World Journal of Gastroenterology®

Volume 18 Number 8
February 28, 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@baishideng.com
http://www.wjgnet.com

ISSN 1007-9327

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

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

Baishideng Publishing Group
www.wjgnet.com

WJG World Journal of Gastroenterology

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Almeida PRC, Ferreira FVA, Santos CC, Rocha-Filho FD, Feitosa RRP, Falcão EAA, Cavada BK, Lima-Júnior RCP, Ribeiro RA. Immunoexpression of cyclooxygenase-2 in primary gastric carcinomas and lymph node metastases.
World J Gastroenterol 2012; 18(8): 778-784
http://www.wjgnet.com/1007-9327/full/v18/i8/778.htm

AIM AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1352 experts in gastroenterology and hepatology from 64 countries.

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.

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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
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

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

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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.i8.727

World J Gastroenterol 2012 February 28; 18(8): 727-735
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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.

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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)
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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.

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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)
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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.

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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 INR: < 1.1 = 0; 1.1-1.4 = 1; > 1.4 = 2 CDS is the sum of the above (possible value, 0-11)
API	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.

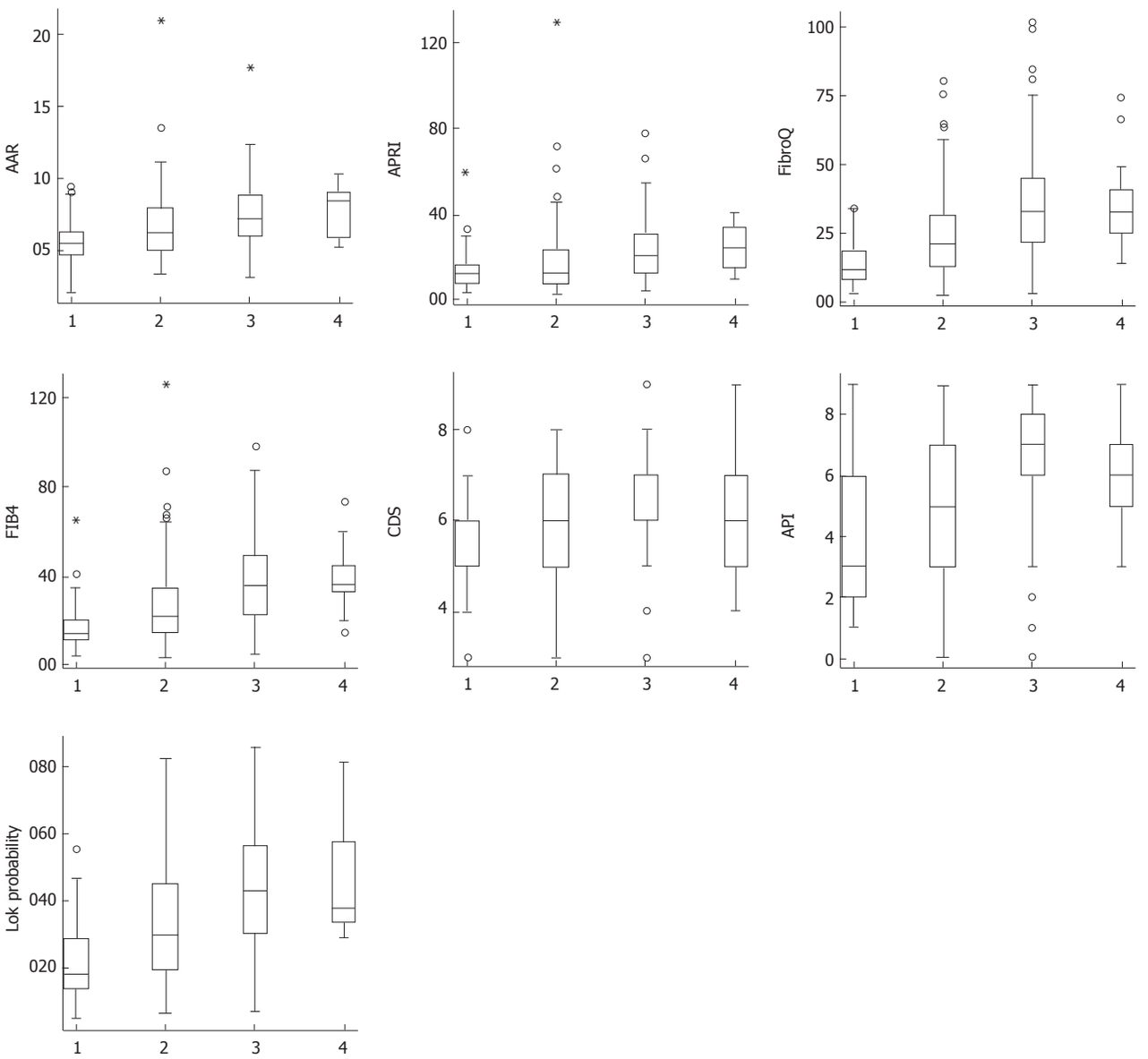

Figure 1 Score values according to Metavir fibrosis stages. Each outlier value is represented by a small circle symbol (o) in the Box Plot graph. If an outlier is more than 3 times the inter-quartile range away from Q1 or Q3, it is classified as an extreme outlier, asterisk sign (*). The top and bottom 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.

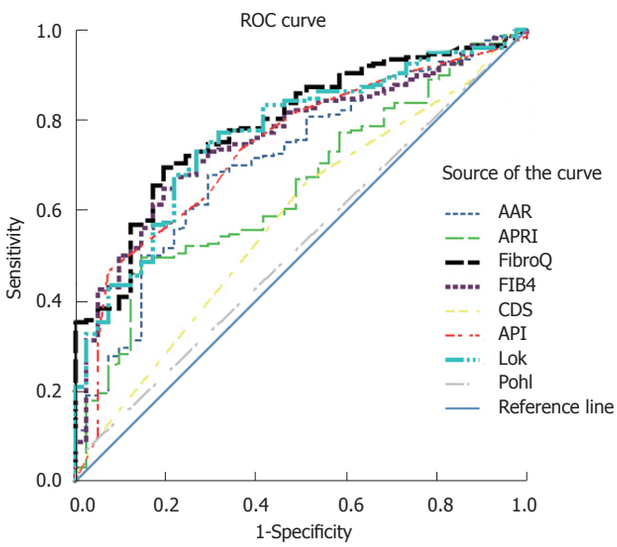

Figure 2 Receiver operating characteristic curves of simple noninvasive tests evaluated for prediction of significant fibrosis (F2, F3 and F4). AAR: AST/ALT ratio; APRI: AST-to-platelet ratio index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; API: Age-platelet index; CDS: Cirrhosis 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.

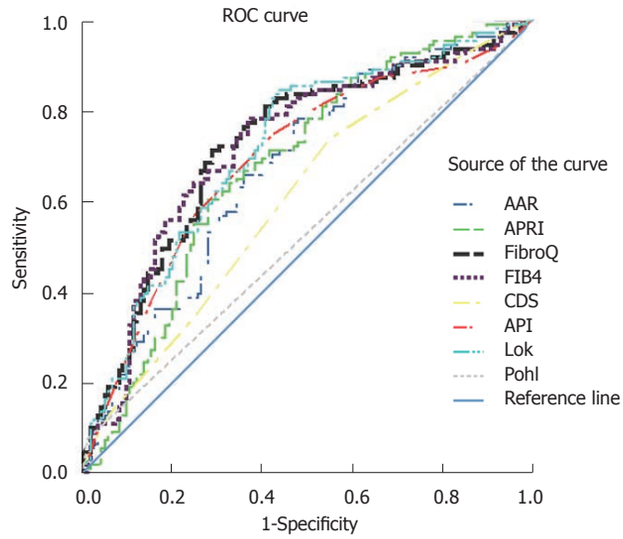

Figure 3 Receiver operating characteristic curves of simple noninvasive tests evaluated for prediction of extensive fibrosis (F3 and F4). AAR: AST/ALT ratio; APRI: AST-to-platelet ratio index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; API: Age-platelet index; CDS: Cirrhosis discriminant 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)
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ORIGINAL ARTICLE

Effects of resistin-like molecule β over-expression on gastric cancer cells *in vitro*

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Author contributions: Tong QS designed the study and drafted the manuscript; Zheng LD, Yang CL, Qi T, Qi M and Tong L performed the research; Zheng LD and Yang CL were also involved in drafting the manuscript and performed statistical analysis.
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.

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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, Mountain 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

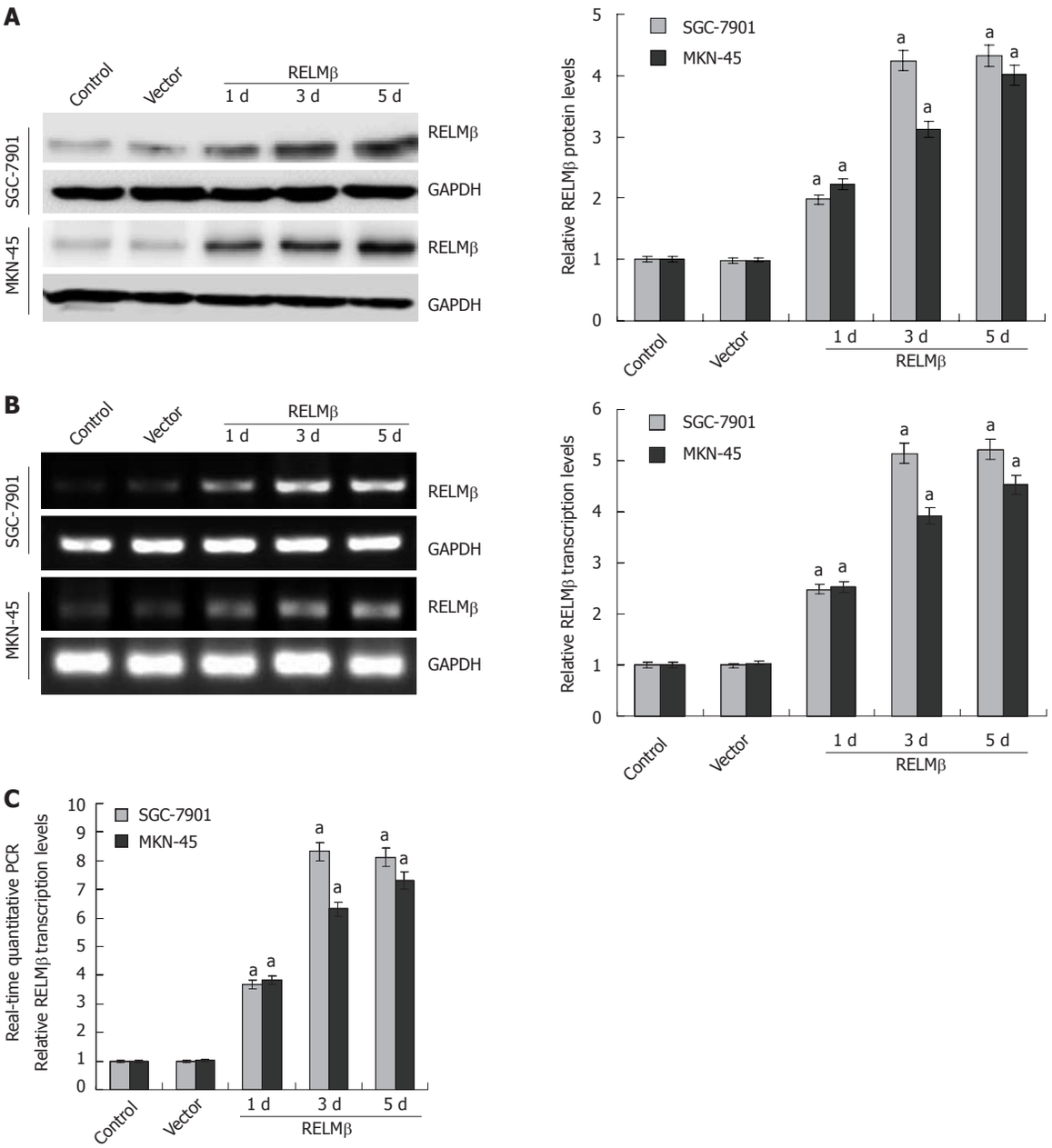

Figure 1 Transient transfection-mediated over-expression of resistin-like molecule β in gastric cancer cells. A: Western blotting indicated that low resistin-like molecule β (RELMβ) protein was detected in the parental SGC-7901 and MKN-45 cells, and transient transfection of the empty vector (mock) did 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 RELMβ 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

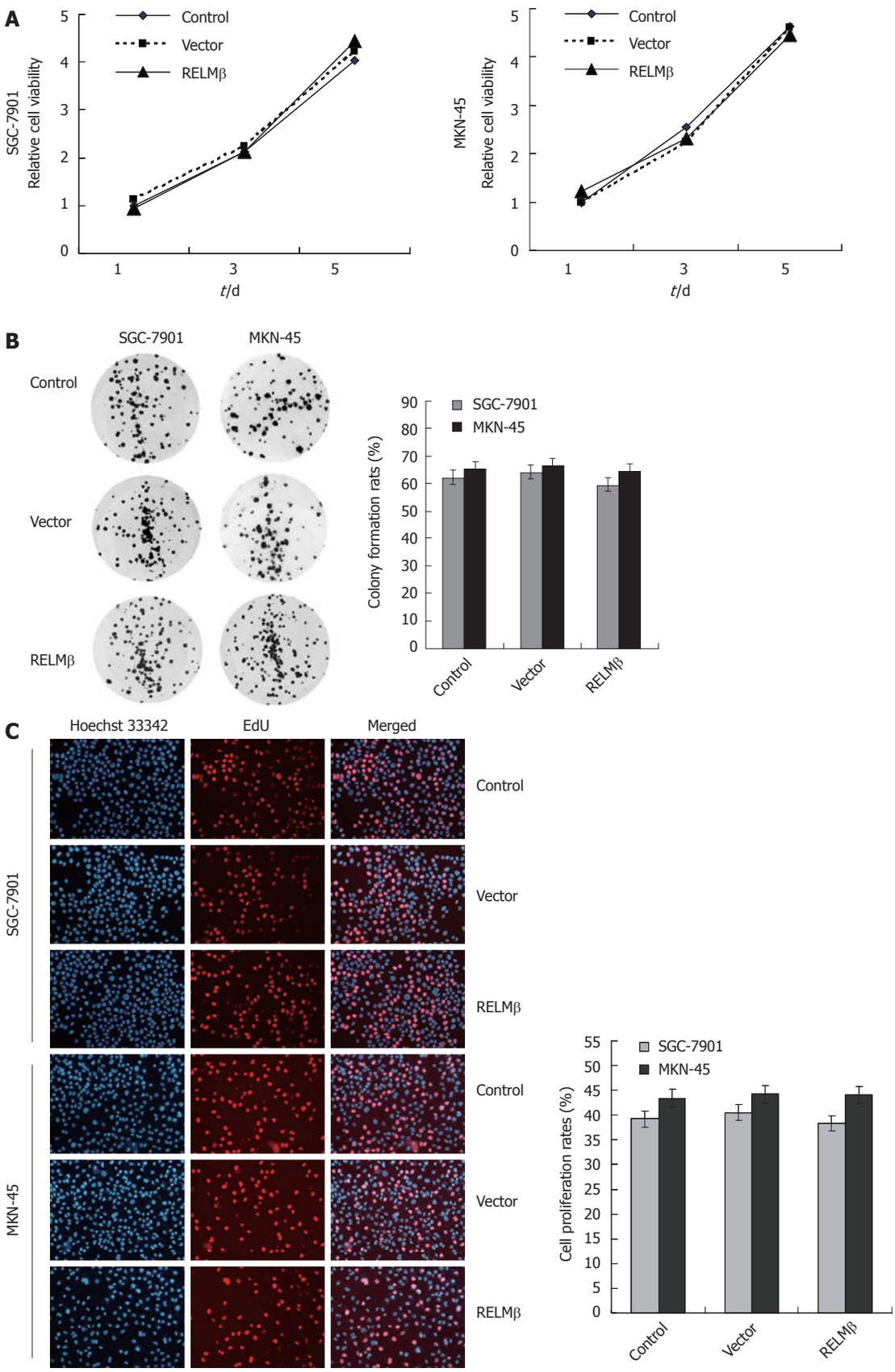

Figure 2 Upregulating resistin-like molecule β expression did not affect the *in vitro* proliferation of gastric cancer cells. A: In 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay, transfection of pcDNA3.1-resistin-like molecule β (RELMβ) or empty vector (mock) for 24 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.

