	LCSAMYVGDLCGSVFLVAQLFTFSPRRHET-VQDCNCSIYP
193 aa total	312 - 351	GHVTGHRMAWDMMMNWSPTAALVVSQLL-RIPQAVVDMVAG
	352 - 383	AHWGVLAGLAYYSMVGNWAKVLIVMLL-FAGVDG
E2	384 - 423	GTYVTGGTMAKNTLGITSLFSP-GSSQKIQLVNTNGSWHIN
aa 384-746	424 - 463	RTALNCNDSLNTGFLAALFYVHKFNSS-GCPERMASCSPID
	464 - 503	AFAQGWGPITYNESHSSDQRPYC-WHYAPRPCGIVPAAQVC
363 aa total	504 - 543	GPVYCFTPSPVVVGTTDRFGVPTYSWGENET-DVLLLNNTR
	544 - 583	PPQGNWFGCTWMNSTGFTKTCGGPPCNIG-GIGNKTLTCPT
	584 - 623	DCFRKHPEATYTKCGSGPWLTPRCLVHY-PYRLWHYPCTVN
	624 - 663	FTIFKVRMYVGGVEHRLEAACNWTRGERCN-LEDRDRSELS
	664 - 703	PLLLSTTEWQVLPCSFTTLPALSTGLIHLHQN-VVDVQYLY
	704 - 743	GIGSAVVSFAIKWEYVLLLFLLLADARVCA-CLWMMLLIAQ
	744 - 746	AEA

used to determine the levels of anti-HCV-immunoglobulin in the sera of the different immunization groups. Following standard protocol 96-well microtiter plates (Millipore, Bedford, United States) were coated with either HCVpp or PepSet™ (Mimotopes, Clayton Victoria, Australia) containing biotinylated overlapping peptides (offset by 8) of the E1 and E2 Protein of the Con1 HCV sequence (Table 2). Again the 69 peptides were pooled by 7 peptides. Mouse serum was added in a 1:100 dilution. Colour development, using an HRP conjugated goat anti-mouse-IgG antibody (Santa Cruz Biotech, Santa Cruz, United States), was read in an automated reader at 450 nm (Microplate Reader 2001, Whittaker Bioproducts, Walkersville, United States).

RESULTS

Dendritic cells are strongly activated by HCVpp

As shown in Figure 1, CD11c positive DC that were used in our study were activated by HCVpp *in vitro*. In the FACS analysis non-activated DC expressed CD86 in 10% and CCR7 in 10%. After 24 h of incubation with HCVpp and the costimulatory factor LPS, HCVpp only, or LPS alone CD86 rates were increased to 41%, 40% or 49%, respectively. Thus, DC were strongly activated. Regarding the more specific migration marker CCR7, incubation with LPS resulted in low levels of expression (12%). In contrast, priming with HCVpp or HCVpp and LPS resulted in a stronger upregulation. Here, CCR7 was found in 25% and 39%, respectively. In all our experiments CCR7 receptor expression was more enhanced whenever we used HCVpp for activation of the DC.

DC treatment is well tolerated in mice

We immunized different groups of BALB/c mice, each consisting of 8 animals (Table 1). For immunization details see Materials and Methods section. All mice were checked daily. Treatment was well tolerated and no mouse was considered ill, lost weight or died.

BALB/c mice immunized with DC pulsed with HCVpp can induce antibody response

Humoral immune responses against HCVpp were assessed by ELISA. Antibody titers were highest in groups of mice vaccinated with HCVpp pulsed DC. However, only slightly significant antibody binding could be demonstrated in the overlapping peptides (PepSets™) spanning the E1 and E2 protein of the HCV Con 1 isolate (Figure 2). Mice vaccinated with HCVpp showed lower antibody binding of the overlapping peptides compared to mice vaccinated with HCVpp pulsed DC. However, using HCVpp as read-out antigen, the measured antibody binding was comparably high in HCVpp pulsed DC and HCVpp vaccinated animals. Overall, highly significant antibody binding ($P < 0.001$) was only observed with HCVpp as read-out antigen, indicating the importance of correct three dimensional folding.

ELISPOT demonstrates a specific T-cell response in BALB/c mice immunized with DC previously incubated with HCVpp

Specific T-cell responses were assessed by IFNγ-ELISPOT analysis. Results are shown as mean SFC/3 × 10^5 splenocytes (Figure 3). T-cells from mice vaccinated with DC pulsed by the combination of HCVpp and LPS showed the highest amount of IFN-γ when stimulated with the peptide pools or HCVpp itself. The responses were significantly higher compared to T-cells from mice vaccinated with HCVpp only. As expected, the two negative control groups (mice vaccinated with saline or 293T supernatant) showed the lowest number of spots. Peak results with SFC/3 × 10^5 splenocytes above 80 were seen in cells stimulated with pools 3 and 7 in the analysis. Pool 3 (85.8 spots/well) represents amino acids 312-379 of the E1 protein and pool 7 (86.3 spots/well) represents amino acids 544-611 of the E2 protein, regarding the sequence of the HCV polyprotein. In contrast to the ELISA assay, there was no significant difference between the read-out antigens used (peptide pools *vs* HCVpp).

DISCUSSION

The hepatitis C virus inhibits intracellular interferon pathways, impairs DC activation and T-cell responses[34-36,50]. In addition, it induces a state of T-cell exhaustion and selects escape variants with mutations in immunodominant T-cell epitopes[51]. This is especially important since

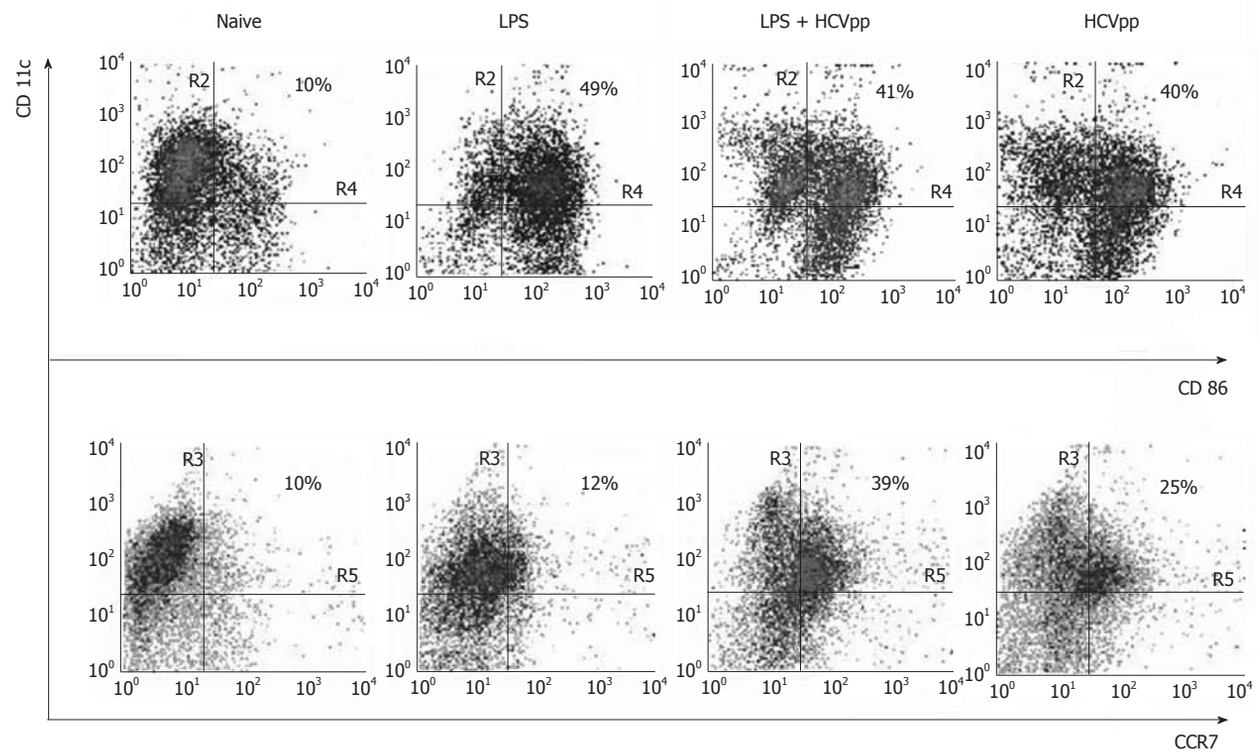

Figure 1 Fluorescence activated cell sorting analysis of bone-marrow derived dendritic cell of BALB/c mice, identified by expression of CD11c, after 7 d of maturation, followed by incubation with different agents. Column one shows the negative control, columns two and three show dendritic cell (DC) incubated with only lipopolysaccharide (LPS) or hepatitis C virus pseudo particles (HCVpp) with the co-stimulator LPS, respectively, and column four shows DC incubated with HCVpp only. Expression of the surface markers CD86 and CCR7 after pulsion of the DC with HCVpp and/or LPS is shown in percent.

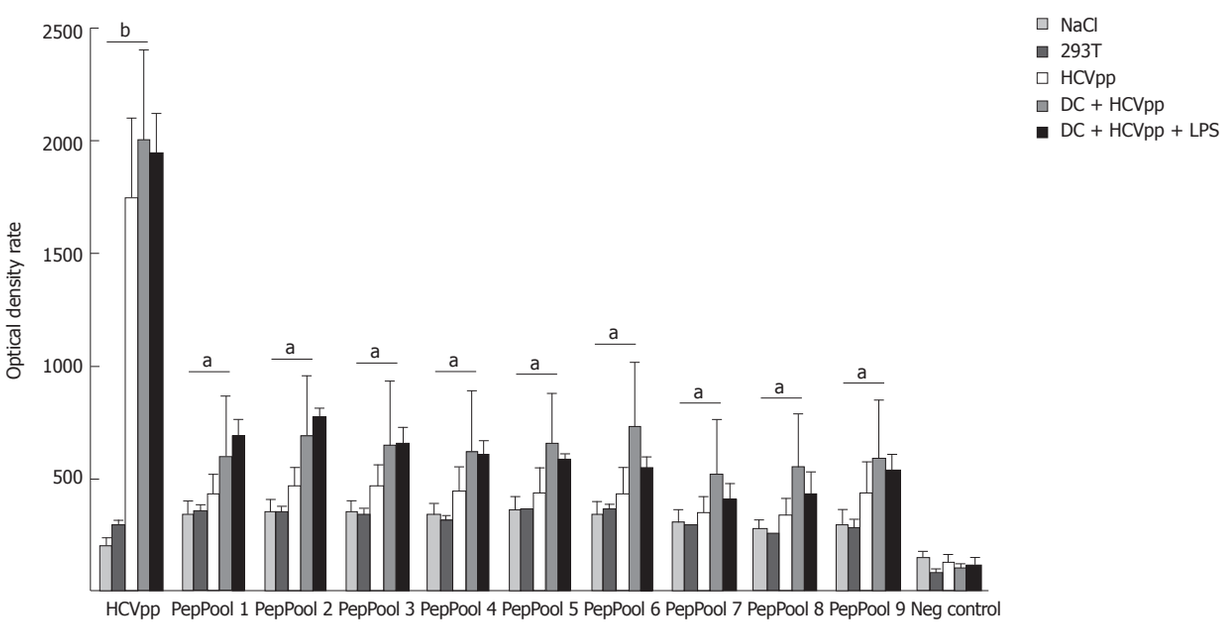

Figure 2 Induction of anti-E1 and anti-E2 antibodies following s.c. immunization of BALB/c mice. All animals specifically vaccinated developed specific antibodies. Highest antibody titers were observed in the two groups of mice which received the dendritic cell (DC) based vaccines. The negative control with phosphate buffered saline showed only very little unspecific binding. PepSets™ Pools 1-9 spanning the E1 and E2 protein of the hepatitis C virus (HCV) Con1 isolate showed considerably lower binding activity in the treatment groups, whereas the negative control groups did not show any differences between the different antigens. Through serial dilutions OD was calculated for HCV pseudo particles (HCVpp) group to be OD 1755, for the DC + HCVpp group OD 2013 and for the DC + HCVpp+ lipopolysaccharide (LPS) group OD 1944. For significance, NaCl groups were compared with DC + HCVpp groups. Pool 1-3 covers most of the E1 protein, pool 4 comprises the last 24 amino acids of the E1 protein and the first 32 amino acids of the E2 protein, and pool 5-9 enclose the rest of the E2 protein. NaCl: Saline; 293T: Cell culture supernatant of 293T-cells. Results are given as means of quadruplicate measurements of eight mice each group. $^{a}P < 0.05$, $^{b}P < 0.001$.

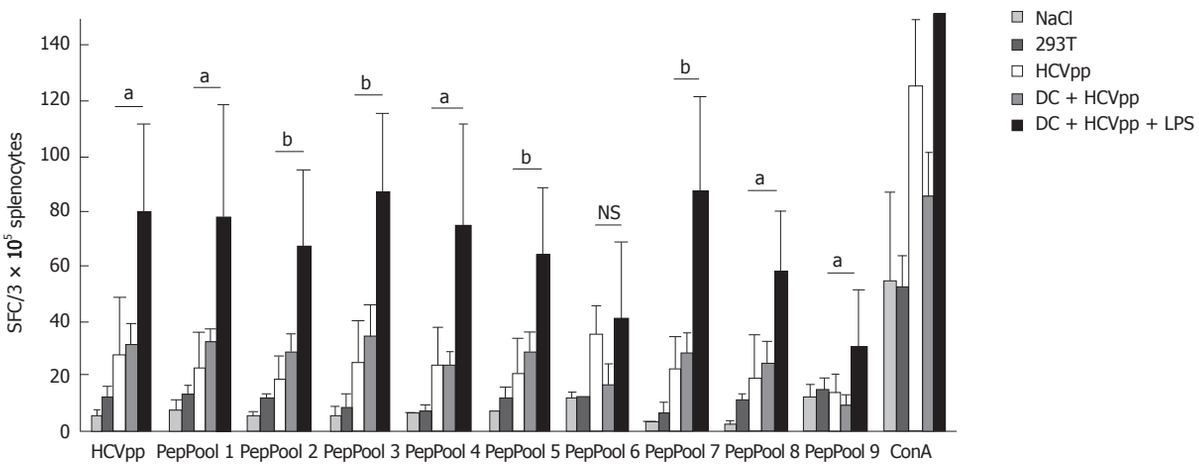

Figure 3 Antigen specific T-cell responses detected by interferon-gamma enzyme-linked immunosorbent spot test. Immune responses were induced by vaccination of different BALB/c mice with different agents. The two negative control groups were vaccinated with saline or 239T supernatant. The third group were mice vaccinated with hepatitis C virus pseudo particles (HCVpp) only. The treatment groups were mice vaccinated with dendritic cell (DC) prior pulsed with HCVpp with or without lipopolysaccharide (LPS). For detection of specific T-cells, spleenocytes were incubated with HCVpp or pooled overlapping peptides covering the E1 and E2 proteins of the HCV Con1 sequence. Convacalin A (ConA) was used as a positive control. Best results were achieved in the group of mice vaccinated with DC prior pulsed with HCVpp and LPS as an adjuvant. For significance HCVpp groups were compared with DC + HCV + LPS groups. NaCl: Saline; 293T: Cell culture supernatant of 293T-cells; NS: Not significant. Results are shown as mean values of 8 mice each group. $^{a}P < 0.05$, $^{b}P < 0.001$.

the clearance of HCV infection requires strong and broadly cross-reactive T-cell and neutralizing antibody responses[52-54]. It has been shown that the development of a multi-specific T-cell response during acute HCV infection is associated with spontaneous clearance of infection[55]. A successful immune response against HCV is therefore based on sufficient innate and adaptive immune responses.

Several approaches in HCV vaccine development have been studied. In chimpanzees it has been shown that the inclusion of structural HCV proteins was more significantly associated with protective immune responses compared to vaccines based on non-structural proteins of HCV[56]. Immunization with recombinant HCV E1 and E2 glycoproteins has been shown to prevent development of chronic infection in chimpanzees[40]. Distinct epitopes in certain regions of the E1 and E2 protein have been shown to drive the production of neutralizing antibodies[11,18,57,58].

We have shown recently that *in vitro* activation of DC followed by immunization with these DC leads to the induction of strong and specific antibody and T-cell responses in the hepatitis B context[59]. For HCV it has been shown that activation of DC by the core or the NS3 protein leads to maturation and stimulation of T-cells[60,61]. In addition, it was shown that DC function was restored in chronic HCV infected patients by the use of IL-10 inhibitors[62]. Thus, re-activation of DC may be an important tool in fighting HCV infection. We activated DC derived from BALB/c mice with HCVpp and were able to induce HCV specific antibodies and T-cells after immunization of mice with these DC. HCVpp were chosen to activate the DC for several reasons. They contain the E1 and E2 proteins and present them as closely to mature virions as possible. Due to that, neutralizing epitopes of the E1 and E2 proteins are potentially presented in the natural three-dimensional fashion. We intended to improve the immune responses using this approach. Immunization with recombinant E1 and E2 proteins as well as synthetic peptides led to limited humoral and cell mediated responses due to the limited number of viral epitopes and the inclusion of incorrectly folded recombinant proteins.

DC were chosen since we and others showed that they can be used to strongly induce immune responses which exceed the responses achieved by immunization with proteins or peptides only[59]. There are many challenges to face using DC as a therapeutic vaccine. The DC must be in the correct maturation state to be sufficiently activated, which may be different regarding the focused target[63]. Early used DC were immunogenic, but suboptimal with regard to their lymph-node homing ability and T-cell stimulatory potential[64]. Besides the maturation state the inflammatory cytokine milieu and the area of origin (plasmacytoid or myeloid) seems to play an important role. Furthermore, a challenge is the site of injection. In some studies intradermally or subcutaneous injected DC only migrated at low levels to the lymph nodes[65]. Reaching the lymph-node DC must show full ability to produce bioactive cytokines to properly activate T- and B-cells[64]. Many DC-based vaccines do not work due to these hurdles and the challenge is to find the right approach for the specific target.

In the present study, we were able to demonstrate that mouse DC can efficiently be activated *in vitro* using HCVpp. Reinjection of these DC into BALB/c mice led to humoral and cellular immune responses, demonstrating that in the HCV context *in vitro* activation of DC induces immune responses. These data lead to the hypothesis that impaired DC function/activation of HCV patients could be restored *in vitro*. This hypothesis is supported by the fact that immunization of the mice with

HCVpp only resulted in less antibody and significantly less IFN-γ production compared to immunization with HCVpp pulsed DC. Interestingly, *in vitro* co-stimulation with LPS led to enhanced T-cell responses but not to enhanced antibody production. The reason for this difference remains elusive. It may be due to a stronger cross-talk between DC and T-cells compared to B-cells. Furthermore, we could not detect significant specific binding of antibodies to recombinant HCV peptides in our experiments. We believe this is due to the lack of a three dimensional read-out using overlapping peptides and not folded proteins. This is supported by the fact that the use of HCVpp as read-out antigen resulted in very strong specific antibody binding. However, HCVpp priming of DC only resulted in slightly higher HCV specific antibody production.

We found relatively few differences between the overlapping peptide pools used for read-out. This is interesting, since we expected to find strong differences between the hypervariable regions in the E proteins compared to other regions. The homogeneity observed in our model suggests that sufficient broad-range immune responses were induced and therefore DC vaccination may be more suitable to potentially match the emergence of escape variants during HCV infection. Moreover, the HCVpp could be engineered for different sub- and quasispecies and thereby even widen the developed immune response after pulsion of DC.

When analyzing T-cell responses, we found two peaks in the immune response. These peaks were seen in pools 3 and 7 of the used overlapping peptides, corresponding to amino acids (aa) 312-379 of the E1 protein and 544-611 of the E2 protein, respectively (Table 2). This is consistent with the described regions aa174-337 and aa527-560, outside the hypervariable region 1[11,17], that have been shown to act as targets of especial interest for the natural immune system fighting HCV infection[18,39].

In conclusion, the data presented in the present study demonstrates that vaccination with HCVpp pulsed DC strongly enhances immune reactions against the structural proteins of HCV in mice. Both specific antibody production and T-cell immunity were enhanced. Furthermore, our data confirms that aa312-379 and aa544-611 of the HCV polyprotein are interesting and strong immunodominant sequences within the structural proteins of HCV. We believe that use of DC as a cellular based therapy is of great interest and should be evaluated further to sufficiently fight chronic HCV infection.

ACKNOWLEDGMENTS

We want to thank Ralf Bartenschlager for providing us with the three plasmids used for the production of the HCVpp and his group for helping us to establish this model in our laboratory. We further want to thank Stefan Urban and his group for providing the basic stock of 293T and Huh-7 cells.

COMMENTS

Background

Due to various genotypes and rapidly evolving quasispecies of the hepatitis C virus (HCV) under selection pressure persistent immune responses are hard to achieve. Most promising to achieve long-term immune responses are cell-based approaches.

Research frontiers

In chronic HCV infection the function of T-cells, B-cells and dendritic cells (DCs) are impaired. Re-activation of DC is probably needed for eradication and long-term immunity against HCV. In this study the authors demonstrate in a mouse model that DC can be re-activated *in vitro* and induce specific T- and B-cell responses *in vivo*.

Innovations and breakthroughs

DC from BALB/c mice were pulsed with pseudo particles from the hepatitis C virus (HCVpp). HCVpp consist of the genotype 1b derived envelope proteins E1 and E2, so the whole "viral surface" is presented to DC to induce immunogenicity. In this study broad-range humoral and cellular immune responses were measured. Furthermore, T-cell responses confirmed two highly immunogenic regions in E1 and E2 outside the hypervariable region 1.

Applications

This study indicates DC as promising vaccination tool to treat chronic HCV infection that should be evaluated further.

Terminology

DCs are antigen-presenting cells involved as key players in the immune response. By up-taking, processing and presentation of proteins/peptides they stimulate T- and B-cells, resulting in cellular and humoral immune response. The envelope proteins are two or three of the structural proteins of the HCV. Together with a lipid layer they form the surface of the HCV particle and are most likely the first proteins of the virus to be recognized by DC. HCVpp are artificial empty particles, consisting mainly of the lipid layer and the envelope proteins, not encapsidating the non-structural proteins of HCV.

Peer review

It describes an immunotherapeutic approach with DC immunization. The study is well organized and written.

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S- Editor Yang XC **L- Editor** O'Neill M **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.794

World J Gastroenterol 2012 February 28; 18(8): 794-799
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Effects of endoscopic sphincterotomy on biliary epithelium: A case-control study

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Received: April 3, 2011 Revised: June 15, 2011
Accepted: June 22, 2011
Published online: February 28, 2012

Abstract

AIM: To study the long-term effects of endoscopic sphincterotomy on biliary epithelium.

METHODS: This is a prospective case-control study. A total of 25 patients with a median age of 71 years (range 49-89 years) and prior endoscopic sphincterotomy (ES) for benign disease formed the first group. The median time from ES was 42 mo (range 8-144 mo). Another 25 patients with a median age of 76 years (range 44-94 mo) and similar characteristics who underwent current endoscopic retrograde cholangiopancreatography (ERCP) and ES for benign disease formed the second group (control group). Brush cytology of the biliary tree with *p53* immunocytology was performed in all patients of both groups. ERCPs and recruitment were conducted at the Endoscopic Unit of Aretaieion University Hospital and Tzaneio Hospital, Athens, from October 2006 to June 2010.

RESULTS: No cases were positive or suspicious for malignancy. Epithelial atypia was higher in the first group (32% *vs* 8% in the second group, $P = 0.034$). Acute cholangitis and previous biliary operation rates were also higher in the first group (acute cholangitis, 60% *vs* 24% in the second group, $P = 0.01$; previous biliary operation, 76% *vs* 24% in the second group, $P = 0.001$). Subgroup analysis showed that previous ES was the main causal factor for atypia, which was not related to the time interval from the ES ($P = 0.407$). Two patients (8%) with atypia in the first group were *p53*-positive.

CONCLUSION: ES causes biliary epithelial atypia that represents mostly reactive/proliferative rather than premalignant changes. The role of *p53* immunoreactivity in biliary atypia needs to be further studied.

Key words: Endoscopic sphincterotomy; Brush cytology; Atypia; Cholangiocarcinoma; *p53* immunocytology

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Kalaitzis J, Vezakis A, Fragulidis G, Anagnostopoulou I, Rizos S, Papalambros E, Polydorou A. Effects of endoscopic sphincterotomy on biliary epithelium: A case-control study. *World J Gastroenterol* 2012; 18(8): 794-799 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/794.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.794

INTRODUCTION

The introduction of endoscopic retrograde cholangiopancreatography (ERCP) with endoscopic sphincterotomy (ES) in 1974 changed the treatment of choledocholithiasis[1]. Today, ES is a widely available endoscopic technique that is used not only for the removal of common bile duct stones but also in the treatment of a wide variety of biliary and pancreatic diseases[2]. An increasing number of young patients with a long life expectancy will undergo ES, and this raises concern for the long-term complications of the procedure and especially the late development of cholangiocarcinoma[3].

Carcinogenesis after surgical sphincteroplasty and biliary-enteric anastomosis has been well documented. Numerous case reports and large series have described a causal association between biliary-enteric drainage procedures for benign disease and the late development of cholangiocarcinoma[4-6]. The hypothesis is that the disruption of the sphincter of Oddi causes prolonged pancreatobiliary and duodenobiliary reflux, bacterial overgrowth and chronic inflammation of the bile ducts. Chronic inflammatory irritation induces hyperplasia, dysplasia and atypia of the epithelium that ultimately can lead to carcinogenesis[5].

ES also causes ablation of sphincter function, which raises concern about the late development of cholangiocarcinoma by the same mechanism as previously mentioned. In a study of long-term consequences of ES, 410 patients were followed for an average of 10 years after ES, and late development of cholangiocarcinoma was documented in 3 patients, which represents an elevated risk of malignancy[7]. Furthermore, the long-term effects of ES on sphincter function are not clear. In a small trial, the function of the biliary sphincter was permanently lost, and this was associated with bacterial colonisation and chronic inflammation of the biliary system[8]. In another study, pancreatobiliary reflux was present for the first year after ES and was minimised after wards[9]. Recently, two large population-based studies demonstrated an increase in the cholangiocarcinoma rate after ERCP mostly in the first five years, but there was no causal association between sphincterotomy and cholangiocarcinoma[10,11].

In the present study, we performed cytological evaluation with additional *p53* immunocytology of the biliary epithelium one to twelve years after ES for benign disease.

MATERIALS AND METHODS

Ethics

The trial was approved by the Science Board of Aretaieion University Hospital and Tzaneio General Hospital and was registered at ClinicalTrials.org (protocol number NCT01135732). All patients gave informed consent for the ERCP procedure and for participation in the trial.

Study design

This was a prospective case-control study. ERCPs and recruitment of the patients were conducted at the Endoscopic Unit of Aretaieion University Hospital and Tzaneio General Hospital, Athens, from October 2006 to June 2010. Cytological evaluation and *p53* immunocytology were performed at the Cytology Department of Tzaneio General Hospital. Analysis and interpretation of the data were completed in January 2011.

Table 1 Demographic characteristics of the two groups *n* (%)

	First group	Second group (control)	*P* value
Patients	25	25	
Sex, male/female	11/**14**	11/**14**	1[3]
Median age (yr)	71 (range 49-89)	76 (range 44-94)	0.49[4]
ERCP indication			
Stent removal	4 (16)	-	
Biliary colic	5 (20)	17 (68)	0.01[3]
Biliary pancreatitis	1 (4)	2 (8)	
Acute cholangitis	15 (60)	6 (24)	
Previous biliary operation[1]	19 (76)	6 (24)	0.001[3]
Dilated bile ducts (ultrasound)	14 (56)	18 (72)	0.23[3]
Median time from ES (mo)[2]	42 (range 8-144)	-	

[1]Previous biliary operations were laparoscopic or open cholecystectomies; [2]This is the median time since prior endoscopic sphincterotomy (ES) that cytology brushing was performed; [3]Estimated *P* values were calculated using the χ^2 test; [4]Estimated *P* value was calculated using the Mann-Whitney test. ERCP: Endoscopic retrograde cholangiopancreatography.

The patients were enrolled in two groups. A total cohort of twenty-five patients who underwent ERCP for benign disease and had a prior ES formed the first group (Table 1). Cytology brushing was performed at a median time of 42 mo from ES within a range of 8 to 144 mo. Exclusion criteria were biliary stricture, liver cirrhosis, viral hepatitis infection, sclerosing cholangitis, choledochal cysts, malignancy of the liver, bile ducts and pancreas, and any other known or suspected malignancy. To provide comparable data, a second group (control group) was formed of twenty-five patients of similar age and gender as the first group who underwent current ES for benign disease. The difference between the two groups was that the patients of the first group had a prior ES, while those of the control group had a current ES. The endoscopic procedures were performed by two specialised endoscopists (Vezakis A and Polydorou A).

Brush cytology

Brush cytology was performed in all patients of the first group to evaluate the biliary epithelium. After cannulation of the common bile duct through the previous sphincterotomy, a 3.0-mm standard endoscopic cytology brush with a catheter sheath (Hobbs Medical, United States) was passed through the endoscope into the biliary tree under fluoroscopy. The brush was then pushed out of the catheter, and cellular material was obtained by moving it back and forth across the common and left or right hepatic ducts approximately ten times. The brush was then withdrawn into the catheter, and the catheter and brush were withdrawn from the endoscope as a unit. Similarly, brush cytology was performed in all patients of

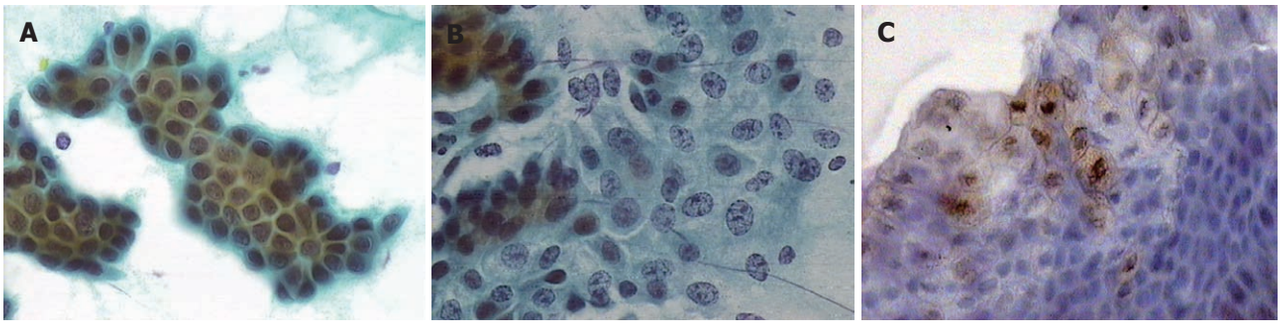

Figure 1 Brush cytology samples. A: This is a sample that was evaluated as negative for malignancy. Note the smooth nuclear shape, the low nuclear/cytoplasmic ratio and the absence of nucleoli (Papanicolaou stain, x 400); B: This is a sample that was classified as atypical-indeterminate. Note the moderate nuclear/cytoplasmic ratio, the granular chromatin pattern with mild clumping and the presence of 1 or 2 small but distinct nucleoli (Papanicolaou stain, x 400); C: This is a *p53*-positive sample from the first group. There are at least five cells with strong nuclear staining (x 400). (Analysis of photos was performed with ACDSee Pro).

Table 2 Diagnostic categories for cytologic diagnosis

Cytological grade	Definition
Unsatisfactory	No diagnostic specimen: acellular or hypocellular; Smears too thick, containing blood, obscured by inflammation or air-drying artefacts
Negative for malignancy	Adequate cellular specimens containing benign cells consistent with normal elements of the target organ
Atypical/indeterminate	Minimally abnormal cellular findings that most likely represent reactive/reparative and inflammatory type changes; Malignancy is unlikely but cannot be completely ruled out
Suspicious for malignancy	Cytologic specimens exhibit abnormal cellular findings that display some, but not all, of the features of malignancy; Specimens containing highly abnormal cells in very limited numbers
Positive for malignancy	Unquestionable cellular features of malignancy

the control group immediately after performing the ES.

The cellular material obtained from each patient was immediately smeared onto five glass slides. Four slides were fixed with a 95% ethanol solution and one was air-dried. The brush was then fixed in cytospin (a solution composed of 50% ethanol, carbowax, and isopropanol) to obtain additional material if needed. Papanicolaou stain was applied to three out of four ethanol-fixed slides, and May-Grunwald-Giemsa stain was applied to the air-dried slide.

p53 immunocytology

p53 immunostaining was performed on the fourth ethanol-fixed slide for all patients in both groups. The immunocytochemical method involved application of an anti-*p53* primary antibody (monoclonal mouse anti-human *p53*, clone DO-7, Dako Athens, Greece, 1:100 dilution).

Cytological evaluation

Samples were classified based on their morphological characteristics into one of five categories (Table 2)[12]. Positive immunocytochemical staining for *p53* mutation was defined as the detection of more than five cells with strong intranuclear staining of *p53* at high magnification (× 400) (Figure 1). Two specialised cytologists separately examined and evaluated the specimens to achieve more objective results.

Statistical analysis

Results were analysed using the χ^2 test, Mann-Whitney test and regression analysis. Differences were considered significant at $P < 0.05$. Statistical analysis was performed with Minitab 16 statistical software.

RESULTS

Cytological evaluation and subgroup analysis

All of the samples had adequate cellularity, and no case was judged too poor to be analysed. In one case, cytological evaluation by the two specialists was different, and an additional evaluation was then performed. No samples were suspect or positive for malignancy (Table 3). However, patients in the first group had a significantly higher proportion of atypia (32% *vs* 8% in the second group, $P = 0.034$). Acute cholangitis and previous biliary operation rates were also higher in the first group (acute cholangitis, 60% *vs* 24% in the second group, $P = 0.01$; previous biliary operation, 76% *vs* 24% in the second group, $P = 0.001$). On the contrary, the presence of dilated bile ducts detected by ultrasound, the extraction of common bile duct stones and the presence of dilated bile ducts without choledocholithiasis by ERCP did not differ between the two groups. A subgroup analysis of the patients with atypia demonstrated that previous ES was the only predisposing factor (Table 4).

There was no correlation between atypia and the time interval from the previous ES ($P = 0.407$). Furthermore, 7 out of 8 cases of atypia in the first group occurred in the first five years after ES (Figure 2).

p53 immunocytology

There were two samples with positive immunocytological staining for *p53* in the first group and none in the second group; both positive samples were from patients

Table 3 Comparative analysis of the two groups *n* (%)

	First group	Second group (control)	*P* value
Patients	25	25	
Cytologic evaluation			
Atypical	8 (32)	2 (8)	0.034[2]
Negative	17 (68)	23 (92)	
Stone extraction on ERCP	13 (52)	16 (64)	0.39[2]
Dilated bile ducts[1] without lithiasis on ERCP	8 (32)	7 (28)	0.75[2]
p53 immunocytology			
Positive	2	0	

[1]Diameter of the common bile duct > 10 mm; [2]Estimated *P* values were calculated using the χ^2 test. ERCP: Endoscopic retrograde cholangiopancreatography.

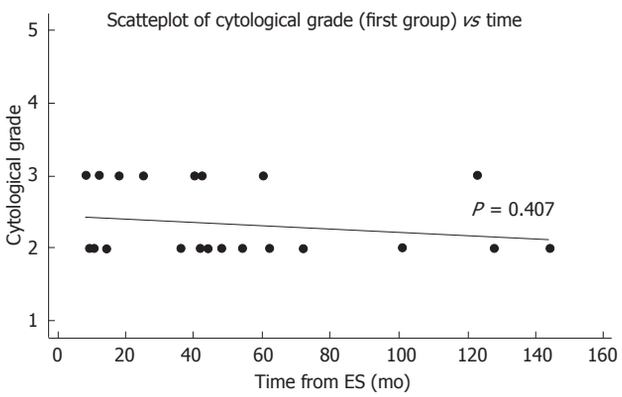

Figure 2 Scatterplot with regression of the cytological evaluation of the first group *vs* time (mo) after endoscopic sphincterotomy. The cytological grade is according to Table 2. 3: Atypical/indeterminate sample; 2: Negative for malignancy sample. ES: Endoscopic sphincterotomy. (Figure design was prepared with Minitab 16 Statistical software).

with epithelial atypia (Table 5).

DISCUSSION

The main outcome of this trial was that no case was suspect or positive for malignancy for a median period of 42 mo after a previous ES. This finding is in agreement with several retrospective studies that examined the long-term complications of ES and did not report late development of cholangiocarcinoma[13-15].

However, there was a significant difference in the reported atypia of the biliary epithelium between the two groups in the present study. Subgroup analysis showed that only previous ES, and not acute cholangitis as one might expect, was directly connected to these changes. This result is not as controversial as it seems. Acute cholangitis as an indication for ERCP is a factor that represents a clinical condition at a specific moment that cannot express chronic subclinical inflammatory irritation of the epithelium. Furthermore, possible previous episodes of acute cholangitis were not included in the present study, as it is extremely difficult to have an accurate estimation. On the contrary, previous ES is a factor that includes and expresses these possible conditions, and the fact that it is

Table 4 Subgroup analysis of the patients with atypia *vs* all others *n* (%)

	Atypical/ indeterminate	Negative for malignancy	*P* value
Patients	10	40	
Median age (yr)	69.5 (SD = 11.3)	74.5 (SD = 12.1)	0.26[2]
Sex, male/female	3/7	20/20	0.25[1]
Previous ES	8 (80)	17 (42)	0.034[1]
Acute cholangitis	5 (50)	16 (40)	0.56[1]
Previous biliary operation	6 (60)	19 (47)	0.48[1]
Stone extraction	5 (50)	22 (55)	0.77[1]
Dilated bile ducts without lithiasis on ERCP	4 (40)	18 (45)	0.78[1]
p53 immunocytology			
Positive	2	0	
Negative	8	40	

[1]Estimated *P* values were calculated using the χ^2 test; [2]Estimated *P* value was calculated using the Mann-Whitney test. ES: Endoscopic sphincterotomy; ERCP: Endoscopic retrograde cholangiopancreatography.

associated with epithelial atypia demonstrates that these epithelial changes are possibly the result of a chronic process.

The reported difference in previous biliary operations between the two groups can be easily explained by the fact that a cholecystectomy was performed after ES for gallbladder lithiasis in most of the patients in the first group.

Cellular atypia may also represent premalignant changes of the biliary epithelium[16]. It is known that carcinogenesis of the bile ducts is a very slow process that involves several cellular pathways such as growth autonomy, escape from senescence, unlimited replication, blockade of growth inhibitory signals, altered microenvironment and evasion of cell death[17]. One of the most commonly affected genes is the *p53* tumour suppressor gene, which is inactivated in 21.7% to 76% of all cholangiocarcinomas[18]. Alterations in *p53* have also been demonstrated in premalignant biliary lesions[19]. For these reasons and because of the availability of its use, *p53* immunocytology was performed in all patients in both groups to assess possible premalignant lesions.

Immunocytological staining with *p53* was positive in two young women who both had cellular atypia and also a clinical history that was negative for malignancy. False-positive *p53* immunocytology results in biliary strictures are not known, as it is a relatively new method with only a few series reported, but it seems that *p53* detection increases the sensitivity of brush cytology in cholangiocarcinoma detection[20,21]. Stewart *et al*[22] reported only one case that was *p53*-positive with cytological features of atypia, in whom clinical follow-up revealed no evidence of malignancy. Villanacci *et al*[21] reported two patients with mild dysplasia who were *p53*-positive and submitted to clinical follow-up. The two *p53*-positive cases in our study had a negative clinical follow-up at approximately 3.5 years. Nevertheless, these changes might represent premalignant conditions, and follow-up should continue.

Another important finding in this study is that the atypia of the biliary epithelium was not related to the

Table 5 Patients with positive p53 immunocytology

	Sex	Age (yr)	Indication ERCP	Previous biliary operation	Stone extraction	Cytological evaluation	Time from ES (mo)	Negative[1] follow-up
Patient 1	Female	49	Acute cholangitis	Yes (open cholecystectomy)	No	Atypia	18	3 yr 5 mo
Patient 2	Female	54	Stent removal	Yes (open cholecystectomy)	Yes	Atypia	25	3 yr 6 mo

[1]No clinical or laboratory findings of bile duct malignancy. ES: Endoscopic sphincterotomy; ERCP: Endoscopic retrograde cholangiopancreatography.

time interval from ES as one could expect. In fact, seven out of eight cases of atypia were noticed in the first five years after ES. It seems that the causal factor for these epithelial changes ceases after a certain period of time. These data are in accordance with the results from the study by Sugiyama *et al*[9], in which the sphincterotomy length, pancreatobiliary reflux and pneumobilia gradually decreased at one and five years after ES. Interestingly, Mortensen *et al*[10], in their population-based study found elevated rates of cholangiocarcinoma within the first five years after ES, with percentage declination afterwards.

A major strength of this trial is the presence of a control group with almost matched sex and age. Brush cytology is an easy and safe technique with minimal complications[23]. A major limitation of this method is the relatively low sensitivity in detecting cholangiocarcinoma (30% to 57% in most published series)[23]. However, in this study, the intention was to collect cells from many sites of the biliary tree and not from a focal stenosis. In addition, *p53* immunocytology is a method that seems to increase the sensitivity of brush cytology[21,22]. Finally, one could argue that the patients of the first group had recurrent biliary complications, and so the results cannot be fully extrapolated in asymptomatic patients with prior ES. The result is that many of these pathologic conditions are long-term complications of the ES. Furthermore, a human clinical trial with asymptomatic patients undergoing ERCP is not ethical.

In conclusion, no cases were positive or suspect for malignancy after a median period of 42 mo (range 8-144 mo) after ES for benign disease. Biliary epithelial atypia is likely to occur as a result of previous ES, primarily during the first five years after the procedure. Cytological findings represent reactive/proliferative rather than premalignant changes, but this must be confirmed by additional future studies. In addition, the role of *p53*-positive immunocytology in epithelial atypia needs to be further studied.

COMMENTS

Background

Carcinogenesis after surgical sphincteroplasty and biliary-enteric anastomosis has been well documented. Endoscopic sphincterotomy (ES) also causes ablation of sphincter function, and this raises concerns about the late development of cholangiocarcinoma.

Research frontiers

Long-term effects of ES on biliary epithelium are not very clear. It seems that the biliary sphincter function is permanently lost, which results in duodenobiliary reflux. This study examines the effects of possible reflux on biliary epithelium and demonstrates that biliary atypia is most likely to occur after ES.

Innovations and breakthroughs

Several retrospective studies have examined the late complications of ES. This is the first prospective, case-control study that examines possible premalignant changes of biliary epithelium after ES using brush cytology and *p53* immunocytology.

Applications

By understanding whether and how ES causes changes in biliary epithelium, tha authors contribute to a better knowledge of this commonly used procedure with emphasis on possible future adverse effects.

Terminology

Brush cytology is an easy and safe technique of cytological examination. Mutation in the *p53* oncogene is one of the most common mutations in the malignancy of biliary epithelium, thus *p53* immunocytology indicates possible mutations in cytological samples that could represent premalignant changes.

Peer review

The authors examined the long-term effects of ES on biliary epithelium. It is an interesting paper on a well-known subject. The results showed that reactive or proliferative change in biliary epithelium primarily occurred by ES.

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S- Editor Yang XC **L- Editor** Logan S **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.800

World J Gastroenterol 2012 February 28; 18(8): 800-805
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Vitamin D improves viral response in hepatitis C genotype 2-3 naïve patients

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Received: February 6, 2011 Revised: April 19, 2011
Accepted: April 26, 2011
Published online: February 28, 2012

Abstract

AIM: To examine whether vitamin D improved viral response and predicted treatment outcome in patients with hepatitis C virus (HCV) genotype 2-3.

METHODS: Fifty patients with chronic HCV genotype 2-3 were randomized consecutively into two groups: Treatment group [20 subjects, age 48 ± 14 years, body mass index (BMI) 30 ± 6, 65% male], who received 180 μg pegylated α-interferon-2a plus oral ribavirin 800 mg/d (Peg/RBV), together with oral vitamin D3 (Vitamidyne D drops; 2000 IU/d, 10 drops/d, normal serum level > 32 ng/mL) for 24 wk; and control group (30 subjects, age 45 ± 10 years, BMI 26 ± 3, 60% male), who received identical therapy without vitamin D. HCV RNA was assessed by reverse transcription polymerase chain reaction. Undetectable HCV RNA at 4, 12 and 24 wk after treatment was considered as rapid virological response, complete early virological response, and sustained virological response (SVR), respectively. Biomarkers of inflammation were measured.

RESULTS: The treatment group with vitamin D had higher BMI (30 ± 6 *vs* 26 ± 3, $P < 0.02$), and high viral load (> 400 000 IU/mL, 65% *vs* 40%, $P < 0.01$) than controls. Ninety-five percent of treated patients were HCV RNA negative at week 4 and 12. At 24 wk after treatment (SVR), 19/20 (95%) treated patients and 23/30 (77%) controls were HCV RNA negative ($P < 0.001$). Baseline serum vitamin D levels were lower at baseline (20 ± 8 ng/mL) and increased after 12 wk vitamin D treatment, to a mean level of (34 ± 11 ng/mL). Logistic regression analysis identified vitamin D supplement [odds ratio (OR) 3.0, 95% CI 2.0-4.9, $P < 0.001$], serum vitamin D levels (< 15 or > 15 ng/mL, OR 2.2, $P < 0.01$), and BMI (< 30 or > 30, OR 2.6, $P < 0.01$) as independent predictors of viral response. Adverse events were mild and typical of Peg/RBV.

CONCLUSION: Low vitamin D levels predicts negative treatment outcome, and adding vitamin D to conventional Peg/RBV therapy for patients with HCV genotype 2-3 significantly improves viral response.

Key words: Hepatitis C; Genotype 2-3; Vitamin D; Sustained viral response; Peg-interferon alpha 2a

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Nimer A, Mouch A. Vitamin D improves viral response in hepatitis C genotype 2-3 naïve patients. *World J Gastroenterol* 2012; 18(8): 800-805 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/800.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.800

INTRODUCTION

The current treatment for chronic hepatitis C virus (HCV) infection is pegylated interferon α combined with ribavi-

rin (Peg/RBV), administered for 24 wk for HCV genotype 2 or 3 or 48 wk for HCV genotype 1[1]. The aim of HCV therapy is a sustained viral response (SVR), which is defined as undetectable serum HCV RNA level at 24 wk after cessation of therapy. For patients with HCV genotype 1, the rate of SVR ranges between 38% and 46%[2,3]. For patients with HCV genotype 2 or 3, the rate of SVR ranges between 74% and 77%. In subgroups of this population (e.g., Hispanics and African Americans), the rate of SVR is even lower, reaching only 19% in genotype 1 and 57% in genotype 2 or 3[4]. These differences in the rate of viral response are not explained by baseline viral load or adherence to treatment. Recent efforts to improve patient outcomes have focused on adding new antiviral therapies that specifically target HCV, including polymerase or protease inhibitors[5]. However, few studies have addressed the issue of improving host factors with immunomodulators.

Vitamin D is a potent immunomodulator that favors innate immunity and cell differentiation[6,7]. Increased production of 1,25-dihydroxy vitamin D3 results in the synthesis of cathelicidin, a peptide capable of destroying many viral infectious agents as well as *Mycobacterium tuberculosis*. Low serum levels of 25-hydroxyvitamin D (< 20 ng/mL) prevent macrophages from initiating this innate immune response, which may explain why African Americans, who are often vitamin D deficient, are more prone to contracting viral infections and tuberculosis than Caucasians are[8]. Moreover, vitamin D improves insulin sensitivity[9], suppresses proinflammatory cytokines, increases anti-inflammatory cytokines, and improves CD4 T cell hyper-responsiveness[10]. Vitamin D deficiency is very common (92%) among patients with chronic liver disease, and at least one-third of them suffer from severe vitamin D deficiency (< 12 ng/mL)[11]. Israeli subjects from various ethnic backgrounds are at higher risk of vitamin D deficiency[12]. Petta *et al*[13] have recently shown a low serum vitamin D level to be related to severe fibrosis and low responsiveness to interferon-based therapy in genotype 1 chronic hepatitis C. More recently, we have shown that adding vitamin D to conventional Peg/RBV therapy for naïve, genotype 1 patients with chronic HCV infection significantly improves SVR[14]. Southern *et al*[15] have retrospectively shown that vitamin D supplementation improves the SVR in patients with HCV genotype 2-3, with mild-to-moderate fibrosis.

The aim of the present study was to assess prospectively the influence of vitamin D supplementation on outcome (SVR) in the treatment of patients with chronic hepatitis C with HCV genotype 2-3.

MATERIALS AND METHODS

Subjects

Study inclusion criteria were age 18-65 years, a chronic genotype 2 or 3 HCV infection, no previous treatment for hepatitis C, seronegative for hepatitis B virus, hepatitis A virus, and HIV infection, an absolute neutrophil count of > 1500/mm^3, a platelet count of > 90 000/mm^3, and a normal hemoglobin level. Liver biopsies were not required prior to study entrance. Exclusion criteria were decompensated liver disease (cirrhosis with Child-Pugh score > 9), another cause of clinically significant liver disease, or the presence of hepatocellular carcinoma. The protocol was approved by the two institutional review boards of both study centers (Ziv Medical Center, and Hilel Yafe Medical Center, Israel), and all patients provided written informed consent to participate in the study.

Study design and organization

This was an intention to treat prospective randomized study. Fifty consecutive chronic hepatitis C (genotype 2-3) treatment-naïve patients were stratified according to ethnic group (i.e., Russian/Jewish/Arab) due to possible difference in vitamin D levels, and alanine aminotransferase levels were enrolled. They were consecutively and randomly assigned to one of two study groups. The treatment group comprised 20 patients [mean age 48 ± 14 years, body mass index (BMI) 30 ± 6, 65% male], who received pegylated interferon α2a (180 μg once weekly by subcutaneous injection) plus ribavirin orally at a dose of 800 mg/d, with vitamin D [Vitamidyne 2000 IU/d (Fischer, Israel), 10 oral drops, target serum level > 32 ng/mL] for 24 wk. The control group of 30 patients (mean age 45 ± 10 years, BMI 26 ± 3, 60% male) received pegylated interferon α2a (180 μg once weekly) plus ribavirin (800 mg/d) without vitamin D for 24 wk. Vitamin D3 was given by oral drops at a dose of (2000 IU/d, 10 drops) to the treatment group for 12 wk before the initiation of antiviral treatment until serum levels reached > 32 ng/mL. The adherence to vitamin D treatment was excellent during the entire course and the vast majority of patients in the treatment group achieved the target level. Vitamin D supplement was maintained during the course of therapy and during follow-up.

Efficacy assessments

Plasma HCV-RNA levels were measured using the COBAS Taq Man HCV assay, version 1.0 (Roche Molecular Systems, Israel), with a lower limit of quantification of 50 IU/mL and a lower limit of detection of 10 IU/mL. HCV-RNA levels were measured at the time of screening and during the treatment period at weeks 4, 12 and 24 for the treatment group, and at 24 wk after treatment. HCV RNA was not measured at weeks 4 and 12 in the control group because this was not included in the health package at the time of study. Subjects had a safety follow-up visit after the completion of treatment. Those who had undetectable HCV RNA levels at the time of treatment completion had follow-up visits 24 wk later, at which time, HCV-RNA levels were measured again. Assessment of efficacy was SVR, i.e., undetectable HCV RNA at 24 wk after treatment. Clearance of HCV RNA was assessed at week 4 (rapid virological response), week 12 (complete early virological response), and at week 24 at the end of treatment response (ETR) for the treat-

Table 1 Baseline demographic, clinical, and virological characteristics of all patients

Baseline demographics	Peg/RBV (n = 30)	Vitamin D + Peg/RBV (n = 20)	P value
Age (yr)	45 ± 10	48 ± 14	0.3
Males (%)	60	65	0.2
BMI (kg/m^2)	26 ± 3	30 ± 6	0.02
HCV virus genotype: 2/3	17/13	11/9	0.01
SVR rate genotype: 2/3	90%/64%	100%/89%	0.01
Vitamin D (ng/mL)	19 ± 6	20 ± 8	0.1
Week 12 baseline	-	34 ± 11	
Baseline HCV RNA > 400 000 IU/mL)	40%	65%	0.01
Baseline ALT (U/L)	50 ± 16	48 ± 10	0.1
Ethnicity (Russian/ Jewish/Arabic)	26/3/1	16/2/2	0.1
CRP	0.5 ± 1.0	0.4 ± 0.5	0.3

BMI: Body mass index; HCV: Hepatitis C virus; SVR: Sustained viral response; ALT: Alanine aminotransferase; CRP: C-reactive protein; Peg/RBV: Pegylated interferon α combined with ribavirin.

ment group. Patients with ETR who tested positive for HCV RNA during follow-up were classified as relapsers. Breakthrough was defined as an increase in HCV RNA level of 1 $\log_{10}$ unit, as compared with the lowest value[3].

Safety assessment

Biochemical assessments were performed at each visit during the treatment period and after treatment a follow-up visits. Data on adverse events were collected and physical examinations were also performed each time. The safety assessment included complete blood count, antinuclear antibody and thyroid stimulating hormone levels. Pegylated interferon α2a was reduced to 90 μg/wk in patients with neutrophil count < 750 cells/μL, and withdrawn temporarily in patients with counts < 500 cells/μL. The same dose reduction was applied if platelet levels fell under 50 000 cells/mm^3, with pegylated interferon being discontinued when the 25 000 cell/mm^3 threshold was reached. In both treatment arms, the ribavirin dose was tapered by 200 mg/d in patients with hemoglobin < 10 g/dL, and discontinued altogether in patients with hemoglobin level < 8.0 g/dL.

Clinical and laboratory measurements

25(OH) vitamin D3 levels were determined by ^{125}I radioimmunoassay (Dia-Sorin, Stillwater, MN, United States)[16]. 25-OH vitamin D is the major circulating form of vitamin D and is used as an indicator of vitamin D status. Vitamin D deficiency was defined as a 25(OH) D serum level < 12 ng/mL, vitamin D insufficiency as 25(OH) D level 12-32 ng/mL, and vitamin D sufficiency as > 32 ng/mL[12]. BMI was calculated as weight in kilograms divided by the square of height in meters [17]. Obesity was defined as BMI > 30 kg/m^2. C-reactive protein (CRP) was determined by the nephlometric method[18]. Thyroid stimulating hormone, antinuclear antibody, glucose, liver enzymes, albumin, bilirubin, prothrombin time, and creatinine were measured by standard biochemical tests.

Statistical analysis

Results were expressed as the mean ± SD. The difference between two groups was assessed by χ^2 test for categorical variables and by Mann-Whitney rank for continuous variables. The Spearman correlation was used to express correlations between variables. The primary study endpoint was evidence of the influence of vitamin D on viral response at week 24 after treatment. Logistic regression analysis was used to document independent variables that predicted SVR. The significance level was set at $P < 0.05$. The statistical analysis was carried out with the WINSTAT software program (Kalmia, San Diego, CA, United States).

RESULTS

Twenty percent of the patients in the treatment group had severe baseline vitamin D deficiency (< 12 ng/mL), 60% showed insufficiency, and 20% had sufficient vitamin D levels. In the control group, 30% of the patients had baseline vitamin D deficiency, 50% had insufficiency, and 20% had sufficient vitamin D levels. Table 1 shows the clinical and biochemical parameters of the patient populations. The treatment group with vitamin D had higher BMI (30 ± 6 *vs* 26 ± 3, $P < 0.02$), and high viral load (> 400 000 IU/mL, 65% *vs* 40%, $P < 0.01$) than patients in the control group. There were no significant differences between the groups in terms of age, HCV genotype, ethnic background, aminotransferases, or CRP levels. Figure 1 shows the rates of viral response in the treatment and control groups: 19/20 (95%) patients in the treated group were HCV-RNA negative at weeks 4 and 12. At 24 wk after treatment (SVR), 19/20 (95%) patients in the treatment group and 23/30 (77%) in the control group were HCV RNA negative ($P < 0.001$). The rate of viral breakthrough and relapse was nill. The rates of non-response were significantly lower in the treatment group compared to the control group [1/20 (5%) *vs* 7/30 (23%), $P < 0.001$]. Figure 2 shows the baseline and week 12 vitamin D levels in the treatment group before the initiation of antiviral therapy. Serum vitamin D levels were significantly lower at base line (20 ± 8 ng/mL) and increased after 12 wk of vitamin D treatment to a mean level of 34 ± 11 ng/mL. Adherence to vitamin D treatment was excellent during the entire course, and all patients in the treatment group achieved the target level. Vitamin D supplementation was maintained during the course of therapy with the same amount (2000 IU/d) as in the lead in phase.

Predictive factors for SVR in patients treated with Peg/RBV combination therapy are shown in Table 2. Logistic regression analysis identified vitamin D supplementation (OR 3.0, 95% CI 2-4.9, $P < 0.001$), serum vitamin D levels (< 15 or > 15 ng/mL; OR 2.2, $P < 0.01$) and BMI (< 30 or > 30, OR 2.6, $P < 0.01$) as independent predictors of viral response. Thus, vitamin D

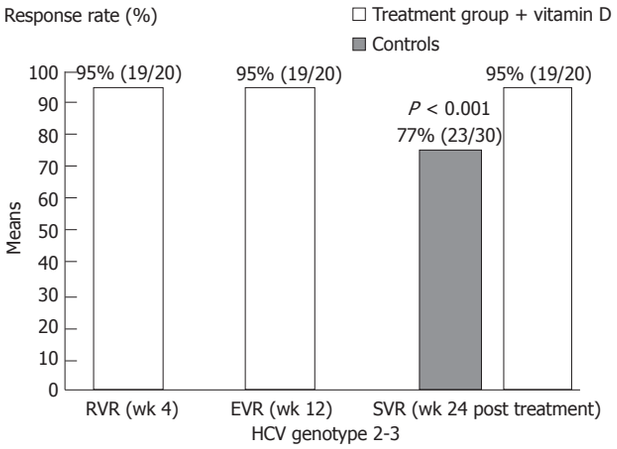

Figure 1 Rate of rapid virologic response, early virologic response and sustained viral response in the treatment (*n* = 20) and control (*n* = 30) groups. Rapid virologic response (RVR) was defined as undetectable hepatitis C virus (HCV) RNA at 4 wk during treatment. Early virologic response (EVR) was defined as undetectable HCV RNA at 12 wk during treatment. Sustained viral response (SVR) was defined as undetectable HCV RNA at 24 wk after cessation of therapy.

Table 2 Viral response, vitamin D levels and biomarkers of inflammation, insulin resistance and oxidative stress in all patients

	OR	95% CI	*P* value
Received vitamin D supplementation (yes *vs* no)	3.0	2.0-4.9	< 0.001
Base line vitamin D (< 15 or > 15 ng/mL)	2.2	1.1-4.3	0.01
Genotype (2 *vs* 3)	2.0	1.2-3.8	0.01
High viral load (< 400 000 or > 400 000 IU/mL)	2.8	1.2-4.0	0.001
Baseline CRP (< 0.5 or > 0.5 mg/dL)	1.0	0.5-1.9	0.5
BMI > 30 (kg/m^2) (yes *vs* no)	2.6	0.8-3.5	0.01

CRP: C-reactive protein; BMI: Body mass index; OR: Odds ratio.

supplementation emerged as being more responsible for higher SVR than the baseline vitamin D level. The SVR rate was significantly different between patients with HCV genotype 2/3 in the treated group versus those in the control group (100%/89% *vs* 90%/64%, $P < 0.01$).

The most common adverse events were mild, similar in both groups, and consistent with typical interferon–ribavirin induced systemic symptoms including nausea, headache, insomnia, myalgia, pyrexia, mild neutropenia, thrombocytopenia, and mild anemia. No serious adverse events were seen. Adherence to Peg/RBV therapy was excellent and there was no difference in dose reduction for Peg/RBV therapy due to adverse events in both groups. No patients discontinued treatment. Changes in laboratory values during the study were consistent with those reported in association with the combined use of Peg/RBV[3].

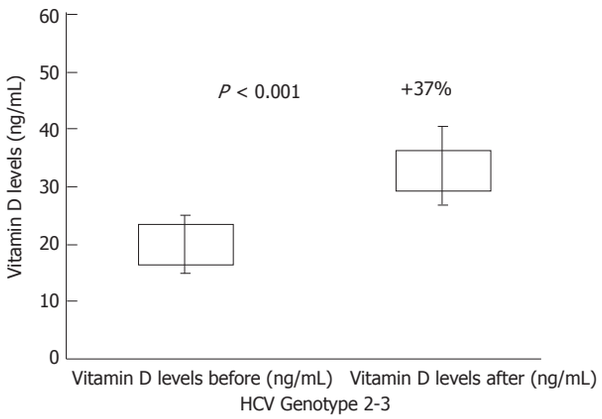

Figure 2 Vitamin D serum levels before and 12 wk after initiation of antiviral treatment (*n* = 30) and vitamin D supplementation (*n* = 20). Percentage change was +37%.

DISCUSSION

The results of this study suggest that the addition of a vitamin D supplement to current standard therapy can significantly improve the rate of SVR in treatment-naïve patients with HCV genotype 2-3, compared to the rates with standard therapy alone. The observed SVR rate in the control group (77%) is consistent with previous reports[2,3]. The overall responses reflect a marked increase in the rate of virological response at week 24 after cessation of therapy (95% *vs* 77%) and a low rate of non-response (5% *vs* 23%) with vitamin-D-based treatment, as compared to the control group[19].

There are only two reports dealing with the association between vitamin D status and outcome of antiviral therapy for chronic HCV viral infection. Petta *et al*[13] have retrospectively analyzed a cohort of 167 patients treated with peg-interferon and ribavirin for hepatitis C, and detected an association between lower vitamin D serum levels and failure to achieve SVR. Our results provide further support to these data. The second study by Bitetto *et al*[20] has shown that vitamin D supplementation improves response to antiviral treatment for recurrent hepatitis C in liver transplant recipients. Several differences between those two studies should be noted. Their HCV patients were immunocompromised and they were supplemented by low-dose vitamin D (800 IU/d) after liver transplantation. In addition, most of their HCV patients (75%) had low vitamin D levels despite treatment. Finally, that study was retrospective and focused on the prevention of osteoporosis and not on the treatment of hepatitis C. Very recently, Southern *et al*[15] have shown the beneficial effect of vitamin D supplementation on the outcome in patients with chronic HCV genotype 2-3 infection. However, this study was retrospective and the authors used Calcichew D3 Forte during the course of treatment without indicating the dose or serum levels of vitamin D.

The exact mechanism of action leading to improved

response to antiviral treatment is unknown in patients receiving vitamin D. Vitamin D is metabolized by the liver and is converted to 1,25 dihydroxyvitamin D3, which is the active form of the vitamin[6,7]. Those with chronic liver disease may have poor conversion from vitamin D3 or any of its other biologically active metabolites[11]. 1,25 vitamin D3 appears to modulate immunity principally via regulating T-cell function[21]. The vitamin D receptor (VDR) is expressed on virtually every type of cell involved in immunity[22]. The immunomodulatory actions of vitamin D are elicited through its direct action on T-cell antigen-presenting cell function[23]. T helper cell type 1 (Th1) cell actions are intensified when vitamin D is insufficient, as in the majority of our patient population, or when signals through VDR are weak. Regulatory T cells and Th2 cells are diminished, thus favoring an autoimmune Th1 response[24]. This is a proinflammatory response that may impair interferon and insulin signaling, thus decreasing viral response[25,26]. A recent study, comprised of 120 patients with chronic infection with HCV genotype 1 reported a Th1 to Th2 ratio < 15.5 (OR, 9.6) was significantly associated with SVR[27]. The overall effect is a switch from the Th1/Th17 response to the Th2/Treg profile[28]. However, Th1 and Th2 measurement was not performed in the present study. Persistent HCV infection modulates the balance between immunostimulatory and inhibitory cytokines that can prolong inflammation and lead to fibrosis and chronic liver diseases[29]. More recently, Gutierrez *et al*[30] have shown that vitamin D3 increases VDR protein expression and inhibits viral replication in cell culture.

It is well known that people of African and Hispanic descent are less likely to respond to standard therapy[31]. This may be due to a polymorphism of the *IL28B* gene and to vitamin D deficiency[13,32]. The vast majority of subjects in the present study had vitamin D insufficiency that was possibly related to low exposure to the sun and/or to a low supply of vitamin D from the diet. Recently, an important study by Lange *et al*[33] has confirmed the association of response to therapy with vitamin D levels and, even more significantly, by describing novel associations between genetic polymorphisms within VDR; especially in the α-hydroxylase promoter region (CYP27B1-1260), and vitamin D levels in HCV patients. The authors showed also that fibrosis alone is not the key to understanding the impact of chronic hepatitis C on vitamin D metabolism.

The role of insulin resistance and supplementation of vitamin D with regards to chronic HCV infection has been investigated previously by our group[14]. Insulin resistance has emerged as one of the most important host factors in the prediction of response in non-diabetic HCV-infected patients treated with Peg/RBV, and is a common denominator to the majority of features associated with difficult-to-treat patients[34]. Vitamin D is also known to help prevent type 2 diabetes and it is possible that low levels of vitamin D lead to insulin resistance[9]. The direct effect of vitamin D may be mediated by binding of its circulating active form to the pancreatic B cell VDR[35,36]. Moreover, oxidative stress leaches calcium, and vitamin D helps absorb calcium[37]. In the current study, increasing levels of vitamin D to > 32 ng/mL increased the response to antiviral therapy. The calcium levels were normal in our patient population.

Multivariate analysis revealed that vitamin D supplementation, baseline vitamin D levels, viral load, and hepatitis C genotype remained as independent predictors. Thus, it can be concluded that vitamin D supplementation is responsible for a higher SVR, rather than the baseline vitamin D level. Limitations of the present study include the small number of patients, and the lack of Th1 and Th2 immune response. The identification of determinants of response such as polymorphisms of the *IL28B* gene and within the VDR may explain the difference in response rates between patients with different ethnic backgrounds. This was not done in our study because data on *IL-28B* and VDR polymorphism were not available. Because of the small number of patients in the present study, a multicenter study with a larger number of patients is warranted. Another limitation was the lack of results that documented the viral response at weeks 4 and 12 in the control group. This test was not included in the health package at the time of study recruitment. Finally, we did not have a dose-response relationship for vitamin D supplementation. The dose of 2000 IU/d was based on previous investigations[38].

In conclusion, the addition of vitamin D to pegylated interferon α2b and ribavirin in naïve patients infected with HCV genotype 2-3 significantly increased the rate of viral response. We suggest routine testing of vitamin D levels prior to combination therapy and replacement during treatment for chronic hepatitis C.

COMMENTS

Background

Treatment of patients with chronic hepatitis C virus (HCV) infection with pegylated interferon + ribavirin (Peg/RBV) achieves virus clearance in < 50% of cases. Thus, there is a need to find new medication.

Research frontiers

Recent efforts have focused on adding new antiviral therapies including polymerase or protease inhibitors to standard therapy. However, these drugs have many side effects like rash and are very expensive. Few studies have addressed the issue of improving the immune system of the patient with immunomodulators, such as vitamin D supplementation.

Innovations and breakthroughs

Adding vitamin D supplements (2000 IU/d) to standard Peg/RBV therapy for patients with HCV genotype 2-3 significantly improves viral response (viral clearance) from 42% to 86% for genotype 1 and from 77% to 95% for genotype 2/3. The addition of vitamin D is cheap and without side effects.

Applications

The authors suggest routine testing of vitamin D levels prior to Peg/RBV therapy and adding vitamin D supplementation for chronic hepatitis C until reaching a blood level > 32 ng/mL.

Terminology

Response rate: Undetectable virus in the blood at 6 mo after discontinuation of therapy; peg-interferon is an immune system modulator that improves immune function and clears virus; ribavirin is a drug that inhibits viral replication.

Peer review

This is an interesting study and supports the previous work from this group on HCV treatment in genotype 1 subjects.

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S- Editor Sun H **L- Editor** Kerr C **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.806

World J Gastroenterol 2012 February 28; 18(8): 806-813
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Simultaneous laparoscopic multi-organ resection combined with colorectal cancer: Comparison with non-combined surgery

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Received: February 22, 2011 Revised: June 9, 2011
Accepted: June 16, 2011
Published online: February 28, 2012

Abstract

AIM: To access the short-term outcomes of simultaneous laparoscopic surgery combined with resection for synchronous lesions in patients with colorectal cancer.

METHODS: Between March 1996 and April 2010 prospectively collected data were reviewed from 93 consecutive patients who had colorectal cancer and underwent simultaneous multiple organ resection (combined group) and 1090 patients who underwent conventional laparoscopic right hemicolectomy or laparoscopic low/anterior resection for colorectal cancer (non-combined group). In the combined group, there were nine gastric resections, three nephrectomies, nine adrenalectomies, 56 cholecystectomies, and 21 gynecologic resections. In addition, five patients underwent simultaneous laparoscopic resection for three organs. The patient demographics, intra-operative outcomes, surgical morbidity, and short-term outcomes were compared between the two groups (the combined and non-combined groups).

RESULTS: There were no significant differences in the clinicopathological variables between the two groups. The operating time was significantly longer in the combined group than in the non-combined group, regardless of tumor location (laparoscopic right hemicolectomy and laparoscopic low/anterior resection groups; $P = 0.048$ and $P < 0.001$, respectively). The other intra-operative outcomes, such as the complications and open conversion rate, were similar in both groups. The rate of post-operative morbidity in the combined group was similar to the non-combined group (combined *vs* non-combined, 15.1% *vs* 13.5%, $P = 0.667$). Oncological safety for the colon and synchronous lesions were obtained in the combined group.

CONCLUSION: Simultaneous laparoscopic multiple organ resection combined with colorectal cancer is a safe and feasible option in selected patients.

Key words: Laparoscopic combined resection; Colorectal cancer; Laparoscopic surgery

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Kim HJ, Choi GS, Park JS, Park SY, Jun SH. Simultaneous laparoscopic multi-organ resection combined with colorectal cancer: Comparison with non-combined surgery. *World J Gastroenterol* 2012; 18(8): 806-813 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/806.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.806

INTRODUCTION

The laparoscopic approach for colorectal cancer was in-

troduced during the last decade, and the oncological safety and short-term outcomes have been demonstrated in randomized prospective trials[1-5]. As a result, the laparoscopic approach has been increasingly used to treat colorectal cancer, and this approach for other abdominal organs has also been widely accepted and performed[6-9]. The remarkable development of the laparoscopic approach is due to its unique benefits over open surgery, such as less pain, shorter hospital stay, faster recovery period, and better cosmetic results.

Simultaneous resection of synchronous abdominal lesions requiring surgical intervention is likely to benefit patients by reducing psychological and physiological stress related to re-operation. Some patients with sporadic colorectal cancer have one or more co-existing intra-abdominal diseases that require simultaneous resection with the primary colorectal cancer. This combined surgery could result in increased tissue injury and morbidity related to the longer operative time, as well as a larger or more radical incision, especially when the target organ is distant from the colorectal cancer. Fortunately, the laparoscopic approach can be applied to almost all intra-abdominal surgical procedures. Well-planned positioning of trocars, and a common mini-incision for retrieval of specimens, would maximize the advantages of laparoscopic surgery, even in cases with simultaneous multi-organ resection. However, the laparoscopic approach for greater than two co-existing abdominal disorders, especially colorectal cancer, is rarely performed and is the subject of few case reports in the literature[10-14]. Furthermore, no studies have compared laparoscopic combined resection in colorectal cancer patients with conventional laparoscopic colorectal resection. Therefore, it remains unknown whether or not a laparoscopic approach is safe and beneficial in patients with colorectal cancer undergoing simultaneous combined resection on synchronous abdominal lesions.

In the present study, we clarified the feasibility of simultaneous laparoscopic surgery combined with resection for synchronous lesions in patients with colorectal cancer. The study will elucidate the oncological outcomes in combined resection by comparing findings in patients who underwent conventional laparoscopic non-combined colorectal resection. We also described the technical details related to laparoscopic multiple organ resection combined with colorectal cancer.

MATERIALS AND METHODS

Patients

Between January 1996 and March 2010, 1657 patients underwent laparoscopic resection for colorectal cancer by one surgeon at the Kyungpook National University Hospital in Daegu, South Korea. Three hundred fifteen patients underwent laparoscopic right hemicolectomy and 937 patients underwent laparoscopic anterior or low anterior resection. Patients who had distant metastases, including metastasis of the liver, *en-bloc* regional resection due to T4 serosal invasive lesions, or patients who underwent palliative resection or emergency operations caused by perforation or obstruction of the colon and rectum, were excluded. Patients who underwent transverse colectomy or left hemicolectomy were also excluded because of the absence of simultaneous resection during these surgical procedures. Therefore, 263 patients who underwent laparoscopic right hemicolectomies were matched to 26 patients who underwent combined organ resections with right hemicolectomies. In addition, 827 patients who underwent single low/anterior resections were matched to 67 patients who underwent simultaneous low/anterior resections and other intra-abdominal organ procedures (Figure 1).

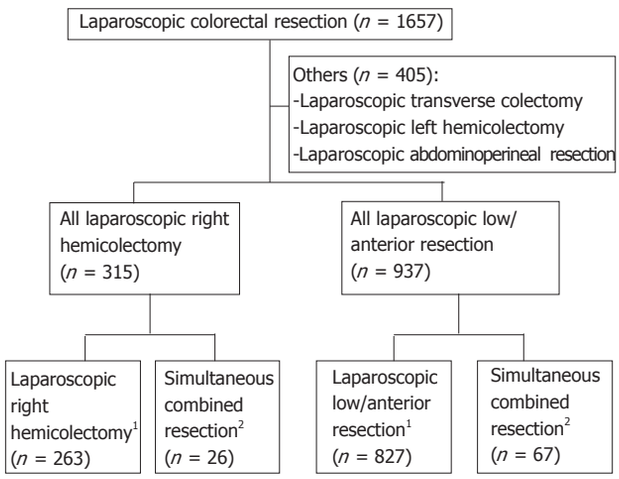

Figure 1 Algorithm of enrolled patients (number of patients). [1]Satisfied with inclusion criteria; [2]Type of combined rection was explained in Figure 2.

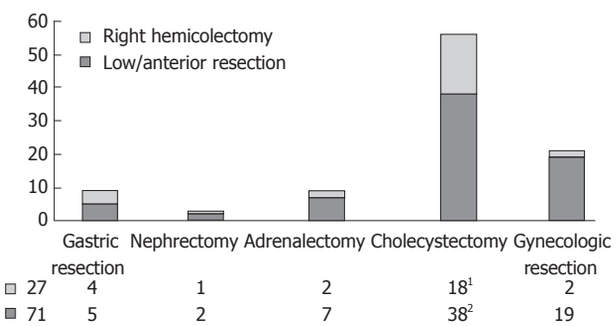

Figure 2 Type of simultaneous combined resection. [1]One case was adrenalectomy with cholecystectomy; [2]Two cases were cholecystectomies with adrenalectomies, 1 case was gastric resection and 1 case was gynecologic resection.

Figure 2 shows the type of simultaneous combined resection according to the location of synchronous abdominal lesions, distinguished between the primary colorectal resections. Five gastric resections were laparoscopically-assisted distal gastrectomies with more than D1+α lymph node dissection for gastric cancer[15], and four were laparoscopic gastric wedge resections for submucosal tumors. Five patients underwent laparoscopic simultaneous resection of three organs.

Data on patient demographics, medical co-morbid-

ities, operative details related to laparoscopic combined resections, short-term post-operative outcomes, and pathological findings were collected prospectively and entered into a database of colorectal malignancies.