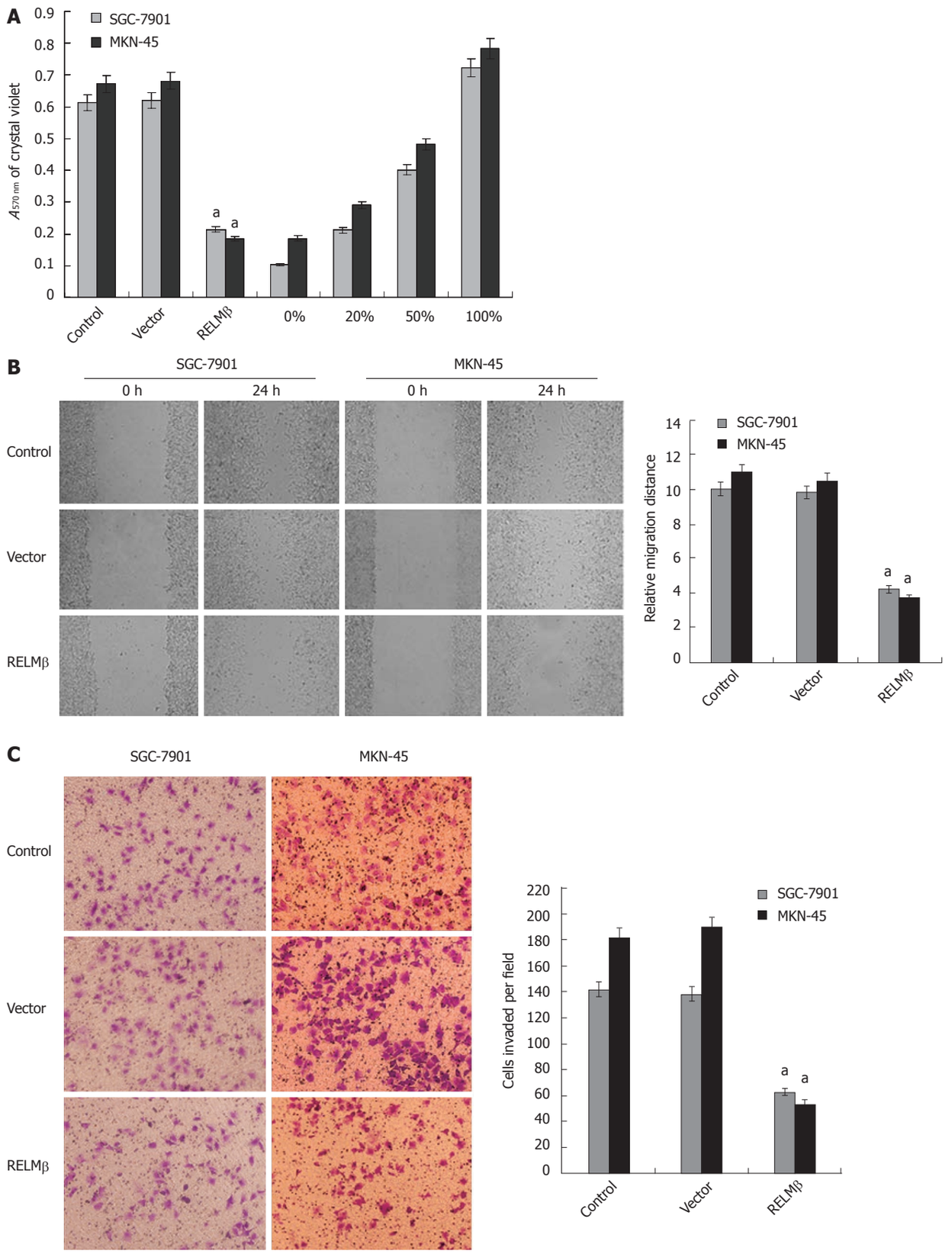

Figure 3 Over-expression of resistin-like molecule β attenuated the adhesion, migration and invasion of gastric cancer cells *in vitro*. A: In the adhesion assay, SGC-7901 and MKN-45 cells transfected with pcDNA3.1-resistin-like molecule β (RELMβ) for 72 h exhibited markedly reduced ability in adhesion 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.

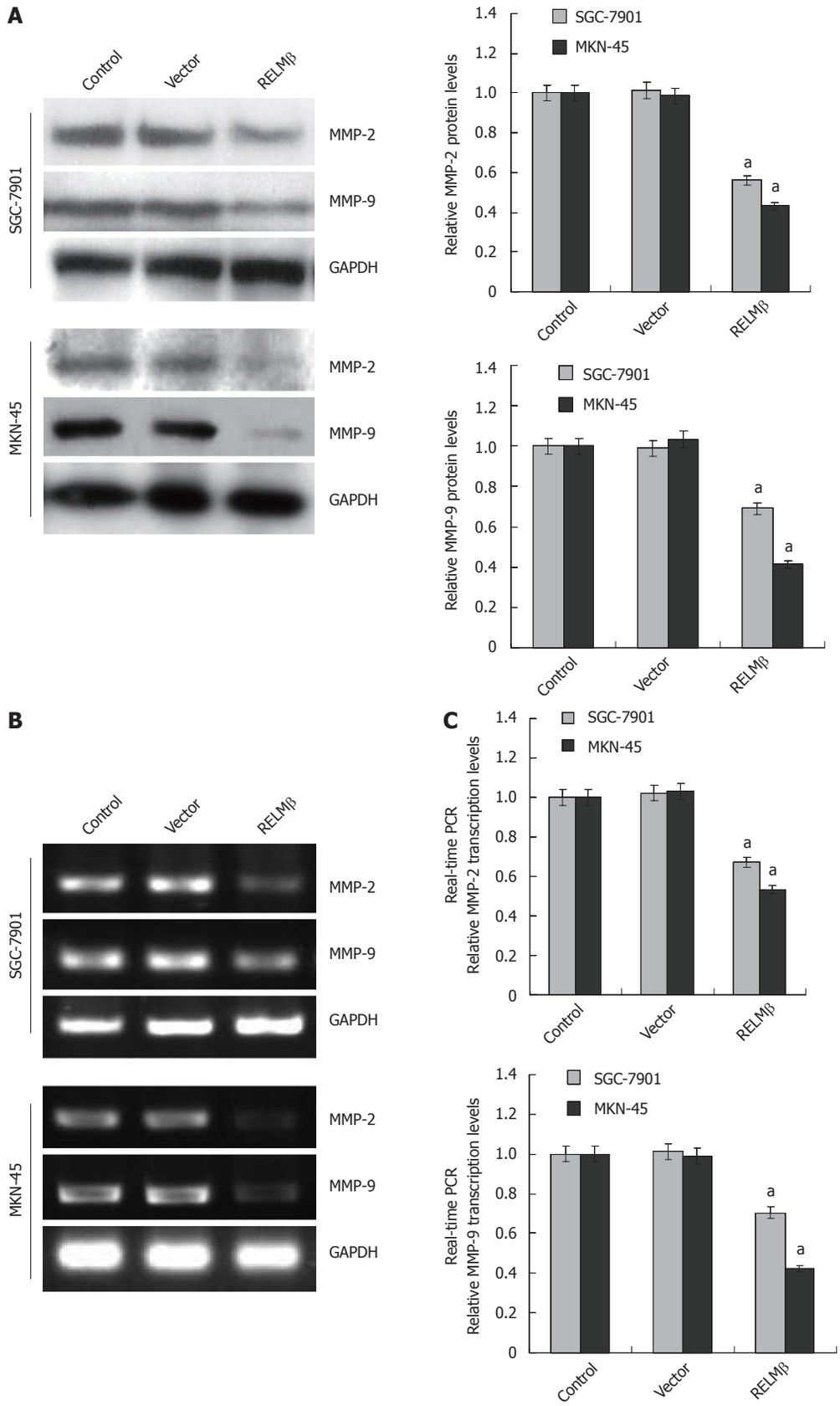

Figure 4 Over-expression of resistin-like molecule β decreased the expression of matrix metalloproteinase-2 and matrix metalloproteinase-9 in gastric cancer cells. A: Western blotting indicated that 72 h after transfection, over-expression of resistin-like molecule β (RELMβ) abolished the expression 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.

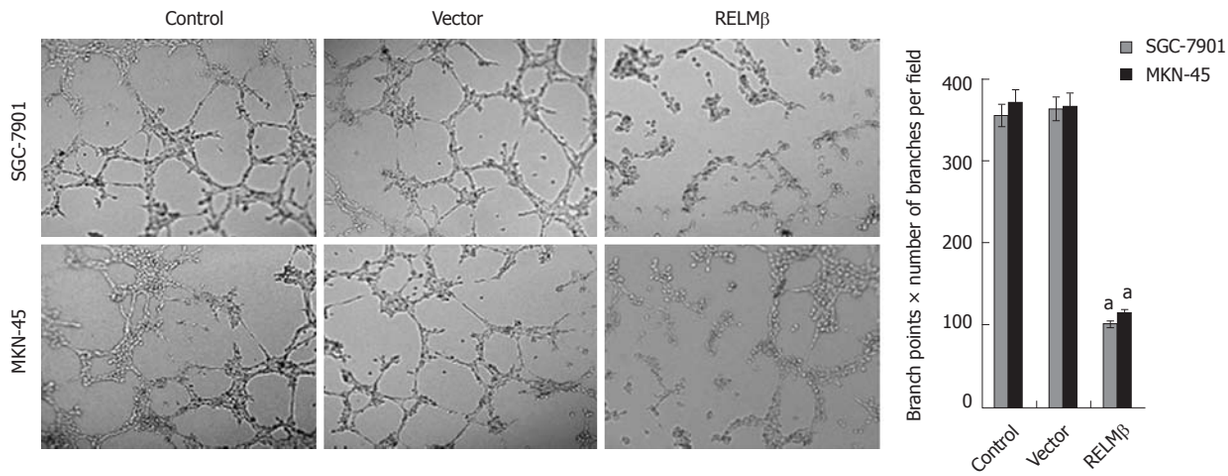

Figure 5 Over-expression of resistin-like molecule β inhibited the *in vitro* angiogenic capabilities of gastric cancer cells. Extensive tube formation of endothelial cells was observed in parental and empty vector (mock) groups. However, when the endothelial cells were treated by the medium preconditioned 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

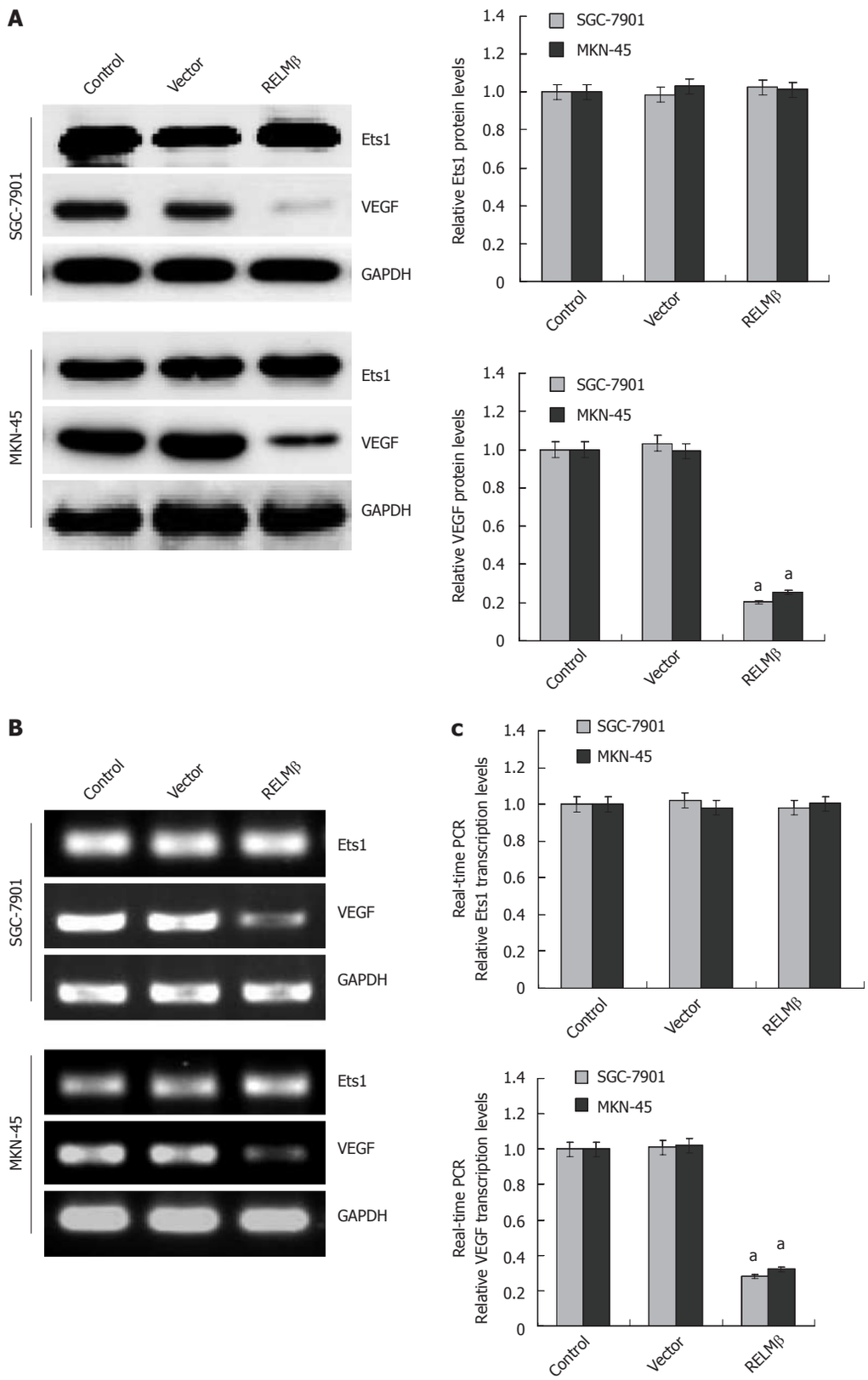

Figure 6 Over-expression of resistin-like molecule β decreased the expression of vascular endothelial growth factor, but not v-ets erythroblastosis virus E26 oncogene homolog 1, in gastric cancer cells. A: Western blotting indicated that 72 h after transfection, over-expression of resistin-like molecule 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)
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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.

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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

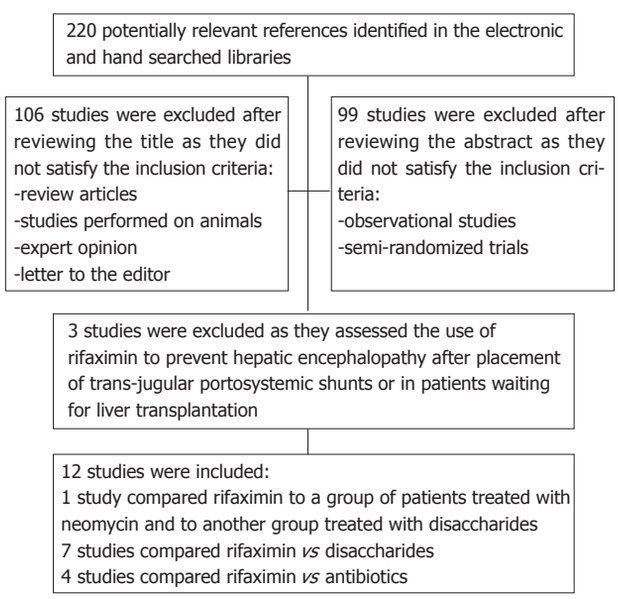

Figure 1 Flowchart of the included studies.

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

Study or subgroup	Rifaximin Events	Rifaximin Total	Control Events	Control Total	Weight (%)	Odds ratio M-H, random, 95% CI
6.1.1 Rifaximin *vs* disaccharides						
Fera *et al*[37]	20	20	16	20	5.4	11.18 [0.56, 222.98]
Festi *et al*[17]	9	9	12	12		Not estimable
Loguercio *et al*[39]	8	14	2	13	12.0	7.33 [1.16, 46.23]
Mas *et al*[36]	40	50	41	53	27.2	1.17 [0.45, 3.01]
Massa *et al*[18]	20	20	18	20	5.0	5.54 [0.25, 123.08]
Paik *et al*[21]	27	32	21	22	8.9	0.26 [0.03, 2.37]
Song *et al*[32]	32	39	18	25	21.4	1.78 [0.54, 5.88]
Subtotal (95% CI)		184		165	80.0	1.92 [0.79, 4.68]
Total events	156		128			
Heterogeneity: Tau² = 0.42; χ^2 = 7.89, df = 5 (*P* = 0.16); I^2 = 37%						
Test for overall effect: *Z* = 1.44 (*P* = 0.15)						
6.1.2 Rifaximin *vs* other antibiotics						
Di Piazza *et al*[33]	4	8	0	6	4.9	13.00 [0.55, 306.21]
Festi *et al*[17]	20	20	15	15		Not estimable
Miglio *et al*[38]	25	25	24	24		Not estimable
Parini *et al*[34]	11	15	10	15	15.2	1.38 [0.29, 6.60]
Pedretti *et al*[25]	0	0	0	0		Not estimable
Subtotal (95% CI)		68		60	20.0	2.77 [0.35, 21.83]
Total events	60		49			
Heterogeneity: Tau² = 0.97; χ^2 = 1.60, df = 1 (*P* = 0.21); I^2 = 38%						
Test for overall effect: *Z* = 0.97 (*P* = 0.33)						
Total (95% CI)		252		225	100.0	1.96 [0.94, 4.08]
Total events	216		177			
Heterogeneity: Tau² = 0.28; χ^2 = 9.56, df = 7 (*P* = 0.22); I^2 = 27%						
Test for overall effect: *Z* = 1.80 (*P* = 0.07)						

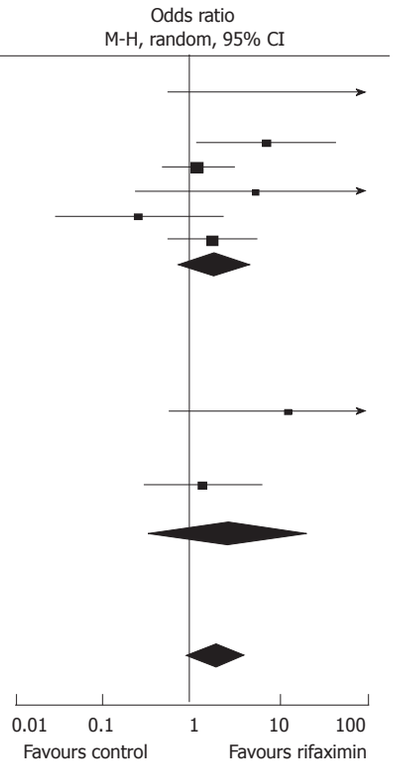

Figure 2 Efficacy of rifaximin *vs* oral disaccharides, rifaximin *vs* other oral antibiotics and rifaximin *vs* control therapy which included the combination of oral disaccharides and other antibiotics. M-H: Mantel Haenszel.

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-

Study or subgroup	Rifaximin Events	Rifaximin Total	Control Events	Control Total	Weight (%)	Odds ratio M-H, random, 95% CI
4.1.1 Diarrhea						
Bass *et al*[47]	15	140	21	159	9.0	0.79 [0.39, 1.60]
Bucci *et al*[35]	0	30	15	28	4.2	0.01 [0.00, 0.26]
Loguercio *et al*[39]	0	14	0	13		Not estimable
Mas *et al*[36]	2	50	1	53	5.0	2.17 [0.19, 24.67]
Massa *et al*[18]	0	20	13	20	4.1	0.01 [0.00, 0.26]
Miglio *et al*[38]	0	25	2	24	3.9	0.18 [0.01, 3.87]
Paik *et al*[21]	0	32	1	22	3.7	0.22 [0.01, 5.67]
Song *et al*[32]	0	39	1	25	3.7	0.21 [0.01, 5.28]
Subtotal (95% CI)		350		344	33.6	0.20 [0.04, 0.92]
Total events	17		54			
Heterogeneity: Tau² = 2.55; χ^2 = 17.61, df = 6 (P = 0.007); I^2 = 66%						
Test for overall effect: Z = 2.07 (P = 0.04)						
4.1.2 Abdominal pain						
Bass *et al*[47]	12	140	13	159	8.8	1.05 [0.46, 2.39]
Bucci *et al*[35]	2	30	1	28	5.0	1.93 [0.17, 22.53]
Fera *et al*[37]	0	20	10	20	4.1	0.02 [0.00, 0.46]
Loguercio *et al*[39]	0	14	0	13		Not estimable
Mas *et al*[36]	1	50	0	53	3.7	3.24 [0.13, 81.47]
Massa *et al*[18]	0	20	3	20	4.0	0.12 [0.01, 2.53]
Paik *et al*[21]	0	32	1	22	3.7	0.22 [0.01, 5.67]
Song *et al*[32]	1	39	0	25	3.7	1.99 [0.08, 50.71]
Subtotal (95% CI)		345		340	33.0	0.60 [0.18, 1.98]
Total events	16		28			
Heterogeneity: Tau² = 0.99; χ^2 = 10.21, df = 6 (P = 0.12); I^2 = 41%						
Test for overall effect: Z = 0.84 (P = 0.40)						
4.1.3 Nausea/anorexia/weight loss						
Bass *et al*[47]	20	140	21	159	9.1	1.10 [0.57, 2.12]
Bucci *et al*[35]	2	30	24	28	6.5	0.01 [0.00, 0.07]
Fera *et al*[37]	0	20	5	20	4.1	0.07 [0.00, 1.34]
Mas *et al*[36]	0	50	1	53	3.7	0.35 [0.01, 8.71]
Massa *et al*[18]	0	20	12	20	4.1	0.02 [0.00, 0.31]
Miglio *et al*[38]	2	25	2	24	5.9	0.96 [0.12, 7.40]
Subtotal (95% CI)		285		304	33.4	0.14 [0.02, 1.04]
Total events	24		65			
Heterogeneity: Tau² = 4.73; χ^2 = 31.41, df = 5 (P < 0.00001); I^2 = 84%						
Test for overall effect: Z = 1.92 (P = 0.06)						
Total (95% CI)		980		988	100.0	0.27 [0.12, 0.59]
Total events	57		147			
Heterogeneity: Tau² = 1.65; χ^2 = 60.65, df = 19 (P < 0.00001); I^2 = 69%						
Test for overall effect: Z = 3.26 (P = 0.001)						
Test for subgroup differences: Not applicable						

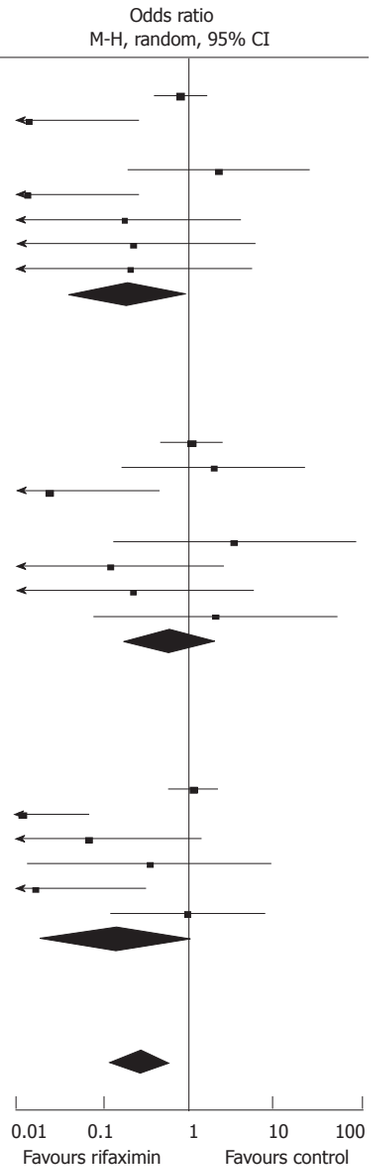

Figure 3 Adverse events experienced by patients treated with rifaximin *vs* control therapy. M-H: Mantel Haenszel.

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.

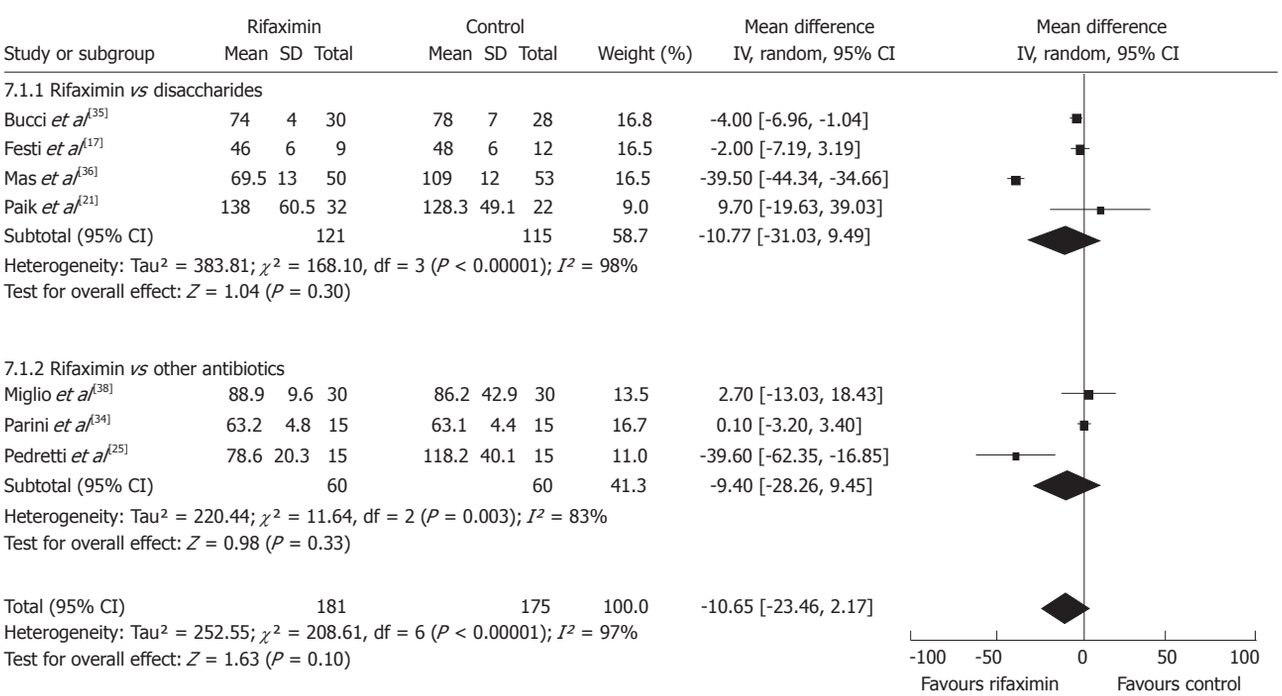

Figure 4 Serum ammonia levels at the end of the treatment protocols: rifaximin *vs* oral disaccharides, rifaximin *vs* other oral antibiotics and rifaximin *vs* control therapy which included the combination of oral disaccharides and other antibiotics. IV: Inverse variance.

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)
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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.

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Key words: Cyclooxygenase-2; Gastric carcinoma; Immunoexpression; Lymph node metastases; Tissue microarray

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Almeida PRC, Ferreira FVA, Santos CC, Rocha-Filho FD, Feitosa RRP, Falcão EAA, Cavada BK, Lima-Júnior RCP, Ribeiro RA. Immunoexpression of cyclooxygenase-2 in primary gastric carcinomas and lymph node metastases. *World J Gastroenterol* 2012; 18(8): 778-784 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/778.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.778

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.