The following tests and studies were performed pre-operatively: tumor markers; colonoscopy; abdominal computed tomography; and positron emission tomography. Endocrine function tests, ultrasonography, and gastroduodenoscopy were also performed when synchronous lesions were noted for surgical planning purposes.

Surgical techniques

All surgeries were performed with curative intent, irrespective of the location of the synchronous lesions. All procedures were performed by one surgeon (Choi GS), and the same surgeon performed the combined multiple abdominal resections.

Laparoscopic right hemicolectomy: The patients were placed in the supine or modified lithotomy position in Trendelenburg slightly tilted to the left side. An 11-mm infra-umbilical camera port was placed, two 5-mm trocars were introduced into the right upper and lower quadrants as operating ports, and two additional 5-mm trocars were placed in the left as assistant ports. The surgical technique for the laparoscopic right hemicolectomy was described in a previous report[16]. After D3 lymph node dissection and full mobilization of the right colon, the surgeon moved to the other abdominal lesions. No additional trocars were required. The surgeon changed his position to the right only when performing an operation on left-side lesions. During gastric resections the heads of the patients were elevated, and during nephrectomies the patients were changed to the lateral decubitus position. Ileocolic anastomosis was performed intra- or extra-corporeally, and the other abdominal organs were resected using a routine approach, as described previously[6-8,15,17,18]. Mini-laparotomies were then performed in the upper midline or midline, to extend the umbilical port through which the specimens were extracted and anastomoses were performed.

Laparoscopic low/anterior resection: Initially, the patient was placed in the modified lithotomy position in Trendelenburg with a slight tilt to the right side. An 11-mm infra-umbilical camera port was placed, a 12-mm trocar was placed in the right lower quadrant, and two additional 5-mm trocars were placed in the right upper and left lower quadrant as operating and assistant ports. The surgical technique used at our institution for the laparoscopic anterior and low anterior resections with total mesorectal excision has been previously described[16,19]. Following transection of the rectum, the surgeon approached the other abdominal lesions. In gastric resections and right nephrectomies, an additional trocar was required in the left upper quadrant. The positions of the surgeon and patients were changed in the same way for laparoscopic right hemicolectomies. Then, mini-laparotomies were performed in the upper midline or midline to extend the umbilical port, or the left lower quadrant transversely. The specimen was extracted and an anastomosis was performed.

Statistical analysis

Data analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, United States). Clinicopathological variables between the groups were compared using the Fisher's exact test for categorical data and the Student's *t* test for continuous variables. A P value < 0.05 was considered statistically significant.

RESULTS

Laparoscopic right hemicolectomy

The clinical characteristics of the patients are summarized in Table 1. All 293 patients underwent elective laparoscopic right hemicolectomy. The two groups (non-combined and combined) were similar with respect to the distribution of age, gender, body mass index and American Society of Anesthesiologists score. Fifty-three (20%) and five patients (19.2%) had a history of previous abdominal surgery in the non-combined and combined groups, respectively ($P = 0.911$).

Table 2 presents data on the surgical aspects and pathological results of primary colon cancer. The mean operating time was significantly longer in the combined group than in the non-combined group [189.6 min (range, 65-397 min) *vs* 166.9 min (range, 210-320 min), $P = 0.048$]. Intra-operative complications and conversion to open surgery were similar in both groups. In the combined group, bile leakage due to gallbladder perforation occurred during cholecystectomy in one patient. Conversion to open surgery was required in one patient to remove common bile duct stones noted during intra-operative cholangiography. In this case, we converted to open surgery followed by choledocolithotomy with T-tube insertion. However, we used a right subcostal incision for biliary surgery as well for retrieval of the specimen and performing an anastomosis. There were no significant differences between the groups in the mean post-operative hospital stay (7.8 d in the non-combined group *vs* 8.6 d in the combined group, $P = 0.363$) or in the overall and major post-operative complications ($P = 0.513$ and $P = 0.910$, respectively).

There were no significant differences between the groups in colon tumor size, proximal and distal margins (all cases had clear resection margins), number of retrieved lymph nodes and American Joint Committee on Cancer stage (6th edition).

Laparoscopic low/anterior resection

The non-combined and combined groups were similar with respect to clinical characteristics of patients who underwent laparoscopic low/anterior resections (Table 1). In laparoscopic resection for primary colorectal cancer, 350 (42.3%) and 29 patients (43.3%) underwent anterior

Table 1 Clinical characteristics of patients

	Right hemicolectomy			Low/anterior resection		
	NC group (*n* = 263)	C group (*n* = 26)	*P* value	NC group (*n* = 827)	C group (*n* = 67)	*P* value
Age at surgery (yr)[1]	62.7 (28-87)	65.1 (39-87)	0.314	62.4 (21-92)	62.3 (38-85)	0.980
Gender (%)			0.893			0.053
Male	125 (47.5)	12 (46.2)		495 (59.9)	32 (47.8)	
Female	138 (52.5)	14 (53.8)		332 (40.1)	35 (52.2)	
BMI (kg/m^2)[1]	23.9 (14-22.9)	23.1 (18.6-30.3)	0.731	23.5 (14.4-40.5)	24.1 (16.8-31.9)	0.105
ASA score (%)			0.510			0.666
Ⅰ	153 (58.2)	15 (57.7)		469 (56.7)	40 (59.7)	
Ⅱ	97 (36.9)	10 (38.5)		313 (37.8)	25 (37.3)	
Ⅲ	13 (4.9)	1 (3.8)		45 (5.4)	2 (3)	
History of abdominal surgery (%)	53 (20.2)	5 (19.2)	0.911	187 (22.6)	16 (23.9)	0.812
Type of resection						0.602
Right hemicolectomy	268 (100)	26 (100)				
Anterior resection				350 (42.3)	29 (43.3)	
Low anterior resection				397 (48)	34 (50.7)	
Colo-anal anastomosis				80 (9.7)	4 (6)	

[1]Values are expressed as the mean (range). NC: Non-combined; C: Combined; BMI: Body mass index; ASA: American Society of Anesthesiologists.

Table 2 Intraoperative and postoperative outcomes

	Right hemicolectomy			Low/anterior resection		
	NC group (*n* = 263)	C group (*n* = 26)	*P* value	NC group (*n* = 827)	C group (*n* = 67)	*P* value
Operation time (min)[1]	166.9 (65-397)	189.6 (120-320)	0.048	178 (60-430)	228.1 (80-480)	< 0.001
Intraoperative complication (%)	6 (2.3)	1 (3.8)	0.621	43 (5.2)	5 (7.5)	0.429
Conversion to open surgery (%)	2 (0.8)	1 (3.8)	0.139	4 (0.5)	1 (1.5)	0.287
Diverting stoma formation (%)	0	0		18 (2.2)	0	0.282
Postoperative hospital stay (d)[1]	7.8 (4-41)	8.6 (5-22)	0.363	7.9 (4-34)	8.8 (5-31)	0.104
Postoperative complication (%)						
Overall	38 (14.4)	5 (19.2)	0.513	109 (13.2)	9 (13.4)	0.953
Major	9 (3.4)	1 (3.8)	0.910	30 (3.6)	4 (6)	0.335
Colon pathology[1]						
Tumor size (cm)	5.3 (1-15)	5.5 (2-12)	0.803	4.0 (0.5-17)	4.2 (0.5-8)	0.468
Proximal margin (cm)	18.8 (2-72)	16.8 (5-45)	0.394	14.1 (1-54.5)	14 (5.5-30)	0.943
Distal margin (cm)	16.9 (1-60)	18 (3-37)	0.598	4.4 (1-30)	4.4 (1-14)	0.945
Retrieved LN	32.5 (2-93)	27.4 (7-62)	0.157	16.5 (1-80)	17.9 (5-65)	0.264
AJCC stage (%)			0.352			0.803
Ⅰ	63 (24)	3 (11.5)		277 (33.5)	20 (29.9)	
Ⅱ	119 (45.2)	14 (53.8)		282 (34.1)	25 (37.3)	
Ⅲ	81 (30.8)	9 (34.6)		268 (32.4)	22 (32.8)	

[1]Values are expressed as the mean (range). NC: Non-combined; C: Combined; LN: Lymph node; AJCC: American Joint Committee on Cancer stage (6th edition).

resections, 397 (48%) and 34 patients (50.7%) underwent low anterior resections, and the remaining 80 (9.7%) and four patients (6%) underwent low anterior resections with colo-anal anastomoses, respectively. No significant differences existed between the two groups ($P = 0.602$).

The mean operating time was significantly longer in the combined group than in the non-combined group [228.1 min (range, 80-480 min) *vs* 178 min (range, 60-430 min), $P \leqslant 0.001$]. The rate of intra-operative complications and conversion to open surgery was similar in both groups. In the combined group, one patient was converted to open surgery because of massive intra-abdominal adhesions associated with two previous operations. There were no cases requiring protective diverting stoma in the combined group. In contrast, 18 patients in the non-combined group required protective diverting stomas ($P = 0.282$). There were no significant differences between the groups in the mean post-operative hospital stay (7.9 d in the non-combined group *vs* 8.8 d in the combined group, $P = 0.104$) or in the overall and major post-operative complications ($P = 0.953$ and $P = 0.335$, respectively).

The two groups were similar regarding primary colorectal pathology. All cases were performed safely in terms of oncological outcomes.

Post-operative morbidity

The post-operative complications in both groups are

Table 3 Perioperative complications

	Non-combined group (n = 1090)	Combined group (n = 93)	P value
Postoperative complication (%)	147 (13.5)	14 (15.1)	0.667
Bleeding	7	0	
Anastomosis leakage	27	3	
Intra-abdominal abscess	9	1	
Ascites	8	1	
Wound problem	15	4	
Delayed bowel movement	29	2	
Bile leakage	0	1	
Urinary retention	10	0	
Respiratory problem	25	1	
Phlebitis in injection site	13	0	
Cerebral infarction	1	1	
Deep vein thrombosis	1	0	
Acute renal failure	1	0	
Urinary tract infection	1	0	

shown in Table 3. There were no significant differences between the groups (P = 0.667). In the combined group, post-operative complications developed in 14 patients (15.1%). Three patients had anastomosis leakages that were controlled by conservative management without the need to perform a temporary stoma. Two patients underwent percutaneous drainage to resolve pelvic fluid collections or intra-abdominal abscesses. One patient had bile leakage related to combined procedures on post-operative 1 d, but this resolved with a Jackson-Pratt (JP) drainage for 1 d without other surgical interventions. Two patients experienced dietary delay because of post-operative ileus controlled by an nasogastric tube. The remaining patient developed a cerebral infarction without post-operative sequelae. There were no 30-d operative mortalities.

Pathological findings of synchronous lesions

The pathological data of synchronous lesions are summarized in Table 4. Five early gastric cancers (EGCs) were detected in the stomach; three gastrointestinal stromal tumors and one schwannoma were also discovered. In the five EGC cases, the entire tumor was confined to the mucosa with 45, 31, 17, 17 and 16 lymph nodes retrieved, respectively. There were no lymph node metastases. The other synchronous lesions also had clear resection margins. Two patients who underwent nephrectomies were diagnosed with renal cell carcinomas (RCCs) and one lesion, which was diagnosed with RCC pre-operatively, was a leiomyoma. Eight lesions were adrenal adenomas, and the remaining lesion was diagnosed with adrenal metastasis. Fifty-five cholecystectomies involved cholelithiases, and one cholecystectomy was a primary gallbladder cancer confined to the mucosa with a clear resection margin, which did not require additional resection. In 21 gynecological resections, two lesions were leiomyomas of the uterus and four were serous cystadenomas; seven lesions were endometriomas, six were ovarian cysts, one was a teratoma, and one was an ovarian abscess.

Table 4 Pathological findings of synchronous lesion

Synchronous lesion pathology (n = 98)			
Stomach (n = 9)		**Gall bladder (n = 56)**	
EGC	5	Primary malignancy	1
GIST	3	Stone	55
Schwannoma	1	**Gynecologic resection (n = 21)**	
Kidney (n = 3)		Uterine leiomyoma	2
RCC	2	Serous cystadenoma	4
Leiomyoma	1	Endometriosis	7
Adrenal gland (n = 9)		Cyst	6
Adrenal metastasis	1	Teratoma	1
Adenoma	8	Abscess	1

EGC: Early gastric cancer; GIST: Gastrointestinal stromal tumor; RCC: Renal cell carcinoma.

DISCUSSION

In the present study, we report on the laparoscopic approach for synchronous multiple abdominal lesions in colorectal cancer patients. Our data showed that this approach is feasible and safe in terms of peri-operative and oncological outcomes.

Minimally invasive laparoscopic surgery has been introduced as an option for patients with colorectal cancer, and many advantages of this surgery have been reported. Attenuated surgical trauma offers faster bowel recovery compared with open surgery, thus reducing post-operative ileus related to decreased expression of inflammatory cytokines[1-3,20]. Furthermore, this approach offers good cosmetic results, reduced post-operative pain and short-term morbidity, and improved quality of life. Combined surgery for synchronous lesions also has potential advantages[10-13]. The patients may minimize the length of hospital stay and have a single, rather than multiple, hospital admission. Cardiac and pulmonary burden related to multiple anesthetic exposures were reduced by combining surgery. Thus, combined surgery reduces the cost related to the hospital stay. If combined surgery for synchronous lesions is performed with a laparoscopic approach, there are also potential advantages over open combined surgery. This approach is available in multi-quadrant abdominal operations with better visualization of the operative field and without additional skin incisions. Furthermore, this approach may reduce the number of trocar incisions by reusing the ports and improving cosmesis. Therefore, there are reduced concerns about delaying the post-operative recovery and impairing post-operative quality of life according to open combined resections.

Simultaneous laparoscopic resection for co-existing abdominal lesions has been reported in several case series, the benefits of which have been previously described[10-13,21]. Most recently, in relation to colorectal cancer patients, laparoscopic resection for synchronous gastric cancer was reported in seven patients with synchronous double primary cancers[11]. All of these reports suggest that simultaneous laparoscopic combined resection is the best choice in patients with co-existing

abdominal lesions, in terms of technical feasibility and oncological safety. However, the previous studies emphasized the need for large-scale studies related to simultaneous laparoscopic combined resection owing to the study limitations that prevented a demonstration of actual benefit[10-13]. This present study overcomes these limitations by including 93 patients who underwent laparoscopic combined resection with colorectal cancer and 1090 patients who underwent conventional laparoscopic colorectal resections at our institution. The number of patients included thus enables a comparison of peri-operative and oncological outcomes, including the presence of synchronous cancer and the multiple locations of co-existing abdominal lesions.

In relation to the technical aspects of the procedure, an additional port was inserted in cases of gastric resection and right renal cell carcinoma only in patients with left colon cancer. We shared the trocars in resection of synchronous multiple organs. It is very important to plan the position of ports and mini-incisions appropriately to share the trocars and incisions, not only for exposure and removal of synchronous lesions, but also for colorectal cancer to minimize the number of unnecessary trocars. We modified the number and location of the ports so that we could accomplish multiple surgeries with a minimum number of trocars, but with the same ease. For example, in the case of a cholecystectomy with left colon cancer, the left trocar as an assistant port was placed at a higher level than in standard cases.

In the present study, the mean operative time in the combined group was significantly longer than in the non-combined group. However, the mean operative times were only 20 and 40 min, respectively, compared to the non-combined conventional laparoscopic right hemicolectomy and laparoscopic low/anterior resection. This time is acceptable, as surgeries for more than two lesions were performed, thus avoiding the need for additional operations at a later stage.

Intra-operative complications occurred in six patients; in two of the cases, conversion to open surgery was necessary because of a common bile duct stone discovered intra-operatively and a massive adhesion due to previous abdominal surgery. Although complications developed post-operatively in 14 patients, none required surgical intervention. Anastomotic leakage, which occurred in three patients, was treated by conservative management with bowel rest, percutaneous drainage insertion, and rectal tube insertion. Anastomotic leakage was reported to occur in 0.5%-27% of patients in published series[22,23]; in the current study, anastomotic leakage occurred in 2.5% of patients in the non-combined group and 3.2% of the patients in the combined group. Furthermore, there was only one complication associated with the combined resection; specifically, bile leakage occurring on postoperative 1 d in the combined group, which was resolved through JP drainage for 1 d without other surgical interventions. Our data, therefore, show that laparoscopic combined resection does not increase peri-operative complications in selected patients.

The results of histopathological examinations of the mean number of retrieved lymph nodes and the length of the distal margins in the combined group were similar to the non-combined group, suggesting that oncological safety for colorectal cancer was obtained in both groups. Furthermore, nine patients with synchronous malignancies had clear resection margins. The median follow-up of these patients was 36.6 mo; there was one recurrence in a patient undergoing adrenalectomy for adrenal metastasis that metastasized to the brain in nine months. For primary colorectal cancer, the recurrence rate between the both groups was not significantly different in both laparoscopic right hemicolectomy (combined *vs* non-combined, 11.5% *vs* 8.7%, $P = 0.716$) and laparoscopic low/anterior resection (combined *vs*. non-combined, 10.2% *vs* 7.2%, $P = 0.289$). The five-year overall survival was also similar in both groups, regardless of tumor location (laparoscopic right hemicolectomy; combined *vs* non-combined, 93.3% *vs* 86.5%, $P = 0.665$, laparoscopic low/anterior resection groups; combined *vs* non-combined, 96.6% *vs* 96.4%, $P = 0.565$). Our study showed oncological safety for both colorectal and synchronous cancers.

The retrospective nature of our study posed some limitations. It is uncertain whether or not the longer operative time in the combined group accounts for the reason why the combined resection was difficult or why the primary tumor resection was technically difficult. The time required for colorectal and synchronous lesion resections was not shown; therefore, we did not estimate the results according to conventional resections of other abdominal lesions. Thus, a comparison of the time required for conventional resection of other organs is needed with the time required for combined laparoscopic resection of synchronous lesions.

Following the recent introduction of the Da Vinci system, robotic surgery has been attempted in various fields. Colorectal surgery has been considered one indication for robotic surgery because of advantages such as a magnified three-dimensional visual field and articulating instruments[24-27]. However, various disadvantages have also been reported, one of which is motion limitation in multi-quadrant operations. Our study included 10 cases of robotic-assisted laparoscopic combined resections. Although seven patients in our study underwent a hybrid-surgical technique that involved colectomies and a laparoscopic approach for combined resection, the remaining three patients underwent complete robotic-assisted combined resection for synchronous lesions, low anterior resection with left nephrectomy and anterior resection with aderenalectomy, and right hemicolectomy with cholecystectomy. During nephrectomy, we re-docked after the position change, but the total operating time (340 min) and peri-operative outcomes were acceptable. Therefore, we sought to determine the possibility of using the robotic system in multi-quadrant surgery.

In conclusion, we have demonstrated that simultaneous laparoscopic resection for multiple synchronous abdominal lesions in colorectal cancer patients is safe and feasible. The advantages of this approach with co-existing abdominal lesions include a reduced number of sequential operations and a reduction in hospital stay and overall morbidity. Compared to conventional non-combined colorectal resection, the combined approach has acceptable peri-operative outcomes. Based on these findings, we suggest that simultaneous laparoscopic resection is the best choice for managing co-existing abdominal lesions in colorectal cancer patients.

COMMENTS

Background

The laparoscopic approach is now routinely employed for colorectal cancer on basis of its unique benefits over open surgery and can be applied to almost all intra-abdominal surgical procedures. Simultaneous resection of synchronous abdominal lesions requiring surgical intervention is likely to benefit patients by reducing psychological and physiological stress related to re-operation. If so, when patients with colorectal cancer have co-existing abdominal lesions, is a laparoscopic combined resection safe and beneficial?

Research frontiers

The laparoscopic approach for synchronous multiple abdominal lesions in colorectal cancer patients laparoscopic combined resection does not increase peri-operative complications and secures the oncological safety for both colorectal and synchronous cancers. Therefore, in selected patients, laparoscopic combined resection is feasible and safe.

Innovations and breakthroughs

Simultaneous laparoscopic resection for co-existing abdominal lesions has been reported in several case series, the benefits of which have been previously described. However, the previous studies emphasized the need for large-scale studies related to simultaneous laparoscopic combined resection owing to the study limitations that prevented a demonstration of actual benefit. This present study overcame these limitations by including 93 patients who underwent laparoscopic combined resection with colorectal cancer and 1090 patients who underwent conventional laparoscopic colorectal resections at the institution. This enabled a comparison of peri-operative and oncological outcomes, including the presence of synchronous cancer and the multiple locations of co-existing abdominal lesions.

Applications

Simultaneous laparoscopic resection is the best choice for managing co-existing abdominal lesions in colorectal cancer patients.

Peer review

This is a comparative study on retrospective data on a topic of current interest, although studies to date have suggested more complications with combined procedures that have been done as open surgery.

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S- Editor Sun H **L- Editor** Stewart GJ **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.814

World J Gastroenterol 2012 February 28; 18(8): 814-820
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Germline mutation analysis of *MLH1* and *MSH2* in Malaysian Lynch syndrome patients

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Supported by USM Research University Grant, No. 1001/CIPPT/813005
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Received: March 15, 2011 Revised: April 26, 2011
Accepted: May 3, 2011
Published online: February 28, 2012

Abstract

AIM: To investigate the protein expression profile of mismatch repair (*MMR*) genes in suspected cases of Lynch syndrome and to characterize the associated germline mutations.

METHODS: Immunohistochemical analysis of tumor samples was performed to determine the protein expression profile of MMR protein. Germline mutation screening was carried out on peripheral blood samples. The entire exon regions of *MLH1* and *MSH2* genes were amplified by polymerase chain reaction, screened by denaturing high performance liquid chromatography (dHPLC) and analyzed by DNA sequencing to characterize the germline mutations.

RESULTS: Three out of 34 tissue samples (8.8%) and four out of 34 tissue samples (11.8%) showed loss of nuclear staining by immunohistochemistry, indicating the absence of MLH1 and MSH2 protein expression in carcinoma cells, respectively. dHPLC analysis followed by DNA sequencing showed these samples to have germline mutations of *MSH2* gene. However, no deleterious mutations were identified in any of the 19 exons or coding regions of *MLH1* gene, but we were able to identify *MLH1* promoter polymorphism, -93G > A (rs1800734), in 21 out of 34 patients (61.8%). We identified one novel mutation, transversion mutation c.2005G > C, which resulted in a missense mutation (Gly669Arg), a transversion mutation in exon 1, c.142G > T, which resulted in a nonsense mutation (Glu48Stop) and splice-site mutation, c.2006-6T > C, which was adjacent to exon 13 of *MSH2* gene.

CONCLUSION: Germline mutations were identified in four Malaysian Lynch syndrome patients. Immunohistochemical analysis of tumor tissue proved to be a good pre-screening test before proceeding to germline mutation analysis of DNA *MMR* genes.

Key words: Denaturing high performance liquid chromatography; DNA sequencing; Germline mutation; Mismatch repair genes; Immunohistochemistry; Lynch syndrome

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Zahary MN, Kaur G, Abu Hassan MR, Singh H, Naik VR, Ankathil R. Germline mutation analysis of *MLH1* and *MSH2* in Malaysian Lynch syndrome patients. *World J Gastroenterol* 2012; 18(8): 814-820 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/814.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.814

INTRODUCTION

Colorectal cancer (CRC) is the most common gastrointestinal cancer in Malaysia and Lynch syndrome accounts for approximately 1%-5% of all colorectal cancers[1-3]. Lynch syndrome is generally divided into type I which describes families presenting with colonic cancers at an early age in the absence of multiple colonic adenomas, whereas type Ⅱ describes similar families who also develop extracolonic cancers particularly those of the female reproductive tract[4]. Lynch syndrome is characterized by autosomal dominant inheritance with multiple members affected in families, inheritance of susceptibility genes (mismatch repair genes) with incomplete penetrance (80%-90%), and early onset of CRC and/or extracolonic cancers such as cancers of the endometrium, ovary, stomach, small intestine, hepatobiliary tract, upper urinary tract, brain and skin[5,6].

Germline mutations in a group of DNA mismatch repair (*MMR*) genes (*MLH1*, *MSH2*, *MSH6*, *PMS1* and *PMS2*) have been found to be associated with susceptibility to Lynch syndrome[5-7]. Germline mutations of *MLH1* and *MSH2* genes account for more than 90% of the mutations identified in Lynch syndrome families[5,8]. The loss of MMR function will lead to a phenomenon known as microsatellite instability (MSI) which is the hallmark of Lynch syndrome.

In addition to tumor MSI testing which is available to evaluate the MSI status and germline mutation analysis using peripheral blood, immunohistochemical testing of tumor tissue for the expression status of MMR protein has emerged as another option available in the genetic testing of Lynch syndrome. Data on germline mutations of *MMR* genes are available from different parts of the world in varying frequencies, however, no reports are available from Malaysia. Hence, this study was undertaken to unravel the spectrum of germline mutations in DNA *MMR* genes in Malaysian Lynch syndrome patients. We investigated the protein expression profile of *MMR* genes in suspected cases of Lynch syndrome and characterized the associated germline mutations. This report focuses on the protein expression profile and germline mutations identified in *MLH1* and *MSH2* gene.

MATERIALS AND METHODS

Patients

This study was approved by the Research and Ethics Committee, School of Medical Sciences, Universiti Sains Malaysia and National Institutes of Health for conducting research under the Ministry of Health, Malaysia. Thirty-four patients who fulfilled any of the following revised Bethesda Guidelines were recruited from three collaborating centres in Malaysia namely Hospital Universiti Sains Malaysia, Hospital Alor Star, Kedah and Hospital Queen Elizabeth, Kota Kinabalu, Sabah. (1) CRC with age less than 50 years old; (2) Presence of synchronous or metachronous colorectal or other hereditary nonpolyposis colorectal cancer (HNPCC)-associated tumors regardless of age; (3) CRC with MSI-positive morphology with age less than 60 years old; (4) CRC with one or more first-degree relatives with CRC or other HNPCC-related tumor, with one of the cancers with age less than 50 years old; and (5) CRC with two or more first- or second-degree relatives with CRC or other HNPCC-related tumor (regardless of age), including cancers (endometrial, stomach, ovarian, cervical, esophageal, leukemia, thyroid, bladder, ureter and renal pelvis, biliary tract, small bowel, breast, pancreas, liver, larynx, bronchus, lung and brain (glioblastoma), sebaceous gland adenomas and keratoacanthomas.

Personal and demographic details of the patients including history of CRC in the family were collected and recorded. CRC patients with a strong family history of CRC among first- or second-degree relatives and who met the selection criteria were subjected to detailed pedigree analysis. Detailed pedigrees of these suspected cases of Lynch syndrome were prepared. A 5 mL blood sample was collected from identified Lynch syndrome patients after obtaining informed consent.

Immunohistochemical analysis of tumor tissues

Paraffin-embedded tissue blocks from each resected bowel specimen containing carcinoma and preferably adjacent non-neoplastic colon were selected. Four μm-thick tissue sections were cut, dewaxed in xylene and rehydrated in graded alcohol concentrations to distilled water. Slides were placed in Tris-EDTA buffer at pH 9.0 in a pressure chamber for antigen retrieval for 2 min at 123 ℃. Peroxidase blocking reagent (DakoCytomation) was used to block endogenous peroxidase activity. Slides were incubated with 150-200 μL of primary monoclonal mouse antibodies: MLH1 antibody (BD Biosciences Pharmingen, United States, Cat. No. 550838) at 1/50 dilution and MSH2 antibody (BD Biosciences Pharmingen, United States, Cat. No. 556349) at 1/200 dilution, Horseradish-peroxidase (HRP) labeled polymer, conjugated to secondary antibody and thereafter DAB substrate chromogen (DAKO Envision detection kit, peroxidase/DAB, Rabbit/Mouse) were applied. Slides were counterstained with hematoxylin.

Normal expression of protein was defined as the presence of nuclear staining in colon cancer cells. Loss of staining in carcinoma with concurrent positive staining in nuclei of normal colon epithelial cells indicated absent expression of protein. Adjacent non-neoplastic colon and stromal inflammatory cells served as internal positive controls. The external positive control was normal colon.

Table 1 List of primer sequences used for polymerase chain reaction amplification of the exons of *MLH1* gene and their respective sizes of polymerase chain reaction products

MLH1 gene	Primer sequence (5′-3′)	Size (bp)	Ta (℃)
Exon 1	5′ cagagttgagaaatttgactgg 3′	339	50
	5′ taagtcgtagcccttaagtgag 3′		
Exon 2	5′ tgtatgagcctgtaagacaaag 3′	315	50
	5′ tagtttccagaacagagaaagg 3′		
Exon 3	5′ gggaattcaaagagatttgg 3′	373	50
	5′ gaggtctctgcaggtaaaatag 3′		
Exon 4	5′ ctcatcgttgccacatatta 3′	319	50
	5′ gtactcaagatctctgccaaaa 3′		
Exon 5	5′ tcccctttgggattagtatctat 3′	306	50
	5′ cccacaaaagccaatagtcat 3′		
Exon 6	5′ cactatcttaagacctcgcttt 3′	325	50
	5′ gctaaaccttgaccagaaacta 3′		
Exon 7	5′ agaggagtctgtgttttgattc 3′	367	50
	5′ atcataaccttatctccaccag 3′		
Exon 8	5′ atagtttgctggtggagataag 3′	306	50
	5′ agcctgtgtatttgactaaagc 3′		
Exon 9	5′ agttagtttatgggaaggaacc 3′	438	50
	5′ aagaaccagactccaacagtc 3′		
Exon 10	5′ atagtaagatagtgggctggaa 3′	432	62
	5′ tacctgtaagaagggacagaac 3′		
Exon 11	5′ tatttgaacacactcagactcg 3′	476	62
	5′ taggaacaacagcacaatacc 3′		
Exon 12	5′ atacagactttgctaccaggac 3′	475	50
	5′ agagagaagatgcaagtgattc 3′		
Exon 13	5′ tgtcagataagcagtctaccag 3′	468	50
	5′ ttagtaaaggaagaggagcttg 3′		
Exon 14	5′ cctcatttgtgttcgttttc 3′	411	50
	5′ gtcgaacttggatttgaaac 3′		
Exon 15	5′ catgtttcagggattacttctc 3′	356	50
	5′ ggctaccaaatgactattttgc 3′		
Exon 16	5′ gtatatgggggatgagtgttac 3′	319	50
	5′ cagaagtataagaatggctgtc 3′		
Exon 17	5′ gtgctgtttgtcaggatgaa 3′	430	50
	5′ tgaatattagtggggatgagag 3′		
Exon 18	5′ gtctgtgatctccgtttagaat 3′	358	50
	5′ ctacatcagcagatggaagtaa 3′		
Exon 19	5′ caggacaccagtgtatgttg 3′	497	50
	5′ ctgtgacatgttcaagacctc 3′		

bp: Base pair; Ta: Annealing temperature.

Table 2 List of primer sequences used for polymerase chain reaction amplification of the exons of *MSH2* gene and their respective sizes of polymerase chain reaction products

MSH2 gene	Primer sequence (5′-3′)	Size (bp)	Ta (℃)
Exon 1	5′ gtctgcttatgattggttgc 3′	632	58
	5′ actccgtgatcacaagttca 3′		
Exon 2	5′ gtggaactataagtccagtaagc 3′	457	50
	5′ cagccaaactgcaacttttt 3′		
Exon 3	5′ agaatcgattgaacccttga 3′	554	62
	5′ gtcaattaaagagcctttcc 3′		
Exon 4	5′ acatcatatcagtgtcttgcac 3′	302	50
	5′ taatccatgtacctgattctcc 3′		
Exon 5	5′ gaaggaacaccaaggaaaat 3′	273	50
	5′ ctgaaaaaggttaagggctctg 3′		
Exon 6	5′ ggtcttaggaagaggaacttttt 3′	337	50
	5′ gtataatcatgtgggtaactgc 3′		
Exon 7	5′ ttgagacttacgtgcttagttg 3′	391	50
	5′ tctgaatgtgtcctaagagtga 3′		
Exon 8	5′ aactttggagacctactgtact 3′	353	50
	5′ ccacaaaggtgctacaattag 3′		
Exon 9	5′ tacatcatcagcactgtaactg 3′	494	53
	5′ ttgacagagatgtgaagtcatc 3′		
Exon 10	5′ cagctttcaagtcagaaact 3′	511	53
	5′ aagaaagcttgactcttacctg 3′		
Exon 11	5′ tcacgtagtacacattgcttct 3′	365	61
	5′ tatgtgcaagagtaacttccag 3′		
Exon 12	5′ caaatgggggggattaaatgt 3′	377	50
	5′ aaaacgttacccccacaaag 3′		
Exon 13	5′ gcagtaactctgtccacatctt 3′	432	62
	5′ cttctcacaggacagagacata 3′		
Exon 14	5′ atgtttgtggcatatccttc 3′	499	50
	5′ cagactgtgaattaaggggtaa 3′		
Exon 15	5′ ctaatgacaaggtgagaaggat 3′	338	50
	5′ ccttcatcttagtgtcctgttt 3′		
Exon 16A	5′ ggaactagacagtgcacacata 3′	428	62
	5′ ctgggatttttcacgtagtaac 3′		
Exon 16B	5′ attattcaggagttcctgtcc 3′	500	50
	5′ ctctgagctatttgtttcttcc 3′		

bp: Base pair; Ta: Annealing temperature.

Germline mutation analysis

Genomic DNA was extracted from blood using the commercially available DNA extraction kit, QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). Specific primers were designed by using Primer3 software[9] to amplify all 19 exons of *MLH1* gene and 16 exons of *MSH2* gene. DNA was subjected to polymerase chain reaction (PCR) amplification of 19 exons of *MLH1* gene and 16 exons of *MSH2* gene using specific primers as listed in Tables 1 and 2, respectively, in a total reaction volume of 20 μL, consisting of 1X PCR reaction buffer (Applied Biosystem, Foster City, CA, United States), 1.875 μmol/L $MgCl_2$ (Applied Biosystem, Foster City, CA, United States), 0.375 μmol/L dNTPs (Applied Biosystem, Foster City, CA, United States), 0.4 μmol/L each of forward and reverse primers, about 100 ng genomic DNA and 1 unit of Ampli*Taq* Gold DNA Polymerase (Applied Biosystem, Foster City, CA, United States). PCR was performed with an initial denaturation step at 96 ℃ for 5 min, followed by 40 cycles of denaturation at 95 ℃ for 1 min, annealing at temperatures as listed in Tables 1 and 2 for 1 min and extension at 72 ℃ for 1 min, followed by a final extension step at 72 ℃ for 7 min.

Amplicons were then visualized by gel electrophoresis in a 2% agarose gel. Three μL of each amplicon was mixed with 3 μL of wild-type sample, denatured at 95 ℃ for 3 min and then gradually reannealed by decreasing the sample temperature from 95 ℃ to 65 ℃ over a period of 30 min to allow the formation of heteroduplex. Mutation analysis was performed employing denaturing high performance liquid chromatography (dHPLC) using ProStar Helix System, Varian Inc, United States and samples were analyzed under the optimum melting temperature determined by using the melting program available online, http://insertion.stanford.edu/melt.html. The wild-type sample for all the exon regions was randomly selected and confirmed by DNA sequencing.

Samples which showed heteroduplex peaks were subjected to DNA sequencing to characterize the mutation. For this, amplicons were purified using the QIAGEN QIAamp PCR purification kit (Qiagen, Hilden, Ger-

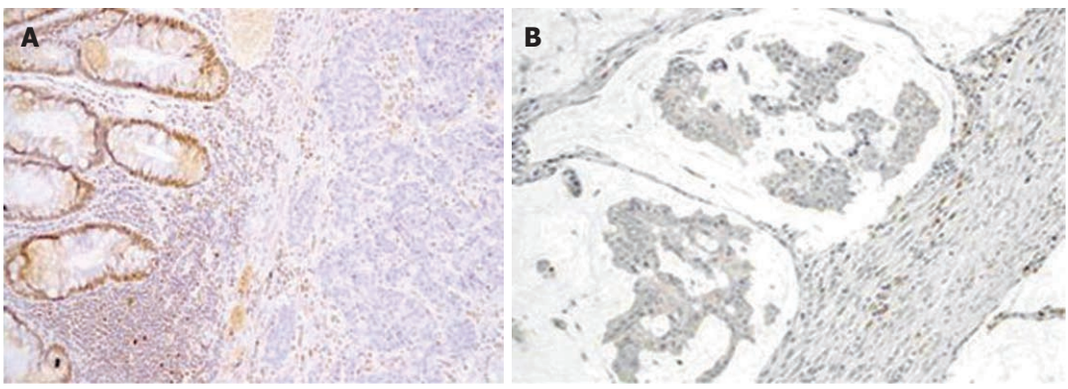

Figure 1 Immunohistochemical staining of mismatch repair proteins. A: *MLH1* gene protein, x 200 magnification. Positive nucleus staining for MLH1 protein in normal colon (left) and absent staining in colon cancer (right); B: *MSH2* gene protein, x 200 magnification. Absent nuclear staining for MSH2 protein in mucinous carcinoma (left) and positive staining in fibrous stromal cells, as internal control (right).

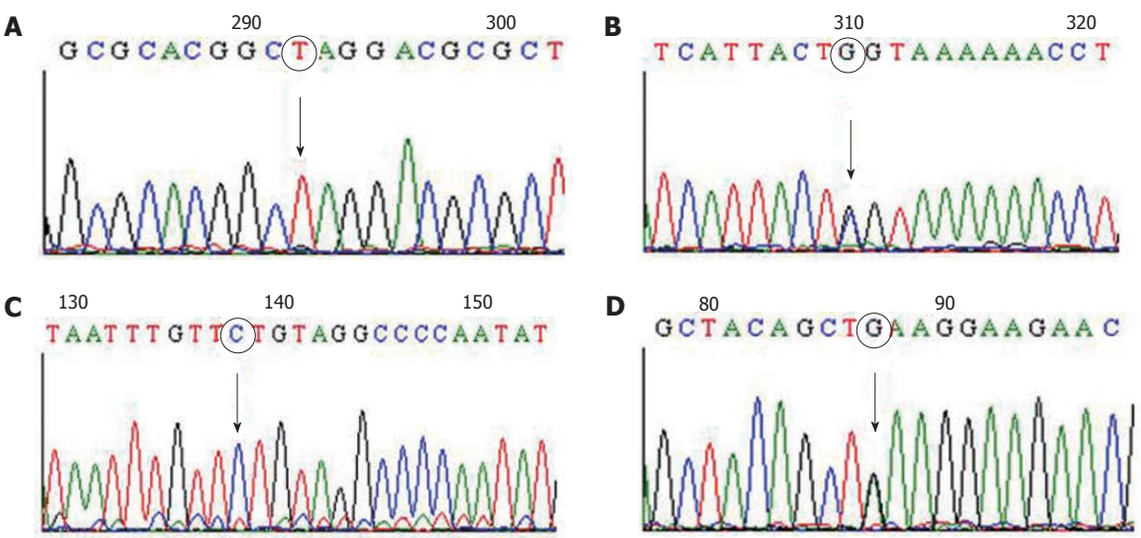

Figure 2 DNA sequencing result showing the germline mutations of *MSH2* gene identified in 4 Lynch syndrome patients. A: Deleterious mutation, c.142G > T, which resulted in a nonsense mutation (Glu48Stop); B: Novel mutation, transversion mutation c.2005G > C, which resulted in a missense mutation (Gly669Arg); C: Splice-site mutation, c.2006-6T > C, which was adjacent to exon 13 of *MSH2* gene; D: *MLH1* promoter polymorphism, -93G > A (rs1800734).

many). Purified amplicons were sequenced using BigDye Terminator v3.1 kit (Applied Biosystem, Foster City, CA, United States) on the automated ABI PRISM 3100 Genetic Analyzer (Applied Biosystem Inc, United States).

RESULTS

Immunohistochemical analysis of tumor tissues

Protein expression of MLH1 and MSH2 antigens were evaluated in tissue samples from 34 Malaysian Lynch syndrome patients. Three out of 34 tissue samples (8.8%) showed absence of nuclear staining for MLH1 and four out of 34 tissue samples (11.8%) showed loss of nuclear staining for MSH2 in cancer epithelium cells. Figure 1A and 1B show the loss of nuclear staining in MLH1 and MSH2 protein, respectively, observed in tumor samples. Patients whose samples showed absence of MLH1 and MSH2 protein expression were subjected to mutation analysis of *MLH1* and *MSH2* genes to detect and characterize germline mutations, if present.

Germline mutation analysis

Nineteen exons of *MLH1* gene and 16 exons of *MSH2* gene were successfully amplified and screened by dHPLC. Samples which showed heteroduplex peaks by dHPLC were subjected to DNA sequencing to characterize the germline mutations. All four Lynch syndrome patients who showed absence of MSH2 protein expression by immunohistochemical analysis were found to harbor germline mutations. Three different types of mutations were identified in *MSH2* gene among these four patients (Table 3). Of these, one was a substitution in exon 1 of *MSH2* gene, c.142G > T, which resulted in a nonsense mutation (Glu48Stop) and is considered a deleterious mutation (Figure 2A). The second type was a transversion mutation, c.2005G > C (Figure 2B), which resulted in a missense mutation (Gly669Arg). This has not been reported before in any of Lynch syndrome mutation databases namely MMR Genes Variant Database (www.med.mun.ca/MMRvariants), International Society for Gastrointestinal Hereditary Tumours mutation database

Table 3 Germline mutations of *MSH2* gene detected in 4/34 Lynch syndrome patients

Gene	Patients ID	Age	Exon/intron region	Nucleotide change	Consequence
MSH2	LS8	50	Exon 12	c.2005G > C	Missense mutation (G669R)
	LS20	43	Exon 12	c.2005G > C	Missense mutation (G669R)
	LS37	41	Intron 12	c.2006-6T > C	Splice-site mutation
	LS41	49	Exon 1	c.142G > T	Nonsense mutation (E48X)

(www.insight-group.org) and Human Gene Mutation Database (www.hgmd.cf.ac.uk/ac/index.php) and could be considered a novel mutation. This novel mutation was identified in two patients who showed absent expression of MSH2 protein by immunohistochemistry. The fourth patient showed a splice-site mutation, c.2006-6T > C (Figure 2C), which was adjacent to exon 13 of *MSH2* gene in one of the patients.

However, no deleterious mutations were identified in any of the 19 exons or coding regions of *MLH1* gene in the three Lynch syndrome patients who showed absence of MLH1 protein expression by immunohistochemical analysis, but *MLH1* promoter polymorphism, -93G > A (rs1800734), was detected in 21 out of 34 patients (61.8%) (Figure 2D).

DISCUSSION

Germline mutations in DNA *MMR* genes particularly of *MLH1* and *MSH2* genes were reported to be responsible for the majority of Lynch syndrome cases[5,8]. The most crucial procedure in confirming Lynch syndrome is the sequencing analysis of DNA *MMR* genes to unravel the germline mutation harbored by the suspected patients. However, the procedure is problematic and challenging especially in large-scale studies, thus the application of a pre-screening method is essential. MSI analysis as well as immunohistochemical analysis of tumor tissue has been shown to be highly predictive for selecting candidates with defective *MMR* genes[10,11]. Immunohistochemical testing of tumor tissue in particular has been proven to be a good diagnostic tool, since it can pinpoint the *MMR* genes most eligible for germline mutation analysis of DNA *MMR* genes[12].

In our study consisting of 34 Malaysian Lynch syndrome patients, immunohistochemical analysis of tumor tissue was applied as a pre-screening method. Using immunohistochemistry, we identified 4 out of 34 patients (11.8%) who showed absent expression of MSH2 protein. One of them harbored a substitution in exon 1 of *MSH2* gene, c.142G > T, which resulted in a nonsense mutation (Glu48Stop). This mutation can be considered as probably deleterious as it results in a premature stop codon and subsequently a nonfunctional and truncated protein. The mutation has been reported in Dutch hereditary nonpolyposis CRC family members who showed absent expression of MSH2 protein[13].

Interestingly, we identified a novel mutation, transversion mutation c.2005G > C, which resulted in a missense mutation (Gly669Arg) in two patients. Functional studies conducted by Acharya *et al*[14] showed that amino acid residues 658-670 is a highly conserved disordered loop region within Walker boxes in ATPase domain (domain V) which play a role in regulating mismatch binding and ATP-dependent clamp formation. Therefore, we strongly believe that changes in protein conformation and subsequent changes in biological properties of MSH2 protein are possible due to this mutation. Currently, we are unable to comment on the real impact of the novel mutation identified, and further functional studies are warranted to elucidate this issue.

In addition, we identified another splice-site mutation, c.2006-6T > C, which was adjacent to exon 13 of *MSH2* gene in one of the patients. The location of this mutation which was in the region of the polypyrimidine tract prompted us to suggest that it might affect normal MSH2 mRNA splicing. Since the polypyrimidine tract plays a major role in promoting the assembly of spliceosome as well as polypyrimidine tract-binding protein[15], the mutation might result in aberrant mRNA splicing during the process of post-transcriptional modification. The skipping of exon 13 which encompasses the most conserved region of *MSH2* gene might occur, leading to diminished MSH2 protein function. However, the impact of the splice-site mutation, c.2006-6T > C, in the pathogenesis of cancer is still controversial due to conflicting evidence. A few reports have shown that this mutation was associated with an increased risk of CRC in patients with ulcerative colitis[16], sporadic CRC[17] and non-Hodgkin lymphomas[18] as well as being described as a polymorphism[19]. It is also possible that the splice-site mutation is in linkage disequilibrium with the other unknown pathogenic mutation. Thus, further extensive studies are warranted to clarify the real impact of splice-site mutation towards the susceptibility to CRC.

Even though no deleterious mutations were identified in any of the 19 exons or coding regions of *MLH1* gene in the three Lynch syndrome patients who showed absence of MLH1 protein expression by immunohistochemical analysis, the possibility of germline epimutation of *MLH1* gene cannot be ruled out. On the other hand, the promoter polymorphism, -93G > A (rs1800734), which we identified in *MLH1* gene in 61.8% of our patients has been reported to be located in the core promoter region, 93 nucleotides upstream from the transcription start site, in the potential region of transcription factor binding sites[20]. The high frequency of this promoter polymorphism, -93G > A, indicates the predominance of this variation among Malaysians. Functional studies have already demonstrated that the *MLH1* promoter region from nucleotide position -184 to the transcription start site, in which the G > A alteration occurs, is essential for transcription of *MLH1*

gene[20]. The fact that the location of the promoter polymorphism is in the region of two potential transcription factor binding sites, GT-motif 2B and interleukin-6-regulated nuclear factor, could greatly enhance the chances of abnormal *MLH1* transcription and expression and subsequently reduce the DNA repair capability[21,22]. On the other hand, there is a possibility that this promoter polymorphism may alternatively be in linkage disequilibrium with another polymorphism in the coding region or intron or with the *MLH1* germline mutation itself which can affect *MLH1* function.

The function of the MMR system is to maintain the fidelity of the genome during replication. The DNA *MMR* genes *via* its intranuclear protein, namely MMR protein, will correct nucleotide base mispairs and small insertions or deletions generated by slippage errors of DNA polymerase during replication. Germline mutation in one of these genes will cause inactivation of the MMR system where the MMR proteins would no longer be expressed resulting in failure to repair the replication errors that occur spontaneously during DNA replication. This defect leads to the accumulation of errors in repetitive DNA sequences (microsatellites) throughout the genome of tumors resulting in microsatellite instability.

In summary, immunohistochemical analysis for MMR protein expression in suspected cases of Lynch syndrome could be considered a pre-screening method and a good strategy prior to germline mutation screening of DNA *MMR* genes. Since no germline mutations were identified in *MLH1* gene in Lynch syndrome patients who showed absence of MLH1 protein expression by immunohistochemical analysis, it is imperative to determine the methylation status of *MLH1* promoter as it may play a role in the other molecular mechanism of Lynch syndrome. Germline mutation analysis of the other *MMR* genes especially *PMS2* with the aim of characterizing the spectrum of DNA *MMR* gene mutation among Malaysian Lynch syndrome patients is also underway.

ACKNOWLEDGMENTS

We would like to thank the patients and their family members who participated in this project. We would also like to thank the staff at Clinical Research Centre, Hospital Sultanah Bahiyah, Alor Setar, Kedah, Malaysia for their contribution to this study and Mariam Azmi, Advanced Medical and Dental Institute, Universiti Sains Malaysia, for her technical expertise in immunohistochemistry.

COMMENTS

Background

Lynch syndrome, the most common form of hereditary colorectal cancer is mainly due to germline mutations in a group of DNA mismatch repair (*MMR*) genes (*MLH1*, *MSH2*, *MSH6*, *PMS1* and *PMS2*), and mutations in *MLH1* and *MSH2* genes alone account for more than 90% of the mutations identified in Lynch syndrome families. Data on germline mutations of DNA *MMR* genes are available from different parts of the world in varying frequencies but no reports are available from Malaysia.

Research frontiers

Mutational analysis of DNA *MMR* genes is the most pivotal step in confirming Lynch syndrome cases in order to unravel the germline mutation harbored by suspected patients. Immunohistochemical analysis of MMR protein expression in suspected cases of Lynch syndrome can be considered a pre-screening method and a good strategy prior to germline mutation screening of DNA *MMR* genes.

Innovations and breakthroughs

There are a number of previous reports available on germline mutations in *MMR* genes in other populations with varying frequencies, but no reports are available from Malaysia. This is the first study to unravel the spectrum of germline mutations in DNA *MMR* genes in Malaysian Lynch syndrome patients. This study also focuses on the protein expression profile of MMR protein and germline mutations identified in *MLH1* and *MSH2* genes. The authors also identified one novel mutation, transversion mutation c.2005G > C, which resulted in a missense mutation (Gly669Arg). The possibility of germline epimutation of *MLH1* gene cannot be ruled out since no deleterious mutations were identified in any of the 19 exons or coding regions of *MLH1* gene in the three Lynch syndrome patients who showed absence of MLH1 protein expression by immunohistochemical analysis.

Applications

The data generated from this study will help to establish a *MMR* gene mutation database of Malaysian Lynch syndrome kindreds.

Terminology

MLH1 and *MSH2* genes are the genes which code for MMR protein and are major elements in the MMR system. The main function of the MMR system is to maintain the fidelity of the genome during replication, where the MMR protein will correct nucleotide base mispairs and small insertions or deletions generated by slippage errors of DNA polymerase during replication.

Peer review

This is a very interesting article.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.821

World J Gastroenterol 2012 February 28; 18(8): 821-832
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Meta-analysis of combined therapy for adult hepatitis B virus-associated glomerulonephritis

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Supported by National Natural Science Foundation, No. 81072914; Major Project Foundation of National Science and Technology, No. 2010ZX9102-204; Traditional Chinese Medicine Research Grant for Military Organization, No. 10ZYZ255
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Received: June 9, 2011 Revised: September 13, 2011
Accepted: October 28, 2011
Published online: February 28, 2012

Abstract

AIM: To investigate the efficacy and safety of combined antiviral and immunosuppressant therapy in adult hepatitis B virus-associated glomerulonephritis (HBV-GN) patients.

METHODS: A computerized literature search was carried out in the PubMed database, Embase, the Cochrane Library, Chinese BioMedical Literature on disc, Chinese Medical Current Contents, Chinese National Knowledge Infrastructure, Wanfang and VIP (Chinese Technological Journal of Database) to collect articles between June 1980 and December 2010 on therapy with immunosuppressants, e.g., glucorticosteroids, mycophenolate mofetil and leflunomide, combined with antivirals, e.g., interferon, lamivudine, entecavir and adefovir dipivoxil, in adult HBV-GN patients. The primary outcomes were remission of proteinuria, clearance of HBV e-antigen, and elevation of serum albumin. The secondary outcomes were blood levels of alanine aminotransferase, serum creatinine, and HBV-DNA titer. Meta-analysis was performed using Review Manager 5.1. Fixed or random effect models were employed to combine the results after a heterogeneity test. The effects of the combined therapy were analyzed for different doses of glucorticosteroid and different types of HBV-GN.

RESULTS: Twelve clinical trials with 317 patients were included. A significantly higher incidence of HBV-GN was found in male patients (relative risk = 2.40, 95% CI: 1.98-2.93). Combined therapy reduced the proteinuria significantly with a mean difference of 4.19 (95% CI: 3.86-4.53) and increased the serum albumin concentration significantly with a mean difference of -11.95 (95% CI: -12.97-10.93) without significant alterations of liver function (mean difference: 4.62, 95% CI: -2.55-11.79) and renal function (mean difference: 10.29, 95% CI: 0.14-20.45). No significant activation of HBV-DNA replication occurred (mean difference: 0.12, 95% CI: -0.37-0.62). There was no significant difference between the high dose glucorticosteroid group and the low dose glucorticosteroid group in terms of proteinuria remission (P = 0.76) and between different pathological types of HBV-GN [membranous glomerulonephritis (MN) *vs* mesangial proliferative glomerulonephritis, P = 0.68; MN *vs* membranoproliferative glomerulonephritis, P = 0.27].

CONCLUSION: Combined antiviral and immunosuppressant therapy can improve the proteinuria in HBV-GN patients without altering HBV replication or damaging liver and renal functions.

Key words: Meta-analysis; Hepatitis B virus-associated glomerulonephritis; Glucocorticoids; Immunosuppressant; Antiviral drug

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Zheng XY, Wei RB, Tang L, Li P, Zheng XD. Meta-analysis of combined therapy for adult hepatitis B virus-associated glomerulonephritis. *World J Gastroenterol* 2012; 18(8): 821-832 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/821.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.821

INTRODUCTION

Hepatitis B virus (HBV) infection is an important public health problem worldwide and especially in developing countries such as China. It is estimated that there are up to 112 million chronic HBV carriers in China, and hepatitis B virus-associated glomerulonephritis (HBV-GN) remains one of the most common secondary glomerular diseases among the Chinese population[1]. Most HBV-GN patients present with the nephrotic syndrome and some show mild to moderate proteinuria with hematuria. The most common histological type of HBV-GN is membranous nephropathy[2,3]. Although spontaneous remission of HBV-GN has been reported in pediatric patients, it is not as successful for adult HBV-GN patients as for children[3]. There is still considerable morbidity and significant mortality in the world; about 30% of adult patients may progress to renal failure, and as many as 10% of these will require maintenance dialysis[2].

HBV-GN treatment includes antiviral drugs, e.g., interferon (IFN), lamivudine (LAM), entecavir (ETV) and adefovir dipivoxil (ADV), or immunosuppressants, e.g., glucocorticosteroids, mycophenolate mofetil (MMF) and leflunomide (LEF). Although antivirals have proven to be effective in clearing the viral antigens and abrogating the proteinuria[2,4], the safety and efficacy of immunosuppressants are still controversial. It has been argued that immunosuppressants are not appropriate for HBV-GN because of concerns such as induction of HBV replication, liver damage, and even deterioration of renal lesions[5-7].

In a recent meta-analysis study, Zhang *et al*[8] showed that treatment of HBV-GN with corticosteroids only (monotherapy) was not effective with regard to proteinuria remission. However, this conclusion was drawn by analyses of data from both adult and pediatric patients, and because spontaneous remission can occur in children, this could have confounded the effectiveness of corticosteroid treatment in adult patients. In addition, the number of adult patients treated with glucorticosteroids in that study was only thirteen. The efficacy of combined therapy was not evaluated[8].

On the other hand, treatment of HBV-GN without immunosuppressants has been reported to lead to progressive kidney disease[9]. In fact, along with antivirals, glucorticosteroids are being widely and empirically used for patients with HBV-GN, especially those in the Asia-Pacific regions[10]. However, to date, there has not been a meta-analysis on the efficacy and safety of this combined therapy in adults.

Thus the purpose of this study was to analyze the published data on the efficacy and safety of combined therapy with antivirals and immunosuppressants for adult patients with HBV-GN. To accomplish this, we performed a systematic review and meta-analysis of all published clinical trials that met our entry criteria.

MATERIALS AND METHODS

Search strategy and data extraction

We searched MEDLINE, EMBASE, Cochrane Library, and the Chinese BioMedical Literature on disc (CBM), Chinese Medical Current Contents (CMCC), Chinese National Knowledge Infrastructure (CNKI), Wanfang and VIP (Chinese Technological Journal of Database). The key words were; "hepatitis B virus", "glomerulonephritis", "prednisolone", "nephrotic syndrome", "mycophenolate mofetil", "immunosuppressants", "drug therapy", and their synonyms and related terms. All articles were identified by searching from June 1980 to December 2010. In addition, manual searches of selected specialty journals were performed to identify all pertinent literature. Qualitative reviews and published clinical trials were also searched.

Criteria for inclusion

Eligible patients had to be adults (age > 18 years) with renal biopsy-proven HBV-GN. We included randomized controlled trials (RCTs), controlled clinical trials (CCTs), prospective and retrospective self-controlled studies, and cohort studies that used immunosuppressants and antivirals to treat HBV-GN. The data had to be published in a full length paper, and all had a follow-up period of at least 6 mo. The primary and secondary outcomes were a decrease of proteinuria, levels of serum albumin and alanine aminotransferase (ALT), renal function, and HBV-DNA titer.

Criteria for exclusion

Studies were excluded if the data on the measurements of the responses were incomplete or the HBV-GN was treated with Chinese herbal drugs. Trials published as abstracts or as interim reports were excluded, but letters and review articles were not excluded. For serial reports of the same patients, only those articles that provided the most comprehensive information were included. Trials with immunosuppressants only were also excluded.

Definitions of outcome measures

The primary outcome measures were proteinuria remission and HBV reactivation. Secondary outcome measures were elevations of ALT, serum creatinine (Scr), serum albumin, and other adverse effects such as a transient increase in transaminases, dizziness, fatigue, and gastrointestinal symptoms. A complete remission (CR) was de-

fined as disappearance of proteinuria after treatment, and partial remission was defined as a 24 h urinary protein decreased more than 50% of the previous value[11]. Cases that had no improvement of symptoms and laboratory tests were classified as non-responsive.

HBV reactivation was defined as the presence of detectable serum HBV DNA, and an ALT elevation was defined as an increase to > 50 U/L[12]. A relapse was defined as the reappearance of > 1+ albuminuria on urinalysis. A low-dose glucorticosteroid (prednisone or prednisolone) was defined as ⩽ 0.5 mg/kg per day, and high-dose glucorticosteroid was defined as ⩾ 1.0 mg/kg per day.

Data extraction and quality assessment

Two reviewers (Zheng XY and Wei RB) independently selected the studies and extracted the data and outcomes according to the inclusion criteria. In cases of disagreement between the two reviewers, a third reviewer (Tang L) examined the data and discussed the choices with the two initial reviewers. The data were incorporated only when the three reviewers reached a consensus.

Statistical analysis

For dichotomous outcomes, the results are expressed as relative risk (RR) with 95% confidence intervals (CI) for individual studies. When the outcomes were measured on a continuous scale, the mean difference (MD) was used to evaluate the difference in the change from the beginning to the end of treatment. The meta-analysis was performed using the fixed-effect or random-effect methods depending on the absence or presence of significant heterogeneity. Heterogeneity was measured using Chi-square (χ^2) and I^2 tests, and statistical significance was considered to be present when $P < 0.05$. In the absence of heterogeneity, the Mantel-Haenszel method of the fixed-effect model was used for the meta-analysis. Otherwise, the DerSimonian and Laird method for the random-effect model was selected[13]. The RR with 95% CI was used to assess the treatment efficacy. The combined results were the average RR and 95% CI weighted according to the standard error of the RR of the trial. A $P < 0.05$ was considered statistically significant. Funnel plots were used to assess the publication bias, and funnel plot asymmetry was tested using the Egger's test and Begg's test[14,15]. All analyses were performed with the Review Manager version 5.1 (RevMan, Cochrane Collaboration, Oxford, England).

RESULTS

Description of trials included in meta-analysis

Our electronic and manual searches identified 774 studies. After a review of the titles and abstracts or full text, 762 articles were excluded and 12 articles[9,16-26] with 317 patients were included based on the criteria mentioned (Cheng CL 2005, Dang YM 2004, Fang YQ 2009, He LS 2007, Liu J 2010, Liu ZH 2008, Sun LL 2005, Sun XP 2010, Tang L 2005, Tang Y 2005, Wu SB 2008 and Xia DQ 2009; Figure 1 and Tables 1-3). The numbers of patients studied in each trial were 20, 24, 58, 13, 25, 30, 11, 29, 9, 21, 35 and 42, respectively. One of these studies was a RCT[23], three were CCT[9,18,24], and the others were prospective and retrospective self-controlled studies[16,17,19-22,25,26]. The characteristics of the 12 clinical trials included are shown in Table 1, and detailed information of the interventions such as dose and duration of medication, main outcomes, and follow-up periods are summarized and tabulated in Tables 2 and 3.

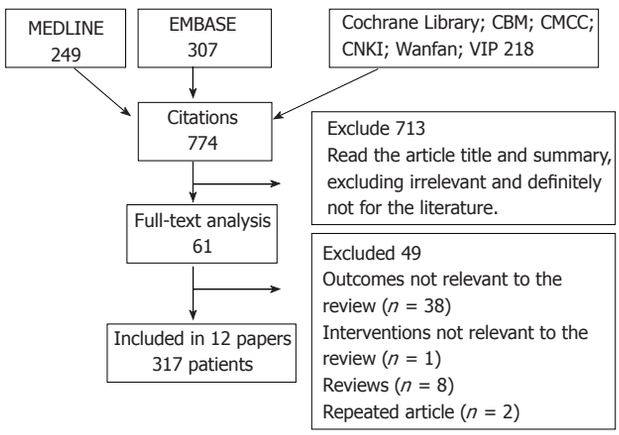

Figure 1 Flowchart showing the abstract screening and study selection process. CBM: Chinese BioMedical Literature on disc; CMCC: Chinese Medical Current Contents; CNKI: Chinese National Knowledge Infrastructure.

Clinical characteristics of gender differences

Gender differences in the incidence of HBV-GN were assessed from the results of 10 trials[16,17,19-26] including 1 RCT[23], 1 CCT[24], and 8 self-controlled studies[16,17,19-22,25,26]. The total number of patients was 286, including 202 male patients and 84 female patients. The χ^2 test of heterogeneity was not significant ($P = 0.37$), therefore the fixed-effect model was selected. A significantly larger number of male patients than female patients had HBV-GN (RR = 2.40, 95% CI: 1.98-2.93; Figure 2).

Efficacy of combined therapy for proteinuria

The efficacy of combined therapy for proteinuria of six months duration was assessed from the results of 8 trials[16,18-21,23-25] including 1 RCT[23], 2 CCT[18,24], and 5 self-controlled studies[16,19-21,25]. The total number of patients was 170, and the χ^2 test of heterogeneity was not significant ($P = 0.62$). Therefore, the fixed-effect model was used. There was a significant decrease in the level of proteinuria after the treatments (mean difference: 4.19, 95% CI: 3.86-4.53, Figure 3).

Efficacy of combined therapy on serum albumin concentration

The efficacy of combined therapy on serum albumin concentration was assessed from the results of 8 trials[16-20,22-24] of 209 patients including 1 RCT[23], 2 CCT[18,24], and 5 self-controlled studies[16,17,19,20,22]. The χ^2 test of heterogeneity was not significant ($P = 0.11$), therefore the fixed-effect

Table 1 Characteristics of 12 included studies

Ref.	Region	Patients		Study design
		Gender	Mean age (yr)	
Fang *et al*[9]	Zhejiang	NA	> 18	CCT study
Cheng *et al*[16]	Guangzhou	21 M, 3 F	29.3 ± 10.3	Self-control study
Dang *et al*[17]	Shandong	38 M, 20 F	41	Self-control study
He *et al*[18]	Beijing	NA	> 18	CCT study
Liu *et al*[19]	Shandong	17 M, 8 F	39.6 ± 11.2	Self-control study
Liu *et al*[20]	Hunan	20 M, 10 F	31.4 ± 13.8	Self-control study
Sun *et al*[21]	Shanghai	9 M, 4 F	34.4 ± 10.4	Self-control study
Sun *et al*[22]	Shandong	22 M, 7 F	> 18	Self-control study
Tang *et al*[23]	Beijing	8 M, 1 F	38.2 ± 11.2	RCT study
Tang *et al*[24]	Guangzhou	16 M, 5 F	31 ± 8.9	CCT study
Wu *et al*[25]	Shanghai	23 M, 12 F	40.5 ± 10.4	Self-control study
Xia *et al*[26]	Shandong	28 M, 14 F	35.4 ± 17.1	Self-control study

M: Male; F: Female; NA: Not available; RCT: Randomized controlled trial; CCT: Clinical controlled trial.

Table 2 Categories of interventions used in individual studies and duration of follow-up

Ref.	Intervention	Duration	Follow-up	Dropout (*n*)
Fang *et al*[9]	Prednisone 0.8-1.0 mg/kg per day + LAM/ETV/ADV	12 mo	40 mo	0
Cheng *et al*[16]	Prednisolone 0.4 mg/kg per day + MMF + LAM	6 mo	6 mo	0
Dang *et al*[17]	Prednisone 0.8-1.0 mg/kg per day + MMF + LAM	6 mo	6 mo	0
He *et al*[18]	Prednisone 40-60 mg/d + MMF	18 mo	12 mo	0
Liu *et al*[19]	prednisolone 0.5-1.0 mg/kg per day + MMF + LAM	12 mo	12 mo	0
Liu *et al*[20]	Prednisone 1.0 mg/kg per day + ETV	9 mo	9 mo	0
Sun *et al*[21]	Prednisone 0.5 mg/kgper two days + MMF + LAM	12 mo	12 mo	2
Sun *et al*[22]	Prednisone 1.0 mg/kg per day + ADV	6 mo	12 mo	0
Tang *et al*[23]	Prednisone 0.5-0.8 mg/kg per day + MMF	6 mo	12 mo	0
Tang *et al*[24]	Prednisolone 0.4 mg/kg per day + MMF + LAM	6 mo	NA	0
Wu *et al*[25]	Prednisolone 0.4 mg/kg per two days + MMF + LAM	6 mo	12 mo	0
Xia *et al*[26]	Prednisone 0.5 mg/kg per day + LEF +LAM	6 mo	12 mo	0

MMF: Mycophenolate mofetil; LAM: Lamivudine; ETV: Entecavir; ADV: Adefovir dipivoxil; LEF: Leflunomide; IFNα: Interferon α; NA: Not available.

Table 3 Various changes of interventions used in individual studies

Ref.	*n*	ALT (U/L)		Scr (μmol/L)		Albumin (g/d)		Proteinuria (g/d)		Proteinuria		HBV-DNA titer	
		Before	After	Before	After	Before	After	Before	After	CR	PR	Before	After
Fang *et al*[9]	20	NA	NA	NA	NA	NA	NA	NA	NA	13/20 (12 mo)	6/20 (12 mo)	NA	NA
Cheng *et al*[16]	24	NA	NA	NA	NA	21.0 ± 4.2	35.4 ± 6.5	6.3 ± 2.3	1.1 ± 1.2	9/24	11/24	5.2 ± 1.6 (12 mo)	5.1 ± 1.7 (12 mo)
Dang *et al*[17]	58	21.0 ± 10.3	21.6 ± 12.5	97.3 ± 38.2	92.5 ± 38.6	26.4 ± 4.9	39.0 ± 4.7	4.2 ± 2.1 (12 mo)	1.3 ± 1.5 (12 mo)	19/58	24/58	NA	NA
He *et al*[18]	13	NA	NA	NA	NA	25.8 ± 6.3	34.7 ± 7.0	7.3 ± 3.5	2.8 ± 1.4	NA	NA	NA	NA
Liu *et al*[19]	25	72.5 ± 13.7	42.1 ± 8.2	NA	NA	26.4 ± 8.1	37.1 ± 2.1	6.0 ± 3.7	1.8 ± 0.9	5/25	15/25	NA	NA
Liu *et al*[20]	30	65.9 ± 23.6	62.6 ± 15.0	135.1 ± 83.5	86.0 ± 24.5	26.9 ± 6.0	37.8 ± 2.3	5.6 ± 3.4	1.9 ± 1.1	12/30 (9 mo)	12/30 (9 mo)	NA	NA
Sun *et al*[21]	11	NA	NA	NA	NA	25.3 ± 2.4	33.7 ± 3.1 (12 mo)	6.7 ± 2.5	2.1 ± 1.7	4/11 (12 mo)	5/11 (12 mo)	NA	NA
Sun *et al*[22]	29	NA	NA	105.0 ± 43.5	93.5 ± 35.2	29.5 ± 7.8	38.3 ± 6.9	85.8 ± 39.0 (mg/kg per day)	15.6 ± 8.4 (mg/kg per day)	NA	NA	NA	NA
Tang *et al*[23]	9	27.4 ± 25.2	22.9 ± 4.3	95.3 ± 33.8	89.6 ± 34.3	26.0 ± 6.2	35.1 ± 5.6	4.9 ± 2.9	1.4 ± 0.7	4/9	4/9	NA	NA
Tang *et al*[24]	21	27.0 ± 17	NA	NA	NA	22.4 ± 4.5	36.8 ± 5.6	5.0 ± 2.1	1.0 ± 1.33	15/21	3/21	5.3 ± 1.7 (12 mo)	5.1 ± 1.7 (12 mo)
Wu *et al*[25]	35	NA	NA	135.3 ± 15.2	78.2 ± 11.5 (12 mo)	20.3 ± 4.9	41.6 ± 4.3 (12 mo)	5.2 ± 1.3	1.1 ± 0.1	20/35	10/35	4.8 ± 1.4 (12 mo)	4.7 ± 1.6 (12 mo)
Xia *et al*[26]	42	63.2 ± 22.9	43 ± 10.2 (12 mo)	126.6 ± 73.9	81.5 ± 13.9 (12 mo)	26.9 ± 6.5	37.8 ± 2 (12 mo)	3.5 ± 2.1	0.7 ± 0.4 (12 mo)	7/42 (12 mo)	29/42 (12 mo)	NA	NA

Unless specified data are shown at 6 mo examination after treatment; ALT: Alanine aminotransferase; Scr: Serum creatinine; NA: Not available; CR: Complete remission; PR: Partly remission; HBV: Hepatitis B virus.

Study or subgroup	Male Events	Male Total	Female Events	Female Total	Weight (%)	Risk ratio M-H, fixed, 95% CI
Cheng *et al*[16]	21	24	3	24	3.6	7.00 (2.40, 20.39)
Dang *et al*[17]	38	58	20	58	23.8	1.90 (1.27, 2.84)
Liu *et al*[19]	17	25	8	25	9.5	2.13 (1.13, 4.00)
Liu *et al*[20]	20	30	10	30	11.9	2.00 (1.14, 3.52)
Sun *et al*[21]	9	13	4	13	4.8	2.25 (0.92, 5.49)
Sun *et al*[22]	22	29	7	29	8.3	3.14 (1.60, 6.19)
Tang *et al*[23]	8	9	1	9	1.2	8.00 (1.24, 51.51)
Tang *et al*[24]	16	21	5	21	6.0	3.20 (1.44, 7.13)
Wu *et al*[25]	23	35	12	35	14.3	1.92 (1.14, 3.22)
Xia *et al*[26]	28	42	14	42	16.7	2.00 (1.24, 3.23)
Total (95% CI)		286		286	100.0	2.40 (1.98, 2.93)
Total events	202		84			

Heterogeneity: χ^2 = 9.74, df = 9 (P = 0.37); I^2 = 8%
Test for overall effect: Z = 8.76 (P < 0.00001)

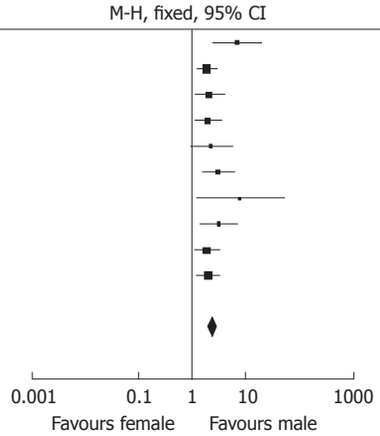

Figure 2 Gender difference in trials. M-H: Mantel-Haenszel.

Study or subgroup	Before Mean	Before SD	Before Total	After Mean	After SD	After Total	Weight (%)	Mean difference IV, fixed, 95% CI
Cheng *et al*[16]	6.3	2.3	24	1.1	1.2	24	10.2	5.20 (4.16, 6.24)
He *et al*[18]	7.3	3.5	13	2.8	1.4	13	2.6	4.50 (2.45, 6.55)
Liu *et al*[19]	6	3.7	25	1.8	0.9	25	4.9	4.20 (2.71, 5.69)
Liu *et al*[20]	5.6	3.4	30	1.9	1.1	30	6.7	3.70 (2.42, 4.98)
Sun *et al*[21]	6.7	2.5	13	2.1	1.7	13	4.1	4.60 (2.96, 6.24)
Tang *et al*[23]	4.9	2.9	9	1.4	0.7	9	2.9	3.50 (1.55, 5.45)
Tang *et al*[24]	5	2.1	21	1	1.33	21	9.7	4.00 (2.94, 5.06)
Wu *et al*[25]	5.2	1.3	35	1.1	0.1	35	58.9	4.10 (3.67, 4.53)
Total (95% CI)			170			170	100.0	4.19 (3.86, 4.53)

Heterogeneity: χ^2 = 5.30, df = 7 (P = 0.62); I^2 = 0%
Test for overall effect: Z = 24.80 (P < 0.00001)

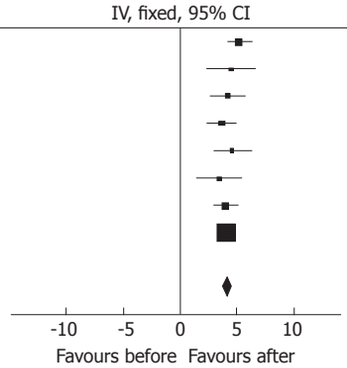

Figure 3 Proteinuria change in steroid combination therapy. IV: Inverse variance.

Study or subgroup	Before Mean	Before SD	Before Total	After Mean	After SD	After Total	Weight (%)	Mean difference IV, fixed, 95% CI
Cheng *et al*[16]	21	4.2	24	35.4	6.5	24	10.9	-14.40 (-17.50, -11.30)
Dang *et al*[17]	26.4	4.9	58	39	4.7	58	34.1	-12.60 (-14.35, -10.85)
He *et al*[18]	25.8	6.3	13	34.7	7	13	4.0	-8.90 (-14.02, -3.78)
Liu *et al*[19]	26.4	8.1	25	37.1	2.1	25	9.7	-10.70 (-13.98, -7.42)
Liu *et al*[20]	26.8	6	30	37.8	2.3	30	19.7	-11.00 (-13.30, -8.70)
Sun *et al*[22]	29.5	7.8	29	38.3	6.9	29	7.2	-8.80 (-12.59, -5.01)
Tang *et al*[23]	26	6.2	9	35.1	5.6	9	3.5	-9.10 (-14.56, -3.64)
Tang *et al*[24]	22.4	4.5	21	36.8	5.6	21	11.0	-14.40 (-17.47, -11.33)
Total (95% CI)			209			209	100.0	-11.95 (-12.97, -10.93)

Heterogeneity: χ^2 = 11.66, df = 7 (P = 0.11); I^2 = 40%
Test for overall effect: Z = 22.96 (P < 0.00001)

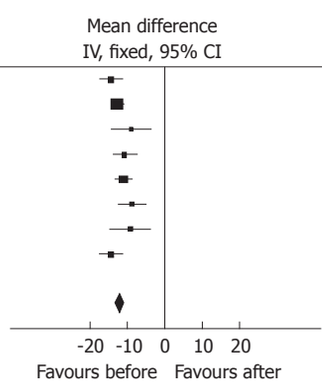

Figure 4 Serum albumin change in combination therapy group. IV: Inverse variance.

model was selected. A significant increase in the level of serum albumin was found after the treatments (mean difference: -11.95, 95% CI: -12.97-10.93, Figure 4).