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.

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.

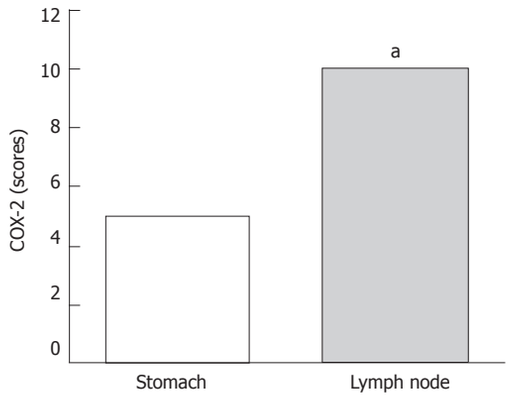

Figure 1 Cyclooxygenase-2 expression in gastric cancer, diffuse histotype. Lymph node expression is significantly higher than in the stomach (Median scores). [a]*P* = 0.0108, Mann-Whitney test. COX-2: Cyclooxygenase-2.

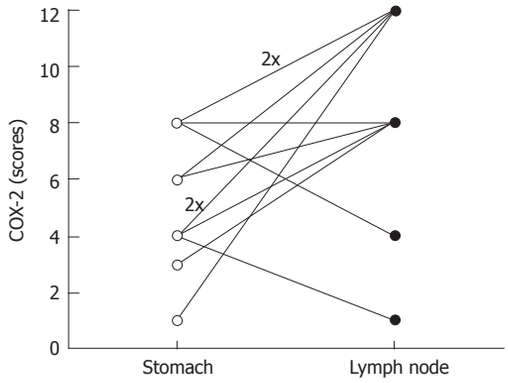

Figure 2 Cyclooxygenase-2 expression in primary diffuse gastric cancer and respective metastasis. Lymph node expression is higher than stomach expression levels in most cases (Individual case scores). *P* = 0.0137, Wilcoxon paired test. 2x means two overlaid cases. COX-2: Cyclooxygenase-2.

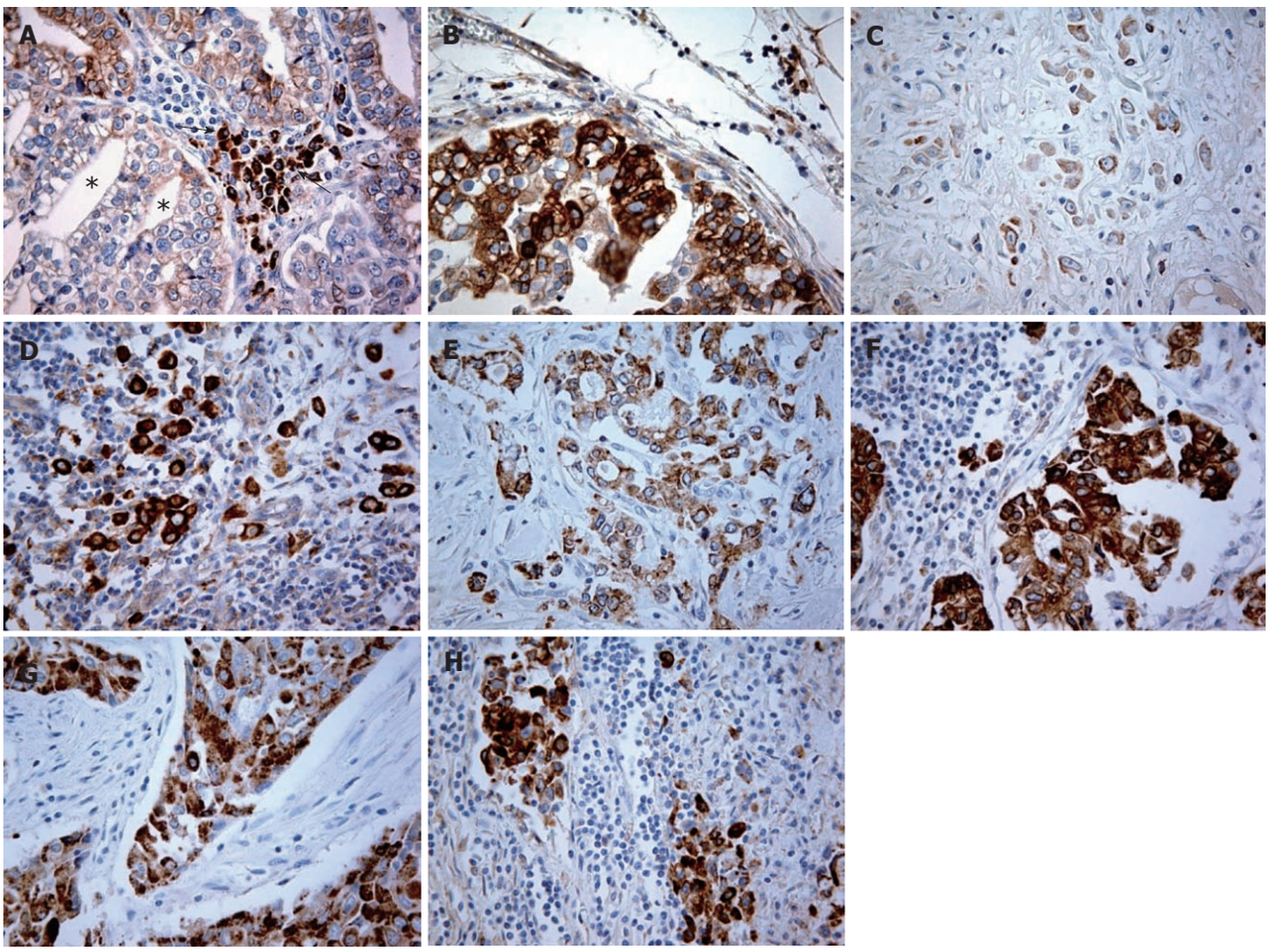

Figure 3 Cyclooxygenase-2 expression in gastric cancer. A, B: Intestinal type, case 12; A: Stomach = score 1, cancer cells were negative or scarcely stained (*). Strong cyclooxygenase-2 staining of inflammatory cells (mononuclear) into the stroma (arrows); B: Lymph node = score 8; C, D: Diffuse type, 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

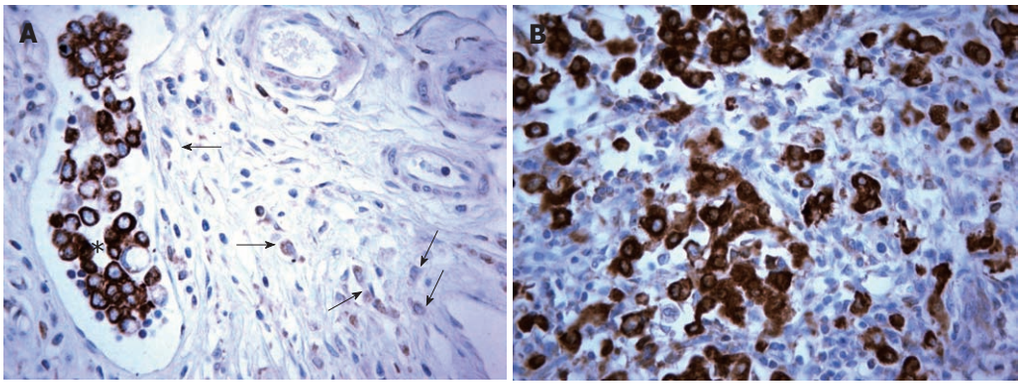

Figure 4 Cyclooxygenase-2 expression in gastric cancer and diffuse type: primary tumor, emboli and metastasis. A: Stomach: Low cyclooxygenase-2 (COX-2) expression in perivascular malignant cells (arrows). Tumor embolus with strongly stained neoplastic cells (*); B: Lymph node: High COX-2 expression at 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)
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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.

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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

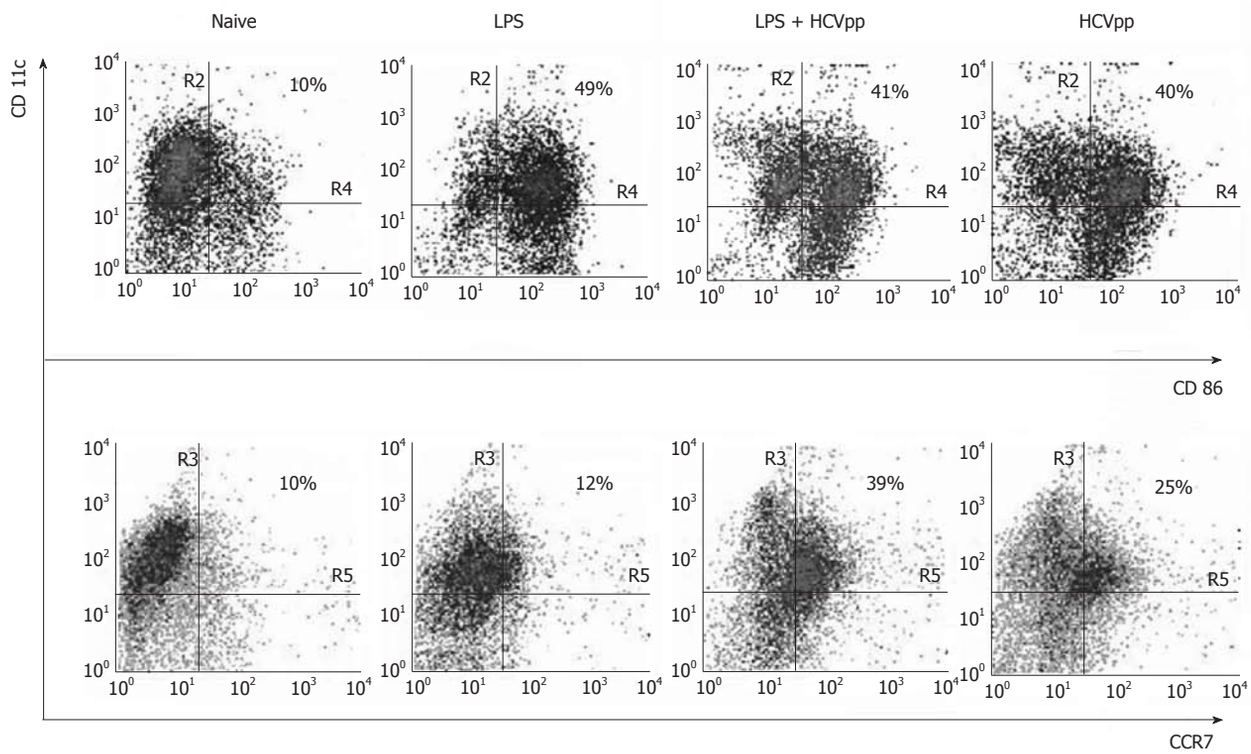

Figure 1 Fluorescence activated cell sorting analysis of bone-marrow derived dendritic cell of BALB/c mice, identified by expression of CD11c, after 7 d of maturation, followed by incubation with different agents. Column one shows the negative control, columns two and three show dendritic cell (DC) incubated 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.

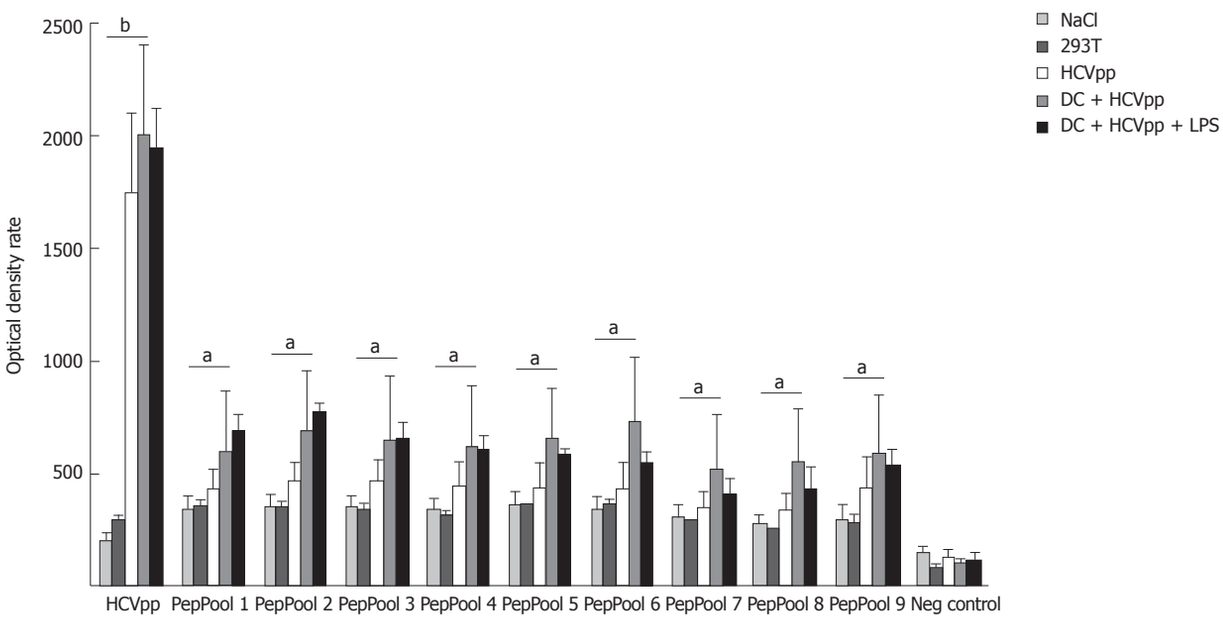

Figure 2 Induction of anti-E1 and anti-E2 antibodies following s.c. immunization of BALB/c mice. All animals specifically vaccinated developed specific antibodies. Highest antibody titers were observed in the two groups of mice which received the dendritic cell (DC) based vaccines. The negative control 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. $^{a}P < 0.05$, $^{b}P < 0.001$.