Safety of combined therapy regarding liver function

Liver function was evaluated by assessing the level of ALT. The change in the ALT level after steroid combined therapy for HBV-GN was assessed from the results of 4 trials[17,19,20,23] including 1 RCT[23] and 3 self-controlled studies[17,19,20]. The total number of patients was 122. The χ^2 test of heterogeneity was significant (P = 0.03), therefore the random-effect model was selected. There was no significant difference after the treatment (P = 0.21, Figure 5); a transient elevation of ALT was noted in 14 patients during the treatment but the treatment was not stopped. Elevation of ALT was the most common side effect of combined therapy and accounted for 4.4% (14/317) of all the participants. These findings demonstrate the ne-

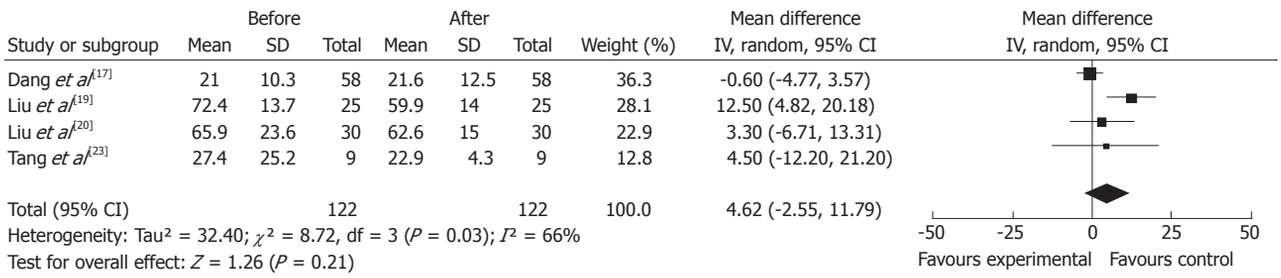

Figure 5 Alanine aminotransferase change in combination therapy group. IV: Inverse variance.

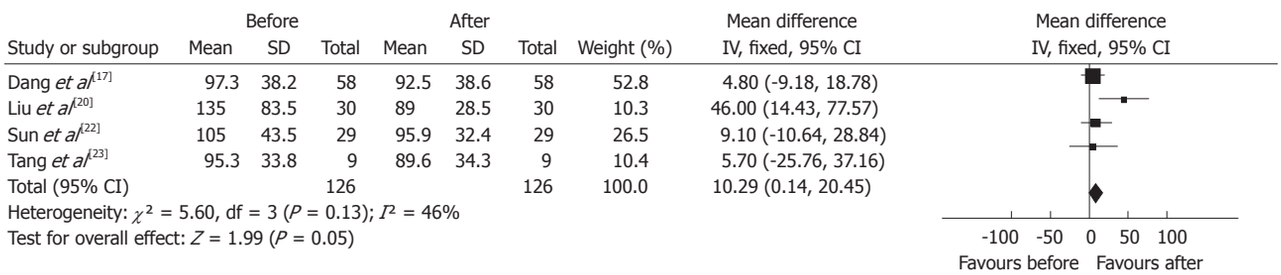

Figure 6 Scr change in combination therapy group. IV: Inverse variance.

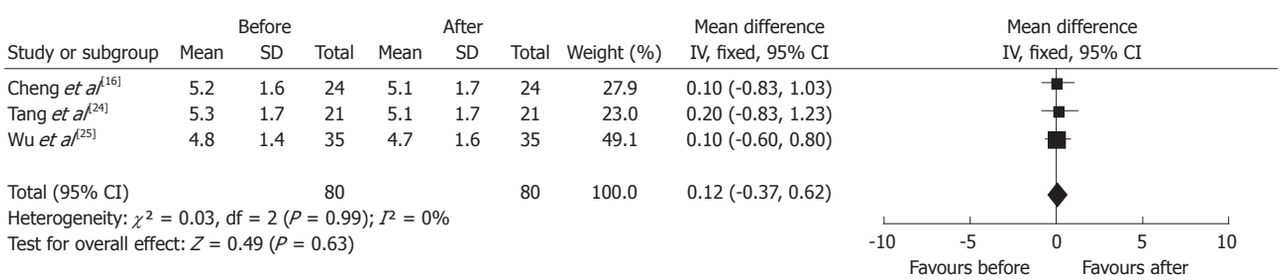

Figure 7 Hepatitis B virus-DNA titer change in combination therapy group. IV: Inverse variance.

cessity of monitoring liver function during the treatment.

Safety of combined therapy regarding renal function

Renal function was assessed by evaluating the change in the serum creatinine (Scr) level. The level of Scr after combined therapy for HBV-GN was assessed from the results of 4 trials[17,20,22,23], including 1 RCT[23] and 3 self-controlled studies[17,20,22]. The total number of patients was 126, and the χ^2 test of heterogeneity was not significant ($P = 0.13$). Therefore, the fixed-effect model was used. There was no significant increase in the level of Scr after the treatments ($P = 0.05$, Figure 6).

HBV-DNA titer

A change in the HBV-DNA titer after combined therapy for HBV-GN was assessed from the results of 3 trials[16,24,25] including 1 CCT[24] and 2 self-controlled studies[16,25]. The total number of patients was 80. The χ^2 test of heterogeneity was not significant ($P = 0.99$), and the fixed-effect model was used. There was no significant difference in the HBV-DNA titer before and after the treatments ($P = 0.63$, Figure 7). However, in one of the 12 articles included in this meta-analysis, one patient was reported to have an increase in the HBV-DNA titer after 5 mo of treatment, but the titer was stabilized following treatment with ADV[9].

Proteinuria in high-dose steroid subgroup vs low-dose steroid subgroup

The change in the proteinuria level in the high-dose steroid therapy group was assessed from the results of 3 trials[18,19,23] including 1 RCT[23], 1 CCT[18], and 1 self-controlled study[19]. The total number of patients was 47, and the χ^2 test of heterogeneity was not significant ($P = 0.77$). Therefore, the fixed-effect model was used. High-dose steroid was found to reduce the proteinuria significantly (mean difference: 4.08, 95% CI: 3.06-5.11, Figure 8). Low-dose steroid therapy was assessed from the results of 4 trials[16,21,24,25] including 1 CCT[24] and 3 self-controlled studies[16,21,25]. The total number of patients was 93, and the χ^2 test of heterogeneity was not significant ($P = 0.25$). Therefore, the fixed-effect model was used. Low-dose steroid was also found to significantly reduce proteinuria (mean difference: 4.25, 95% CI: 3.88-4.61, Figure 8).

The two subgroups were merged for analysis, and the χ^2 test of heterogeneity of the two subgroups was not

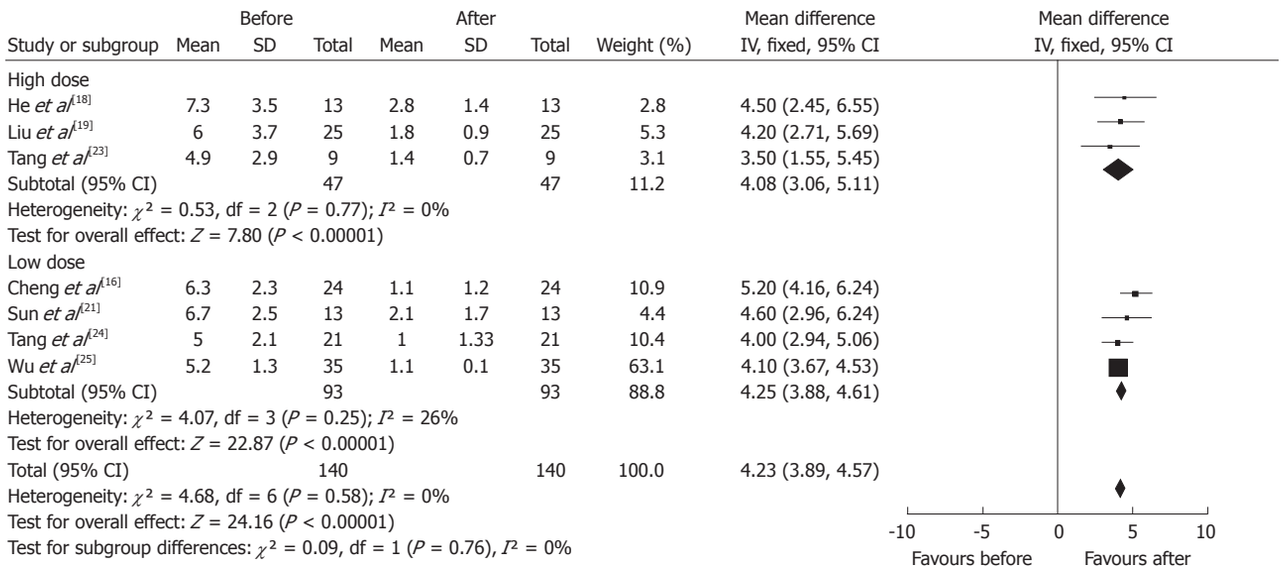

Figure 8 Proteinuria remission rate in different dose glucorticosteroids subgroups. IV: Inverse variance.

significant (P = 0.58). Therefore, the fixed-effect model was used. The combined therapy reduced proteinuria significantly after treatment (mean difference: 4.23, 95% CI: 3.89-4.57, Figure 8), and there was no significant difference between the two subgroups (P = 0.76, Figure 8).

Proteinuria changes in membranous glomerulonephritis subgroup vs mesangial proliferative glomerulonephritis subgroup

Comparisons of the rate of remission or the rate of complete remission in proteinuria between the membranous glomerulonephritis (MN) and mesangial proliferative glomerulonephritis (MsPGN) subgroups were assessed from the results of 6 trials[16,19,21,22,24,26] including 1 CCT[24] and 5 self-controlled studies[16,19,21,22,26]. The total number of patients was 73 for the MN group and 35 for the MsPGN group (Table 4). The χ^2 test of heterogeneity was not significant (P = 0.79 and P = 0.62, respectively), therefore the fixed-effect model was selected. The proteinuria remission rate did not differ between the two subgroups (RR = 0.94, 95% CI: 0.78-1.12, Figure 9). Also the complete remission rate of proteinuria did not differ between the two subgroups (RR = 0.84, 95% CI: 0.51-1.37, Figure 9).

Change in level of proteinuria in MN subgroup vs membranoproliferative glomerulonephritis subgroup

Comparisons of the remission rate and the complete remission rate of proteinuria between MN and membranoproliferative glomerulonephritis (MPGN) subgroups were assessed from the results of 5 trials[16,21,22,24,26] including 1 CCT[24] and 4 self-controlled studies[16,21,22,26]. The total number of patients was 79; 58 in the MN subgroup and 21 in the MPGN subgroup (Table 4). The χ^2 test of heterogeneity was not significant (P = 0.73 and 0.94, respectively), and therefore the fixed-effect model was used. The proteinuria remission rate did not differ between the two subgroups (RR = 1.13, 95% CI: 0.81-1.56, Figure 10), and also the complete remission rate did not differ between the two subgroups (RR = 1.84, 95% CI: 0.81-4.19, Figure 10).

Other adverse events

None of the studies assessed any other serious adverse events associated with the combined treatments. Almost all patients showed good tolerance although some patients had a transient increase in transaminases (4.4%), diarrhea (1.9%), loss of appetite (1.26%), influenza-like illness (0.3%), dizziness (0.3%), and anemia (1.26%). These side effects did not affect the completion and follow-up of the treatments.

Risk of bias in included studies

The overall quality of the studies included in this meta-analysis was suboptimal. Three studies had adequate allocation concealment and in all the rest the allocation concealment method was unclear. No study masked the participants or investigators to the intervention. Masking of the outcome assessors was done in only two studies and almost all the studies included did not analyze the results on an intention-to-treat basis. Because different outcomes and different comparisons were reported, and often without full statistical details, it was not possible to meta-analyze all the data. Risk of bias graph and summary are shown in Figures 11 and 12.

Publication bias

The funnel plots of the study results against precision are shown in Figure 13 with the 95% confidence limits. No obvious publication bias was found in our meta-analysis.

DISCUSSION

HBV-GN is an uncommon but a well-described complication of chronic hepatitis B. There is a high incidence of morbidity and mortality although remission with

Study or subgroup	MN Events	MN Total	MsPGN Events	MsPGN Total	Weight (%)	Risk ratio M-H, fixed, 95% CI
R						
Cheng *et al*[16]	3	5	5	6	7.8	0.72 (0.32, 1.60)
Liu *et al*[19]	11	15	6	7	14.1	0.86 (0.56, 1.31)
Sun *et al*[21]	3	4	3	3	6.7	0.80 (0.40, 1.58)
Sun *et al*[22]	11	12	5	6	11.5	1.10 (0.74, 1.64)
Tang *et al*[24]	6	7	6	6	11.9	0.88 (0.59, 1.29)
Xia *et al*[26]	28	30	6	7	16.7	1.09 (0.79, 1.50)
Subtotal (95% CI)		73		35	68.6	0.94 (0.78, 1.12)
Total events	62		31			
Heterogeneity: χ^2 = 2.41, df = 5 (P = 0.79); I^2 = 0%						
Test for overall effect: Z = 0.71 (P = 0.48)						
CR						
Cheng *et al*[16]	1	5	2	6	3.1	0.60 (0.07, 4.83)
Liu *et al*[19]	2	15	3	7	7.0	0.31 (0.07, 1.46)
Sun *et al*[21]	1	4	1	3	2.0	0.75 (0.07, 7.73)
Sun *et al*[22]	7	12	2	6	4.6	1.75 (0.51, 5.98)
Tang *et al*[24]	5	7	6	6	11.9	0.74 (0.44, 1.23)
Xia *et al*[26]	6	30	1	7	2.8	1.40 (0.20, 9.85)
Subtotal (95% CI)		73		35	31.4	0.84 (0.51, 1.37)
Total events	22		15			
Heterogeneity: χ^2 = 3.55, df = 5 (P = 0.62); I^2 = 0%						
Test for overall effect: Z = 0.71 (P = 0.48)						
Total (95% CI)		146		70	100.0	0.90 (0.74, 1.10)
Total events	84		46			
Heterogeneity: χ^2 = 6.67, df = 11 (P = 0.83); I^2 = 0%						
Test for overall effect: Z = 1.00 (P = 0.32)						
Test for subgroup differences: χ^2 = 0.17, df = 1 (P = 0.68), I^2 = 0%						

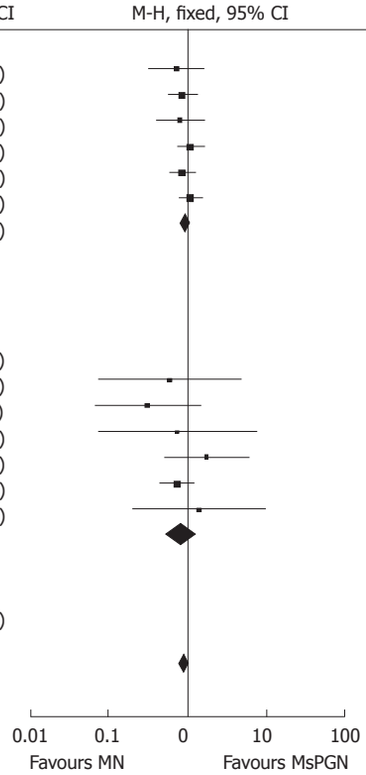

Figure 9 Proteinuria remission rate in membranous glomerulonephritis and mesangial proliferative glomerulonephritis subgroups. M-H: Mantel-Haenszel; MN: Membranous glomerulonephritis; MsPGN: Mesangial proliferative glomemlonephrits; CR: Complete remission; R: Remission.

Study or subgroup	MN Events	MN Total	MPGN Events	MPGN Total	Weight (%)	Risk ratio M-H, fixed, 95% CI
R						
Cheng *et al*[16]	3	5	6	7	19.8	0.70 (0.32, 1.52)
Sun *et al*[21]	3	4	1	2	5.3	1.50 (0.34, 6.70)
Sun *et al*[22]	11	12	7	9	31.7	1.18 (0.40, 1.58)
Tang *et al*[24]	6	7	1	2	6.2	1.71 (0.41, 7.08)
Xia *et al*[26]	28	30	1	1	11.2	1.23 (0.55, 2.75)
Subtotal (95% CI)		58		21	74.1	1.13 (0.81, 1.56)
Total events	51		16			
Heterogeneity: χ^2 = 2.01, df = 4 (P = 0.73); I^2 = 0%						
Test for overall effect: Z = 0.70 (P = 0.48)						
CR						
Cheng *et al*[16]	1	5	1	7	3.3	1.4 (0.11, 17.45)
Sun *et al*[21]	1	4	0	2	2.5	1.80 (0.10, 31.52)
Sun *et al*[22]	7	12	3	9	13.6	1.75 (0.62, 4.95)
Tang *et al*[24]	5	7	0	2	2.9	4.13 (0.31, 54.06)
Xia *et al*[26]	6	30	0	1	3.7	0.84 (0.07, 10.18)
Subtotal (95% CI)		58		21	25.9	1.84 (0.81, 4.19)
Total events	20		4			
Heterogeneity: χ^2 = 0.81, df = 4 (P = 0.94); I^2 = 0%						
Test for overall effect: Z = 1.46 (P = 0.14)						
Total (95% CI)		116		42	100.0	1.31 (0.93, 1.84)
Total events	71		20			
Heterogeneity: χ^2 = 4.22, df = 9 (P = 0.90); I^2 = 0%						
Test for overall effect: Z = 1.57 (P = 0.12)						
Test for subgroup differences: χ^2 = 1.20, df = 1 (P = 0.27), I^2 =16.5%						

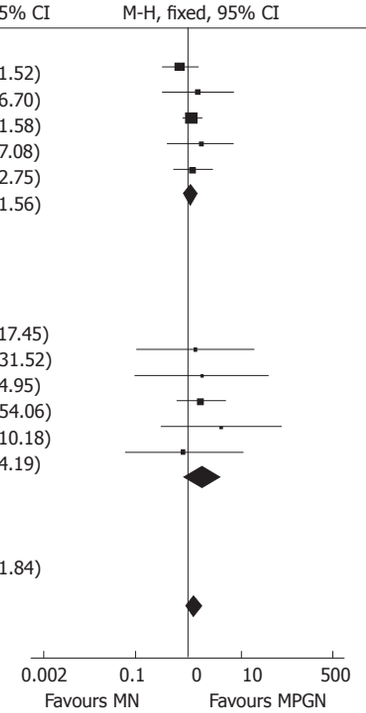

Figure 10 Proteinuria remission rate in membranous glomerulonephritis and membranoproliferative glomerulonephritis subgroups. M-H: Mantel-Haenszel; MN: Membranous glomerulonephritis; MPGN: Membranoproliferative glomerulonephritis; CR: Complete remission; R: Remission.

preservation of renal function has been reported[2]. HBV-GN has been reported from all over the world[10,27-31], but China is known to be the most endemic area with a high incidence of progressive HBV-GN and poor prognosis in

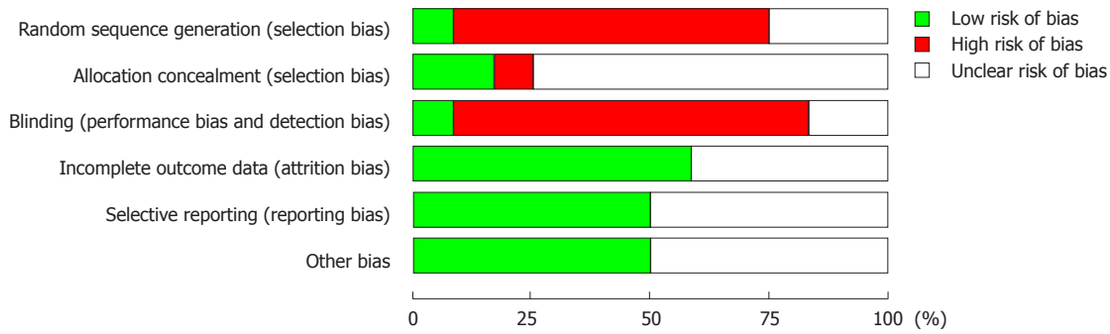

Figure 11 Risk of bias graph: Review authors' judgments about each risk of bias item presented as percentages across all included studies.

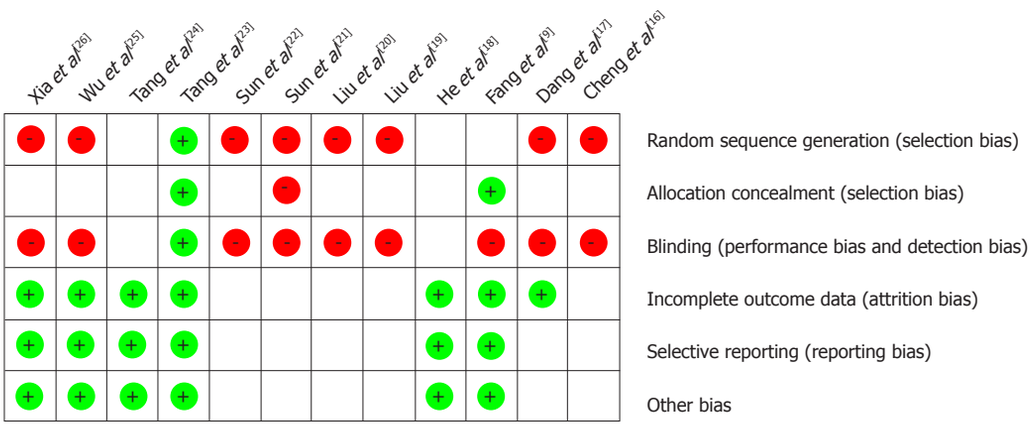

Figure 12 Risk of bias graph: Review authors' judgments about each risk of bias item for each included study.

Table 4 Pathological type and proteinuria remission rate

Ref.	Proteinuria remission rate	Pathological type and proteinuria remission rate								
		MN			MPGN			MsPGN		
		R	CR	PR	R	CR	PR	R	CR	PR
Cheng *et al*[16]	20/24	3/5	1/5	2/5	6/7	1/7	5/7	5/6	2/6	3/6
Liu *et al*[19]	20/25	11/15	2/15	9/15	0	0	0	6/7	3/7	3/7
Sun *et al*[21]	9/11	3/4	1/4	2/4	1/2	0/2	1/2	3/3	1/3	2/3
Sun *et al*[22]	26/29	11/12	7/12	4/12	7/9	3/9	4/9	5/6	2/6	3/6
Tang *et al*[24]	18/21	6/7	5/7	1/7	1/2	0/2	1/2	6/6	6/6	0/6
Xia *et al*[26]	36/42	28/30	6/30	22/30	1/1	0/1	1/1	6/7	1/7	5/7

MN: Membranous glomerulonephritis; MPGN: Membranoproliferative glomerulonephritis; MsPGN: Mesangial proliferative glomemlonephrits; R: Remission; CR: Complete remission; PR: Partly remission.

adults[32]. Case reports and small scale reports have been published, but unfortunately a standardized treatment protocol and justification for the current therapeutic regimen are lacking.

Antiviral drugs have been recommended for treatment of HBV-GN because they can inhibit HBV replication and reduce proteinuria[31,33-35]. The mechanisms by which antivirals including IFN and the nucleoside analogues, e.g., lamivudine and entecavir, reduce the nephrotic syndrome and decrease the proteinuria are known to be by their viral suppression and HBeAg seroconversion, reduction of serum HBV DNA, normalization of serum alanine transaminase[36]. On the other hand, the virological features of HBV, i.e., the genotype or viral load, genetic barrier, drug potency, patient adherence, and the duration of HBV infection, could play important roles in viral resistance even with nucleoside analogues that target the HBV DNA polymerase[37]. For patients who do not respond well to the antiviral therapy or/and have little signs of proteinuria remission, immunosuppressants, especially the glucorticosteroids, are often empirically used in clinics. This is even true for cases treated in developing countries such as China[38]. In fact, immune complexes have been identified in the kidney indicating that the pathogenesis of HBV-GN may be associated with an immune reaction. This could be supportive for the use of immunosuppressants. However, the use of steroids is still controversial because of the risk of activating viral infec-

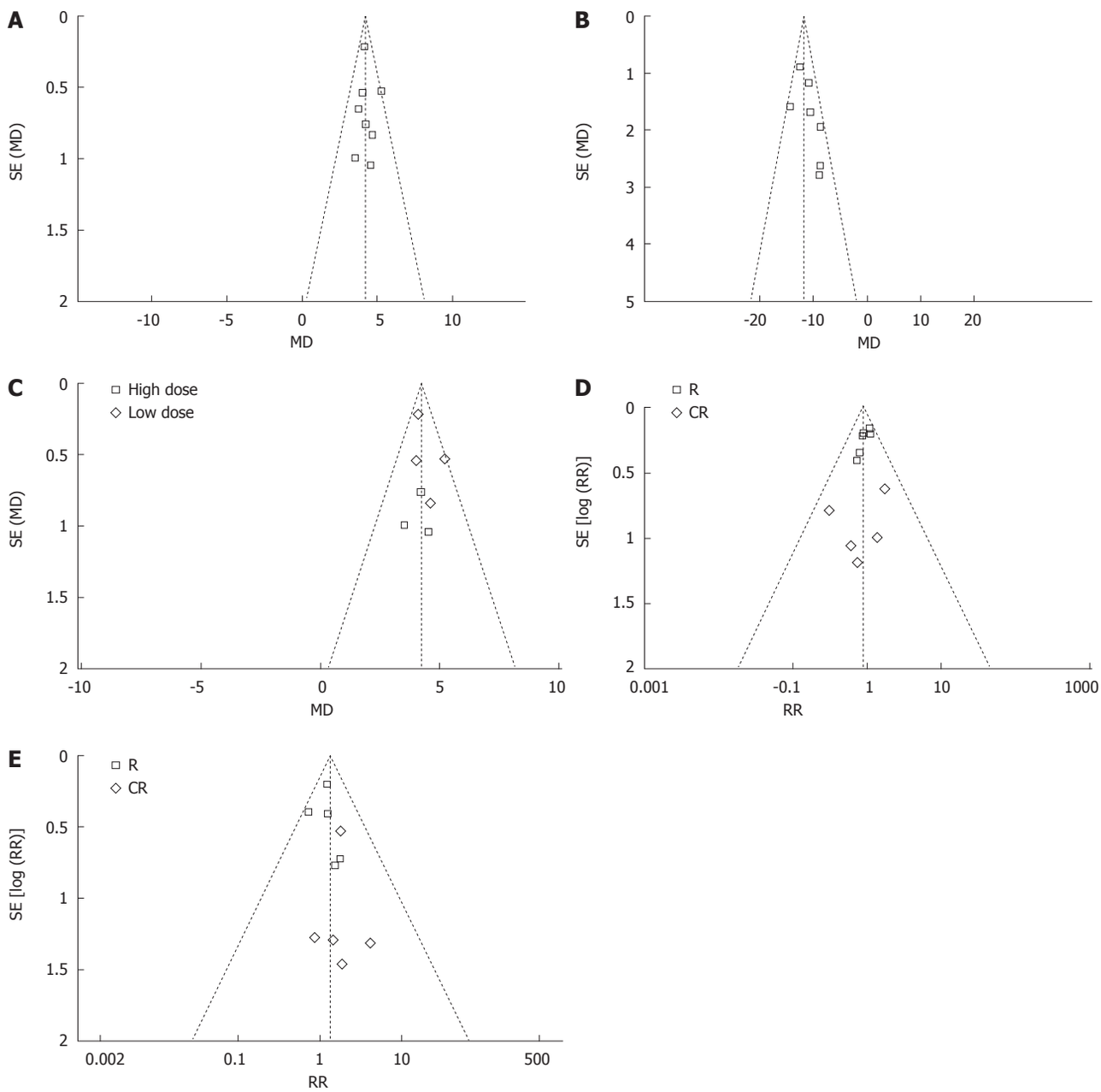

Figure 13 Publication bias analysis. A: Funnel plots of proteinuria treatment with steroid combination therapy; B: Funnel plots of serum albumin change with steroid combination therapy; C: Funnel plots of proteinuria change under different combined glucorticosteroids drugs; D: Funnel plots of remission rate *vs* complete remission rate of MN and MsPGN groups; E: Funnel plots of remission rate *vs* complete remission rate of MN and MsPGN groups. MN: Membranous glomerulonephritis; MsPGN: Mesangial proliferative glomemlonephrits; R: Remission; CR: Complete remission; RR: Relative risk; MD: Mean difference.

tions[3]. In addition, the efficacy of steroids has not been definitively determined.

It has been claimed that HBV-GN occurs predominantly in children and mainly in male patients[39]. The incidence of male patients has been reported to be about 1.5 to 2 times that of female patients[40]. Our meta-analysis of 286 adult patients from 10 studies showed that there were 2.4 times more male patients than female patients. Because all of the trials in this study were from China and considering the fact that China has a relatively larger population of men than women (Census Bureau released in 2011: Chinese male to female population ratio = 120:100), our analysis showed a still higher incidence of male patients.

The use of prednisone has been reported to cause a significant increase in the levels of HBeAg and HBV-DNA[5]. One recent meta-analysis also claimed that glucocorticoid monotherapy did not significantly improve proteinuria[8]. However, favorable effects of glucocorticoids have also been reported in reduction of proteinuria in MsPGN/MN cases following HBV infection[10]. In addition, the proteinuria remission rate of HBV-GN after glucocorticoid treatment in adults has been reported to be 75% which is much higher than that for antiviral treatment (28.6%)[10].

Our analysis showed that most patients with HBV-

GN were successfully treated with combined antiviral and immunosuppressant therapy with an overall estimated rate for proteinuria remission of 83%. Only 2 patients in the treated group dropped out but both were due to economic reasons. Our analyses also demonstrated that the combined therapy can effectively elevate the level of serum albumin. To the best of our knowledge, few reports exist in literature regarding the comparison of combined therapy with monotherapy of either antivirals or immunosuppressants. Thus, it could be an overstatement to conclude that the combined therapy is superior to antivirals or steroids. However, at least our meta-analysis provides evidence showing the efficacy and safety of this combined therapy of antivirals and immunosuppressants, which is actually a widespread practice in China.

Low-dose steroid therapy is aimed at reducing HBV replication while minimizing the risk of HBV activation. Both high-dose and low-dose groups showed similar efficacy of proteinuria reduction following treatment, indicating that low-dose steroid is effective and should be recommended for its safety. In addition, all of the reviewed patients tolerated steroid therapy well without occurrence of liver dysfunction or renal insufficiency.

Our analysis also revealed that no significant increase in the viral titers was observed after combined therapy. However, because the observation time was relatively short, long term effects of the combined therapy should be evaluated in the future.

The most common pathological type of HBV-GN is membranous nephropathy (MN; 50%), followed by MPGN and MsPGN[3]. Our study of 6 trials (152 patients) showed that MN accounted for 48% and MPGN + MsPGN for 36.8% of the phenotypes of HBV-GN. To determine the efficacy of combined therapy for the different pathological types, the trials were divided into three subgroups: MN, MsPGN, and MPGN. The proteinuria remission rate and complete remission rate were compared between the MN group and the MPGN group. There was no significant difference between the two groups. Comparisons of other pathological types could not be included in this analysis because the number of patients was small. Further studies of larger sample sizes are needed to solve this question.

As with all meta-analyses, our study had some biases. Firstly, negative trials are sometimes less likely to be published. To overcome this, we tried to obtain data from as many sources as possible. We used Funnel plots to test our review for publication bias. The risk of having missed trials was acceptably low. Secondly, the number of high quality clinical trials and number of studied patients were limited. Thirdly, we found that randomization and masking were not satisfactory and the allocation concealment of many studies was not available. Therefore, selection bias cannot be completely ruled out. More adequately powered RCTs with sufficient follow-up periods are definitely needed in future studies. Such RCTs should assess clinically important outcomes such as mortality, long-term relapse rate, and long-term HBV-DNA titer. Furthermore, the antiviral therapy should also be in a standard format in conformity with the APASL guidelines (Seoul 2008)[41], which would be beneficial for future analysis.

In summary, our meta-analysis of 12 clinical trials showed that combined therapy with antivirals and immunosuppressants is an effective and safe regimen for adult patients diagnosed with HBV-GN. Low-dose steroid is effective and can be recommended.

COMMENTS

Background

Hepatitis B virus-associated glomerulonephritis (HBV-GN) is one of the most common secondary glomerular diseases in developing countries such as China. To clear the viral antigen and abrogate the proteinuria is crucial for liver and renal function preservation and for decreasing the morbidity and mortality of this disease.

Research frontiers

HBV-GN treatment includes antiviral drugs, e.g., interferon and lamivudine, or immunosuppressants, e.g., glucocorticosteroids and mycophenolate mofetil. Although antivirals have been proven to be effective in clearing the viral antigens and attenuating the proteinuria, the safety and efficacy of immunosuppressants are still controversial.

Innovations and breakthroughs

A recent meta-analysis study showed that treatment of HBV-GN with corticosteroids only was not effective in proteinuria remission. However, this conclusion was drawn by analysis of data from both adult and pediatric patients, and because spontaneous remission can occur in children, this could have confounded the effectiveness of corticosteroid treatment in adult patients. To date, the current meta-analysis is the first to study the efficacy and safety of combined therapy with antivirals and immunosuppressants in adult HBV-GN patients.

Applications

The results of this meta-analysis should be beneficial for investigators and clinicians for obtaining information for the therapy of HBV-GN. Antivirals combined with immunosuppressants are effective and safe for adult patients and low-dose steroid can be recommended. All of the evidence in this paper supports the use of immunosuppressants which are being empirically used in the Asia-Pacific regions.

Terminology

HBV-GN is a clinical entity in which viral antigen and immune complexes have been identified in kidney, indicating that the pathogenesis of this disease may be associated with immune reaction. HBV-GN can be mainly separated into membranous glomerulonephritis, mesangial proliferative glomerulonephritis and membranoproliferative glomerulonephritis subgroups.

Peer review

This paper has major clinical implications and may change clinical policy.

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Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.833

World J Gastroenterol 2012 February 28; 18(8): 833-839
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

D2 dissection in laparoscopic and open gastrectomy for gastric cancer

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Supported by The Capital Medical Development Research Fund, No. 2009-2093
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Received: September 2, 2011 Revised: October 19, 2011
Accepted: January 18, 2012
Published online: February 28, 2012

Abstract

AIM: To evaluate the radicalness and safety of laparoscopic D2 dissection for gastric cancer.

METHODS: Clinicopathological data from 209 patients with gastric cancer, who underwent radical gastrectomy with D2 dissection between January 2007 and February 2011, were analyzed retrospectively. Among these patients, 131 patients underwent laparoscopy-assisted gastrectomy (LAG) and 78 underwent open gastrectomy (OG). The parameters analyzed included operative time, blood loss, blood transfusion, morbidity, mortality, the number of harvested lymph nodes (HLNs), and pathological stage.

RESULTS: There were no significant differences in sex, age, types of radical resection [radical proximal gastrectomy (PG + D2), radical distal gastrectomy (DG + D2) and radical total gastrectomy (TG + D2)], and stages between the LAG and OG groups ($P > 0.05$). Among the two groups, 127 cases (96.9%) and 76 cases (97.4%) had 15 or more HLNs, respectively. The average number of HLNs was 26.1 ± 11.4 in the LAG group and 24.2 ± 9.3 in the OG group ($P = 0.233$). In the same type of radical resection, there were no significant differences in the number of HLNs between the two groups (PG + D2: 21.7 ± 7.5 *vs* 22.4 ± 9.3; DG + D2: 25.7 ± 11.0 *vs* 22.3 ± 7.9; TG + D2: 30.9 ± 13.4 *vs* 29.3 ± 10.4; $P > 0.05$ for all comparisons). Tumor free margins were obtained in all cases. Compared with OG group, the LAG group had significantly less blood loss, but a longer operation time ($P < 0.001$). The morbidity of the LAG group was 9.9%, which was not significantly different from the OG group (7.7%) ($P = 0.587$). The mortality was zero in both groups.

CONCLUSION: Laparoscopic D2 dissection is equivalent to OG in the number of HLNs, regardless of tumor location. Thus, this procedure can achieve the same radicalness as OG.

Key words: Gastric cancer; Laparoscopy; Gastrectomy; D2 dissection; Lymph node

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Cui M, Xing JD, Yang W, Ma YY, Yao ZD, Zhang N, Su XQ. D2 dissection in laparoscopic and open gastrectomy for gastric cancer. *World J Gastroenterol* 2012; 18(8): 833-839 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/833.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.833

INTRODUCTION

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related deaths worldwide[1,2]. In the Far East countries such as China[3], Korea[4] and Japan[5], gastric cancer is the most prevalent malignancy, and the leading cause of cancer-related deaths. Since the first report of laparoscopic gastrectomy in 1992[6], laparoscopy-assisted gastrectomy (LAG) has been carried out not only in distal gastrectomy, but also in proximal gastrectomy and total gastrectomy[7-9]. Several randomized control trials (RCTs) have shown that LAG can be performed in early gastric cancer (EGC)[10-15]. However, LAG for the treatment of advanced gastric cancer (AGC) has remained controversial, mainly due to a lack of evidence from large-scale studies demonstrating that laparoscopic D2 dissection, the standard lymphadenectomy for AGC, is equivalent to open surgery. Recently, some studies have evaluated the outcome of D2 lymph node dissection in LAG and open surgery for gastric cancer[16-19]. The range of lymph node involvement differs due to the tumor location, thereby making the appropriate extent of D2 dissection vary as well[20]. However, little effort has been made to distinguish between tumors in different locations, all being simply regarded as "gastric cancer", or just evaluated as one type of LAG. In this study, we evaluated the overall radicalness of the laparoscopic D2 dissection of gastric cancer, and compared the differences between distal gastrectomy, proximal gastrectomy, and total gastrectomy.

MATERIALS AND METHODS

Patients

This retrospective study involved 221 consecutive patients with gastric cancer treated in the Department of Minimally Invasive Gastrointestinal Surgery, Peking University Cancer Hospital, between January 2007 and February 2011. The exclusion criteria included: (1) invasion of adjacent structures; (2) conglomeration of lymph nodes and no R0 resection; (3) distant metastases; and (4) absence of consent signing before operation. Blood tests, chest X-rays, enhanced computed tomography scans of the abdomen and pelvis, double-contrast upper gastrointestinal X-ray studies, and gastric endoscopy were performed before operation. All tumors were diagnosed as adenocarcinomas by biopsy. Informed consent was signed prior to surgery from each case. Among the 221 patients, 12 were excluded: 7 could not undergo R0 resection (3 with tumor invasion of adjacent structures, 4 with conglomeration of lymph nodes), 4 had distant metastases, and 1 refused operation. The study population thus included 209 cases that successfully underwent radical gastrectomy with D2 dissection. One hundred and thirty-one cases received LAG (LAG group) and 78 cases received open gastrectomy (OG group). All operations were performed by the same surgical team.

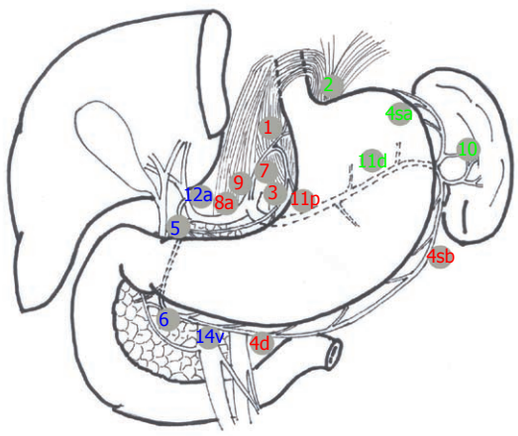

Figure 1 Lymph node stations of the stomach. The groups of lymph node in red, green and blue should be dissected in radical total gastrectomy; the groups of lymph node in red and green should be dissected in radical proximal gastrectomy; the groups of lymph node in red and blue should be dissected in radical distal gastrectomy.

Anesthesia and trocar placement

General anesthesia was administered with epidural analgesia to all the patients. The patients were placed in the supine position with legs apart. A 12-mm trocar for laparoscopy was introduced using the open technique below the umbilicus, and pneumoperitoneum at 10-13 mmHg was induced with carbon dioxide. Another 12-mm trocar was inserted at the left preaxillary line, 2 cm below the costal margin, to serve as a major hand port. Then a 5-mm trocar was placed at the left midclavicular line parallel to the umbilicus, and another 5-mm trocar was inserted at the contralateral site as an accessory port. The last 5-mm trocar was then placed at the right preaxillary line, 2 cm below the costal margin, also serving as another accessory port. The operator stood on the left side of the patient. The first assistant stood on the patient's right. The camera assistant stood between the patient's legs.

Surgical procedures

According to the guidelines of the Japanese Gastric Cancer Association (JGCA), the stomach was divided anatomically into three portions: upper, middle, and lower. The three portions were defined by subdividing both the lesser and greater curvatures into three equal lengths[20]. The type of gastrectomy and extent of D2 dissection were determined by tumor location (Figure 1)[20]. The aim of any oncological resection was to achieve en-bloc resection of gastric segment and surrounding lymph nodes, in order to obtain adequate oncological clearance.

Laparoscopy-assisted radical total gastrectomy with D2 dissection: This procedure was performed for gastric cancer involving more than 2/3 of the stomach. The greater omentum was first dissected, using the ultrasonicactivated scissors (Ultracision-Harmonic Scalpel; Ethicon Endo-Surgery Inc, Cincinnati, OH, United States), along the border of the transverse colon. The

left gastroepiploic vessel was vascularized, clamped with double Hem-o-lok clips (Teleflex Medical RTP, NC, United States) and cut at its origin. The gastrosplenic ligament was then divided and resected along the edge of spleen. These processes allowed the removal of No. 4sb, 4sa, and 10 lymph nodes. Then removal of No. 2 lymph nodes was performed. The next step was to resect the superior leaf of the mesocolon and the anterior leaf of the pancreas rightward the pylorus. The superior mesenteric vein, the Henle's trunk, the right colic vein, and the right gastroepiploic vessel were exposed allowing dissection of No. 14v lymph nodes. Then the right gastroepiploic vessel was clamped at its origin using double Hem-o-lok clips and cut, allowing No. 6 and 4d lymph nodes to be removed. In order to expose the gastropancreatic fold, the stomach was turned headward with the greater omentum folded up on the anterior aspect of the stomach. Along the gastroduodenal artery, the common hepatic artery could be skeletonized easily. The right gastric artery was divided and cut at its origin, using double Hem-o-lok clips, from the proper hepatic artery. Then dissection of No. 8a and 5 lymph nodes was completed. Once No. 8a lymph nodes were dissected, the procedure was continued leftward along the artery in order to remove the lymph nodes located along the celiac trunk (No. 9) and the left gastric artery (No. 7). The left gastric artery was cut from the celiac trunk using triple Hem-o-lok clips. Afterward, the celiac trunk was skeletonized. So, No. 7 and 9 lymph nodes were dissected. Then the splenic artery was skeletonized from its origin to the end in order to remove No. 11 lymph nodes. After returning the stomach and the greater omentum to normal position, the lesser omentum could be resected along the liver edge to the esophagogastric junction, with dissection of No. 1 and 3 lymph nodes. The last step of lymph node dissection was to skeletonize the proper hepatic artery, so No. 12a lymph nodes could be dissected. After standard D2 dissection was completed, an upper midline incision (about 5 cm) was made. The gastrectomy was performed and gastrointestinal continuity was restored in a Roux-en-Y fashion though this incision.

Laparoscopy-assisted radical proximal gastrectomy with D2 dissection: This procedure was performed for lesions located in the upper third of the stomach. The standard D2 dissection, including No. 1, 2, 3, 4, 7, 8a, 9, 10 and 11, was conducted using the same procedure described above. Esophagogastric anastomosis was performed to rebuild gastrointestinal continuity.

Laparoscopy-assisted radical distal gastrectomy with D2 dissection: This procedure was performed for lesions located in the lower third of stomach with or without involving the middle third of the stomach. The standard D2 dissection, including No. 1, 3, 4sb, 4d, 5, 6, 7, 8a, 9, 11p, 12a, and 14v, was conducted using the same procedure described above. Gastroduodenal anastomosis (Billroth Ⅰ) or gastrojejunal anastomosis (Billroth Ⅱ) was performed to rebuild gastrointestinal continuity.

Conventional open operation: The premedication and anesthetic techniques used were similar to the LAG group. Patients lay in the supine position, and the operator stood on the right side of the patient. An upper midline incision (about 20 cm) was made, and a standard gastrectomy with D2 dissection and reconstruction were performed in the same manner as in LAG.

Postoperative management

Nasogastric tube and nasojejunal tube were inserted routinely in operation. Enteral nutrition started through nasojejunal tube on the first postoperative day (POD). Gastroenterography, with the use of compound meglumine diatrizoate, was performed routinely to observe anastomosis on the 7th POD. If there was no evidence of anastomotic leakage by gastroenterography, the nasogastric tube could be removed and liquid diet was taken on the 8th POD.

Outcome evaluation

During surgery, operative time, blood loss (estimated by the volume of suction and the weight of gauze), and the amount of blood transfusion were recorded. Postoperative complications, categorized as surgical and nonsurgical complications, occurred during the hospital stay, and included fluid or abscesses needing drainage, intra-abdominal or anastomotic bleeding needing transfusion or reoperation, ileus, delayed gastric emptying, lymphatic leakage, and anastomotic leakage. Nonsurgical complications included cardiac, pulmonary, urinary, renal and hepatic complications. Mortality was defined as any death that occurred during hospital stay. The depth of tumor invasion, tumor size, margins, the number of harvested lymph nodes (HLNs), and positive lymph nodes were determined by pathological analysis. Histological staging was classified according to the 7th edition of the American Joint Committee on Cancer Staging Manual.

Statistical analysis

The data of patient's age, operation time, blood loss, and the number of lymph nodes were presented as $\chi \pm s$. Differences were compared in sex, type of resection, stage, and complications between the two groups using Chi-square test. Independent-sample *t* test was used to estimate differences in age, operation time, blood loss, and the number of HLNs between the two groups. *P* values less than 0.05 were considered statistically significant. All statistical analyses were performed with SPSS software, version 11.0 (SPSS Inc, Chicago, United States).

RESULTS

Patient clinicopathologic characteristics

Demographic details of the two groups are shown in Table 1. There were 88 males and 43 females in the LAG group, and the mean age of patients was 59.5 (range,

Table 1 Demographic characteristics of the two groups

Variable	LAG (n = 131)	OG (n = 78)	P value
Sex (male:female)	88:43	52:26	0.940
Age (yr)	59.5 ± 12.9	60.6 ± 10.3	0.523
Operation time (min)	259.1 ± 58.6	213.9 ± 37.6	0.000
Blood loss (mL)	111.1 ± 83.7	230.1 ± 96.8	0.000
Types of resections			
PG + D2	33	17	0.856
DG + D2	64	40	
TG + D2	34	21	
Stages			
Ⅰ (Ⅰa:Ⅰb)	29 (9:20)	12 (6:6)	0.253
Ⅱ (Ⅱa:Ⅱb)	28 (14:14)	13 (2:11)	
Ⅲ (Ⅲa:Ⅲb:Ⅲc)	74 (33:21:20)	53 (10:19:24)	

P values were calculated by independent-sample t test or by χ^2 test as appropriate. LAG: Laparoscopy-assisted gastrectomy; OG: Open gastrectomy; PG + D2: Radical proximal gastrectomy with D2 dissection; DG + D2: Radical distal gastrectomy with D2 dissection; TG + D2: Radical total gastrectomy with D2 dissection.

Table 2 Number of harvested lymph nodes in different types of radical resections

Groups	Number	Mean ± SD	P value
LAG	131	26.1 ± 11.4	0.233
OG	78	24.2 ± 9.3	
PG + D2	50	21.9 ± 8.1	0.000
DG + D2	104	24.4 ± 10.0	
TG + D2	55	30.3 ± 12.3	

P values were calculated by independent-sample t test. HLNs: Harvested lymph nodes; LAG: Laparoscopy-assisted gastrectomy; OG: Open gastrectomy; PG + D2: Radical proximal gastrectomy with D2 dissection; DG + D2: Radical distal gastrectomy with D2 dissection; TG + D2: Radical total gastrectomy with D2 dissection.

26-80) years. The OG group included 52 males and 26 females, with a mean age of 60.6 (range, 33-79) years. There were no significant differences in sex and age between the two groups (P = 0.940 and 0.523, respectively). Compared with the OG group, the LAG group had a significantly less blood loss (111.1 ± 83.7 mL in LAG *vs* 230.1 ± 96.8 mL in OG, P < 0.001), and a longer operation time (259.1 ± 58.6 min in LAG *vs* 213.9 ± 37.6 min in OG, P < 0.001). No blood transfusion was administered during surgery in either group.

Radical proximal gastrectomy with D2 dissection (PG + D2) was performed in 50 cases (33 in LAG and 17 in OG), radical distal gastrectomy with D2 dissection (DG + D2) in 104 cases (64 in LAG and 40 in OG), and radical total gastrectomy with D2 dissection (TG + D2) in 55 cases (34 in LAG and 21 in OG). There were no significant differences in the type of radical resection between the two groups (P = 0.856). Tumor free margins were obtained in all the patients. In the LAG group, there were 29 cases in stage Ⅰ, 28 cases in stage Ⅱ, and 74 cases in stage Ⅲ. In the OG group, there were 12 cases in stage Ⅰ, 13 cases in stage Ⅱ, and 53 cases in stage Ⅲ. There were no significant differences in pathological stages between the two groups (P = 0.253).

Table 3 Comparison of number of harvested lymph nodes between laparoscopy-assisted gastrectomy and open gastrectomy

Types of resections	Number	Mean ± SD	P value
PG + D2			
LAG	33	21.7 ± 7.5	0.770
OG	17	22.4 ± 9.3	
DG + D2			
LAG	64	25.7 ± 11.0	0.091
OG	40	22.3 ± 7.9	
TG + D2			
LAG	34	30.9 ± 13.4	0.653
OG	21	29.3 ± 10.4	

P values were calculated by independent-sample t test. HLNs: Harvested lymph nodes; LAG: Laparoscopy-assisted gastrectomy; OG: Open gastrectomy; PG + D2: Radical proximal gastrectomy with D2 dissection; DG + D2: Radical distal gastrectomy with D2 dissection; TG + D2: Radical total gastrectomy with D2 dissection.

Table 4 Postoperative complications in the two groups

Complications	LAG (n = 131)	OG (n = 78)	P value
Delayed gastric emptying	5	3	
Lymphatic leakage	3	2	
Anastomotic leakage	2	1	
Large pleural effusions	1		
Anastomotic bleeding	1		
Acute myocardial infarction	1		
Total (%)	13 (9.9)	6 (7.7)	0.587

P value was calculated by χ^2 test. LAG: Laparoscopy-assisted gastrectomy; OG: Open gastrectomy.

Number of harvested lymph nodes in different types of gastrectomies

Details of the number of HLNs are shown in Tables 2 and 3. In the LAG and OG groups, 127 (96.9%) cases and 76 cases (97.4%) had 15 or more HLNs. The average number of HLNs was 26.1 ± 11.4 in the LAG group and 24.2 ± 9.3 in the OG group (P = 0.233). The number of HLNs was 21.9 ± 8.1 in PG + D2, 24.4 ± 10.0 in DG + D2, and 30.3 ± 12.3 in TG + D2 (P < 0.001) (Table 2). In the same type of resection, there was no significant difference in the number of HLNs between the LAG and OG groups (PG + D2: 21.7 ± 7.5 in LAG *vs* 22.4 ± 9.3 in OG, DG + D2: 25.7 ± 11.0 in LAG *vs* 22.3 ± 7.9 in OG, TG + D2: 30.9 ± 13.4 in LAG *vs* 29.3 ± 10.4 in OG). P value was 0.770, 0.091 and 0.653, respectively (Table 3).

Morbidity and mortality after operation

Postoperative complications are listed in Table 4. Delayed gastric emptying (n = 5), lymphatic leakage (n = 3), anastomotic leakage without reoperation (n = 2), large pleural effusion needed drainage (n = 1), anastomotic bleeding needed transfusion (n = 1), and acute myocardial infarction (n = 1) occurred in the LAG group. Postoperative complications in the OG group included delayed gastric emptying (n = 3), lymphatic leakage (n = 2), and anastomotic leakage without reoperation (n = 1). There

were no significant differences in the morbidity between the two groups (9.9% *vs* 7.7%, $P = 0.587$). Mortality was zero in both groups.

DISCUSSION

For the treatment of AGC, surgical procedures include gastrectomy and lymphadenectomy. However, the extent of lymph node dissection has remained controversial worldwide[21]. In Eastern Asian countries such as Japan, China, and Korea, D2 dissection has been the standard operation[21]. However, in Western countries, D2 dissection is thought to be accompanied by significant mortality and morbidity, with no survival advantage[22,23]. Hartgrink *et al*[23] reported the results of a Dutch gastric cancer group trial in 2004, which included 711 patients who underwent randomly assigned treatment with curative intent (380 in D1 and 331 in D2). Both the postoperative morbidity (25% *vs* 43%, $P < 0.001$) and mortality (4% *vs* 10%, $P = 0.004$) were significantly higher in patients who underwent D2 dissection, while there was no difference in the 11-year overall survival (30% *vs* 35%, $P = 0.53$) between the two groups. Those results were similar to that of the Medical Research Council Gastric Cancer Surgical Trial[22]. However, the conclusions drawn from those two famous RCTs were questioned by Eastern investigators. The main concern was that 80 centers participated in the Dutch gastric cancer group trial, so the mean number of patients who underwent D2 dissection in each center was less than 5. Thus, the discommenders considered it very difficult to perform safe and standard D2 dissections in each center. Unexpectedly, in the 15-year follow-up from the Dutch gastric cancer group trial, published in 2010[24], the gastric-cancer-related death rate of the D2 group was significantly lower than that of the D1 group (37% *vs* 48%, $P = 0.01$), local recurrence was 12% in the D2 group *vs* 22% in D1, and regional recurrence was 13% in D2 *vs* 19% in D1. Thus, the authors recommended D2 dissection as the standard surgical approach for resectable gastric cancer. Currently, more and more evidences have proved D2 dissection as a feasible and safe procedure with survival advantages as compared with the D1 dissection[25-27], and D2 dissection has been gradually accepted by Western investigators. In the 2010 National Comprehensive Cancer Network guidelines, the panel recommended that gastric cancer surgery should remove D2 lymph nodes with the goal of examining 15 or more lymph nodes.

Although D2 dissection is performed in AGC as a standard procedure, more and more investigators have emphasized the need for D2 dissection in EGC because of pre-operative understaging[28-30]. In our hospital, about 90% of gastric cancers were initially diagnosed as AGC, and since endoscopic ultrasonography is not routinely performed, it is difficult to diagnose EGC preoperatively. Therefore, standard D2 dissection is routinely performed in all patients with gastric cancer in our hospital.

Laparoscopic surgery is a minimally invasive operation and is proved to be an acceptable alternative to open surgery in patients with colorectal cancer[31-33]. However, in gastric cancer, laparoscopic surgery has not yet been validated, and thus, was only performed in a limited number of patients with EGC in six small-scale RCTs[10-13,15,34]; this was due to the difficulties in systematic lymph node dissection, especially in the standard D2 dissection.

The number of HLNs is regarded as an important short-term oncological outcome of laparoscopic D2 dissection. Several recent retrospective studies have shown that laparoscopic D2 dissection is both a safe and oncologically feasible procedure, with a similar number of HLNs compared with open dissection[16-19,28,35,36]. Du *et al*[16] evaluated 82 patients with AGC who underwent laparoscopy-assisted total gastrectomy with D2 dissection compared with 94 patients who received open surgery; a similar number of HLNs was obtained in both groups (34.2 ± 13.5 *vs* 36.4 ± 19.1, $P = 0.331$). Huang *et al*[17] analyzed 66 cases of laparoscopy-assisted distal gastrectomy (LADG) with D2 dissection for AGC and 69 cases of open distal gastrectomy (ODG); no significant differences were found in the number of HLNs between the two groups (25.8 ± 12.5 *vs* 27.5 ± 10.3, $P = 0.401$). The morbidity in LADG was lower than that in ODG (6.1% *vs* 15.9%). Lee *et al*[18] evaluated 64 patients who underwent LADG with D2 dissection. The compliance rate, defined as cases with no more than one missing lymph node station according to JGCA lymph node grouping, was similar to that of ODG (67% *vs* 66%). The mean number of HLNs was 50.1 (range, 20-100), and the surgical morbidity and mortality were acceptable (3.1%, 0%, respectively).

There was still debate that the number of HLNs in laparoscopic D2 dissection was less than in the open D2 dissection[37,38]. Jeong *et al*[37] reported a study of 398 patients who underwent radical gastrectomy with R0 resection (261 in LAG and 138 in OG). The number of HLNs was significantly smaller in LAG than in OG (25 ± 13 *vs* 30 ± 14, $P < 0.01$). Lee *et al*[38] reported similar results in the number of HLNs (31.3 ± 11.1 *vs* 40.4 ± 17.9, $P < 0.001$). Unfortunately, in these two studies, the distribution of stages and the extent of lymph node dissection were not balanced between the two groups. The percent of AGC and D2 dissection in OG was higher than in LAG. This variability might explain the different number of HLNs between the two groups.

In this study, all patients successfully underwent radical surgery with D2 dissection. And 127 cases (96.9%) in the LAG group and 76 cases (97.4%) in the OG group had 15 or more HLNs. The mean number of HLNs was comparable between the LAG and OG groups ($P = 0.233$). The extent of D2 dissection should be decided in accordance with tumor location, so the number of HLNs was significantly different in different types of radical gastrectomies ($P < 0.001$). In addition, we analyzed the number of HLNs in different subgroups (PG + D2, DG + D2 and TG + D2) between LAG and OG, and found no significant differences ($P = 0.770$, 0.091

and 0.653, respectively). To our knowledge, this is the first published report that systematically compares the number of HLNs in different types of radical gastrectomies between LAG and OG. Similar to other studies, there was less blood loss, longer operation time, and comparable morbidity in LAG compared with OG.

Our team had completed more than 30 cases of LAG until this study. All operations, including LAG and OG, were performed by the same surgical team, thus allowing consistency of treatment, and all D2 dissections were completed successfully. The stages and types of radical resections were matched between the two groups. These are favorable conditions for comparing the number of HLNs, regardless of tumor location, between LAG and OG in a nonrandomized study.

However, there were some limitations in this study. First, this is a retrospective analysis. Second, there might be a selection bias as a result of comparing these nonrandomized groups to a retrospective profile. Third, there is no survival data. Thus, long-term oncological outcomes of LAG need to be evaluated in future studies.

In conclusion, our data suggests that the number of harvested lymph nodes of laparoscopic D2 dissection is equivalent to open surgery, regardless of the tumor location. Laparoscopic D2 dissection is safe, with less blood loss than open surgery, and can achieve the same radicalness as open surgery for gastric cancer. Large-scale RCTs with a longer follow-up period should be carried out in future studies to prove that LAG with D2 dissection is a good alternative to OG in selected patients.

COMMENTS

Background

Gastric cancer is one of the leading fatal malignancies worldwide, particularly in China and other Far Eastern countries. Laparoscopic surgery is a minimally invasive operation, which can be performed in colorectal cancer, and has been proved by many randomized control trials (RCTs). However, few studies have evaluated the role of laparoscopy-assisted gastrectomy (LAG) in gastric cancer.

Research frontiers

Several RCTs have shown that LAG can be performed in early gastric cancer. However, LAG for the treatment of advanced gastric cancer (AGC) has remained controversial. This is mainly due to a lack of evidence from large-scale studies showing that laparoscopic D2 dissection, the standard lymphadenectomy for AGC, is equivalent in outcome to open surgery.

Innovations and breakthroughs

In recent years, some studies have analyzed D2 dissection in LAG and open surgery for gastric cancer. Although the range of lymph node involvement differs by tumor location, making the extent of D2 dissection differ as well, little effort has been made to distinguish between tumors in various locations, with all simply being regarded as "gastric cancer" or just evaluated as one type of LAG. Thus, this study evaluated the overall radicalness of laparoscopic D2 dissection in gastric cancer, and further compared the difference among distal gastrectomy, proximal gastrectomy, and total gastrectomy.

Applications

This paper confirms the equivalent radicalness of D2 dissection between LAG and open surgery, thus providing preliminary evidence of the feasibility of applying LAG in gastric cancer, even in AGC. Long-term outcomes of LAG should be further studied.

Terminology

D2 dissection is the standard lymphadenectomy for AGC. The regional lymph nodes of the stomach are classified into three groups according to the guidelines of the Japanese Gastric Cancer Association. D2 dissection includes dissection of all Group 1 and Group 2 nodes. The extent of D2 dissection is different due to different tumor locations.

Peer review

The paper can be accepted for publication. The authors compared the radicalness of gastrectomy by laparoscopic and open approaches.

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S- Editor Shi ZF **L- Editor** Ma JY **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.840

World J Gastroenterol 2012 February 28; 18(8): 840-846
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Expression of fibroblast activation protein in human pancreatic adenocarcinoma and its clinicopathological significance

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Supported by The National Key Project of Scientific and Technical Supporting Programs of China, No. 2006BAI02A14; and National Natural Science Foundation of China, No. 30770996 and No. 81172310
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Received: April 14, 2011 Revised: September 1, 2011
Accepted: October 28, 2011
Published online: February 28, 2012

Abstract

AIM: To examine fibroblast activation protein (FAP) expression in pancreatic ductal adenocarcinoma (PDAC) and to analyze its relationship with the clinicopathology of PDAC.

METHODS: FAP expression was examined in 134 PDAC specimens by immunohistochemistry, and in four pancreatic cancer cell lines (SW1990, Miapaca-2, AsPC-1 and BxPC-3) by Western blotting assay. We also analyzed the association between FAP expression in PDAC cells and the clinicopathology of PDAC patients.

RESULTS: The results showed that the FAP was expressed in both stromal fibroblast cells (98/134, 73.1%) and carcinoma cells (102/134, 76.1%). All 4 pancreatic cancer cell lines expressed FAP protein at different levels. Protein bands corresponding to the proteolytically active 170-kDa seprase dimer and its 88-kDa seprase subunit were identified. Higher FAP expression in carcinoma cells was associated with tumor size ($P < 0.001$), fibrotic focus ($P = 0.003$), perineural invasion ($P = 0.009$) and worse clinical outcome ($P = 0.0085$).

CONCLUSION: FAP is highly expressed in carcinoma cells and fibroblasts in PDAC tissues, and its expression is associated with desmoplasia and worse prognosis.

Key words: Pancreatic ductal adenocarcinoma; Cancer-associated fibroblasts; Fibroblast activation protein; Prognosis

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Shi M, Yu DH, Chen Y, Zhao CY, Zhang J, Liu QH, Ni CR, Zhu MH. Expression of fibroblast activation protein in human pancreatic adenocarcinoma and its clinicopathological significance. *World J Gastroenterol* 2012; 18(8): 840-846 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/840.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.840

INTRODUCTION

Pancreatic carcinoma is one of the most aggressive human malignancies. Currently pancreatic cancer ranks fourth in cancer-related deaths in Western countries and sixth in China[1,2]. As one of the most lethal malignancies, human pancreatic ductal adenocarcinoma (PDAC) results in an overall 5-year survival of the patients of less than 5%, a rate with no substantial improvement over the past 25 years[3,4]. The poor prognosis is mainly associated with early local invasion, a high incidence of recurrence and a

poor response to chemotherapy and radiotherapy. Failure of the conventional therapies for PDAC might be due to our limited understanding of the role of tumor-stromal interactions in cancer progression[5].

In many solid tumors, the stroma is increasingly recognized to be important in promoting tumor proliferation, invasion, metastasis, and chemoresistance[6]. PDAC is one of the most highly invasive of the solid cancers and is characterized by an extensive desmoplastic stromal response. Several studies revealed that the elements in desmoplasia played important roles in tumorigenesis of PDAC[7-9] and was associated with a poor prognosis of the patients[10,11]. Mounting evidence suggests that cancer-associated fibroblasts (CAF), the predominant stromal cell type, actively communicate with and stimulate tumor cells, thereby contributing to tumor development and progression.

Fibroblast activation protein (FAP), or seprase, a cell surface glycoprotein belonging to the serine protease family, is a 170 kDa dimer that is catalytically active and has dipeptidase and gelatinase activities. FAP was identified as being expressed in reactive fibroblasts during embryonic development[12], in healing wounds[13], in chronic inflammation, in liver cirrhosis[14], and most importantly, in the CAFs of many human carcinomas[15-19]. As a marker of CAFs, FAP can enhance stromal cell proliferation and invasiveness, affect cell apoptosis, and is closely correlated with poor prognosis of a variety of tumors[20-23]. Recently, Kraman *et al*[23] reported that stromal cells expressing FAP may be involved in tumor immune system suppression. FAP expression has been described to be present predominantly in the tumor stroma of epithelial malignancies, and its presence has been associated with cancer invasion, tumor angiogenesis, and subsequent growth and metastasis[15-19]. FAP was also reported to be expressed in carcinoma cells of the stomach[20], colorectum[21], breast[22] and uterine cervix[24]. Cohen *et al*[15] found that FAP was expressed predominantly in the fibroblasts in PDAC, and was shown to be statistically highly correlated with a worse clinical outcome. However, there has been no study of the expression of FAP in pancreatic cancer cells and its role in pancreatic cancer development and progression[20]. In the present study, we aimed to investigate the expression of FAP in PDAC carcinoma cells and its association with desmoplasia and patient survival, which may shed light on the role of FAP in desmoplasia in PDAC, and more importantly, signify a potentially new therapeutic target in PDAC.

MATERIALS AND METHODS

Patients and specimens

The PDAC specimens were collected with informed consent from 134 patients undergoing radical resection of primary PDAC in Changhai Hospital, Second Military Medical University during 2005-2007. Of the 134 specimens, 74 were used for tissue array study, and the remaining 60 were used to prepare standard pathological sections. All the experimental procedures were approved by the Research Ethics Committee of the Second Military University. The medical records of the patients were retrospectively reviewed, and the demographic, clinicopathological and treatment data were also collected. Tumor location and size were derived from the surgical report. The patients receiving preoperative chemotherapy or radiotherapy were excluded from the study. All patients were followed up after the operation, and the follow-up of the survivors at the time of analysis ranged from 1-33 (median 12) mo postoperatively. The patients who were alive at the time of the last follow-up were censored for overall survival analysis.

Cell lines

Four pancreatic cancer cell lines (SW1990, Miapaca-2, AsPC-1 and BxPC-3) maintained in our laboratory were incubated in the presence of 5% CO_2 in a humidified atmosphere at 37 ℃. AsPC-1, SW1990 and BxPC-3 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories GmbH, Linz, Austria), and Miapaca-2 cells were maintained in DMED supplemented with 10% FBS and 2.5% horse serum (Gibco BRL, Invitrogen Corp, CA, United States).

Assessment of fibrotic focus and perineural invasion

Sixty standard slides were observed under the microscope after hematoxylin and eosin staining. A fibrotic focus was defined as a scar-like fibrosclerotic area located within the tumor containing a number of fibroblasts admixed with collagen fibers. The fibroblasts and collagen fibers were arranged in a storiform-like or irregular pattern[25]. The degree of perineural invasion was determined by two independent observers as described previously[26,27].

Immunohistochemistry

Tissue microarrays were constructed using the 74 paraffin-embedded tumor tissue specimens by a precision arraying instrument (Beecher Instruments, Silver Spring, MD, United States). In each case, three tumor cores and two surrounding non-tumor tissues were selected. The standard slices of the other 60 specimens and the sections from the tissue microarray blocks were deparaffinized, rehydrated and then heated in 0.01 mol/L sodium citrate buffer (pH 6.0) for 8 min at 95 ℃. After incubation with 0.3% hydrogen peroxide in methanol for 15 min at room temperature and treatment with normal goat serum (Invitrogen, Carlsbad, CA), the sections were incubated overnight at 4 ℃ with rabbit anti-human FAP polyclonal antibody (LifeSpan BioSciences Inc, United States) at a dilution of 1:70 in solution (Zymed Labroatories, Invitrogen Corp, CA). Slides were rinsed for 10 min in phosphate buffered saline (PBS) wash solution and incubated for 30 min with the horseradish peroxidase (HRP)-labeled polymer conjugated secondary antibody (EnVision+; DakoCytomation, Carpintera, CA, United States) was added according to the manufacturer's instructions. A known positive endometrial cancer tissue

Table 1 The clinical data of 134 pancreatic ductal adenocarcinoma patients

Items	Category	*n* (%)
Age (yr)	≤ 60	63 (47.0)
	> 60	71 (53.0)
Gender	Male	92 (68.7)
	Female	42 (31.3)
Location of tumor	Head	97 (72.4)
	Body/tail	37 (27.6)
Size of tumor (cm)[1]	≤ 2.0	17 (13.2)
	> 2.0	112 (86.8)
Histological grade	G1	3 (2.2)
	G2	116 (86.6)
	G3	15 (11.2)
Perineural invasion	Absent	25 (18.7)
	Present	109 (81.3)
pT[1]	T1	10 (7.6)
	T2	43 (32.8)
	T3	46 (35.1)
	T4	32 (24.4)
pN[1]	Absent	84 (65.1)
	Present	45 (34.9)
pTNM[1]	I	32 (24.8)
	II	30 (23.3)
	III	33 (25.6)
	IV	34 (26.4)

[1]Some records are missing. pTNM: Pathological tumor-node-metastasis.

biopsy sample was used as the positive control. PBS and non-immune serum were used instead of the primary antibody for the blank control and negative control samples, respectively.

Evaluation of FAP staining was carried out by two independent observers, and the stained area and intensity were scored separately. Specifically, a score of 0 was assigned to a stained area with ≤ 10% of the tumor cells, 1 for an area with > 11% to ≤ 25% of tumor cells, 2 for > 26% to ≤ 50% tumor cells, and 3 for > 51% tumor cells. For the staining intensity, a score of 0 was assigned for absent/weak staining (negative control), 1 for a weak staining obviously stronger than the negative control level, 2 for moderately intense staining, and 3 for intense staining. The final grade of the section was derived from the sum of the stained area and intensity scores. A final score ≥ 3 was recognized to indicate positive expression.

Western blotting

Upon reaching 80%-90% confluence, the cultured cells were harvested and lysed in ice-cold lysis buffer (50 mmol/L Tris-HCl (pH8.0), 150 mmol/L NaCl, 0.02% NaN_3, 1% NP-40, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/mL aprotinin), and the protein level was quantified according to the manufacturer's instructions of the BCA protein assay kit (Tiangen Biotech Co, China). Total protein was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were then blocked with 5% skim milk, incubated with 1:1000 rabbit anti-human FAP polyclonal antibody (Abcam, United States) overnight at 4 ℃, washed in Tris-buffered saline Tween-20 buffer, and then incubated with HRP-conjugated goat anti-rabbit IgG (MR Biotech, China) for 1 h at room temperature. The protein bands were visualized by chemiluminescence (Tiangen Biotech Co, China).

Statistical analysis

All the analysis were carried out using SPSS 11.5 software. The χ^2 test was used to analyze the correlation between FAP expression and clinicopathological features of PDAC. The Spearman correlation was used to evaluate the correlation of fibrotic focus and FAP expression. The Kaplan-Meier method was used for survival analysis and the difference in the survival curves was evaluated using the log-rank test. The Cox proportional hazards regression model in a stepwise manner was used to analyze the independent prognostic factors. All the tests were conducted with a 5% type I error.

RESULTS

Clinical data

We examined a total of 134 specimens. The patients consisted of 92 males and 42 females, age range 29-80 (median, 59) years. The patients' characteristics are summarized in Table 1.

Fibrotic focus in PDAC

A fibrotic focus was detected in 47 (78.3%) of the 60 PDAC specimens; in the fibrotic focus, active fibroblasts and collagen fibers were radially aligned around the tumor cells (Figure 1A), a pattern different from that in the surrounding PDAC stroma, where the fibroblasts and collagen fibers were arranged in an orderly fashion (Figure 1B). There was no significant correlation between the presence of a fibrotic focus and other clinicopathological factors.

Patterns of FAP expression in PDAC tissues

FAP was strongly expressed not only in activated fibroblasts within the PDAC tissues (98/134, 73.1%), but also in carcinoma cells in 102 of the 134 specimens (76.1%). Three patterns of FAP immunostaining were found in PDAC tissues, which included carcinoma cells (15/134, Figure 2A), stroma cells (11/134, Figure 2B), both carcinoma and stroma cells (87/134, Figure 2C). FAP immunostaining was localized mainly on the cell membrane and in the cytoplasm, and occasionally in the gland lumens. The expression presented with a diffuse or focal distribution in the carcinoma tissues. The FAP-positive fibroblasts were mainly located adjacent to the carcinoma tissue. The adjacent noncancerous pancreatic ducts showed virtually no positive staining for FAP, and the fibroblasts away from the carcinoma tissues rarely expressed FAP (Figure 2D).

FAP expression in pancreatic cancer cell lines

To further investigate the expression of FAP in pancreatic cancer cells, we performed Western blotting assays

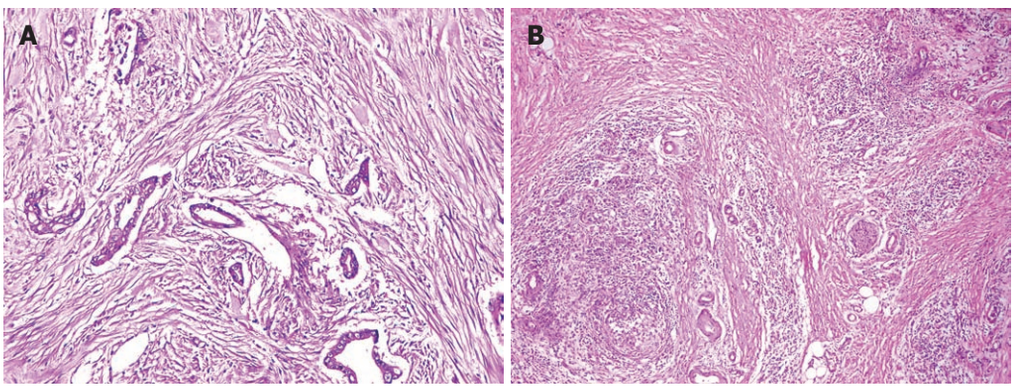

Figure 1 Fibrotic focus in pancreatic ductal adenocarcinoma. A: In the fibrotic focus, fibroblasts and collagen fibers are radially aligned around the tumor cells (× 200); B: A pattern different from that in the surrounding pancreatic ductal adenocarcinoma stroma where the fibroblasts and collagen fibers are arranged in an orderly fashion (× 100).