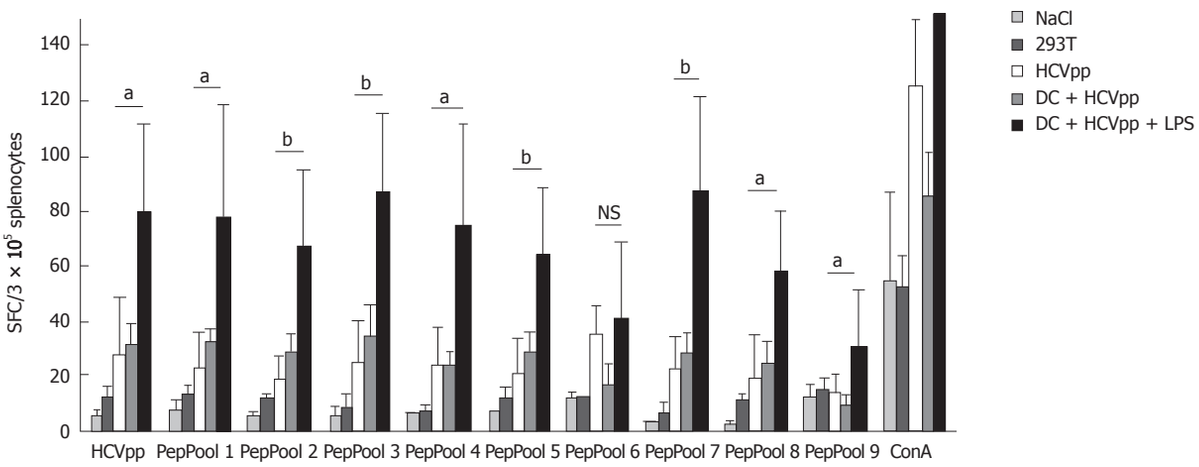

Figure 3 Antigen specific T-cell responses detected by interferon-gamma enzyme-linked immunosorbent spot test. Immune responses were induced by vaccination of different BALB/c mice with different agents. The two negative control groups were vaccinated with saline or 239T supernatant. The third group 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)
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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.

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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

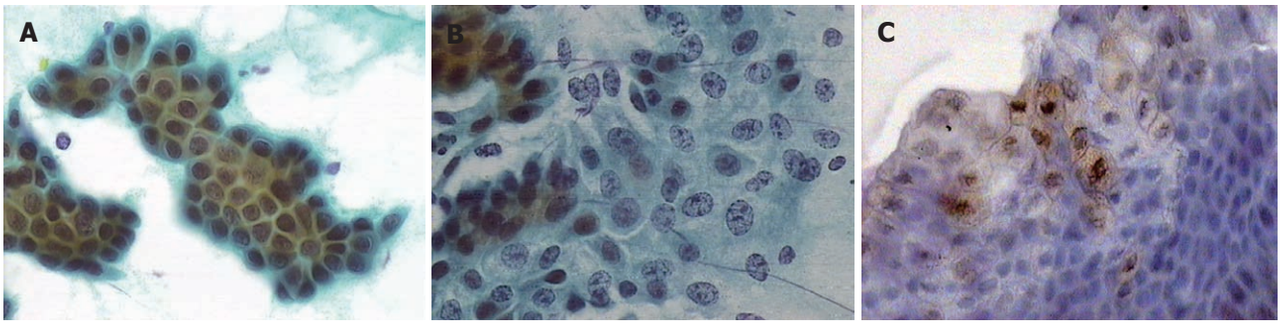

Figure 1 Brush cytology samples. A: This is a sample that was evaluated as negative for malignancy. Note the smooth nuclear shape, the low nuclear/cytoplasmic ratio and the absence of nucleoli (Papanicolaou stain, x 400); B: This is a sample that was classified as atypical-indeterminate. Note the moderate 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.

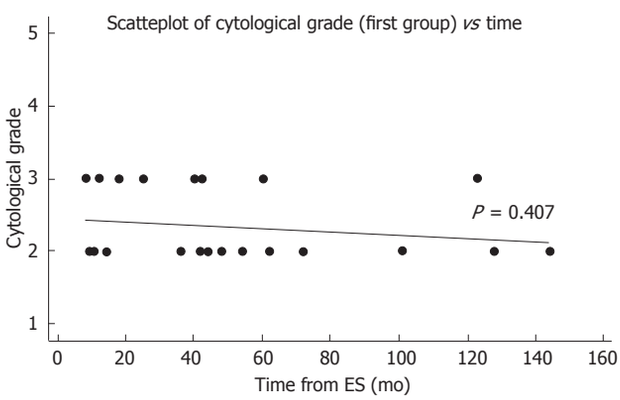

Figure 2 Scatterplot with regression of the cytological evaluation of the first group *vs* time (mo) after endoscopic sphincterotomy. The cytological grade is according to Table 2. 3: Atypical/indeterminate sample; 2: Negative for malignancy sample. ES: Endoscopic sphincterotomy. (Figure design was prepared 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)
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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.

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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

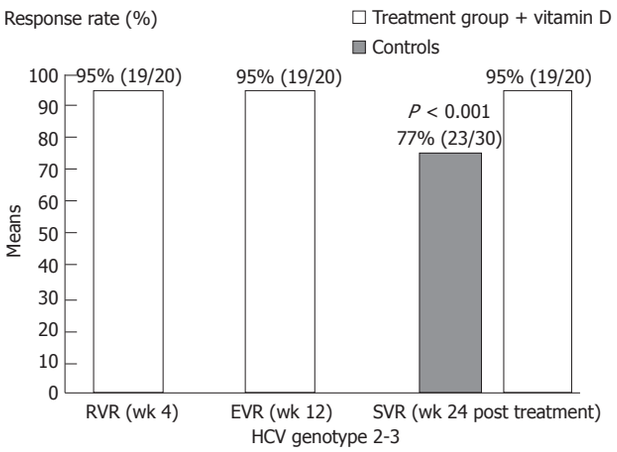

Figure 1 Rate of rapid virologic response, early virologic response and sustained viral response in the treatment (*n* = 20) and control (*n* = 30) groups. Rapid virologic response (RVR) was defined as undetectable hepatitis C virus (HCV) RNA at 4 wk during treatment. Early virologic response (EVR) was 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].

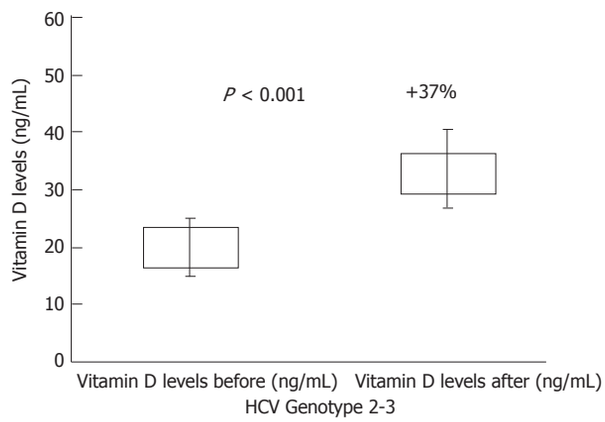

Figure 2 Vitamin D serum levels before and 12 wk after initiation of antiviral treatment (*n* = 30) and vitamin D supplementation (*n* = 20). Percentage change was +37%.

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)
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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.

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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).

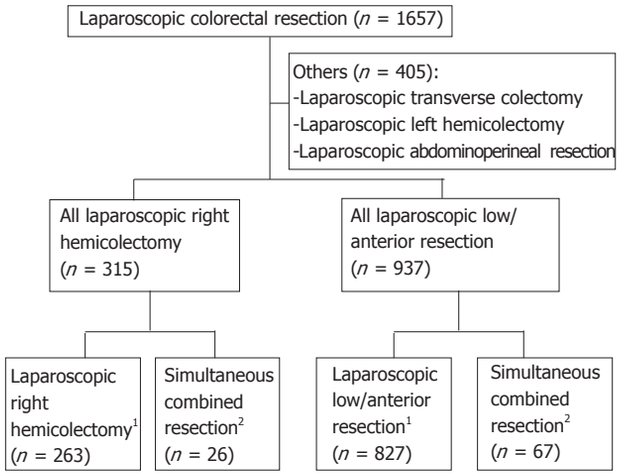

Figure 1 Algorithm of enrolled patients (number of patients). [1]Satisfied with inclusion criteria; [2]Type of combined rection was explained in Figure 2.

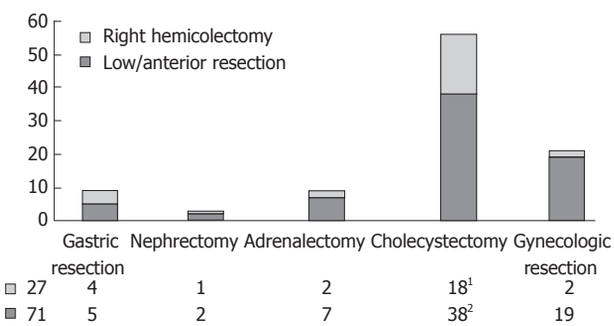

Figure 2 Type of simultaneous combined resection. [1]One case was adrenalectomy with cholecystectomy; [2]Two cases were cholecystectomies with adrenalectomies, 1 case was gastric resection and 1 case was gynecologic resection.

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 preoperatively: 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)
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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.

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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′ 5′ taagtcgtagcccttaagtgag 3′	339	50
Exon 2	5′ tgtatgagcctgtaagacaaag 3′ 5′ tagtttccagaacagagaaagg 3′	315	50
Exon 3	5′ gggaattcaaagagatttgg 3′ 5′ gaggtctctgcaggtaaaatag 3′	373	50
Exon 4	5′ ctcatcgttgccacatatta 3′ 5′ gtactcaagatctctgccaaaa 3′	319	50
Exon 5	5′ tccccttgggattagtatctat 3′ 5′ cccacaaaagccaatagtcat 3′	306	50
Exon 6	5′ cactatcttaagacctcgcttt 3′ 5′ gctaaaccttgaccagaaacta 3′	325	50
Exon 7	5′ agaggagtctgtgttttgattc 3′ 5′ atcataaccttatctccaccag 3′	367	50
Exon 8	5′ atagtttgctggtggagataag 3′ 5′ agcctgtgtatttgactaaagc 3′	306	50
Exon 9	5′ agttagtttatgggaaggaacc 3′ 5′ aagaaccagactccaacagtc 3′	438	50
Exon 10	5′ atagtaagatagtgggctggaa 3′ 5′ tacctgtaagaagggacagaac 3′	432	62
Exon 11	5′ tatttgaacacactcagactcg 3′ 5′ taggaacaacagcacaatacc 3′	476	62
Exon 12	5′ atacagactttgctaccaggac 3′ 5′ agagagaagatgcaagtgattc 3′	475	50
Exon 13	5′ tgtcagataagcagtctaccag 3′ 5′ ttagtaaaggaagaggagcttg 3′	468	50
Exon 14	5′ cctcatttgtgttcgttttc 3′ 5′ gtcgaacttggatttgaaac 3′	411	50
Exon 15	5′ catgtttcagggattacttctc 3′ 5′ ggctaccaaatgactattttgc 3′	356	50
Exon 16	5′ gtatatgggggatgagtgttac 3′ 5′ cagaagtataagaatggctgtc 3′	319	50
Exon 17	5′ gtgctgtttgtcaggatgaa 3′ 5′ tgaatattagtggggatgagag 3′	430	50
Exon 18	5′ gtctgtgatctccgtttagaat 3′ 5′ ctacatcagcagatggaagtaa 3′	358	50
Exon 19	5′ caggacaccagtgtatgttg 3′ 5′ ctgtgacatgttcaagacctc 3′	497	50

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′ 5′ actccgtgatcacaagttca 3′	632	58
Exon 2	5′ gtggaactataagtccagtaagc 3′ 5′ cagccaaactgcaacttttt 3′	457	50
Exon 3	5′ agaatcgattgaacccttga 3′ 5′ gtcaattaaagagcctttcc 3′	554	62
Exon 4	5′ acatcatatcagtgtcttgcac 3′ 5′ taatccatgtacctgattctcc 3′	302	50
Exon 5	5′ gaaggaacaccaaggaaaat 3′ 5′ ctgaaaaaggttaagggctctg 3′	273	50
Exon 6	5′ ggtcttaggaagaggaactttt 3′ 5′ gtataatcatgtgggtaactgc 3′	337	50
Exon 7	5′ ttgagacttacgtgcttagttg 3′ 5′ tctgaatgtgtcctaagagtga 3′	391	50
Exon 8	5′ aactttggagacctactgtact 3′ 5′ ccacaaaggtgctacaattag 3′	353	50
Exon 9	5′ tacatcatcagcactgtaactg 3′ 5′ ttgacagagatgtgaagtcatc 3′	494	53
Exon 10	5′ cagctttcaagtcagaaact 3′ 5′ aagaaagcttgactcttacctg 3′	511	53
Exon 11	5′ tcacgtagtacacattgcttct 3′ 5′ tatgtgcaagagtaacttccag 3′	365	61
Exon 12	5′ caaatgggggggattaaatgt 3′ 5′ aaaacgttacccccacaaag 3′	377	50
Exon 13	5′ gcagtaactctgtccacatctt 3′ 5′ cttctcacaggacagagacata 3′	432	62
Exon 14	5′ atgtttgtggcatatccttc 3′ 5′ cagactgtgaattaaggggtaa 3′	499	50
Exon 15	5′ ctaatgacaaggtgagaaggat 3′ 5′ ccttcatcttagtgtcctgttt 3′	338	50
Exon 16A	5′ ggaactagacagtgcacacata 3′ 5′ ctgggatttttcacgtagtaac 3′	428	62
Exon 16B	5′ attattcaggagttcctgtcc 3′ 5′ ctctgagctatttgtttcttcc 3′	500	50

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-

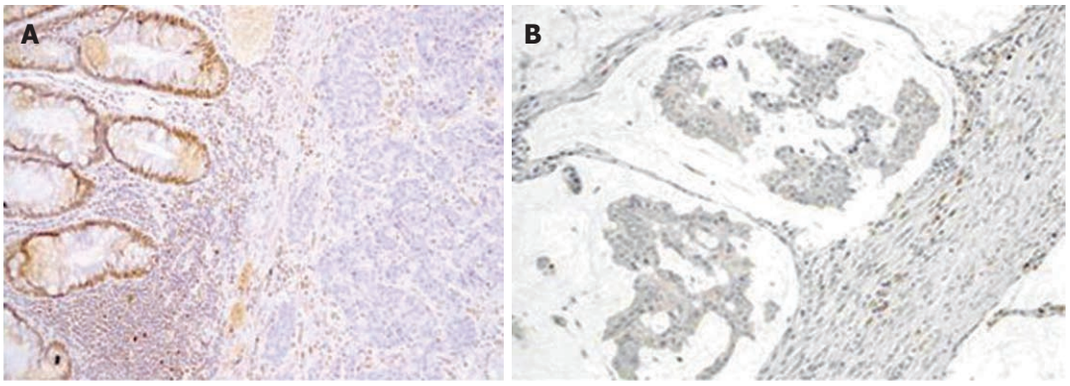

Figure 1 Immunohistochemical staining of mismatch repair proteins. A: *MLH1* gene protein, x 200 magnification. Positive nucleus staining for MLH1 protein in normal colon (left) and absent staining in colon cancer (right); B: *MSH2* gene protein, x 200 magnification. Absent nuclear staining for MSH2 protein in mucinous carcinoma (left) and positive staining in fibrous stromal cells, as internal control (right).

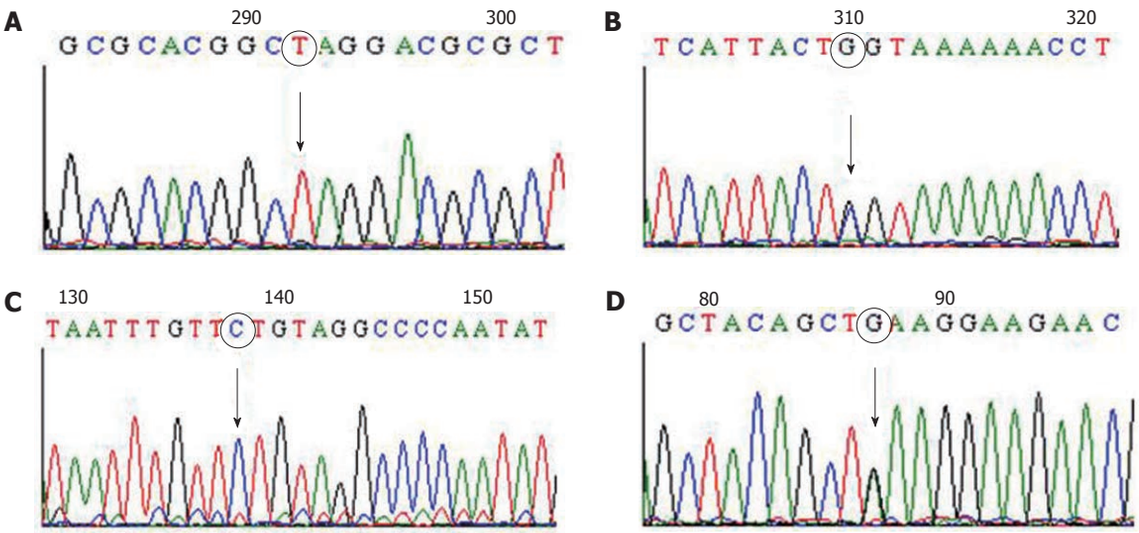

Figure 2 DNA sequencing result showing the germline mutations of *MSH2* gene identified in 4 Lynch syndrome patients. A: Deleterious mutation, c.142G > T, which resulted in a nonsense mutation (Glu48Stop); B: Novel mutation, transversion mutation c.2005G > C, which resulted in a missense mutation (Gly669Arg); 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)
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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.

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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.

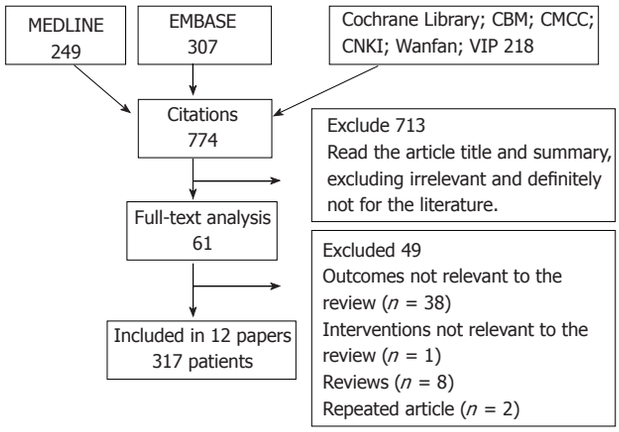

Figure 1 Flowchart showing the abstract screening and study selection process. CBM: Chinese BioMedical Literature on disc; CMCC: Chinese Medical Current Contents; CNKI: Chinese National Knowledge Infrastructure.

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)

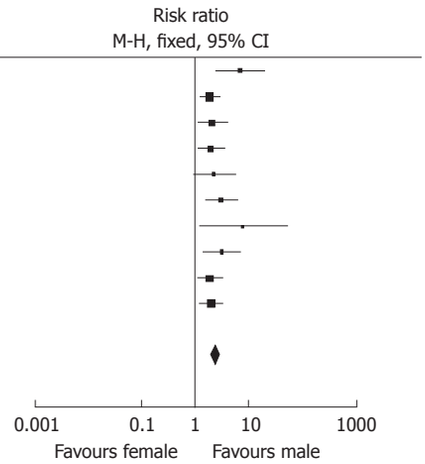

Figure 2 Gender difference in trials. M-H: Mantel-Haenszel.

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)

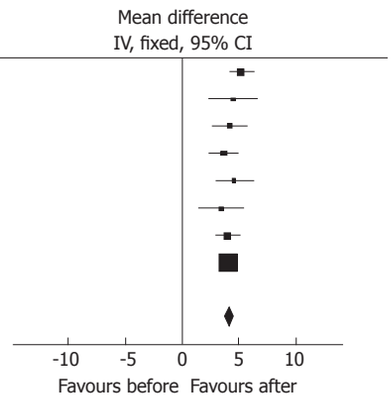

Figure 3 Proteinuria change in steroid combination therapy. IV: Inverse variance.

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)

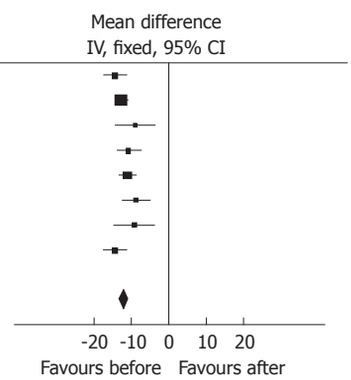

Figure 4 Serum albumin change in combination therapy group. IV: Inverse variance.

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-

Study or subgroup	Before Mean	Before SD	Before Total	After Mean	After SD	After Total	Weight (%)	Mean difference IV, random, 95% CI
Dang *et al*[17]	21	10.3	58	21.6	12.5	58	36.3	-0.60 (-4.77, 3.57)
Liu *et al*[19]	72.4	13.7	25	59.9	14	25	28.1	12.50 (4.82, 20.18)
Liu *et al*[20]	65.9	23.6	30	62.6	15	30	22.9	3.30 (-6.71, 13.31)
Tang *et al*[23]	27.4	25.2	9	22.9	4.3	9	12.8	4.50 (-12.20, 21.20)
Total (95% CI)			122			122	100.0	4.62 (-2.55, 11.79)

Heterogeneity: Tau² = 32.40; χ^2 = 8.72, df = 3 (P = 0.03); I^2 = 66%
Test for overall effect: Z = 1.26 (P = 0.21)

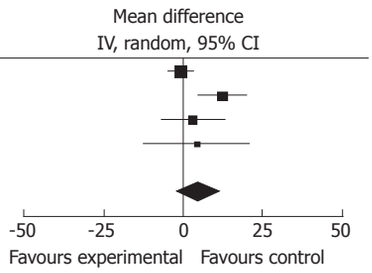

Figure 5 Alanine aminotransferase change in combination therapy group. IV: Inverse variance.

Study or subgroup	Before Mean	Before SD	Before Total	After Mean	After SD	After Total	Weight (%)	Mean difference IV, fixed, 95% CI
Dang *et al*[17]	97.3	38.2	58	92.5	38.6	58	52.8	4.80 (-9.18, 18.78)
Liu *et al*[20]	135	83.5	30	89	28.5	30	10.3	46.00 (14.43, 77.57)
Sun *et al*[22]	105	43.5	29	95.9	32.4	29	26.5	9.10 (-10.64, 28.84)
Tang *et al*[23]	95.3	33.8	9	89.6	34.3	9	10.4	5.70 (-25.76, 37.16)
Total (95% CI)			126			126	100.0	10.29 (0.14, 20.45)

Heterogeneity: χ^2 = 5.60, df = 3 (P = 0.13); I^2 = 46%
Test for overall effect: Z = 1.99 (P = 0.05)

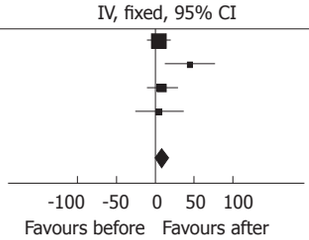

Figure 6 Scr change in combination therapy group. IV: Inverse variance.

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]	5.2	1.6	24	5.1	1.7	24	27.9	0.10 (-0.83, 1.03)
Tang *et al*[24]	5.3	1.7	21	5.1	1.7	21	23.0	0.20 (-0.83, 1.23)
Wu *et al*[25]	4.8	1.4	35	4.7	1.6	35	49.1	0.10 (-0.60, 0.80)
Total (95% CI)			80			80	100.0	0.12 (-0.37, 0.62)

Heterogeneity: χ^2 = 0.03, df = 2 (P = 0.99); I^2 = 0%
Test for overall effect: Z = 0.49 (P = 0.63)

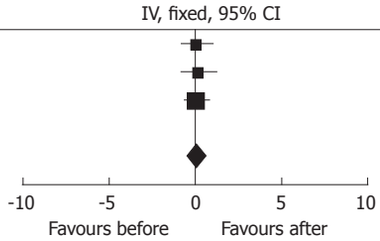

Figure 7 Hepatitis B virus-DNA titer change in combination therapy group. IV: Inverse variance.

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

Study or subgroup	Before Mean	Before SD	Before Total	After Mean	After SD	After Total	Weight (%)	Mean difference IV, fixed, 95% CI
High dose								
He *et al*[18]	7.3	3.5	13	2.8	1.4	13	2.8	4.50 (2.45, 6.55)
Liu *et al*[19]	6	3.7	25	1.8	0.9	25	5.3	4.20 (2.71, 5.69)
Tang *et al*[23]	4.9	2.9	9	1.4	0.7	9	3.1	3.50 (1.55, 5.45)
Subtotal (95% CI)			47			47	11.2	4.08 (3.06, 5.11)
Heterogeneity: χ^2 = 0.53, df = 2 (P = 0.77); I^2 = 0%								
Test for overall effect: Z = 7.80 (P < 0.00001)								
Low dose								
Cheng *et al*[16]	6.3	2.3	24	1.1	1.2	24	10.9	5.20 (4.16, 6.24)
Sun *et al*[21]	6.7	2.5	13	2.1	1.7	13	4.4	4.60 (2.96, 6.24)
Tang *et al*[24]	5	2.1	21	1	1.33	21	10.4	4.00 (2.94, 5.06)
Wu *et al*[25]	5.2	1.3	35	1.1	0.1	35	63.1	4.10 (3.67, 4.53)
Subtotal (95% CI)			93			93	88.8	4.25 (3.88, 4.61)
Heterogeneity: χ^2 = 4.07, df = 3 (P = 0.25); I^2 = 26%								
Test for overall effect: Z = 22.87 (P < 0.00001)								
Total (95% CI)			140			140	100.0	4.23 (3.89, 4.57)
Heterogeneity: χ^2 = 4.68, df = 6 (P = 0.58); I^2 = 0%								
Test for overall effect: Z = 24.16 (P < 0.00001)								
Test for subgroup differences: χ^2 = 0.09, df = 1 (P = 0.76), I^2 = 0%								

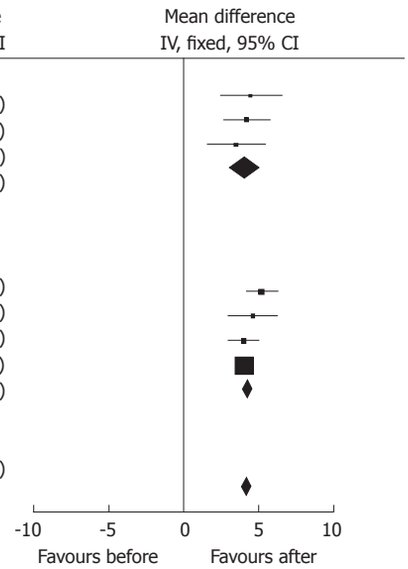

Figure 8 Proteinuria remission rate in different dose glucorticosteroids subgroups. IV: Inverse variance.

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%						

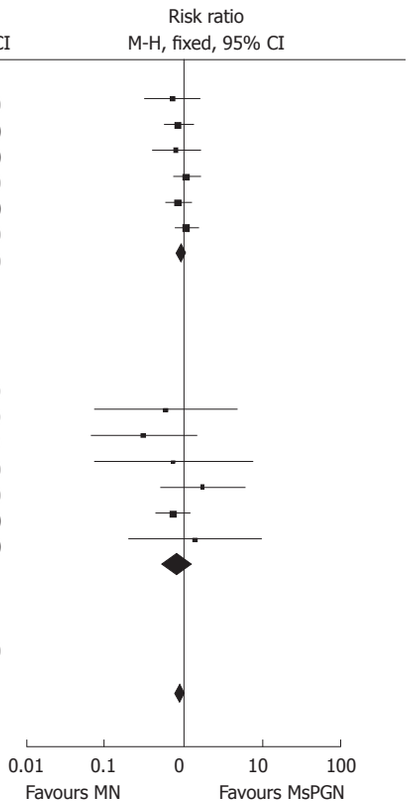

Figure 9 Proteinuria remission rate in membranous glomerulonephritis and mesangial proliferative glomerulonephritis subgroups. M-H: Mantel-Haenszel; MN: Membranous glomerulonephritis; MsPGN: Mesangial proliferative glomemlonephrits; CR: Complete remission; R: Remission.

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%						

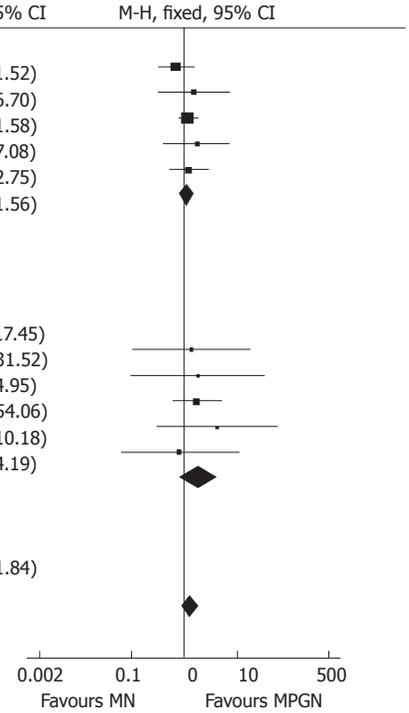

Figure 10 Proteinuria remission rate in membranous glomerulonephritis and membranoproliferative glomerulonephritis subgroups. M-H: Mantel-Haenszel; MN: Membranous glomerulonephritis; MPGN: Membranoproliferative glomerulonephritis; CR: Complete remission; R: Remission.