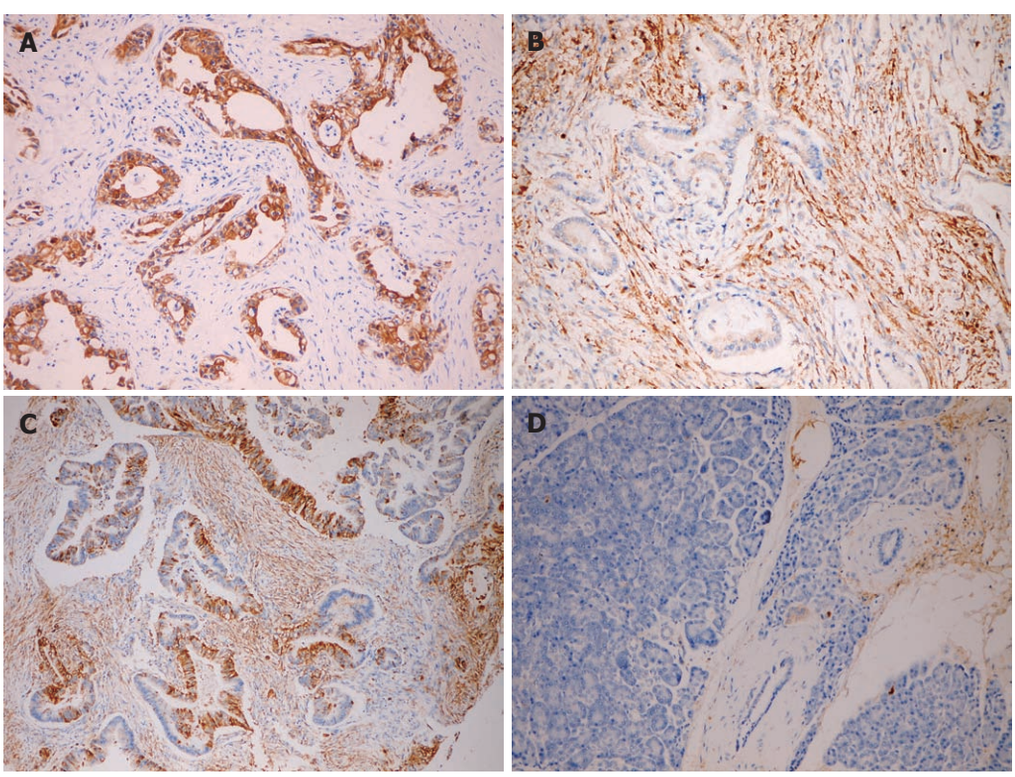

Figure 2 Fibroblast activation protein expression in pancreatic ductal adenocarcinoma. A: Fibroblast activation protein (FAP) expression in carcinoma cells is located mainly on the cell membrane and in the cytoplasm (× 200); B: FAP-positive fibroblasts are mainly located adjacent to carcinoma tissue (× 200); C: FAP is expressed in carcinoma cells and in fibroblasts adjacent to carcinoma tissue (× 100); D: The adjacent noncancerous pancreatic ducts show no positive staining for FAP and fibroblasts away from the tumor tissues rarely express FAP (× 200).

on cancer cells *in vitro*. The results showed that all four pancreatic cancer cell lines expressed FAP protein at different levels. Protein bands corresponding to the proteolytically active 170 kDa seprase dimer and its 97 kDa seprase subunit protein were identified (Figure 3). This result suggests that pancreatic cancer cells can secrete FAP by an autocrine mechanism.

Correlation of FAP expression with clinicopathological features of PDAC patients

The χ^2 test indicated significant correlations of FAP expression in the carcinoma cells from PDAC patients with patient age ($P < 0.001$), tumor size ($P < 0.001$), fibrotic focus ($P = 0.003$) and perineural invasion ($P = 0.009$) (Table 2). The Spearman correlation revealed that FAP expression was positively correlated with fibrotic focus, with a Pearson correlation coefficient of 0.379 ($P = 0.003$). We also found that PDAC patients with high expression of FAP in the carcinoma cells had a significantly shorter median overall survival compared to those with low expression of FAP (10 mo *vs* 33 mo, $P = 0.0085$) (Figure 4). In univariate analyses, the following param-

Table 2 Correlation of fibroblast activation protein expression with clinicopathological features of pancreatic ductal adenocarcinoma patients *n* (%)

Items	Category	*n*	FAP negative	FAP positive	*P* value
Age (yr)	≤ 60	63	19 (30.2)	44 (69.8)	0.000
	> 60	71	13 (18.3)	58 (81.7)	
Gender	Male	92	22 (23.9)	70 (76.1)	0.990
	Female	42	10 (23.8)	32 (76.2)	
Location of tumor	Head	97	27 (27.8)	70 (72.2)	0.082
	Body/tail	37	5 (13.5)	32 (86.5)	
Size of tumor (cm)[1]	≤ 2.0	17	10 (58.8)	7 (41.2)	0.000
	> 2.0	112	21 (18.8)	91 (81.2)	
Histological grade	G1	3	1 (33.3)	2 (66.7)	0.889
	G2	116	27 (23.3)	89 (76.7)	
	G3	15	4 (26.7)	11 (73.3)	
Fibrotic focus	Absent	13	7 (53.8)	6 (46.2)	0.003
	Present	47	7 (14.9)	40 (85.1)	
Perineural invasion	Absent	25	11 (44.0)	14 (56.0)	0.009
	Present	109	21 (19.3)	88 (80.7)	
pT[1]	T1	10	5 (50.0)	5 (50.0)	0.170
	T2	43	8 (18.6)	35 (81.4)	
	T3	46	12 (26.1)	34 (73.9)	
	T4	32	6 (18.8)	26 (81.2)	
pN[1]	Absent	84	21 (25.0)	63 (75.0)	0.725
	Present	45	10 (22.2)	35 (77.8)	
pTNM[1]	I	32	7 (21.9)	25 (78.1)	0.895
	II	30	8 (26.7)	22 (73.3)	
	III	33	9 (27.3)	24 (72.7)	
	IV	34	7 (20.6)	27 (79.4)	

[1]Some records are missing. pTNM: Pathological tumor-node-metastasis; FAP: Fibroblast activation protein.

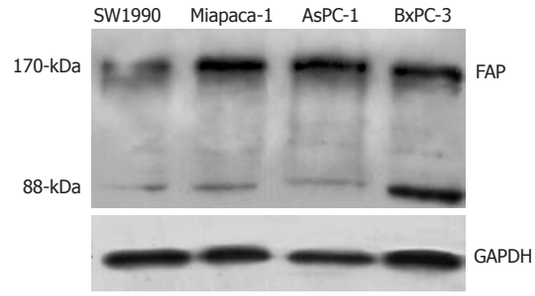

Figure 3 Western blotting analysis of fibroblast activation protein in pancreatic cancer cell lines. All four cell lines express fibroblast activation protein (FAP) with protein bands corresponding to the proteolytically active 170-kDa seprase dimer and its 88-kDa seprase subunit protein. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

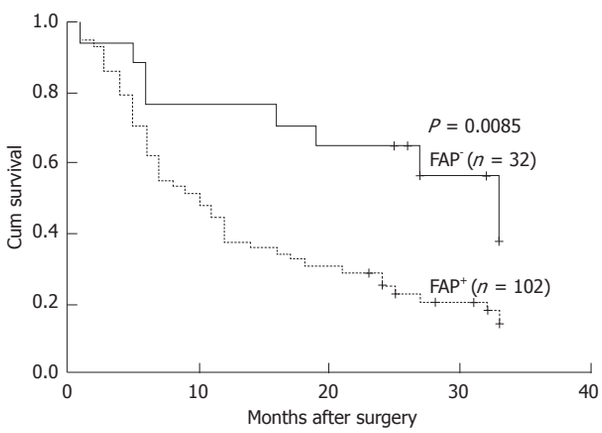

Figure 4 Kaplan-Meier survival curves of pancreatic ductal adenocarcinoma patients. The prognosis was significantly worse in the fibroblast activation protein (FAP)-positive group compared with the FAP-negative group (P = 0.0085).

eters were significantly associated with overall survival in PDAC: patient age (P = 0.013), tumor size (P = 0.008), fibrotic focus (P = 0.023) and perineural invasion (P = 0.009). The multivariate survival analysis further revealed that high expression of FAP was a predictor of overall survival (P = 0.017; relative risk, 2.513) independent of patient age (P = 0.029), tumor size (P = 0.011), fibrotic focus (P = 0.037) or perineural invasion (P = 0.020).

DISCUSSION

In this study, we confirm and extend previous findings that FAP expression is significantly elevated in PDAC tissues. FAP overexpression was positively correlated with patient age, tumor size, fibrotic focus, perineural invasion and poor survival, implying its involvement in PDAC development. Moreover, the protein product of FAP was found in all four pancreatic cancer cell lines and in PDAC tumor cells. To our knowledge, this was a novel finding of interest in the current study.

Solid tumors are a composite of cancer cells, endothelial cells, inflammatory cells, and fibroblasts. Although the relevance of fibroblasts in cancer progression is increasingly being recognized, little is known about their origin. Activated pancreatic stellate cells (myofibroblast-like cells), connective tissue-type fibroblasts, and mesenchymal cells were originally identified as the source of the fibrosis in chronic pancreatitis[9], and are now thought to be responsible for the dense stroma associated with pancreatic adenocarcinoma[9,28]. Cancer cells were surrounded by extremely dense collagen bundles associated with abundant reactive stromal fibroblasts, which were closely related to tumor prognosis[29,30]. The presence of a fibrotic focus correlated positively with disease progression, greater tumor size, the presence of lymph node metastases, and poor outcome in breast[31-33] and pancreatic[29,34] cancers. We found that the fibrotic focus was positive in 78.3% (49/60) of the PDAC tissues examined. Nevertheless, statistical analysis supported no correlation between the fibrotic focus and the prognosis of PDAC, which might be due to the fact that the majority of patients in our study were at an advanced stage, when a fibrotic focus would be common; the high short-term mortality rate of PDAC patients following surgery may also contribute to this result.

FAP expression has been described to be present predominantly in the tumor stroma of epithelial malignancies, and its presence has been associated with epithelial cancer invasion, tumor angiogenesis, and subsequent growth and metastasis[15-19,23]. FAP staining of the epithelial neoplasm has also been seen in some of studies, mainly located in the cytoplasm and the cell membrane

in carcinoma cells of the stomach[20], colorectum[18,21], breast[22], ovaries[35] and uterine cervix[24], and increased expression of FAP was also associated with histological grade, invasion and metastatic progression. Cohen *et al*[15] reported that FAP expression was seen mainly in the fibroblasts immediately adjacent to PDAC tissues, but was extremely rare in the carcinoma cells. In contrast, we found in the present study that FAP was highly expressed in the carcinoma cells (76.1%) as well as in the fibroblasts (73.1%) of PDAC tissues. The results of the Western blotting assay also demonstrated the presence of FAP protein expression in four human pancreatic cancer cell lines. Our findings suggested that PDAC cells might directly contribute to stroma desmoplasia through an autocrine mechanism of the FAP protein. We further analyzed the correlation of FAP expression in PDAC cells with patient survival. As we expected, FAP expression in the tumor cells was correlated with a shorter patient survival and served as an independent prognostic indicator for PDAC. These data suggest that FAP acts through an autocrine and/or paracrine mechanism in human pancreatic cancer, and may be a potential new therapeutic target in the treatment of PDAC.

As noted, we obtained different results concerning FAP expression in PDAC and its relation to desmoplasia from those reported previously[15]. We used a commercial primary antibody, and a non-biotinylated secondary antibody, so there would not be a false impression that FAP was localized to carcinoma cells. Although we do not know the reasons for this difference in expression, it may be related to use of a different source of FAP antibody. According to a recent study, the presence of collagen structures radially aligned with tumor cells was suggested to promote tumor progression and invasion[36]. The difference in FAP expression may reflect distinct biological features of these types of cancer.

To conclude, we found that PDAC cells, as well as CAFs, can also express FAP, which is closely associated with desmoplasia in PDAC. This suggests that PDAC cells may be directly responsible for the desmoplastic reaction through an autocrine mechanism of FAP production, and may partly explain why desmoplasia is particularly conspicuous in PDAC compared with the other carcinomas. This suggests that carcinoma cells may play a direct role, through an autocrine and/or paracrine mechanism, in desmoplasia in PDAC development and progression. Further research is needed to investigate the mechanism and some studies are already in progress. Our findings also provide evidence for ongoing clinical investigations with FAP as a therapeutic target for PDAC.

COMMENTS

Background

Fibroblast activation protein (FAP) is expressed on reactive fibroblasts during embryonic development, in healing wounds, in chronic inflammation, in liver cirrhosis, and in cancer-associated fibroblasts (CAFs) of many human carcinomas.

Research frontiers

FAP can enhance stromal cell proliferation and invasiveness, affect cell apoptosis, and is closely correlated with poor prognosis of a variety of tumors. Stromal cells expressing FAP may be involved in tumor immune system suppression. FAP expression has been found to be present predominantly in the tumor stroma of epithelial malignancies, and its presence has been associated with cancer invasion, tumor angiogenesis, and subsequent growth and metastasis.

Innovations and breakthroughs

FAP was expressed in both stromal fibroblast cells and carcinoma cells. All four pancreatic cancer cell lines expressed FAP protein at different levels. Higher FAP expression in carcinoma cells was associated with tumor size, fibrotic focus, perineural invasion and worse clinical outcome.

Applications

FAP is highly expressed in carcinoma cells and fibroblasts in pancreatic adenocarcinoma tissues, and its expression is associated with desmoplasia and worse prognosis. This suggests that carcinoma cells may play a direct role, through an autocrine and/or paracrine mechanism, in desmoplasia in pancreatic ductal adenocarcinoma (PDAC) development and progression, and FAP may serve as a potential therapeutic target in PDAC.

Terminology

FAP, or seprase, a cell surface glycoprotein belonging to the serine protease family, is a 170 kDa dimer that is catalytically active and has dipeptidase and gelatinase activities; CAF, is a predominant stromal cell type, which actively communicates with and stimulates tumor cells, thereby contributing to tumor development and progression.

Peer review

This is an interesting work.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.847

World J Gastroenterol 2012 February 28; 18(8): 847-854
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Interleukin-10-1082G/A polymorphism and acute liver graft rejection: A meta-analysis

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Received: May 25, 2011 Revised: September 2, 2011
Accepted: October 28, 2011
Published online: February 28, 2012

Abstract

AIM: To investigate the association between interleukin (IL)-10-1082 (G/A) promoter polymorphism and acute rejection (AR) in liver transplant (LT) recipients.

METHODS: Two investigators independently searched the Medline, Embase, China National Knowledge Infrastructure, and Chinese Biomedicine Databases. Summary odds ratios (ORs) and 95% CIs for IL-10-1082 G/A polymorphism and AR were calculated in a fixed- and a random-effects model as appropriate.

RESULTS: This meta-analysis included seven case-control studies, which comprised 652 cases of LT recipients in which 241 cases developed AR and 411 cases did not develop AR. Overall, the variant A allele was not associated with AR risk when compared with the wild-type G allele (OR = 0.94, 95% CI: 0.64-1.39). Moreover, similar results were observed when the AA genotype was compared with the AG/GG genotype (OR = 1.05, 95% CI: 0.55-2.02). When stratifying for ethnicity, no significant association was observed among either Caucasians or Asians. Because only one study was performed in Asian patients, the result of subgroup analysis by ethnicity would not be reliable for Asians. Limiting the analysis to the studies with controls in the Hardy-Weinberg equilibrium, the results were persistent and robust. No publication bias was found in the present study.

CONCLUSION: This meta-analysis suggests that IL-10-1082 G/A polymorphism may be not associated with AR risk in LT recipients among Caucasians.

Key words: Liver transplantation; Acute rejection; Interleukin-10; Gene polymorphism; Meta-analysis

Peer reviewers: Satoshi Yamagiwa, MD, PhD, Division of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, 757 Asahimachi-dori, Chuo-ku, Niigata 951-8510, Japan; Valentin Fuhrmann, MD, Department of Internal Medicine 4, Intensive Care Unit, Medical University Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria; Virendra Singh, MD, DM, Additional Professor, Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

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INTRODUCTION

Liver transplantation is regarded as an effective therapeutic option for end-stage liver disease as survival after liver transplantation has dramatically improved during

the last two decades. Despite this success, graft dysfunction occurs in up to 13% of the patients during the first year after transplantation and rises to 35% in 5 years[1,2]. Acute rejection (AR) and recurrence of disease are two major immunological complications, which may lead to graft dysfunction. The inflammatory microenvironment within the graft may play a role in the precipitation of rejection[3], although the underlying mechanisms involved in such events remain unclear. A network of short-acting cytokines and growth factors in turn determines this environment. Cytokines have a central role in the immunologic events that occur after transplantation and are intimately implicated in graft rejection.

Interleukin-10 (IL-10), whose encoding gene is located on chromosome 1 (1q31-1q32), is an immunoregulatory cytokine produced by Th2 cells, monocytes/macrophages, and regulatory T cells, and is capable of downregulating T-cell activation and major histocompatibility complex expression on antigen-presenting cells *in vitro*[4]. Previous studies have suggested that IL-10 mRNA levels are increased just before a rejection episode[5]. The production of cytokines (including IL-10) is under genetic control and varies among individuals as a function of polymorphisms within the regulatory regions of the various genes that determine the transcriptional activation[6-9]. The promoter of the *IL-10* gene contains three biallelic polymorphisms at positions -1082 (base G to A, dbSNP no. rs1800896), -819 (base C to T, dbSNP no. rs1800871), and -592 (base C to A, dbSNP no. rs1800872) from the transcription start site, and these influence the capacity of cells to produce IL-10[10]. For example, the G-to-A polymorphism at position -1082 of the IL-10 promoter reduces IL-10 production[7]. Alloimmune responses and variations in susceptibility to rejection may be influenced by individual variations in cytokine genes. Associations between cytokine gene polymorphisms and rejection of kidney[11,12], heart[13], and lung[14] have been reported.

Over the last two decades, a number of studies have assessed the association between the IL-10-1082 (G/A) promoter polymorphism and AR in liver transplant (LT) recipients in different populations; however, the results are inconsistent and inconclusive[15-22]. In 2005, Warlé *et al*[23] published findings from a meta-analysis of the IL-10-1082 (G/A) polymorphism and AR risk in LT recipients (based on five studies). The pooled results by Warlé *et al*[23] suggested that the IL-10 polymorphism at position -1082 was a genetic risk factor for acute liver graft rejection, and that LT recipients carrying the IL-10-1082. A allele displayed a lower rejection rate. However, this manuscript had some limitations mainly due to the small sample size and data retrieval. In order to derive a more comprehensive estimation of the association between IL-10-1082 polymorphism and AR risk in LT recipients, we conducted a meta-analysis to re-evaluate the association.

MATERIALS AND METHODS

Literature search strategy

We searched the PubMed, Embase, CNKI (China National Knowledge Infrastructure) and Chinese Biomedicine databases for all articles on the association between IL-10 polymorphisms and AR risk in LT recipients (last search update 20th March 2011). The following key words were used: "interleukin-10" or "IL-10"; "acute rejection" or "early graft rejection"; "liver transplantation". The search was performed without restriction on language, but conducted on human subjects. The reference lists of reviews and retrieved articles were hand searched at the same time. We did not consider abstracts or unpublished reports. If more than one article was published by the same author using the same case series, we selected the study where the most individuals were investigated.

Inclusion and exclusion criteria

We reviewed abstracts of all citations and retrieved studies. The following criteria were used to include published studies: (1) evaluation of the association between IL-10-1082 G/A polymorphism and AR in LT recipients; (2) a case-control or cohort design; and (3) sufficient genotype data presented to calculate the odds ratio (OR) with 95% confidence interval (CI). Major reasons for exclusion of studies were: (1) duplicate data; (2) an abstract, comment, review or editorial; and (3) no sufficient data were reported.

Data extraction

Two investigators (Liu F and Li B) extracted information from all eligible publications independently according to the inclusion criteria listed above. Disagreements were resolved by discussion between the two investigators. The following information was collected from each study: first author, year of publication, transplant period, indication for transplantation, patient characteristics (age, gender, *etc*), definition of AR, immunosuppressive regimen, country of the first or corresponding author, ethnicity, number of AR cases and controls (non-AR), genotyping methods and evidence of Hardy-Weinberg equilibrium (HWE). Ethnicities were categorized as Asian or Caucasian.

Statistical analysis

We first assessed HWE in the controls for each study using the goodness-of-fit test (χ^2 or Fisher's exact test) and a $P < 0.05$ was considered as significant disequilibrium. The strength of the association between AR and the IL-10-1082 G/A polymorphism was estimated using the OR and corresponding 95% CI. For the -1082G/A polymorphism, we estimated the risk of the variant A allele compared with the wild-type G allele, and then evaluated the risk of AA *vs* (AG + GG) which assumed a recessive effect of the variant A allele. We also carried out the stratified analyses by ethnicity (Caucasians/Asians) and HWE in controls (yes/no).

Both the Cochran Q statistic[24] to test for heterogeneity and the I^2 statistic to quantify the proportion of the total variation due to heterogeneity[25] were calculated. A P value of more than the nominal level of 0.10 for the Q

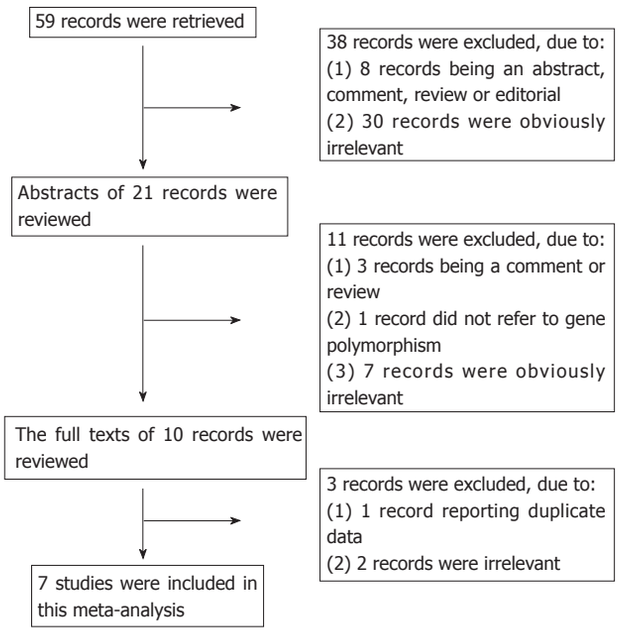

Figure 1 Flow chart of selection of studies and specific reasons for exclusion from the meta-analysis.

statistic indicated a lack of heterogeneity across studies, allowing for the use of a fixed-effects model (the Mantel-Haenszel method)[26]; otherwise, the random-effects model (the DerSimonian and Laird method) was used[27]. Sensitivity analysis was performed to assess the reliability of the results.

Several methods were used to assess potential publication bias. Visual inspection of funnel plot asymmetry was conducted. The Begg's rank correlation method[28] and the Egger's weighted regression method[29] were used to statistically assess publication bias ($P < 0.05$ was considered statistically significant). All analyses were done using STATA software, version 11.0 (STATA Corp., College Station, TX, United States). All the *P* values were two-sided.

RESULTS

Characteristics of studies

There were 59 papers relevant to the search words. *Via* steps of screening the title and reading the abstract, 10 studies were identified[15-22,30,31]. Of these, three studies were excluded (two did not report the association between IL-10-1082 G/A polymorphism and AR in LT recipients[22,30]; two articles[20,31] were published by a different first author using the same case series, and we selected the latest study[20]); thus, seven studies[15-21] which included 241 AR cases and 411 non-AR cases were found to match our inclusion criteria. The flow chart of selection of studies and reasons for exclusion is presented in Figure 1. Characteristics of studies included in the meta-analysis are presented in Tables 1 and 2.

There were six studies of Caucasian descendents, one study of Asian descendents. Studies had been carried out in China, Turkey, the United States, Netherlands, Israel and the United Kingdom. All studies defined rejection as biopsy-proven episodes of AR during the early post-transplant period (AR within first 4-8 wk), treated with high-dose steroids, except for the study of Karasu *et al*[16]. Immunosuppressive regimens in all studies consisted of a calcineurin inhibitor (cyclosporin or tacrolimus) and prednisone with or without azathioprine. Mycophenolate mofetil was only used in a subgroup of patients studied by Xie *et al*[15] and Mas *et al*[17]. Most studies extracted DNA from peripheral blood, and only two studies[15,17] from surgically explant liver tissue from recipients. Several genotyping methods were used, including PCR-RFLP, PCR-SSP, direct sequencing, ARMS-PCR and AS-PCR. The genotype distributions among the controls of all studies were consistent with HWE except for Tambur's study[20].

Quantitative synthesis

Overall, the variant A allele was not associated with AR risk when compared with the wild-type G allele (OR_{random} = 0.94, 95% CI: 0.64-1.39, $P_{heterogeneity}$ = 0.07) (Figure 2). When the AA genotype was compared with AG/GG genotype (recessive model), no significant association was observed (OR_{random} = 1.05, 95% CI: 0.55-2.02, $P_{heterogeneity}$ = 0.01) (Figure 3). When stratified for ethnicity, no significant association was observed among either Caucasians or Asians (for Caucasians: A allele *vs* G allele, OR_{random} = 0.95, 95% CI: 0.61-1.47, $P_{heterogeneity}$ = 0.04; AA *vs* AG/GG, OR_{random} = 1.07, 95% CI: 0.49-2.32, $P_{heterogeneity}$ = 0.01; for Asians: A allele *vs* G allele, OR_{random} = 0.96, 95% CI: 0.34-2.68; AA *vs* AG/GG, OR_{random} = 0.96, 95% CI: 0.33-2.77). Because only one study was performed in Asian patients, the result of subgroup analysis by ethnicity could not be reliable for Asians.

In Tambur's study, the distribution of *IL-10-1082* genotypes among controls was not in HWE. Limiting the analysis to the studies within HWE, the estimated association remained unchanged (A allele *vs* G allele, OR_{fixed} = 0.81, 95% CI: 0.61-1.07, $P_{heterogeneity}$ = 0.13; AA *vs* AG/GG, OR_{random} = 0.98, 95% CI: 0.46-2.11, $P_{heterogeneity}$ = 0.009).

Publication bias

Begg's funnel plot and Egger's test were performed to evaluate the publication bias of studies of AR in LT recipients. Figures 4 and 5 display funnel plots that examined the IL-10-1082 polymorphism and overall AR risk included in the meta-analysis. The shape of funnel plots did not reveal any evidence of funnel plot asymmetry. The statistical results did not show publication bias (A allele *vs* G allele: Begg's test P = 0.55, Egger's test P = 0.26; AA *vs* AG/GG: Begg's test P = 0.76, Egger's test P = 0.67).

DISCUSSION

In spite of major advances in the field of immunosuppressive therapy, acute hepatic allograft rejection remains an important problem after liver transplantation. Almost 30%-50% of patients experience at least one episode of rejection within the first year[32]. Cytokines, a group

Table 1 Baseline characteristics of studies included in the meta-analysis

Ref.	Transplant period	Indications for transplantation	Patients characteristics (age, gender)	Definition of acute rejection	Immunosuppression regimens
Bathgate *et al*[21]	1992-1998	ALD, PBC, PSC, chronic viral hepatitis, acute liver failure, autoimmune hepatitis, other	Not described	Liver biopsy and treatment with high-dose steroids	CsA/tacrolimus + prednisone + azathioprine
Tambur *et al*[20]	Not described	Hepatitis B and/or hepatitis C, PBC, PSC, cryptogenic, other	20-69 yr, M/F: 32/36	Liver biopsy (AR within first 6 wk)	CsA/tacrolimus + prednisone with or without azathioprine.
Warlé *et al*[18]	1992-1999	Hepatitis B, hepatitis C, PBC, PSC, ALD, other	AR group: 47 ± 11 yr, M/F: 22/19 Non-AR group: 49 ± 12 yr, M/F: 20/28	Liver biopsy and treatment with high-dose steroids (AR within first 4 wk)	CsA/tacrolimus + prednisone Maintain target therapeutic blood levels of 100-200 ng/mL for CsA or 5-10 ng/mL for tacrolimus
Fernandes *et al*[19]	Not described	Not described	19-73 yr, M/F: 26/27	Liver biopsy and treatment with high-dose steroids	Tacrolimus + prednisolone
Mas *et al*[17]	1999-2000	Hepatitis B, Hepatitis C, PSC, HCC, ALD, Cryptogenic, other	24-60 yr, M/F: 44/33	Liver biopsy (AR within first 8 wk)	CsA/tacrolimus + steroids + MMF
Karasu *et al*[16]	2002-2003	Viral, nonviral	AR group: 44.4 ± 12.7 yr, M/F: 17/9 Non-AR group: 37.4 ± 11.8 yr, M/F:11/6	Treatment with high-dose steroids (AR within first 8 wk)	CsA/tacrolimus+steroids Maintain target therapeutic blood levels of 5-10 ng/mL for tacrolimus
Xie *et al*[15]	2003-2005	HBV-related cirrhosis, HBV-related HCC, fulminant hepatitis B	AR group: 43.6 ± 9.0 yr, M/F: 35/6 Non-AR group: 46.5 ± 9.0 yr, M/F:130/15	Liver biopsy (AR within first 4 wk)	CsA/tacrolimus + prednisolone + MMF

ALD: Alcoholic liver disease; PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis; HCC: Hepatocellular carcinoma; HBV: Hepatitis B viral; CsA: Cyclosporine A; MMF: Mycophenolate mofetil; AR: Acute rejection.

Table 2 Characteristics of studies included in the meta-analysis

Ref.	Country	Ethnicity	No. of	Case		Control		Genotyping methods	HWE in controls
			case/control	AA	AG/GG	AA	AG/GG		
Bathgate *et al*[21]	United Kingdom	Caucasian	68/76	16	52	22	54	PCR-SSP	Yes
Tambur *et al*[20]	Israel	Caucasian	33/30	19	14	14	16	PCR-SSP	No
Warlé *et al*[18]	Netherlands	Caucasian	41/48	6	35	17	31	ARMS-PCR	Yes
Fernandes *et al*[19]	United States	Caucasian	13/40	4	9	15	25	AS-PCR	Yes
Mas *et al*[17]	United States	Caucasian	19/55	12	7	12	43	DNA-sequencing	Yes
Karasu *et al*[16]	Turkey	Caucasian	26/17	12	14	8	9	PCR-SSP	Yes
Xie *et al*[15]	China	Asian	41/145	36	5	128	17	PCR-RFLP	Yes

PCR-SSP: Polymerase chain reaction and sequence-specific primer typing; ARMS-PCR: Amplification refractory mutation system-polymerase chain reaction; AS-PCR: Allele-specific polymerase chain reaction; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; HWE: Hardy-Weinberg equilibrium.

of small, soluble, or cell membrane-bound protein or glycoprotein molecules, play an essential role in the regulation of inflammatory and immune responses. Despite the many variables that influence acute rejection, previous reports indicate that cytokine genotypes that result from polymorphisms can sometimes correlate with acute allograft rejection[33,34]. Alloimmune responses and variations in susceptibility to rejection may be influenced by individual variations in cytokine genes. An association between susceptibility to graft rejection and polymorphism in cytokine gene promoters in kidney, heart, lung, and bone marrow recipients has been reported by some centers[11-14,35], although others have not confirmed this[36-38].

IL-10 is an antiinflammatory cytokine, which can inhibit the production of tumor necrosis factor-α, IL-1, IL-6, IL-8, and IL-12 in monocytes/macrophages and interferon-γ in T cells[4]. Therefore, in the context of allograft rejection, local IL-10 release may have inhibitory properties on macrophages, T cells, and cytokines. However, the role of IL-10 in LT patients remains controversial. For example, some studies have suggested that IL-10 *mRNA* levels are increased just before a rejection episode[5], while others have indicated that IL-10 levels are unchanged during rejection of the LT[39]. In animal models, overexpression of IL-10 by gene transfer prolonged graft survival of orthotopic LTs[40]. Since some studies[33,34] reported that cytokine genotypes that result from polymorphisms can sometimes correlate with acute

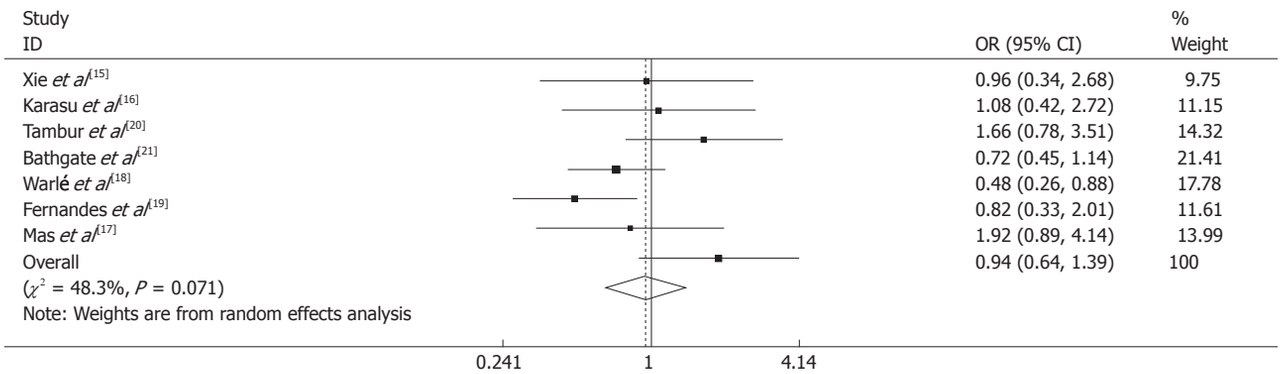

Figure 2 Odds ratios and 95% CI of individual studies and pooled data for the association of the interleukin-10-1082 G/A polymorphism and acute rejection comparing A allele with G allele. OR: Odds ratios.

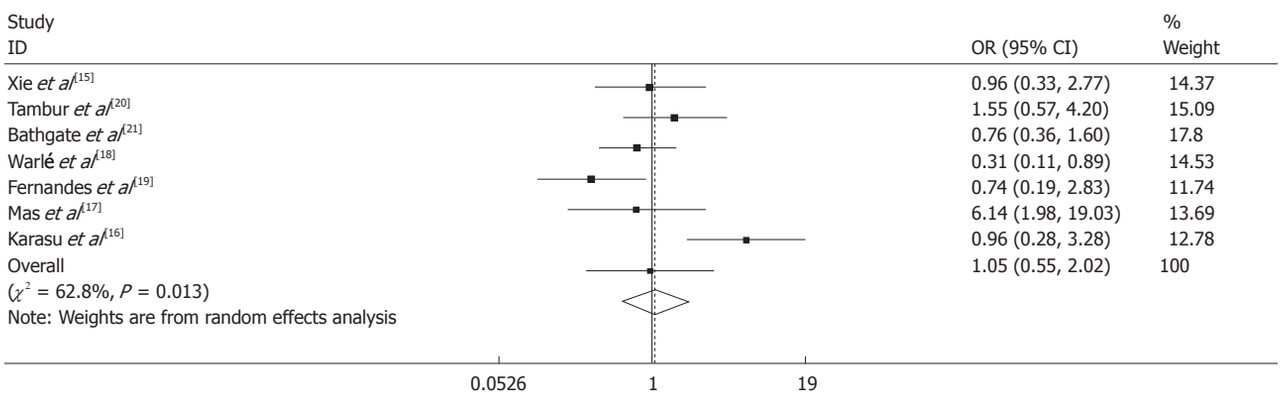

Figure 3 Odds ratios and 95% CI of individual studies and pooled data for the association of the interleukin-10-1082 G/A polymorphism and acute rejection comparing AA genotype with AG/GG Genotype. OR: Odds ratios.

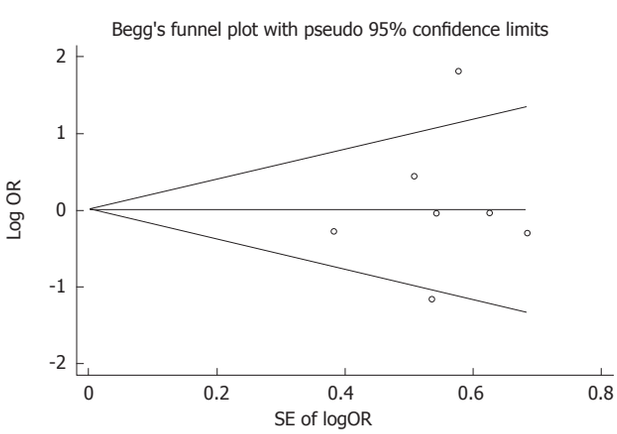

Figure 4 Begg's funnel plot of interleukin-10-1082 G/A polymorphism and acute rejection risk in liver transplant recipients (AA *vs* AG/GG). OR: Odds ratios.

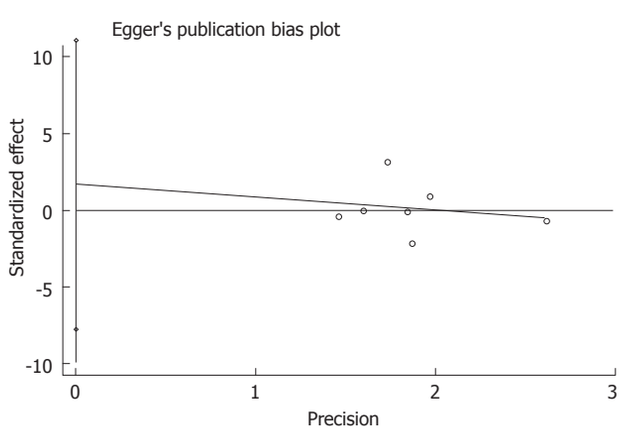

Figure 5 Egger's publication bias plot of interleukin-10-1082 G/A polymorphism and acute rejection risk in liver transplant recipients (AA *vs* AG/GG).

allograft rejection, a number of studies have assessed the association between the IL-10-1082 promoter polymorphism and AR in LT recipients in different populations. However, some of the results were conflicting, even in the same population, and thus a systematic review and meta-analysis of the association between *IL-10-1082* G/A polymorphism and AR risk was of great value.

A meta-analysis can overcome some problems caused by a single study, such as small sample size, low test power and selection bias; however, some concerns have to be addressed before aggregating data. First, the definition of AR, as the main outcome measure for this analysis, should be consistent among included studies. In six studies, AR was defined as "early" biopsy-proven AR within the first 4-8 wk after liver transplantation, treated with high-dose steroids. However, Karasu *et al*[16] defined AR as an increase in liver enzymes in the absence of vascular or biliary problems, associated with an improvement

after treatment by increasing the dose of immunosuppressive drugs or pulse steroid therapy within the first 8 wk. In the overall meta-analysis performed in this study, the number of patients from the Karasu *et al*[16] study is small, suggesting that this factor probably had little effect on the overall estimates. Moreover, the immunosuppressive regimen among different studies included is also an important factor which should be addressed. All LT patients included in this meta-analysis received more or less the same type of immunosuppression: a calcineurin inhibitor and prednisone, with or without azathioprine. However, there were some differences in the type of induction therapy, dosages and maintenance of target levels in blood, which can provide a possible explanation for significant heterogeneity in a recessive model.