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

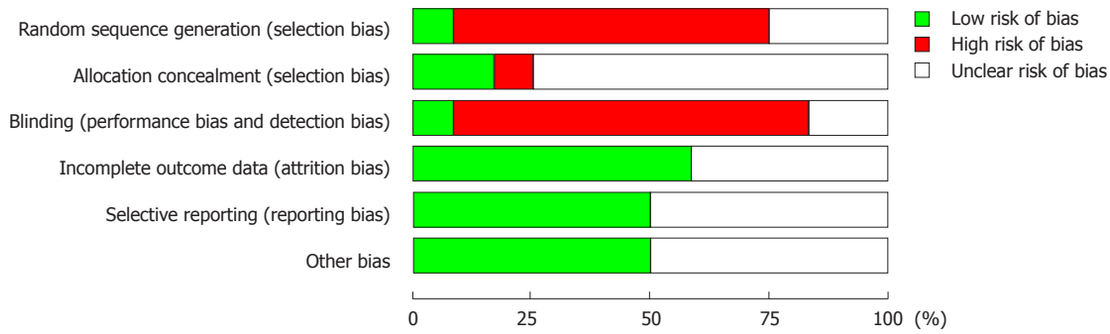

Figure 11 Risk of bias graph: Review authors' judgments about each risk of bias item presented as percentages across all included studies.

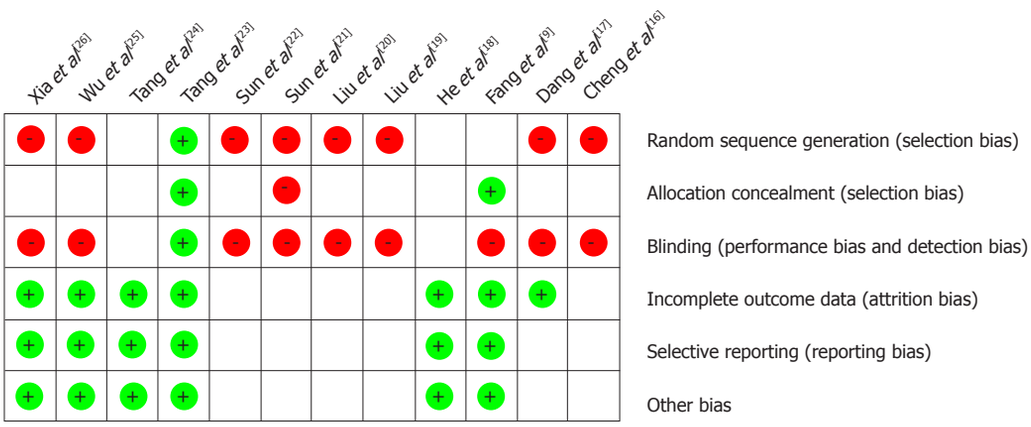

Figure 12 Risk of bias graph: Review authors' judgments about each risk of bias item for each included study.

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-

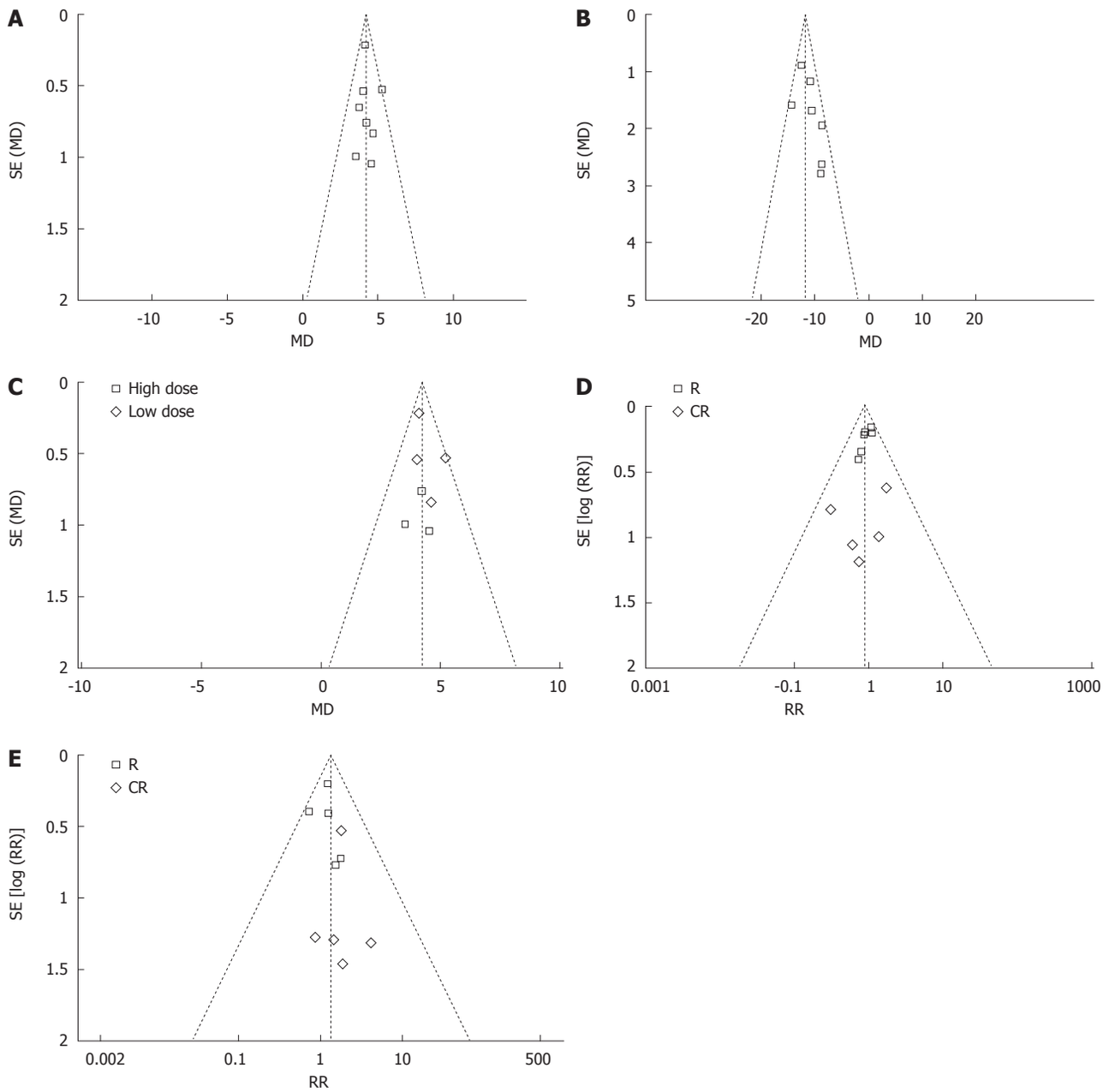

Figure 13 Publication bias analysis. A: Funnel plots of proteinuria treatment with steroid combination therapy; B: Funnel plots of serum albumin change with steroid combination therapy; C: Funnel plots of proteinuria change under different combined glucorticosteroids drugs; D: Funnel plots of remission 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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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.i8.833

World J Gastroenterol 2012 February 28; 18(8): 833-839
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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.

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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.

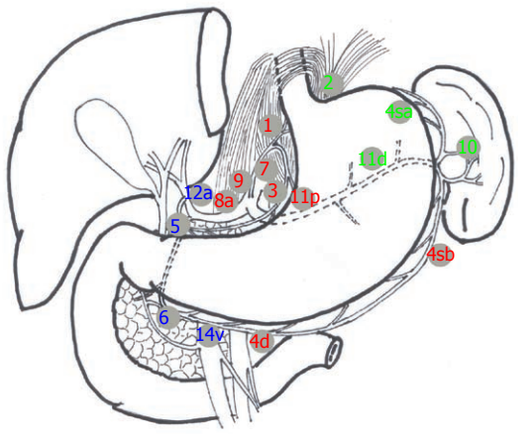

Figure 1 Lymph node stations of the stomach. The groups of lymph node in red, green and blue should be dissected in radical total gastrectomy; the groups of lymph node in red and green should be dissected in radical proximal gastrectomy; the groups of lymph node in red and blue should be dissected in 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)
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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.

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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

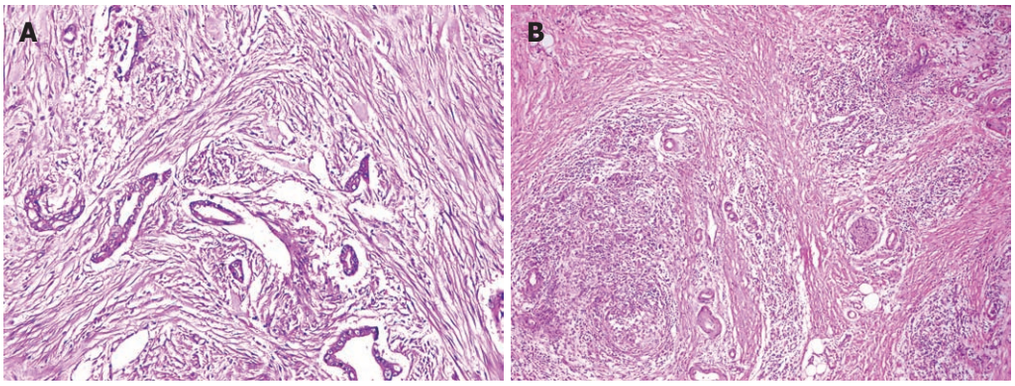

Figure 1 Fibrotic focus in pancreatic ductal adenocarcinoma. A: In the fibrotic focus, fibroblasts and collagen fibers are radially aligned around the tumor cells (× 200); B: A pattern different from that in the surrounding pancreatic ductal adenocarcinoma stroma where the fibroblasts and collagen fibers are arranged in an orderly fashion (× 100).

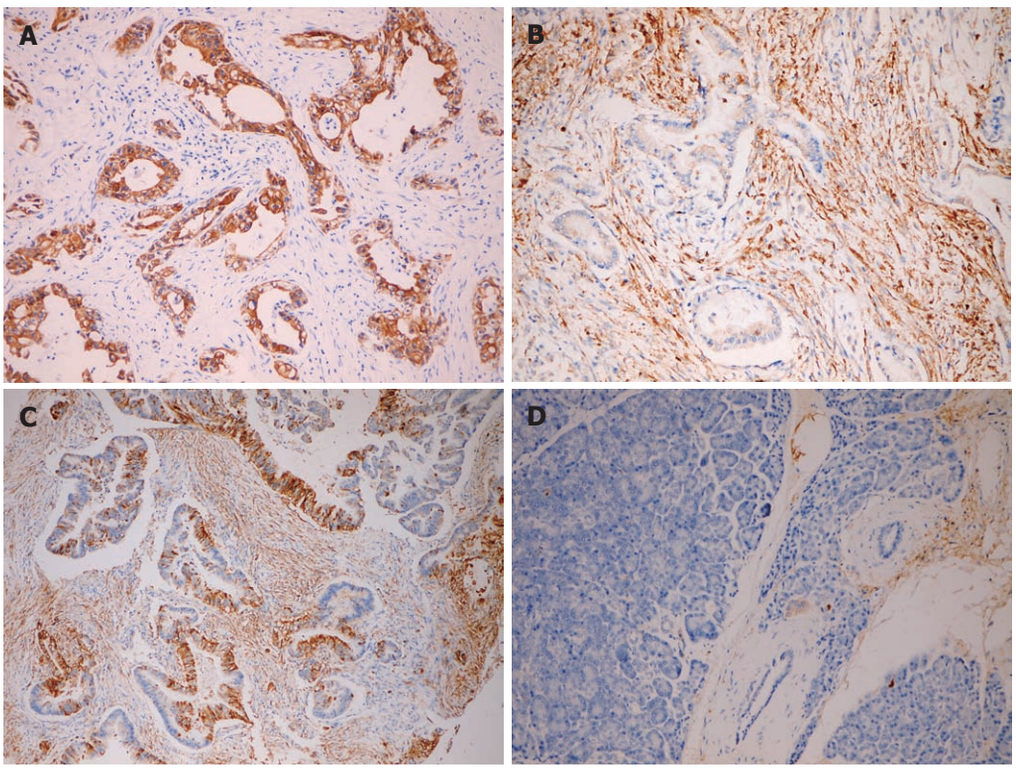

Figure 2 Fibroblast activation protein expression in pancreatic ductal adenocarcinoma. A: Fibroblast activation protein (FAP) expression in carcinoma cells is located mainly on the cell membrane and in the cytoplasm (× 200); B: FAP-positive fibroblasts are mainly located adjacent to carcinoma tissue (× 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.

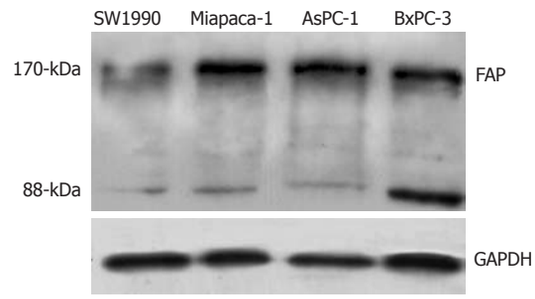

Figure 3 Western blotting analysis of fibroblast activation protein in pancreatic cancer cell lines. All four cell lines express fibroblast activation protein (FAP) with protein bands corresponding to the proteolytically active 170-kDa seprase dimer and its 88-kDa seprase subunit protein. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

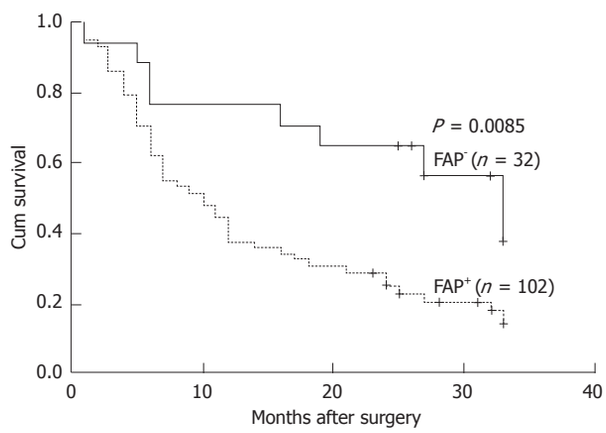

Figure 4 Kaplan-Meier survival curves of pancreatic ductal adenocarcinoma patients. The prognosis was significantly worse in the fibroblast activation protein (FAP)-positive group compared with the FAP-negative group (P = 0.0085).

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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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.i8.847

World J Gastroenterol 2012 February 28; 18(8): 847-854
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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.

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Key words: Liver transplantation; Acute rejection; Interleukin-10; Gene polymorphism; Meta-analysis

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Liu F, Li B, Wang WT, Wei YG, Yan LN, Wen TF, Xu MQ, Yang JY. Interleukin-10-1082G/A polymorphism and acute liver graft rejection: A meta-analysis. *World J Gastroenterol* 2012; 18(8): 847-854 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i8/847.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i8.847

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

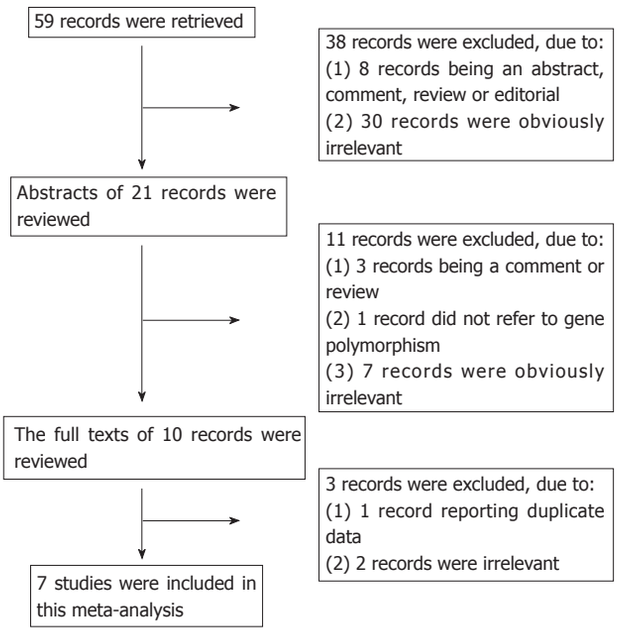

Figure 1 Flow chart of selection of studies and specific reasons for exclusion from the meta-analysis.

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	Case		Control		Genotyping methods	HWE in controls
				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

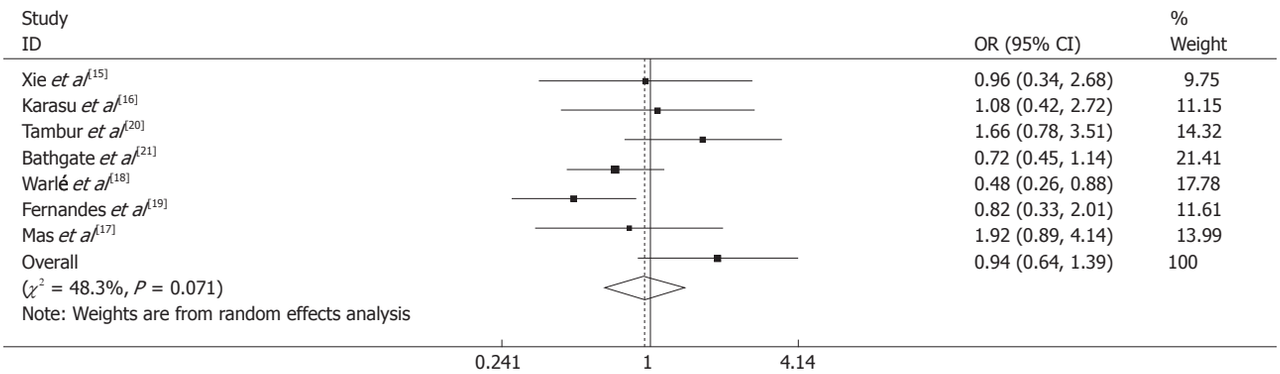

Figure 2 Odds ratios and 95% CI of individual studies and pooled data for the association of the interleukin-10-1082 G/A polymorphism and acute rejection comparing A allele with G allele. OR: Odds ratios.

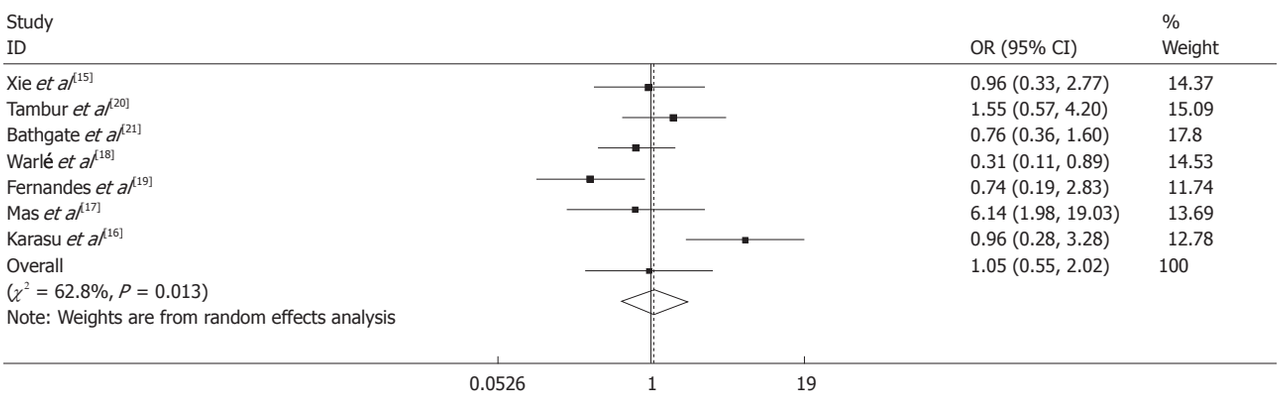

Figure 3 Odds ratios and 95% CI of individual studies and pooled data for the association of the interleukin-10-1082 G/A polymorphism and acute rejection comparing AA genotype with AG/GG Genotype. OR: Odds ratios.

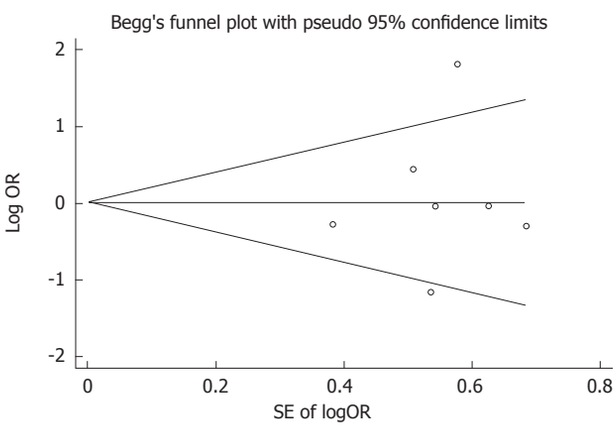

Figure 4 Begg's funnel plot of interleukin-10-1082 G/A polymorphism and acute rejection risk in liver transplant recipients (AA *vs* AG/GG). OR: Odds ratios.

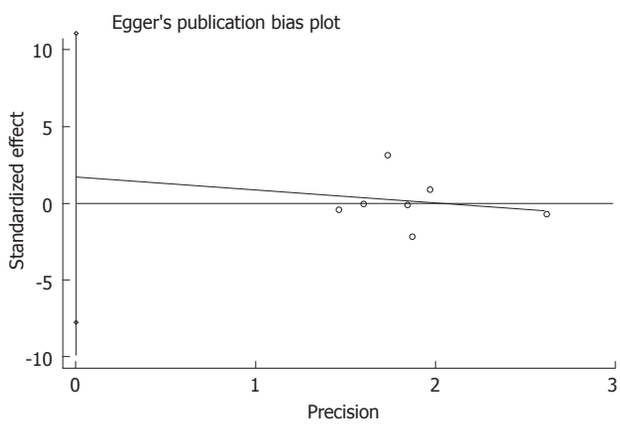

Figure 5 Egger's publication bias plot of interleukin-10-1082 G/A polymorphism and acute rejection risk in liver transplant recipients (AA *vs* AG/GG).

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)
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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.

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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		*P* value (*vs* ANT)	*P* value (*vs* Dukes A and B)
		Amplification			
		⩽ 2	> 2		
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 ($P = 0.0112$).

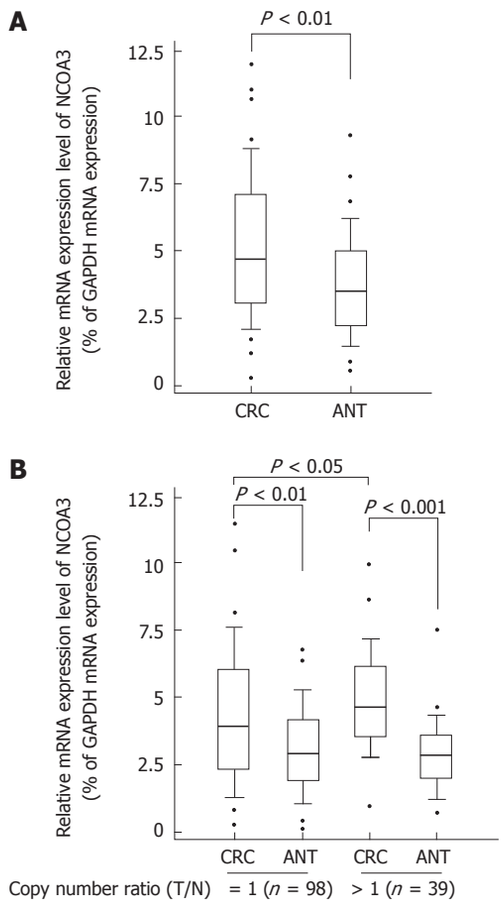

Figure 1 Real-time polymerase chain reaction assay was carried out as described under Materials and Methods section, and the results were obtained from indicated groups of samples. Boxplots of relative copy number of *NCOA3* mRNA measured with real-time polymerase chain reaction analysis showing the 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)
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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*).

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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.

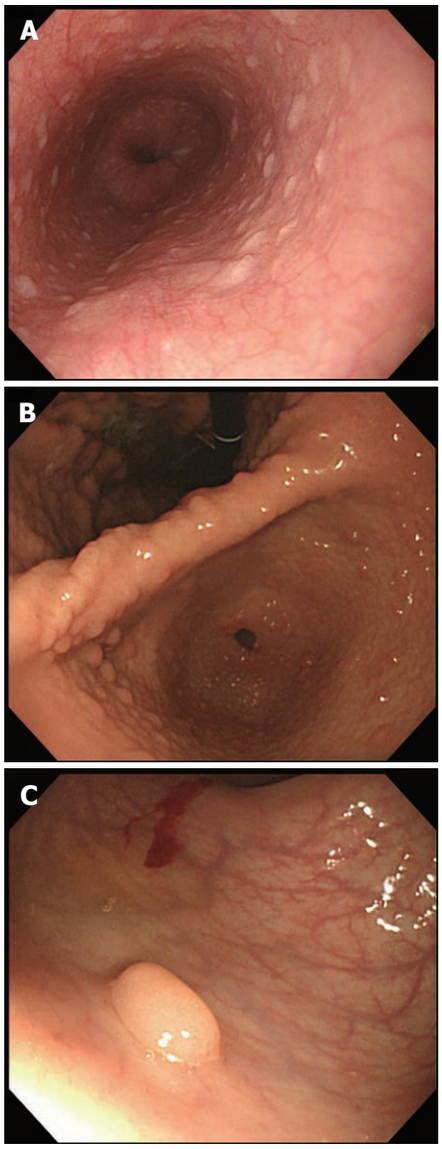

Figure 1 Endoscopic examination. A: Esophageal acanthosis was noted; B: Multiple gastric polyps were noted on entire stomach; C: Several polyps were observed at the rectum.

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

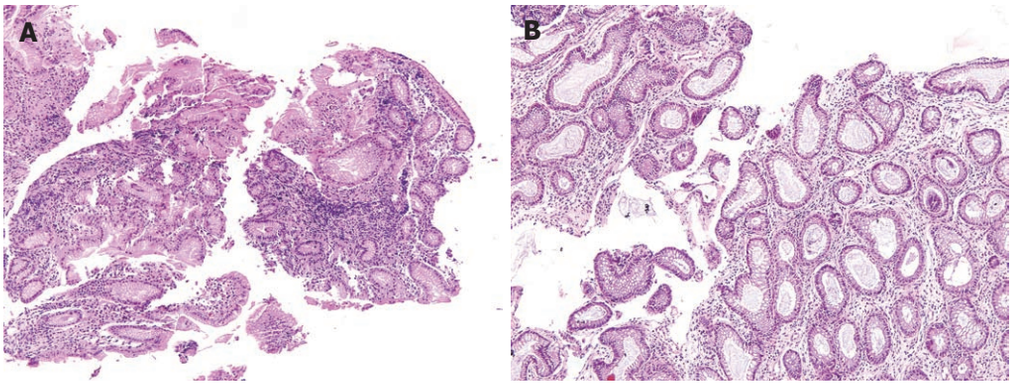

Figure 2 Microscopic examination (hematoxylin and eosin stain, × 100). A: Gastric polyp shows hyperplastic foveolar epithelium; B: Colon polyp exhibits dilated and branched crypts with slightly fibrotic stroma.

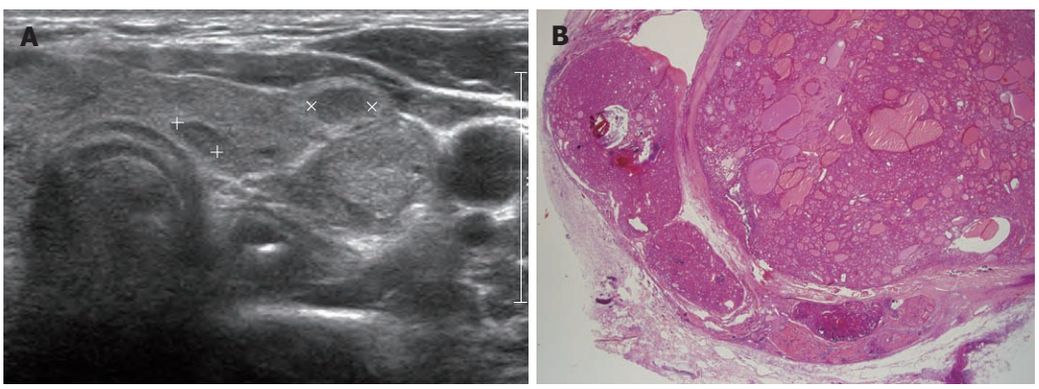

Figure 3 Thyroid masses. A: Multiple thyroid masses in thyroid ultrasonography; B: Postoperative thyroid pathology showed invasive follicular carcinoma (hematoxylin and eosin stain, × 12.5).

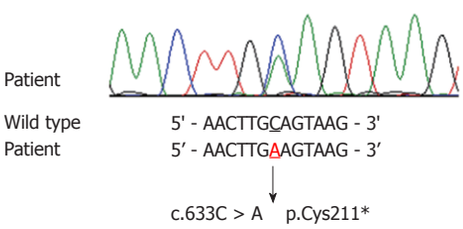

Figure 4 Sequencing result of the phosphatase and tensin homologue mutation. A heterozygous transition of C to A at nucleotide 633 in exon 6 (c.633C > A), making a stop codon (p.Cys211*).

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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World J Gastroenterol 2012 February 28; 18(8): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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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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World J Gastroenterol 2012 February 28; 18(8): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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Events Calendar 2012

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

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

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

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

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

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

May 19-22, 2012
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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

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Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

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Dnepropetrovsk, Ukraine

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The Liver Meeting
San Francisco, CA 94101, United States

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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)

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

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

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

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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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