This meta-analysis was based on seven case-control studies and showed that *IL-10-1082* G/A polymorphism was not associated with the risk of AR in LT recipients. Our result is not consistent with a previous systemic review[23]. This is most probably because the previous meta-analysis had a relatively small sample size (the Warlé *et al*[23] meta-analysis included only five studies for *IL-10-1082* G/A polymorphism and AR risk in LT recipients) and may have generated a very rough risk estimate. The G-to-A polymorphism at position -1082 of the IL-10 promoter reduces IL-10 production[7], and individuals with the *IL-10-1082*-GG genotype showed the greatest IL-10 production after *in vitro* stimulation, whereas *IL-10-1082*-GA and -AA showed intermediate and low production, respectively[7,41]. Moreover, previous studies[42,43] showed that Th2 cytokines, such as IL-10, are associated with graft tolerance. Therefore, it can be deduced that patients with an IL-10 genotype corresponding to low IL-10 production are more susceptible to rejection, whereas the IL-10 genotype corresponding to high production is found mainly among nonrejectors. However, our result is inconsistent with the above hypotheses. This is probably because the notion, derived mainly from animal studies, that IL-10 has a role in human allograft tolerance needs re-evaluation. In addition, since the effect of the *IL-10-1082* promoter polymorphism on *in vitro* and thus *in vivo* cytokine production is still inconclusive[7,44,45], its biological effect on acute liver graft rejection remains speculative.

As previously described, ethnicity can strongly influence the distribution of cytokine gene polymorphisms[46]. In Caucasian patients[17], the IL-10 AA genotype at position -1082 occurred in 32.5%, while among Asian patients[15] it occurred in 88.2%. Therefore, there may be different associations between IL-10-1082 promoter polymorphism and AR in LT recipients among different ethnicities. Nevertheless, our results were inconsistent with our hypothesis. When stratifying for ethnicity, no significant association was observed among either Caucasians or Asians. The null result may be due to the limited number of studies with only one study (based on Asian patients) available in this meta-analysis, and there is a very high risk of reporting bias for the relationship of the *IL-10-1082* G/A polymorphism and AR risk in the Asian population. In future, additional studies based on Asian patients should be performed to re-evaluate the association between IL-10-1082 G/A polymorphism and AR risk in this population.

It seemed that selection bias could have played a role because the genotype distribution of -1082 G/A polymorphism among control subjects disobeyed the HWE in one study[20]. It is widely believed that deviation from HWE may be due to genetic reasons including non-random mating, or the alleles reflect recent mutations that have not reached equilibrium, as well as methodological reasons including biased selection of subjects from the population or genotyping errors[47,48]. Apart from the reasons for disequilibrium, the results of genetic association studies might be spurious if the distribution of genotypes in the control groups were not in HWE[49,50]. Thus we carried out subgroup analysis by HWE in controls. When excluding the study that was not within HWE, the estimated pooled OR did not change at all, suggesting that this factor probably had little effect on the overall estimates.

However, there some limitations remain in this meta-analysis: (1) our meta-analysis was based on unadjusted OR estimates because not all published studies presented adjusted ORs or when they did, the ORs were not adjusted by the same potential confounders, such as age, sex, ethnicity and exposures. Lack of the information for the data analysis may cause serious confounding bias; (2) there was significant between-study heterogeneity from studies of the *IL-10-1082* G/A polymorphism, and the genotype distribution also showed deviation from HWE in one study; (3) the number of studies and the number of subjects in the studies included in the meta-analysis were small; (4) we must emphasize the fact that this meta-analysis adds more studies and increases the sample size but that it is an update that is not important because it adds patients (small importance); it is important because the statistical analysis reflects that IL-10 polymorphisms are not relevant in AR in liver transplantation; and (5) meta-analysis is retrospective research that is subject to methodological limitations. In order to minimize bias, we developed a detailed protocol before initiating the study, and performed a meticulous search for published studies by using explicit methods for study selection, data extraction and data analysis. Nevertheless, our results should be interpreted with caution.

In conclusion, this meta-analysis suggests that IL-10 -1082 G/A polymorphism may be not associated with AR risk in LT recipients among Caucasians. Since only one study was from an Asian population, it is critical that larger and well-designed multicenter studies based on Asian patients should be performed to re-evaluate the association.

COMMENTS

Background

Interleukin (IL)-10 is an antiinflammatory cytokine, which can inhibit the production of tumor necrosis factor-alpha, IL-1, IL-6, IL-8 and IL-12 in monocytes/

macrophages and interferon-γ in T cells. Therefore, in the context of allograft rejection, local IL-10 release may have inhibitory properties on macrophages, T cells, and cytokines. The production of cytokines (including IL-10) is under genetic control and varies among individuals as a function of polymorphisms within the regulatory regions of the various genes that determine the transcriptional activation. The G-to-A polymorphism at position -1082 of the IL-10 promoter reduces IL-10 production. Alloimmune responses and variations in susceptibility to rejection may be influenced by individual variations in cytokine genes.

Research frontiers

To date, a number of studies (including meta-analysis) have assessed the association between the IL-10 1082 G/A polymorphism and acute rejection (AR) risk in liver transplant (LT) recipients among different populations; however, the results are inconsistent and inconclusive.

Innovations and breakthroughs

Contrary to the finding of the previous meta-analysis, this study suggests that IL-10-1082 G/A polymorphism may be not associated with AR risk in LT recipients among Caucasians.

Applications

It can be seen from this paper that IL-10-1082 G/A polymorphism could not alter susceptibility to AR risk in LT recipients. It suggests that a common variant in the functional region of a meaningful gene had little effect on human disease.

Peer review

This appears to be a well-done meta-analysis study.

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S- Editor Tian L **L- Editor** Cant MR **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.855

World J Gastroenterol 2012 February 28; 18(8): 855-860
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Amplifications of *NCOA3* gene in colorectal cancers in a Chinese population

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Supported by Fundamental Research Plan of Shenzhen City, No. JC201005260215A
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Received: May 14, 2011 Revised: August 8, 2011
Accepted: August 31, 2011
Published online: February 28, 2012

Abstract

AIM: To investigate the copy number variation of *NACO3* gene in colorectal cancer (CRC) and its correlation with tumor progression.

METHODS: A total of 142 samples of case-matched CRC tissues and adjacent normal tissues were obtained from patients undergoing bowel resection. Quantitative real-time polymerase chain reaction method was used to investigate the copy number variations of NCOA3 as well as gene expression in the collected tissues.

RESULTS: Copy number gains of NCOA3 were detected in 39 CRC samples (27.5%) and were correlated with tumor progression (χ^2 = 6.42, P = 0.0112). Moreover, there was a positive correlation between copy number gain and mRNA over-expression of NCOA3 in CRCs (P = 0.0023). Expression level of NCOA3 mRNA was also enhanced in the CRC samples with unaltered copy numbers (3.85 ± 1.23 *vs* 2.71 ± 0.64, P < 0.01).

CONCLUSION: Sporadic colorectal cancers exhibit different mechanisms of NCOA3 regulation.

Key words: Colorectal cancer; *NCOA3*; Gene copy number; Gene expression; Tumor progression

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Li Z, Fang ZY, Ding Y, Yao WT, Yang Y, Zhu ZQ, Wang W, Zhang QX. Amplifications of *NCOA3* gene in colorectal cancers in a Chinese population. *World J Gastroenterol* 2012; 18(8): 855-860 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/855.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.855

INTRODUCTION

The p160 steroid receptor coactivator (SRC) family is critical to the transcriptional activation function of nuclear hormone receptors. A key member of this family is nuclear receptor coactivator 3 (NCOA3 and also known as AIB1 and SRC-3), initially found to be amplified and expressed in breast cancer, and has subsequently been shown to express in malignant diseases arising from a wide range of other organs[1-3]. Over-expression of NCOA3 has been implicated in tumor proliferation, invasiveness[4], and resistance to chemoradiotherapy[5] in various kinds of tumors. Increased expression of NCOA3 is

also correlated with advanced colorectal carcinoma[6].

Colorectal cancer (CRC) is currently the third most common cancer in Chinese population, responsible for about 130 000 deaths per year. Both genetic and environmental factors contribute to disease etiology, with about one-third of disease variance attributed to inherited genetic factors[7]. The implication of copy-number variations (CNVs) in cancers has become a hot spot over the past few years[8-10]. Studies using SNP arrays and aCGH have suggested that DNA amplification at chromosome position 20q12, also the chromosome locus of NCOA3, is frequent in CRC[11-13]. Moreover, there might be a correlation between CNVs and NCOA3 over-expression in CRCs. However, most of the aCGH experiments focused on the genome-wide screening of CNVs and the data obtained are generally informative but not definitive. Therefore, a study comprehensively examining CNVs in relation to NCOA3 expression or prognosis should be performed using a larger number of tumors.

In the present study, we collected 142 colorectal cancer samples with matched adjacent normal tissues for CNV analysis. Copy number gains of NCOA3 gene were observed in a relatively high percentage of CRC samples and were correlated with cancer lymph node involvement or metastasis. There was also a positive correlation between gene copy number gains and mRNA over-expression of NCOA3. These findings suggested the potential role of CNVs of NCOA3 in CRCs.

MATERIALS AND METHODS

Patients and tissue collection

CRC samples were obtained from 142 patients undergoing bowel resection at the Department of General Surgery, Henan Province Tumor Hospital. Adjacent normal tissue (ANT) samples located at least 2 cm from the macroscopically unaffected margins of the tumor (polyp or carcinoma) were defined as normal controls. The collected samples were stored in liquid nitrogen. There were 133 adenocarcinomas and 9 mucinous carcinomas (when >50% of the tumor volume was composed of mucin). CRCs were staged according to the Dukes classification system: Dukes A (T_1-T_2, N0, and M_0; n = 42), Dukes B (T_3-T_4, N0, and M_0; n = 37), Dukes C (any T, N_{1-2}, M_0; n = 48) and Dukes D (any T and any N and M_1; n = 15). Case-matched samples of colorectal carcinomas (n = 142) and normal colonic mucosa (n = 142) were immediately stored in liquid nitrogen after operation. For each sample, a portion of the tissue was disposed to make frozen sections and then micro-dissected by the Eppendorf Microdissection System (Siskiyou MX160L micromanipulator and PixeLink PL-A662 color Charge Coupled Device firewire camera). Then the dissected tissues were subjected to real-time polymerase chain reaction (PCR) analysis. All patients were informed about the aims of specimen collection and gave signed written consent in accordance with the ethical guidelines of Zhengzhou University. Peripheral blood samples from 152 healthy controls were collected at the Department of Histology and Embryology, Basic Medical College of Zhengzhou University. The study was approved by the Ethics Committee of Zhengzhou University.

DNA extraction and quantification of copy numbers

Genomic DNA was isolated from the tissues using the Genomic DNA Extraction Kit (Innocent, Shenzhen, China) according to the manufacturer's instructions. The concentration of purified DNA was determined by measuring the absorbance at 260 nm and 280 nm in a spectrophotometer. Pure preparations of DNA have A_{260}/A_{280} > 1.8. Quantitative PCR was performed using the BioRad Chromo4 real-time PCR system. The primers for RNAse P are: forward: 5'-AGA CTA GGG TCA GAA GCA A-3' and reverse: 5'-CAT TTC ACT GAA TCC GTT C-3'[14]. The primers for NCOA3 are: forward: 5'-AAA GTA AAC AGA ATG GAT TG-3' and reverse: 5'-AGT GTG CCT TGG AGT TGA AA-3'. The primer sets for *NCOA3* gene were designed by advanced real time PCR primer design tool (GenScript United States Inc.). A melting curve analysis with a temperature gradient of 0.2 K × S^{-1} from 72 ℃ to 95 ℃ was performed following each PCR amplification to confirm that only specific product was amplified.

Average copy numbers of RNAse P in normal candidates (copy numbers = 2) were used as controls[14,15]. The copy numbers of NCOA3 were calculated by the comparative C (T) method[16]. Cut-off values of 0.25, 0.75, 1.25 and 1.75 were used to define the copy numbers as 0, 1, 2 and 3, respectively. Fold change of each sample was presented as follows: fold change = relative expression level/average expression level in the group with 2 copies of DNA.

A standard curve was prepared using 2 μL of crude DNA solutions, including serially diluted samples (original, 2-, 4-, 8-, 16-diluted). The slopes of Ct and efficiency of each primer were calculated by the BioRad Chromo4 real-time PCR system and Microsoft Excel 2007 for Windows. Relative quantification of NCOA3 was performed by the 2^{-ddCt} method[17].

RNA extraction and real-time PCR

Total RNA was isolated from tissues using the AxyPrepTM Blood Total RNA MiniPrep Kit (Axygen) according to the manufacturer's instructions. First strand cDNA was synthesized with the RevertAidTM First Stand cDNA Synthesis Kit (Fermentas). Quantitative PCR was performed through the BioRad Chromo4 real-time PCR system. At the end point of PCR cycles, melt curves were made to check product purity. The mRNA levels of NCOA3 were expressed as a ratio relative to the GAPDH mRNA in each sample. Exploratory data analysis using box plot was applied to visually identify the expression level of target mRNA.

Statistical analysis

Statistical analysis was performed with the SPSS Software

Table 1 Comparison of copy number variations of *NCOA3* between adjacent normal tissues and healthy normal controls from peripheral blood

Samples	*n*	Copy number					*P* value *vs* HNC
		Deletion			Amplification		
		0	1	2	3	> 3	
HNC	152	2	8	135	6	1	
ANT	142	3	7	124	5	3	0.776

ANT: Adjacent normal tissues; HNC: Healthy normal controls.

Table 2 Copy number variations of *NCOA3* in colorectal cancer tissues and matched adjacent normal tissues

CNVs, population	*n*	Copy numbers Amplification ⩽ 2	Amplification > 2	*P* value (*vs* ANT)	*P* value (*vs* Dukes A and B)
Total					
ANT	142	134	8	-	-
CRC		103	39	< 0.0001	-
Duke's A and B					
ANT	79	74	5	-	-
CRC		64	15	0.0167	-
Duke's C and D					
ANT	63	60	3	-	-
CRC		39	24	< 0.0001	0.0112

CRC: Colorectal cancers; CNVs: Copy number variations; ANT: Adjacent normal tissues.

(version 12). Data were analyzed by the χ^2 test or Fisher exact test. *P* values less than 0.05 were considered statistically significant. Results of the NCOA3 mRNA expression in normal and tumor tissue samples were compared using two-way repeated measurement ANOVA. One-way, repeated measurement analysis of variance (ANOVA-RM) was performed at a significance level of $P = 0.05$ to determine differences from controls within each group. Two-way analysis of variance (ANOVA-2) was performed after baseline subtraction, at a significance level of $P = 0.05$, to determine differences between the groups with amplified and unaltered NCOA3 copy number.

RESULTS

Gene copy number gains of NCOA3 in CRC samples

Since no statistical difference of CNVs between ANTs and healthy normal controls was observed, the adjacent normal tissues could be used as controls for the cancer tissues in this study (Table 1). Table 2 shows CNVs of NCOA3 in paired samples of CRCs and ANTs. A total of 142 CRC samples were examined. Thirty-nine (27.5%) of CRC samples showed gains of NCOA3. Less than 20% of the CRC tissue samples from patients with early-stage CRC (Dukes A and B) had either three, four, or more than four copies of the *NCOA3* gene, whereas more than 20% of the samples from patients with advanced (Dukes C and D) had either three, four, or more than four copies of NCOA3. There was a correlation between gene copy number gains and the tumor phenotypes ($P = 0.0112$).

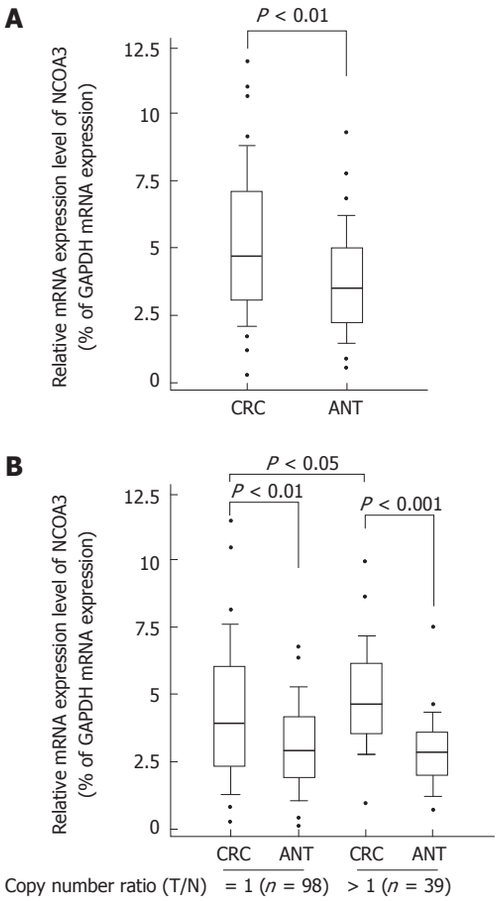

Figure 1 Real-time polymerase chain reaction assay was carried out as described under Materials and Methods section, and the results were obtained from indicated groups of samples. Boxplots of relative copy number of *NCOA3* mRNA measured with real-time polymerase chain reaction analysis showing the median; box: 25th-75th percentile; bars: largest and smallest values within 1.5 box lengths; little circles: outliers. A: mRNA expression level of NCOA3 in all the colorectal cancers (CRC) samples compared with adjacent normal tissues; B: mRNA expression level of NCOA3 in groups with amplified or unaltered DNA copies. ANT: Adjacent normal tissue; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase..

Positive correlation between copy number and mRNA over-expression of NCOA3 in CRCs

To find whether CNVs of NCOA3 have genotype-phenotype correlation, we compared the mRNA expression levels of NCOA3 between the CRC samples and matched adjacent normal tissues by quantitative real-time RT-PCR. As shown in Figure 1A, increased mRNA expression level of NCOA3 was observed in collected CRC tissues, which was consistent with some previous findings[6,18].

Gene CNVs can contribute to qualitative and quantitative diversities to their gene products. Next, we selected the samples with increased or unaltered copies of NCOA3 and tested whether the NCOA3 mRNA expression was correlated with the copy numbers. The samples with decreased copies of NCOA3 (5 of 142) were not included due to the small sample size. As shown in Figure 1B, the CRC samples in the group with increased or

unaltered copies of NCOA3 both showed an increased expression of mRNA compared with adjacent normal tissues ($P < 0.01$). There was a significant statistical difference between the CRC samples in the groups with increased and unaltered copies of NCOA3 ($P = 0.0023$). Thus the DNA copy gain at least plays a partial role in the over-expression of NCOA3 in CRCs.

DISCUSSION

Although cancer is mostly regarded as an acquired disease, familial predisposition plays a significant role in many cancer types. Several highly aggressive cancer predisposing genes correlated with CRC have been identified (DNA mismatch repair genes[19], APC, SMAD4, BMPR1A and MUTYH[20]). As yet, however, these genes explain only a fraction of the familial and/or hereditary cases of cancer. CNVs identified by CGH and array technology have clearly been shown to have the potential to directly or indirectly influence a healthy individual's susceptibility to cancer, for example by varying the gene dosage of tumor suppressors or oncogenes[21,22]. However, there are many discrepancies among previous studies which used high-resolution approaches to screen CNVs[23-27]. Thus, validation of such CNVs by a larger amount of clinical samples is required. In the present study, CNVs of NCOA3 were examined in a relatively high number of case-matched CRC samples. The results obtained here were more definitive than previous reports.

Examination of the CNVs of oncogenes or tumor suppressor genes is a starting point for investigations into the role of gene amplification in the colorectal carcinogenic process. In this study, no differences of NCOA3 gene copy number were found between the healthy normal controls and ANTs from CRC patients. Thus the CNVs of NCOA3 in CRCs were more likely acquired DNA aberrations. The frequency of gains of NACO3 in CRCs was consistent with previous studies, where gains of chromosomes 20q12 were reported in 20%-60% of CRCs, respectively[11,12]. There was a correlation between the frequency of DNA copy-number gains of NCOA3 and Dukes classifications of the CRC samples ($P = 0.0112$), which suggested the potential role of altered copy number of *NCOA3* gene in the progression of CRCs.

It is expected that the CNVs do have a genotype-phenotype correlation. Phenotypic effects of genetic differences, such as CNVs, are supposedly brought about by changes in expression levels[28,29]. We investigated the correlation between the NCOA3 mRNA expression and the copy numbers of its DNA. Contrary to our expectation, the correlation was not as positive as predicted, although a statistical difference was found. mRNA expression of NCOA3 was increased in both the groups of increased and unchanged DNA copies. There was a statistical difference of mRNA expression between the groups of increased and unaltered DNA copies ($P < 0.05$). Thus CNVs did play a role of over-expression of the NCOA3 mRNA in CRCs while there were also other mechanisms involved. This is consistent with two recent reports which assessed an over-representation of differentially expressed genes among CNV-mapping transcripts, showing a weak yet significant positive correlation between relative expression level and gene dosage[30,31].

In a small percentage of samples, the recorded relative expression levels were inversely correlated with copy numbers (data not shown here). Such kinds of inverse correlation were also observed by other previous studies[30,31]. The mechanism of this phenomenon is still poorly understood but may be explained by two models. In the first model, a negative correlation between the number of copies and relative expression is explained by immediate early genes, which induced the expression of a repressor directly or indirectly. This expression, by a negative feedback loop, reduces or even abolishes the expression of the *CNV* gene. In the second model, the extra copies of a gene impair, through steric hinderance, their access to a specific transcription factory, where this particular locus should be transcribed.

Taken together, our findings showed that the CNVs of NCOA3 had a correlation with CRC progression as well as gene expression and might have the potential to serve as a prognostic marker for colorectal malignancies. However, the functional consequences of CNVs, the different features of CNVs between colorectal and other gastrointestinal malignancies and the underlying mechanisms of the heterogeneous expression levels, need to be extensively investigated in the future.

ACKNOWLEDGMENTS

We thank Shenzhen Biomedical Research Support Platform for the technical help.

COMMENTS

Background

NCOA3 is a member of the p160 nuclear receptor co-activator family and is considered an important oncogene. The chromosome locus of *NCOA3* gene, 20q12, is frequently amplified in colorectal cancers (CRCs) and the functional impact of such regions needs to be extensively investigated in a large size of clinical samples.

Research frontiers

Fluorescence *in situ* hybridization and array-CGH are the regular methods for the detection of genomic imbalances in tumors. However, the obtained data from these approaches are generally informative but not definitive. Recently, studies using quantitative real-time polymerase chain reaction can provide more precise data on the analysis of gene copy number.

Innovations and breakthroughs

To date, there has been a limited number of studies on the copy number variation (CNV) of *NCOA3* gene in tumors. The present study employed a more sensitive method to investigate the copy number variation of the targeted gene in a larger number of CRC samples. Furthermore, the authors elucidated the potential correlation between gene copy number and tumor progression.

Applications

This study indicated the different mechanisms of NCOA3 regulation in CRCs. This may impact the efficacy of NCOA3 targeted therapies.

Terminology

CNVs are alterations of the DNA of a genome that results in the cell having an abnormal number of copies of one or more sections of the DNA. CNVs cor-

respond to relatively large regions of the genome that have been deleted (fewer than the normal number) or duplicated (more than the normal number) on certain chromosomes.

Peer review

The authors investigated the role *NCOA3* gene in CRC. Authors detected copy number gain of NCOA3 in a relatively high percentage of CRC samples and correlated them with Dukes stage of CRC. Moreover, they observed a positive correlation between CNV gain and mRNA levels of *NCOA3* gene. The study is original and potentially interesting.

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S- Editor Tian L **L- Editor** Ma JY **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i8.861

World J Gastroenterol 2012 February 28; 18(8): 861-864
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

A case of Cowden syndrome diagnosed from multiple gastric polyposis

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Received: July 29, 2011 Revised: September 23, 2011
Accepted: January 7, 2012
Published online: February 28, 2012

Abstract

Cowden syndrome is a rare autosomal dominant disorder that is characterized by multiple hamartomas in a variety of tissues and this is associated with germline mutations in the phosphatase and tensin homologue (*PTEN*) gene, which is the tumor suppressor gene located on chromosome 10q23.3. It is characterized by multiple hamartomatous neoplasms of the skin, oral mucosa, gastrointestinal (GI) tract, bones, central nervous system, eyes, and genitourinary tract. Cowden syndrome does not have increased risk of GI malignancy; however, it has an increased risk of breast, thyroid and endometrial cancer development. Here the authors report a rare case of Cowden syndrome incidentally diagnosed from multiple gastric polyposis. A 29-year-old woman presented with multiple gastric polyps. The laboratory results were normal except for mild anemia, with a hemoglobin level of 11.9 g/dL. Esophagogastroduodenoscopy revealed multiple gastric, duodenal polyps and esophageal acanthosis. Colonoscopy revealed possible hamartomatous polyps in the rectum. Under the suspicion of Cowden syndrome, sonography of the thyroid and breasts was carried out, which revealed multiple thyroid masses. Subsequent fine-needle aspiration biopsy revealed the presence of clusters of follicular epithelial cells, and due to the possibility of malignancy, the patient underwent total thyroidectomy. The pathology was reported as invasive follicular carcinoma. A gene study by direct sequencing showed the presence of a *PTEN* mutation (c.633C > A /p.Cys211*).

Key words: Cowden syndrome; Gastric polyposis; Phosphatase and tensin homologue mutation; Esophageal acanthosis; Thyroid cancer

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Ha M, Chung JW, Hahm KB, Kim YJ, Lee W, An J, Kim DK, Kim MG. A case of Cowden syndrome diagnosed from multiple gastric polyposis. *World J Gastroenterol* 2012; 18(8): 861-864 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/861.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.861

INTRODUCTION

Cowden syndrome is a rare autosomal dominant disorder that is characterized by multiple hamartomas in a

variety of tissues, and is associated with germline mutations in the phosphatase and tensin homologue (*PTEN*) gene, which is a tumor suppressor gene located on chromosome 10q23.3[1,2]. It is characterized by multiple hamartomatous neoplasms of the skin, oral mucosa, gastrointestinal (GI) tract, bones, central nervous system, eyes and genitourinary tract. Cowden syndrome does not have an increased risk of GI malignancy; however, it has an increased risk of developing breast, thyroid and endometrial cancer[3]. Here we present a case of Cowden syndrome which was confirmed through genetic testing of the *PTEN* gene in a 29-year-old female patient with multiple gastric polyps on esophagogastroduodenoscopy (EGD), and thyroid cancer.

CASE REPORT

A 29-year-old woman presented with multiple gastric polyps, which were detected at a private clinic. On presentation, she complained of no symptoms other than dyspepsia; her vital signs were stable and the laboratory results were normal except for mild anemia, with a hemoglobin level of 11.9 g/dL.

Multiple gastric, duodenal polyps and esophageal acanthosis were observed on EGD, and colonoscopy revealed possible hamartomatous polyps in the rectum and oral mucosal papillomatosis (Figure 1). The polyps, observed through EGD and colonoscopy, were shown upon pathologic analysis to be hamartomatous polyps accompanied by mild inflammation and fibrosis (Figure 2). She also had papillomatous papules around her lips and oral mucosal papillomatosis. Because of a high index of suspicion of Cowden syndrome, ultrasonography (USG) of the thyroid and breasts was carried out, which revealed multiple thyroid masses (Figure 3A). Fine-needle aspiration biopsy revealed the presence of clusters of follicular epithelial cells. Although benign lesions were expected on USG, because of the possibility of malignancy, left lobectomy and right enucleation with ipsilateral central node dissection were carried out. Pathology showed the lesions to be invasive follicular carcinoma, and right lobectomy with ipsilateral central node dissection (completion total thyroidectomy) was carried out (Figure 3B).

Genetic analysis of the *PTEN* genes was performed. Genomic DNA was extracted from the patient's whole blood. Polymerase chain reaction direct sequencing with primers targeting all the coding regions and flanking introns was performed. Sequencing analysis of the *PTEN* gene revealed a heterozygous transition of C to A at nucleotide 633 in exon 6 (NM_000314.4 c.633C > A), which is a nonsense mutation, making a stop codon (p.Cys211*) (Figure 4). This mutation has been previously reported and seems to be the cause of the disorder in this patient[4]. During the course of the evaluation of the patient, the authors investigated other members of the patient's family, and a history of endometrial cancer was identified in the patient's mother. However, she refused further evaluation related to Cowden syndrome, and thus further examination could not be carried out.

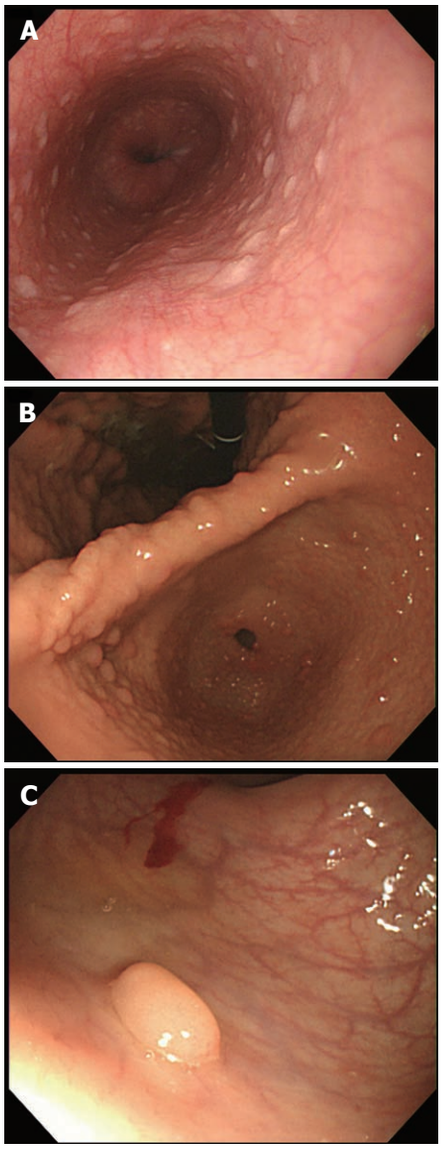

Figure 1 Endoscopic examination. A: Esophageal acanthosis was noted; B: Multiple gastric polyps were noted on entire stomach; C: Several polyps were observed at the rectum.

DISCUSSION

Cowden syndrome was first described by Lloyd and Dennis in 1963 in a case report on a patient named Rachel Cowden[5]. Later, in 1972, Weary *et al*[6] confirmed the existence of this new syndrome through examination of five additional patients with similar clinical findings. The hamartomatous lesions observed in Cowden syndrome can arise in any of the three embryonic germ cell layers and thus it may be ectodermal, mesodermal or endodermal in origin[7]. These lesions can arise in the derivatives of any of the three embryonic germ cell layers[3,7]. The cardinal manifestations of Cowden syndrome include facial trichilemmomas, which are hamartomas

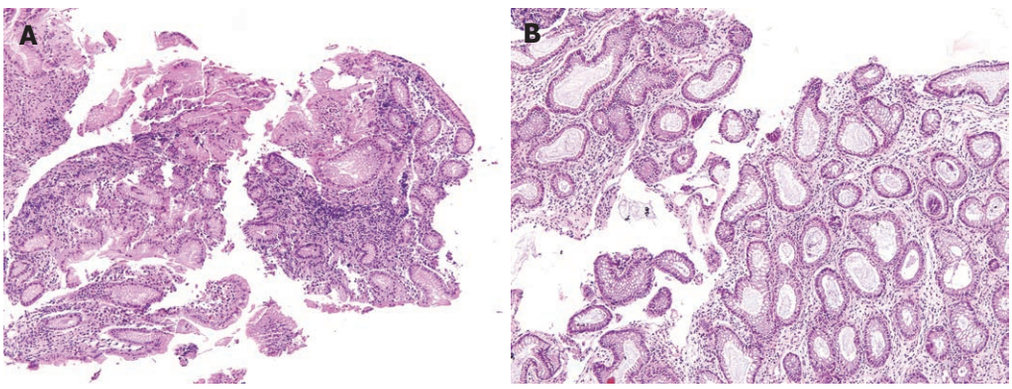

Figure 2 Microscopic examination (hematoxylin and eosin stain, × 100). A: Gastric polyp shows hyperplastic foveolar epithelium; B: Colon polyp exhibits dilated and branched crypts with slightly fibrotic stroma.

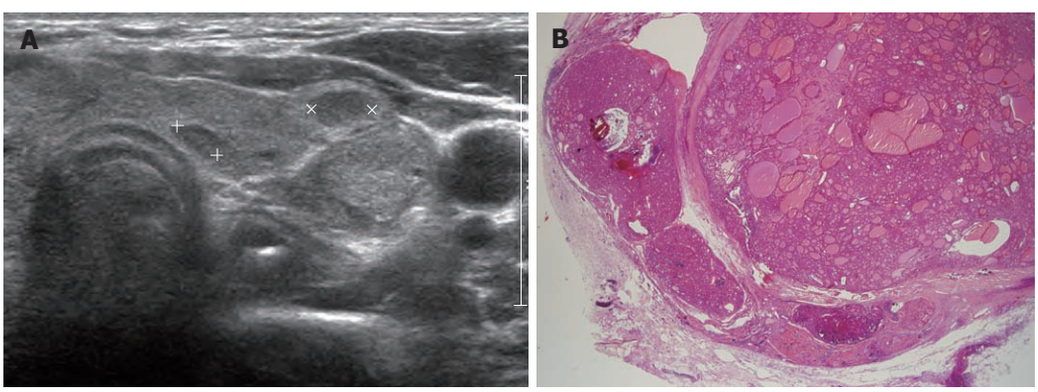

Figure 3 Thyroid masses. A: Multiple thyroid masses in thyroid ultrasonography; B: Postoperative thyroid pathology showed invasive follicular carcinoma (hematoxylin and eosin stain, × 12.5).

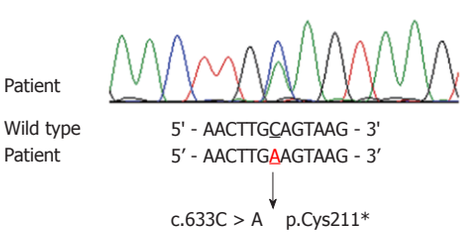

Figure 4 Sequencing result of the phosphatase and tensin homologue mutation. A heterozygous transition of C to A at nucleotide 633 in exon 6 (c.633C > A), making a stop codon (p.Cys211*).

of the infundibulum of the hair follicle, acral keratoses and mucocutaneous papillomatous papules[8-10]. The incidence of Cowden syndrome was estimated to be 1:1 000 000[1]. However, after identification of the relevant gene, the estimate for the prevalence of Cowden syndrome has been increased to between 1 in 200 000 and 1 in 250 000[11,12]. The diagnosis of Cowden syndrome is mainly based on clinical criteria. Nowadays the criteria of the International Cowden Consortium are commonly used for making the diagnosis[7]. The first set of clinical criteria were proposed by Salem *et al*[8], and have subsequently been revised, most recently in the United States by the National Comprehensive Cancer Network, which published the Cowden syndrome testing criteria based on pathognomonic criteria along with major and minor diagnostic criteria. In 1996, the International Cowden Consortium identified germline *PTEN* mutations as being the cause of Cowden syndrome[1,2]. Cowden syndrome is now well recognized as a highly variable, autosomal-dominant hereditary cancer susceptibility syndrome that is characterized by multiple hamartomas and an increased risk of developing malignant transformations.

Cowden syndrome is an autosomal dominant inherited disorder with incomplete penetrance and variable expressivity. Since the first case report of this disorder in Korea in 1997[2], 20 such cases have been reported, but most of these cases involved breast cancer and gynecological cancers, with few involving colon polyps[13]. In this current case study, Cowden syndrome was identified in a patient with multiple gastric polyps, as was reported in 2010, and in addition, thyroid cancer was diagnosed through screening and surgically resecting the tumor, and the *PTEN* mutation was confirmed through genetic testing (c.633C > A/p.Cys211*)[14].

Considering the worldwide prevalence of Cowden syndrome, the number of cases reported in Korea appears to be disproportionately small. Although this difference must be examined in the context of ethnicity, it

may also imply that some cases have gone undetected. However, since Cowden syndrome, as aforementioned, may involve malignancies in several organs, aggressive diagnostic evaluation should be carried out in suspected cases, and screening should be performed in light of the autosomal dominant inheritance of this disorder.

Finally, as was the case with this patient, adequate screening for the organs known to develop malignancies in Cowden syndrome should be carried out in suspected patients. Even when benign lesions are suspected, the potential for malignant lesions must be kept in mind, and further pathologic examination and surgical resection should be aggressively performed without reservation.

In conclusion, this case is a reminder of the importance of early screening for patients suspected of having Cowden syndrome, and female patients should be evaluated for lesions in the breasts and thyroid. Even if benign lesions are suspected, there have been published reports on cases diagnosed with cancer postoperatively; consequently, the possibility of malignancy should always be considered.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
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www.wjgnet.com

World J Gastroenterol 2012 February 28; 18(8): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2012 February 28; 18(8): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

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American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

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American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

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Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

November 9-13, 2012
American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel Diseases
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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2012 February 28; 18(8): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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Name of journal

World Journal of Gastroenterology

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

Editorial office
World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
Ocean International Center,
No. 62 Dongsihuan Zhonglu,
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-59080039
Fax: +86-10-85381893

Indexed and abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifer, and Directory of Open Access Journals. ISI, Thomson Reuters, 2010 Impact Factor: 2.240 (35/71 Gastroenterology and Hepatology).

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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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